GENEESKUNDE
master in de biomedische wetenschappen: milieu en gezondheid

Masterproef
The role of CD28null T cells in autoimmune diseases

Promotor :
Prof. dr. Niels HELLINGS

Marijke Brulmans
Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting milieu en gezondheid

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Preface

This thesis is the result of the studies conducted during my 8 month internship at the Biomedical research institute at Diepenbeek. It has been a very fulfilling experience, but all good things must come to an end.

I would sincerely like to thank my supervisor, Bieke Broux, for the excellent guidance she has given me during my stay at Biomed, for the corrections and improvements she has provided me for my thesis, and for her pleasant company throughout my internship.

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Special thanks go to Christel Boken, my fellow students and everybody at Biomed who has helped me with my questions and experiments.

Last but definitely not least, I would like to thank my parents. They made it possible for me to go to university, and they have supported me throughout my entire studies, especially these last few months. I would never have gotten this far without them.

Content

List of Abbreviations

AICD: Activation-induced cell death

AD: Alzheimer's disease

BSA: Bovine serum albumin

BBB: Blood brain barrier

CD40L: CD40 Ligand

CMV: Cytomegalovirus

CNS: Central nervous system

EAE: Experimental autoimmune encephalomyelitis

EBV: Epstein-Barrvirus

ECM: Extracellular matrix

EDSS: Expanded disability status scale

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

FACS: Fluorescence-activated cell sorting

FBS: Fetal Bovine serum

G-CSF: Granulocyte colony-stimulating factor

HCMV: Human Cytomegalovirus

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HRP: Horseradish peroxidase

HSV: Herpes Simplex Virus

IFN-β: Interferon-β

IFN-γ: Interferon-γ

IL-12: Interleukine-12

IL-2: Interleukine-2

KIR: Killer imunoglobin like receptor

MBP: Myelin basic protein

MHC: Major histocompatibility complex

MS: Multiple sclerosis

NAWM: Normal appearing white matter

NDC: Non-demented control

NK: Natural killer

PBS: Phosphate buffered saline

P/S: Penicilline streptomycin

PHA: Phytohemaglutinin

RA: Rheumatoïd arthritis

RPMI: Roswell Park Memorial Institute

TBS: Tris-buffered saline

TBS-T: Tris-buffered saline/0,03% Triton X-100

TCR: T-cell receptor

TMB: Tetramethylbenzidine

TNF-α: Tumor necrosis factor α

VSV: Vesicular stomatitis virus

Abstract

Objective: During aging and chronic infections, T cells lose the costimulatory molecule CD28 and show various similarities with NK cells, such as proinflammatory functions characterized by the production of high levels of IFN-γ, TNF-α, and IL-2. They are cytotoxic and resistant to apoptotic cell death. An increase in CD4$^+$CD28^{null} T cells represents an important amplification mechanism in several autoimmune diseases, leading to more severe disease symptoms. CD8$^+$CD28^{null} T cells are also detrimental for the host, but have been shown to have some beneficial effects as well. We aim to further elucidate the role of both CD8$^+$CD28^{null} and CD4$^+$CD28^{null} T cells in immunopathology, and hypothesize that CD4$^+$CD28^{null} T cells actively contribute to chronic inflammatory diseases such as MS.

Methods: PBMC of HC and patients with MS were stained and analyzed with flow cytometry for CD28, CD4, CD8 and a range of cytokines to assess the cytotoxicity, degranulation, chemotactic capacity, and proliferation rate of both CD28^{null} T cell populations. A cytokine array was performed to identify the range of cytokines produced by CD4$^+$CD28^{null} T cells in HC and MS patients. Brain tissue of NDC, MS and AD patients were stained for the presence of CD4$^+$CD28^{null} and CD8$^+$CD28^{null} T cells, as well as for the presence of CX$_3$CR1, using immunohistochemistry

Results: The presence of CD4$^+$CD28^{null} and CD8$^+$CD28^{null} T cells in the peripheral blood is strongly correlated, although CD8$^+$CD28^{null} T cells are more common and show increased frequencies with chronic infections as well as during normal aging. Both cell populations express CX$_3$CR1 and are able to migrate towards an increasing fractalkine gradient. CX$_3$CR1 is expressed by microglia and oligodendrocytes in the brains of NDC and AD patients, but not by neurons as in the brains of MS patients. Eight major proinflammatory cytokines were produced by CD4$^+$CD28^{null} T cells: IL-1α, IL-1β, IL-6, G-CSF, TNF-α, IFN-β, CCL2 and CCL4. The production of proinflammatory cytokines IFN-γ and IL-17, and cytotoxic molecules NKG2D, perforin and granzyme B was elevated after IL-15 stimulation.

Conclusion: CD4$^+$CD28^{null} T cells share many similarities with NK cells and are considered cytotoxic and inflammation-seeking cells. They can infiltrate the brains of MS patients through the expression of CX$_3$CR1 and contribute to the disease by releasing an arsenal of cytoxic and proinflammatory cytokines. The contribution of CD8$^+$CD28^{null} T cells is not so clear, due to the fact that CD8$^+$CD28$^+$ T cells are already considered cytolytic.

Abstract

Inleiding en doel: T cellen verliezen de expressie van de costimulator CD28 tijdens het ouder worden en door chronische infecties. Hierdoor vertonen ze gelijkenissen met NK cellen, zoals de productie van IFN-γ, TNF-α en IL-2. Ze zijn cytotoxic en resistent tegen apoptose. Een toename in het aantal $CD4^{+}CD28^{null}$ T cellen zorgt ervoor dat vele auto-immuun ziekten verergeren. $CD8^{+}CD28^{null}$ T cellen zijn ook schadelijk voor de gastheer, maar hebben eveneens enkele voordelen. In deze studie proberen we de rol van beide $CD28^{null}$ T celpopulaties in de immunopathologie te ontrafelen. De hypothese dat $CD4^{+}CD28^{null}$ T cellen bijdragen aan chronische ontstekingsreacties in ziekten zoals MS zal hierbij onderzocht worden.

Methoden: PBMC van gezonde personen en MS patiënten werden gekleurd en geanalyseerd d.m.v. flowcytometrie. Dit werd gedaan voor CD28, CD4, CD8 en een reeks van cytokines om de cytotixiciteit, degranulatie, chemotactische capaciteit en proliferatie van beide $CD28^{null}$ T celpopulaties te onderzoeken. Er werd een cytokine array verricht om te bepalen welke cytokines worden geproduceerd door $CD4^{+}CD28^{null}$ T cellen. Als laatste werden hersencoupes van gezonde personen, MS en AD patiënten gekleurd op de aanwezigheid van $CD4^{+}CD28^{null}$, $CD8^{+}CD28^{null}$ T cellen en $CX_{3}CR1$ d.m.v. immunohistochemie.

Resultaten: De aanwezigheid van $CD4^{+}CD28^{null}$ en $CD8^{+}CD28^{null}$ T cellen in het bloed is sterk gecorreleerd aan elkaar, hoewel $CD8^{+}CD28^{null}$ T cellen veel vaker voorkomen, ook door het natuurlijk ouderdomsproces. Beide celpopulaties vertonen expressie van $CX_{3}CR1$ en kunnen naar een verhoogde fraktalkinegradiënt migreren. $CX_{3}CR1$ wordt ook tot uiting gebracht door microglia en oligodendrocyten in de hersenen van gezonde personen en AD patiënten, maar niet door neuronen zoals het geval is voor MS patiënten. Er werden 8 belangrijke cytokines gedetecteerd in het cytokine array, namelijk IL-1α, IL-1β, IL-6, G-CSF, TNF-α, IFN-β, CCL2 en CCL4. De productie van proinflammatoire cytokines IFN-γ en IL-17, en cytotoxische moleculen NKG2D, perforine en granzyme B was verhoogd na stimulatie met IL-15.

Conclusie: $CD4^{+}CD28^{null}$ T cellen vertonen veel gelijkenissen met NK cellen en zijn cytotoxische en ontsteking-zoekende cellen. Ze kunnen de hersenen van MS patiënten infiltreren door de expressie van $CX_{3}CR1$, en bijdragen aan de ziekte door de productie van een arsenaal aan cytotoxische en proinflammatoire cytokines. De bijdrage van $CD8^{+}CD28^{null}$ T cellen is niet zo duidelijk omdat $CD8^{+}CD28^{+}$ T cellen al als cytolytisch beschouwd worden.

Introduction

Introduction to the immune system

Immunity is defined as resistance to disease, specifically infectious disease. The physiological function of the immune system, which is the collection of cells, tissues and molecules that mediate resistance to infections, is to prevent and eradicate infections. The importance of the immune system for health is illustrated by the frequent observation that individuals with defective immune responses are susceptible to serious, often life-threatening infections. Host defense mechanisms consist of innate immunity, which mediates the initial protection against infections, and adaptive immunity, which develops more slowly and mediates the later, even more effective, defense against infections. The first line of defense in innate immunity is provided by epithelial barriers and by specialized cells and natural antibiotics present in epithelia. The innate immune system consists of phagocytes and natural killer cells (NK cells) which react against microbes, but not against noninfectious foreign substances. Many microbes that are pathogenic to humans have evolved to resist innate immunity. Defense against these infectious agents is the task of the adaptive immunity, and this is why defects in the adaptive immune system result in increased susceptibility to infections. The adaptive immune system consists of lymphocytes and their products, such as antibodies. These lymphocytes express receptors that specifically recognize different substances produced by microbes as well as non infectious molecules. These substances are called antigens. There are two types of adaptive immunity, humoral immunity, which is mediated by antibodies produced by B lymphocytes, and cell-mediated immunity which is mediated by T lymphocytes.[1]

Figure 1: Activation of T cells

Lymphocytes are the only cells that produce specific receptors for antigens and are thus the key mediators of adaptive immunity. Among T lymphocytes, CD4$^+$ T cells are called T helper cells because t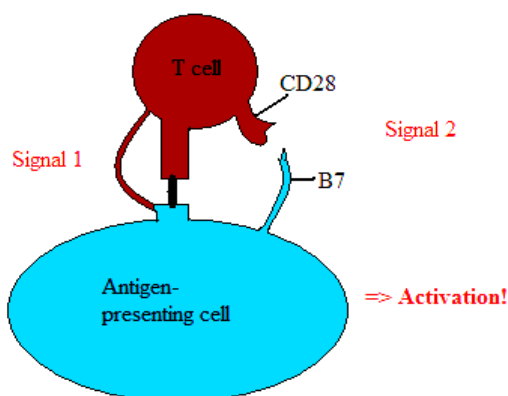hey help B lymphocytes to produce antibodies and phagocytes to destroy ingested microbes. CD8$^+$ T cells are called cytotoxic or cytolytic T cells because they kill cells harboring intracellular microbes.[1] Activation of T cells requires the recognition of a specific antigen,

on an antigen-presenting cell, by the T cell receptor (TCR).[2] Triggering of the TCR by the appropriate major histocompatibiliy-antigen complex (MHC) is insufficient to induce T cell proliferation unless it is accompanied by costimulatory signals derived from accessory cells. (Figure 1) The costimulatory signal determines the activation threshold and the functional outcome of the antigen-specific activation. Dysregulation of this activation threshold may play a critical role in the activation of autoreactive T cells.

Immunosenescence: Aging of the immune system

The efficiency of the immune system of the elderly declines with age, resulting in an increased susceptibility to infectious diseases and pathological conditions relating to inflammation or autoreactivity. This overall change in immunity is termed 'immunosenescence'. The individual factors contributing to immunosenescence are diverse, due to the multi-factorial complexity of the immune system. The interplay between B cells and T cells is crucial for an effective response, so if one subset is affected, this will change the function of the other. Immunosenescence is a descriptive term covering the deleterious age-associated changes to immunity observed in all mammal studies so far. Immunity evolved to protect against infectious diseases, and appropriate immunity is essential for 'normal' longevity. While all components of innate and adaptive immunity change with age, the clinical impact of these changes is not clear, and mechanisms and markers for immunosenescence are controversial.[3] For example, Cytomegalovirus (CMV) is a β-herpesvirus that continuously activates the immune system and is present in approximately 50% of the adult population and 90% of the elderly. In healthy hosts, CMV infection is asymptomatic. Maintaining protective immunity against CMV is essential, but may have a significant impact on the overall adaptive immunity due to the repeated stimulation of T cells.[4,5] There is epidemiological evidence for excess mortality in CMV-positive populations, which is further increased in those co-infected with hepatitis A en B as well.[3] The marked influence of CMV on immune signatures is illustrated in the finding that cross-sectional studies on several different European populations clearly indicate that the consensus view of T cell immunosenescence is indeed true. This entails that the fraction of naive CD8 cells decreases in the elderly and the fraction of late-differentiated memory cells increases, but only for people who are infected with CMV. Such individuals also have higher levels of C-reactive protein, indicating that they are more likely to suffer from inflammatory diseases, as well as general frailty and increased mortality.[3]

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Infection with other persistent herpes viruses, at least Epstein-Barr virus (EBV), Herpes simplex virus (HSV) and vesicular stomatitis virus (VSV), does not appear to have any similar effect. CMV is unique in the context that there is some advantage in early life to being CMV-positive, due to the enhanced pro-inflammatory status in infected people, which might have a protective effect against infection with other pathogens. Although immunosenescence is not caused by CMV, because not all elderly people are CMV-positive, this infectious agent seems to have a large impact on immune parameters in later life and contributes to increased morbidity and eventual mortality.[3]

Immunosenescence: $CD28^{null}$ T cells

The CD28 molecule is currently considered the primary costimulatory receptor functional in transducing signals that augment T cell proliferation and lymphokine production.[6] CD28 is expressed on $CD4^+$ and $CD8^+$ T cells, and receives signals from its ligands CD80/CD86.[7,8] This signal is important in T cell activation, proliferation and survival, together with interleukin-2 (IL-2) production. During aging and chronic infections, T cells lose this receptor and become $CD28^{null}$ T cells.[9,10] The loss of CD28 expression in T cells has been attributed to repeated antigenic stimulation which can also be observed in $CD28^+$ T cells after repeated antigen stimulation in vitro.[11,12] This is particularly relevant for $CD8^+$ T cells, which show increased frequencies of $CD28^{null}$ T cells in patients with chronic infections as well as during normal aging. In contrast, the emergence of $CD28^{null}$ T cells with the $CD4^+$ compartment appears to be the exception rather than the rule.[13] The presence of $CD4^+CD28^{null}$ T cells has been shown in patients with diseases such as multiple sclerosis (MS), reumathoid arthritis (RA), Wegener's granulomatosis, unstable angina, dermatomyositis and polymyositis.[14,15,16] $CD28^{null}$ T cells show specificity for human cytomegalovirus (HCMV) antigens, and their presence was clearly associated with HCMV seropositivity.[17] These cells are also involved in mechanisms increasing the risk of acute ischemic stroke.[18] Lack of CD28 expression on T cells has also been reported to increase with age, particularly within the CD8 subset.[19,20]

T cells that lose the expression of CD28 undergo in vivo clonal expansion[21] and gain several properties that are similar to those of NK cells, such as pro-inflammatory functions characterized by the production of high levels of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). (Tabel 1) These T cells are cytotoxic and resistant to apoptotic cell death. The primary form of apoptosis of clonally expanded T cells is activation-induced cell death (AICD), which is mainly controlled by the Fas (CD95) system. In the absence of appropriate

costimulation, TCR signaling induces Fas and Fas ligand expression. Ligation of Fas initiates the recruitment of Fas-associated death domain protein and caspase-8, which then triggers the proteolytic caspase cascade, resulting in the cleavage of various proteins and finally apoptotic cell death. Several mechanisms exist to counterregulate death processes either at the receptor, mitochondrial, or caspase level. The longevity of CD28[null] T cells is not due to defective Fas ligand expression, but is attributed to resistance against apoptosis which correlates with enhanced expression of the anti-apoptotic molecule Bcl-2.[22,23] The gain of cytolytic function by CD4^{+}CD28[null] T cells is supported by the elevated production of the key cytolytic molecules including perforin, granzyme B and granzyme A.[10,24] They also express the chemokine receptor CCR5, and upregulate CD161, a molecule that facilitates tissue invasion. Expression of activating killer immunoglobin-like receptors (KIR) on CD4^{+}CD28[null] T cells has been associated with severe disease manifestations in RA patients.[15,25,26]

Table 1: Characteristics of CD4^{+}CD28[null] T cells (compared to CD4^{+}CD28^{+} T cells)

Characteristics of CD4^{+}CD28[null] T cells	
Gained	**Lost**
Strong expression CD57 (NK cell surface marker)	Expression CD7 (Lymphocyte marker)
KIR	Expression CD28 (Costimulation)
Production IFN-γ and TNF-α	Expression CD40L (B cell help)
Expression cytolytic molecules (Granzymes A and B, Perforin)	Expression CD27 (Costimulation)
Expression chemokine receptor CCR5	Production IL-2 (B cell differentiation)
Upregulation CD161 (tissue invasion)	
Expression NKG2D	
Expression fractalkine receptor CX$_3$CR1	
Restricted TCR repertoire	
Enhanced expression Bcl-2	

Despite the deficiency of CD28, human CD4^{+}CD28[null] T cells are functionally active.[27] They are highly oligoclonal, long-lived lymphocytes with aberrant functions that are thought to contribute to disease-related immune-dysfunctions. These T cells have lost their classic helper function because of an accompanying inability to upregulate CD40 ligand,[27] and they are incapable of promoting B cell differentiation because of a deficiency in IL-2 production. [28,29]

Numerous receptors that control the function of CD4$^+$CD28^{null} T cells are primarily expressed on NK cells. These receptors include the KIR, NKG2D, the fractalkine receptor CX$_3$CR1, and Ig-like transcripts. NKG2D is an activating receptor that is expressed on most NK cells and CD8$^+$ T cells, but not on CD4$^+$ T cells.[30] When expressed by CD4$^+$CD28^{null} T cells, NKG2D can provide a costimulatory signal as identified in patients with RA, as well as in patients with Wegener's granulomatosis.[9] The expression of NKG2D on these T cells is most likely as a result of their exposure to TNF-α and IL-15, which is structurally similar to IL-2 and is secreted by mononuclear phagocytes following infection by viruses.[30] The fractalkine receptor CX$_3$CR1 is a highly relevant marker to identify CD4$^+$CD28^{null} T cells because they strongly express this receptor while it is almost absent on CD4$^+$CD28$^+$ T cells. This is less clear in CD8$^+$ T cells, where the expression of CX$_3$CR1 is elevated on CD8$^+$CD28^{null} T cells, but is also present on CD8$^+$CD28$^+$ T cells. Loss of CD28 expression is also accompanied with profound changes in T cell functions within the CD8 compartment, such as a lack of antibody production following immunization in old age, an inability to mount a robust proliferative response to stimulation, defects in cytokine production including IL-2, IL-4, IL-10 and IFN-γ following stimulation, and a loss of antigen repertoire diversity.[31] Accumulating evidence shows that NK cell receptors are also expressed in CD8$^+$CD28^{null} T cells. Both CD8$^+$CD28^{null} and CD4$^+$CD28^{null} T cells have shorter telomeres than their CD28$^+$ counterparts, indicative for their repeated clonal expansions.[2,32] In general, CD28^{null} T cells are an inflammation-seeking effector-memory T cell population with cytotoxic properties, and have features that are hallmarks of an aged immune system.[2]

CD4$^+$CD28^{null} T cells and autoimmunity

CD4$^+$CD28^{null} T cells do not depend on the CD28 pathway for activation, they are incapable of activating B cells, have significant cytolytic activity, and express high levels of IFN-γ. Thus, the presence of significant numbers of CD4$^+$CD28^{null} T cells could shift immune response from B cell activation and production of immunoglobulins toward production of IFN-γ and involvement of macrophages releasing matrix-degrading proteases.[33] These deviating characteristics of CD4$^+$CD28^{null} T cells make it likely that they are not just a marker of an aging immune system, but contribute actively or passively to autoimmune disease pathology. The functionality of the immune system is highly dependent on the diversity of the naive T cell pool. If the T cell pool is composed for a considerable part of CD4$^+$CD28^{null} T cells, this could have serious implications for immune function. The accumulation of

CD4$^+$CD28^{null} T cells also limits the peripheral space which is otherwise available for functional naive T cells, competitor T cells or regulatory T cells. This alteration in T cell composition may cause an imbalanced immune system with a changed threshold for T cell activation.[19] An increase in CD4$^+$CD28^{null} T cells represents an important amplification mechanism in MS and other immune diseases, leading to more severe disease symptoms rather than being involved in the initial break of tolerance. These cells are readily triggered by inflammation and subsequently perpetuate and re-enforce the inflammatory process. The presence of these T cells has been shown in several autoimmune diseases, such as MS and RA, were they actively contribute to the inflammation when present. Fractalkine is upregulated in the cerebrospinal fluid of clinically isolated syndrome patients, and in the brain of MS patients which enables the CD4$^+$CD28^{null} T cells to migrate to the sites of inflammation, through the expression of CX$_3$CR1 on their surface. [34] Chemokine receptors and effector molecules such as perforin and granzyme B uniquely expressed on CD4$^+$CD28^{null} T cells may enable these cells to infiltrate tissue and to cause tissue damage. The expression of stimulatory receptors lowers the activation threshold for antigen-specific stimulation.[9] Mechanisms that restore CD28 expression and reduce the pool of CD4$^+$CD28^{null} T cells are therefore potential therapeutic targets in these auto-immune diseases.

A wide variety of inhibitory T cell populations exist within the CD8$^+$ T cell compartment. Means of induction (innate/adaptive), phenotypes (CD28$^+$/CD28^{null}), and suppressive mechanisms are highly diverse. A barrier in investigating CD8$^+$ T cells is the lack of a specific cell surface marker. Lack of CD28 cannot be used as a marker because the CD8$^+$CD28^{null} T cell population contain both cytotoxic and suppressor cells. A series of CD4$^+$ and CD8$^+$ T cells have been described which can disrupt immune responses, including the pathogenic immune responses causing autoimmune diseases, but it is currently unknown whether these two classes of T cells have similar or distinct roles in regulating immune responses. Recent evidence shows that they are functionally connected, but their roles in immune diseases need further elucidation.[35]

CD8$^+$CD28^{null} T cells

CD8$^+$ T cells are responsible for inducing apoptosis of intracellular pathogen-infected or transformed cells. The presence of senescent CD8$^+$ T cells may have a variety of effects on the immune system. They influence the quality and composition of the memory T cell pool, and due to their resistance to apoptosis, they accumulate progressively over time. CD28^{null} T

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cells are usually part of oligoclonal expansions, which leads to a reduction in the overall spectrum of antigenic specificities within the T cell pool. A more direct effect of $CD8^+CD28^{null}$ T cells lies in the area of suppressor cell activity. A population of $CD8^+CD28^{null}$ T cells functions to suppress immune reactivity by inducing antigen-presenting cells to become tolerogenic to helper T cells with the cognate antigen specificity. This same subset of T cells has been implicated in the tolerance to allogeneic organ transplants. This suggests a possible suppressive function which is beneficial in reducing the reactivity against the allograft. Other immune suppressive functions might not be so beneficial, such as the mediation of liver damage in hepatitis C infection, and stimulation of head and neck tumors by a population of TNF-α producing $CD8^+CD28^{null}$ T cells. $CD8^+$ T cells also seem to be involved in the pathogenic mechanisms of disease. Failure to generate non-antigen-specific $CD8^+$ T cells is associated with the development of relapses in patients with MS.[36] Expanded populations of $CD8^+CD28^{null}$ T cells have also been reported in ankylosing spondylitis patients, and seem to correlate with a more severe course of this autoimmune disease. Many of the effects of $CD8^+$ T cell replicative senescence seem to be deleterious, however, it is possible that these cells may have some beneficial effects in some contexts such as in organ transplantation.[37]

CD28^{null} T cells as a possible target for therapy

The pro-inflammatory cytokine IL-15, is essential for the development and activation of $CD8^+$ T cells and NK cells. IL-15 is known as a regulator of haematopoiesis, cell survival and proliferation of different cell types, and can be presented in two ways. Via the classic way, IL-15 is released from one cell and captured by another, but it can also be presented in trans, when it is still bound to the membrane of the first cell. Monocytes/macrophages have been reported as the main source of this cytokine.[38] Increased local expression of IL-15 has been suggested to contribute to the immunopathology of several human inflammatory diseases including rheumatoid arthritis. Previous studies have demonstrated that IL-15 is upregulated on PBMC of MS patients, but the precise contribution of IL-15 to MS immunopathogenesis has not been elucidated.[39] A critical cytokine in the regulation of NK cells is interleukin-12 (IL-12), a proinflammatory cytokine highly expressed in an inflammatory environment. Previous experiments found that IL-12 has an indirect effect on the de novo transcription and translation of the CD28 gene.[40] Previous studies also demonstrated that IFN-β, a cytokine

that both activates and induces proliferation in NK cells, decreases the inflammation[41,42] and suppresses the proliferation of $CD4^+CD28^{null}$ T cell populations in MS patients.[8]

In this study, we will investigate the role of both $CD4^+CD28^{null}$ and $CD8^+CD28^{null}$ T cells by performing a series of experiments on both cell populations. Through these experiments, we can gain a better understanding of how these cells work in the immune system, and how they are involved in the course and development of autoimmune diseases such as MS. By looking at the direct effects of previously described substances on $CD4^+CD28^{null}$ T cells, we can gain a better understanding of how these work and can eventually be implemented in therapeutic treatments. This can result in a more personalized therapy for patients with chronic diseases such as MS and RA. We will work with a subgroup of healthy subjects and MS patients in whom a $CD4^+CD28^{null}$ T cell population is present. Understanding the role of these cells in immunity will also lead to better therapeutic interventions in several autoimmune diseases.

Materials & Methods

Study Subjects

Peripheral blood samples were collected from a total of 27 healthy controls (HC) and 34 patients with MS. Clinical data of MS patients and HC are summarized in table 2. This study was approved by the local Medical Ethical Committee and informed consent was obtained from all study subjects.

Table 2: Study subjects

	Total MS group (n=34)	Relapsing remitting MS (n=14)	Secondary progressive MS (n=8)	Primary progressive MS (n=3)	Unknown type/treatment (n=9)	Healthy controls (n=27)
Age (range)*	49(33-65)	49(38-65)	52(36-64)	53(47-60)	44(33-63)	33(22-53)
Female/male	19/15	8/6	3/5	3/0	5/4	21/6
Disease duration (range)*	13(3-31)	15(3-31)	9.7(3-14)	19.5(19-20)	8(3-13)	/
EDSS (range) **	3.6(1.5-6.5)	3(1.5-6)	4.6(2-6.5)	2.5(2-3)	1.2(3-6)	/
Treated(IFN-β/Rebif/Other)	20(3/3/14)	12(2/2/8)	6(1/0/5)	2(1/0/1)	/	/

*In years **Expanded disability status scale (EDSS)

Cell preparation and culture

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by Histopaque (Sigma-Aldrich, St. Louis, MO, USA) density gradient separation. After several washing steps, these mononuclear cells were counted after Trypan blue (0.4%, Sigma-Aldrich) dead cell exclusion. The cells were cultured in RPMI-1640 medium, (GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with Penicilline and streptomycin (P/S, 0.5%, Invitrogen), Fetal bovine serum (FBS 10%, Hyclone Europe, Erembodegem, Belgium), Sodium pyruvate (1%, Invitrogen) and non-essential amino acids (1%, Invitrogen), and incubated at 37°C and 5% CO_2.

The wanted concentrations of IL-12 (Peprotech, Rocky Hill, NJ, USA) and IFN-β (PBL Interferon Source, Piscataway, NJ, USA) were added to 2×10^5 PBMC of 2 HC per well in a

96 well plate with U-shaped wells (NUNC, Roskilde, Denmark), and expression of CD4, CD28 and 7-AAD was monitored at different time points by using flow cytometry.

For the IL-15 assay, PBMC of 5 HC were placed in two 96 well plates at a concentration of 1.5×10^5 cells per well in culture medium. Culture medium with 15 ng/ml IL-15 (R&D systems, Minneapolis, MN, USA) was added to two-thirds of the cells, of which one third was supplemented with 5 µg/well anti-IL-15, and one third with 5 µg/well isotype control. Plain culture medium was added to the remaining cells. Cells were stained at day 0,1,3 and 5 for the NKG2D receptor, CD69, perforin, granzym B, IFN-γ, IL-17, CD4 and CD28. In two other plates, the same concentrations were used, but the cells were activated with 25 µl/ml PMA (Sigma), 10 µl/ml CaI (Sigma) and 1 µl/ml Golgi-stop (BD) before staining.

To assess the production of IL-15 by monocytes, PBMC of 2 HC and 1 MS patient were placed in a 24 well culture plate (Cellstar, Greiner Bio-One) at a concentration of 1.5×10^6 cells per well. After two hours of incubation, monocytes were fixed to the bottom and top cells were removed. These monocytes were subsequently activated with 100 ng/ml LPS (Calbiochem VWR international, Leuven, Belgium) and 10 ng/ml IFN-γ (BD) (10^5 monocytes per well in a 96 well plate). Expression of IL-15 and IL-15 receptor, together with the expression of 3 markers for activation: CD80, CD86 and MHCII was monitored before and after stimulation.

A CFSE assay was performed by adding an equal volume of 4 µM CFSE (Invitrogen) to PBMC dissolved at 20×10^6 cells per ml in PBS/0.1% BSA. After an incubation period of 7 minutes at 37°C, cells were dissolved in 300 µl culture medium. After a second incubation period of 15 minutes at 37°C, cells were dissolved in culture medium at 2×10^6 cells per ml and seeded in a 96 well plate at a concentration of 2×10^5 cells per well. 100 µl of culture medium containing following substances was added to every well: plain culture medium, culture medium supplemented with 4 µg/ml aCD3, culture medium supplemented with 4 U/ml IL-2, culture medium supplemented with 100 ng/ml IL-15, culture medium supplemented with 4 µg/ml aCD3 and 4 U/ml IL-2 or culture medium supplemented with 4 µg/ml aCD3 and 100 ng/ml IL-15. After 5 days of incubation at 37°C, cells were stained for CD4-PERC and CD28-PE. This was done for 5 HC.

For some experiments, cell lines were made. Here, PBMC were labeled with CD4-FITC and CD28-PE (both BD Biosciences, Franklin Lakes, NJ, USA) and FACS sorted with the FACSAriaII high speed cell sorter (BD Biosciences) in a 96 well plate into CD4$^+$CD28^{null} and

CD4$^+$CD28$^+$ T cells (5 cells per well). 1x10^5 irradiated feeder cells were added to the sorted cells. Cells were stimulates using Phytohemaglutinin (5 µg/ml, PHA, Sigma-Aldrich) and IL-2 (5 U/ml, Roche) Medium was refreshed twice a week with culture medium containing 5 U/ml IL-2. When pellets became too big, they were split up into 2 new wells on a new 96 well plate.

For the chemotaxis assay, an enrichment for CD8$^+$ T cells was performed on PBMC of 6 HC by following the protocol enclosed in the EasySep Human CD8 positive selection kit (StemCell Technologies, Grenoble, France).

ELISA

For optimization of the cytokine array conditions, supernatant from 1 HC was used in an enzyme-linked immunosorbent assay (ELISA) for IFN-γ production, following the protocol enclosed in the human IFN gamma ELISA Ready-SET-Go! Kit (eBioscience Inc, San Diego, CA, USA). The ELISA plate was coated with capture antibody in coating buffer and washed with wash buffer (1xPBS/0.05% Tween-20), which was repeated after blocking with Assay diluent. The supernatant of the sample was added to the wells (100 µl/well), and a standard was made by serial dilution of a 500 pg/ml standard solution in assay diluent. After an incubation period of 2 hours, detection antibody in assay diluent was used for detection, and avidin-horseradish peroxidase (HRP) in assay diluent for conjugation. Finally, a Tetramethylbenzidine (TMB) solution caused a color reaction and the plate was read at 450 nm on the BIO-RAD Benchmark Microplate Reader (Bio-Rad Laboratories N.V., Nazareth, Ghent)

Cytokine Array

Enriched CD4$^+$ T cells from 4 HC and 4 MS patients were obtained using the EasySep Human CD4 positive selection kit (StemCell Technologies, Grenoble, France) following the manufacturer's protocol. Briefly, PBMC were dissolved in EasySep buffer (2% FBS/1mM EDTA, Invitrogen, in phosphate buffered saline (PBS,

Figure 2: Schematic Drawing of EasySep® TAC Magnetic Labeling of Human Cells (Stemcell Technologies)

BioWittaker, Verviers, Belgium)) and labeled for CD4$^+$ T cells with 100 µl/ml EasySep Human CD4 Positive Selection Ab cocktail. After incubation, 50 µl/ml EasySep Magnetic
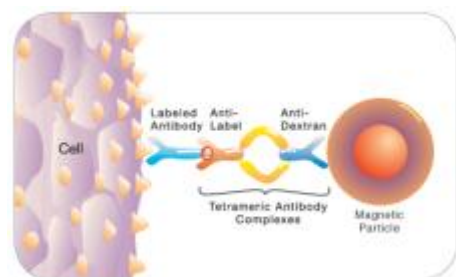
Nanoparticles were added, which attached to the labeled cells, making them magnetic. (figure 2) By Using the EasySep magnet, the labeled CD4$^+$ T cells were pulled towards the sides of the tube, while the other cells could be poured out. (figure 3) The CD4$^+$ T cells were subsequently labeled with CD28-PE (BD Biosciences) and a CD4 antibody clone that binds to a different CD4 epitope on CD4$^+$ T cells, CD4v4-FITC. (StemCell Technologies) In a 96 well plate, $1x10^5$ sorted cells were seeded, together with $1x10^5$ irradiated feeder cells per well. Half of the wells contained feeder cells pulsed with Myelin basic protein (MBP, Biomed, Diepenbeek, Belgium, made according to Deibler et al. 1972[42]). The other half contained unpulsed feeder cells and 4 µg/ml anti-CD3 (Clone 2G3, Biomed) was added to both conditions. Supernatant was removed after 5 days incubation for the cytokine array and cytrometric bead assay, and stored at -20°C.

The cytokine array was performed following the protocol enclosed in the Human Cytokine Array kit-Panel A (R&D Systems, Minneapolis, MN, USA). Briefly, the cytokine membranes were blocked for one hour in array buffer 4 while samples of CD4$^+$CD28^{null} T cells were incubated with Cytokine Array Panel A Detection Antibody Cocktail for one hour. The samples were subsequently added to the membranes and incubated overnight. After washing, Streptavidin-HRP

Figure 3: Schematic representation of the EasySep procedure. (StemCell Technologies)

was added to the membranes. After an incubation period of 30 minutes, membranes were washed and exposed to chemiluminescent reagents with the ECL Plus Western Blotting Detection System (GE Healthcare). The membranes were subsequentlyexposed to an X-ray film in an X-ray film cassette for 30 seconds up to 10 minutes. Finally, the films and dots were semi-quantitavely analyzed using ImageJ and GraphPad Prism 4 software.

Results from the cytokine array were validated using a cytometric bead assay, according to the protocol enclosed in the Human Soluble Protein Master Buffer Kit (BD). This allows to calculate the percentage of a given cytokine produced by the cells. Flex set standards were prepared by pooling standard spheres of all cytokines of interest in one tube, and lyophilizing them with assay diluent. A series dilution was made by transferring 500 µl of the standard into tubes containing 500 µl assay diluent. Solutions of capture beads with capture bead diluent and detection reagents with detection reagent diluent were made according to calculations given in the protocol. Subsequently, 50 µl of the mixed capture beads were added
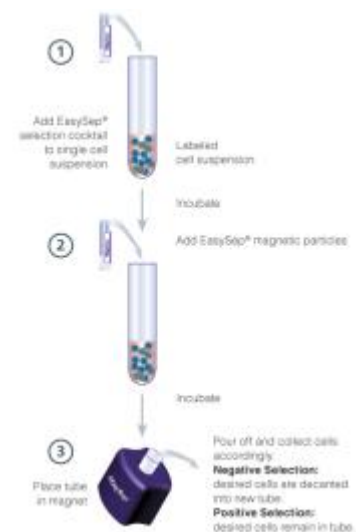
to every tube, with 50 µl standard or sample. After a one hour incubation period, 50 µl of the detection reagent mix was added to every assay tube. 1 ml of wash buffer was added after two hours of incubation, and after centrifugation, the supernatant was removed. Pellets were resuspended in wash buffer and samples were analyzed on the FACSAriaII high speed cell sorter.

Chemotaxis assay

$CD8^+$ T cells were isolated from PBMC of 6 HC with the EasySep procedure. A transwell system with a pore size of 5 µm (Corning, Lowell, MA, USA) was used for the migration assay. Fractalkine (Peprotech, Rocky Hill, NJ, USA) was added in the bottom compartment to chemotaxis buffer (0.5% BSA (US Biological, Swamscott, MA, USA) /RPMI-1640) in concentrations of 0; 0.1; 0.5; 1 and 10 ng/ml. In each insert, 5×10^5 $CD8^+$ T cells were added. The total number of cells which had migrated to the bottom compartment was counted after 4 hours of incubation at 37°C and 5% CO_2. The percentages of $CD8^+CD28^+$ and $CD8^+CD28^{null}$ T cells were then determined by using flow cytometry on the FACSCalibur. The chemotactic index of both cell populations was calculated by dividing the number of migrated cells with fractalkine by the number of migrated cells without fractalkine.

Flow cytometry

For monitoring the expression of CD4, CD28 and 7-AAD, cells were dissolved in FACS buffer (2% FBS/0.1% Sodium Azide in PBS) and labeled in a 96 well plate with V-shaped wells (Greiner BIO-One GmbH, Frickenhausen, Germany) with CD4-FITC, CD28-PE and 7-AAD (all BD Biosciences). After an incubating period in the dark for 15 minutes, they were scanned with the FACSCalibur flow cytometer (BD Biosciences). Subsequent analysis was done by using the BD CellQuest pro software. For monitoring the expression of CD4 or CD8 together with CD28 and CX_3CR1, CD4-PerCP or CD8-PerCP was combined with CD28-FITC (All BD) and CX_3CR1-PE (MBL International, Woburn, MA, USA). To assess the production of IL-15 on monocytes upon stimulation, cells were stained with HLA-DR,DP,DQ-FITC, CD14-PerCP, CD80-PE, CD86-FITC (all BD), IL-15-PE and IL-15receptor-PE (both R&D systems) before and after activation. For the IL-15 assay, cells were stained with combinations of CD314-PE, CD69-PE, Perforin-PE, GranzymeB-FITC, IL-17A-FITC, CD28-FITC, CD4-PerCP, CD28-APC, $\gamma2a/\gamma1-$control (all BD) and IFN-γ-PE (Immunotools, Friesoythe, Germany). For the degranulation assay, cells were stained with

combinations of CD107a-PerCP, CD4-PerCP, CD28-FITC, CD8-PerCP, CD28-PE, CD4-FITC, CD8-FITC and CD69-PE. (all BD) To assess the presence of the IL-15 receptor, cells were stained with IL15R-PE (R&D systems), CD4-PerCP and CD28-FITC. (All BD)

Immunohistochemistry

Frozen brain material from 6 MS patients, 1 Alzheimers Disease patient and 1 non-demented control (NDC) was cut with a Leica CM1900UV cryostat (Leica Microsystems, Wetzlar, Germany) into 10 μm sections. Slides were fixed in acetone for 10 minutes and dried. After washing in Tris-buffered saline/0.03% Triton X-100 (TBS-T, Sigma-Aldrich), they were blocked for 20 minutes with Protein Block Dakocytomation (Dako, Glostrup, Denmark) to prevent aspecific binding.

To identify $CD8^+CX_3CR1^+$ T cells in these tissues, slides were incubated overnight with a rat anti-human CX_3CR1 antibody (MBL International, dilution factors and incubation periods are presented in table 3) and washed again. Slides were subsequently incubated with an Alexa-555 labeled goat anti-rat IgG antibody (Invitrogen). After washing, slides were incubated overnight with a mouse anti-human CD8 antibody (AbCam, Cambridge, UK), followed by a donkey anti-mouse Alexa-488 antibody (Invitrogen).

To assess the presence of $CD4^+CD28^{null}$ T cells in the brains of healthy people or patients with Alzheimer's disease (AD), slides were stained with a rat anti-human CX_3CR1 antibody, an Alexa-555 labeled goat anti-rat IgG antibody and subsequently with an Alexa-488 labeled mouse anti CD4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Brain tissue of patients with AD and NDC patients were stained with CX_3CR1 in combination with a mouse anti GFAP antibody (Sigma-Aldrich), a mouse anti APC (CC-1) antibody (AbCam), a mouse anti NeuN antibody (Millipore, Billerica, MA, USA) or a rabit anti Iba-1 antibody (Wako Chemicals, Osaka, Japan) to determine the presence of fractalkine on astrocytes, oligodendrocytes, neurons and microglia, respectively, in the brains of these patients.

Table 3: Dilution factors and incubation periods of antibodies used for staining.

Primary	Secundary	Streptavidin
Rat anti-human CX$_3$CR1: 1/500 ON	Goat anti-rat Alexa 555: 1/400 1.5h	/
Mouse anti-human CD8: 1/200 ON	Donkey anti-mouse Alexa 488: 1/200 1.5h	/
CD4-Alexa 488: 1/200 1.5h	/	/
GFAP: 1/500 ON	Rabit anti-mouse biotin: 1/400 1h (Dako)	Strep Alexa 488: 1/2000 1.5h
NeuN: 1/100 ON	Rabit anti-mouse biotin: 1/400 1h	Strep Alexa 488: 1/2000 1.5h
CC-1: 1/500 ON	Rabit anti-mouse biotin: 1/400 1h	Strep Alexa 488: 1/2000 1.5h
Iba-1: 1/300 (4°C) ON	Swine anti-rabit biotin: 1/400 1h (Dako)	Strep Alexa 488: 1/2000 1.5h

Control staining was performed by using only the secondary antibodies. After incubation, slides were washed and a nuclear staining was performed using 4,6'-diamino-2-phenylindole (DAPI, Molecular Probes, Invitrogen) for 10 minutes. After blocking for 10 minutes with 0.1% Sudan Black in 70% ethanol, slides were dipwashed in 70% ethanol and TBS. A final washing step was performed with TBS and water and cover slides were subsequently applied with fluorescent mounting medium (Dako). After drying, slides were analyzed on a Nikon eclipse 80i microscope using NIS Elements BR 3.10 software (Both Nikon, Tokyo, Japan).

Degranulation assay

To assess the capacity of CD4$^+$CD28^{null} and CD8$^+$CD28null T cells to expel cytotoxic granules in response to stimulation with CMV, a degranulation assay was set up. PBMC of 3 HC were seeded in a 96 well plate at a concentration of $2x10^5$ cells per well. . Cells were either stimulated with 500 ng/ml CMV pp65 (Peptivator, Miltenyi Biotec), or left unstimulated. 2 µl anti-CD107a-PerCP and 1 µl Golgi-Plug (diluted 1/5 in culture medium, BD) were added during the last four hours of culture before analyzing samples on a FACSCalibur (BD). Cells were analyzed after 4 and 24 hours of stimulation.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 4.03. Two-tailed unpaired Student's T test (with Welch's correction if necessary) was performed to compare two groups. A P value of less than 0.05 was considered significant. A P value between 0.1 and 0.05 was considered a trend.

Results

1. *Frequency of $CD4^+CD28^{null}$ T cells and making of Cell lines*
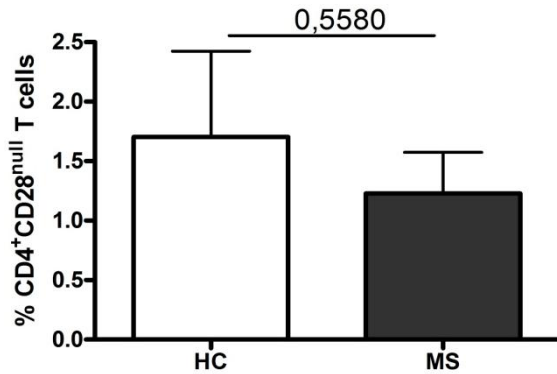

Figure 4: The frequency of senescent CD4+ T cells in the peripheral blood of 20 HC and 34 MS patients was determined by flow cytometry. There is no significant difference in frequency between HC and MS patients.

The frequency of $CD4^+CD28^{null}$ T cells in peripheral blood of 34 MS patients and 20 HC was determined using flow cytometry, and ranged between 0 and 2.5%. (figure 4) Because $CD4^+CD28^{null}$ T cells are only present in the blood in small percentages, we attempted to make cell lines to reach a greater amount of cells to work with. Cells did not grow as expected, and after several failed attempts to make a cell line of $CD4^+CD28^{null}$ T cells, these experiments were replaced by others. The reason why cells did not grow, is unclear. According to protocol, cells should grow after two weeks while replacing the medium twice a week. After over four weeks of incubation, cells still did not multiply enough, and started to die. After three attempts, this experiment was abandoned and replaced by alternative experiments on $CD28^{null}$ T cells.

2. *Cytokine production by $CD4^+CD28^{null}$ T cells*

$CD4^+CD28^{null}$ T cells are known to produce IFN-γ [7], but production of other cytokines and chemokines has not yet been fully examined. To assess the range of cytokines that are produced by $CD4^+CD28^{null}$ T cells, a cytokine array was performed. This array allowed the simultaneous detection of 36 different cytokines, chemokines, and acute phase proteins in a single sample.

2.1 Optimization of stimulation conditions

To determine the optimal conditions for the cytokine array, an ELISA for the production of IFN-γ was performed. IFN-γ was chosen because it is known to be produced by $CD4^+CD28^{null}$ T cells [7]. First, different culture plates and cell concentrations were tested, and the amount of IFN-γ in the supernatant of the culture was analyzed after stimulation with anti-CD3. In a 48 well plate, $2x10^5$ $CD4^+CD28^{null}$ T cells were combined with the same amount of feeder cells,

and, in a 96 well plate, the same was done for 1×10^5 CD4$^+$CD28^{null} T cells and feeder cells. Compared to the IFN-γ concentration in the supernatant of the 48 well plate (15.74 pg/ml), the concentration in the 96 well plate was higher (32.35 pg/ml), but still not optimal for the cytokine array. To increase the IFN-γ production, IL-2 was added to the culture. This increased the concentration of IFN-γ in the supernatant of the 96 well plate to 234.4 pg/ml. Therefore, this setup was chosen for the cytokine array.

2.2 Cytokine array

Figure 5 shows the four cytokine membranes representing the production of different cytokines in the four conditions used. CD4$^+$CD28^{null} T cells of 4 HC and 4 MS patients were either stimulated with MBP-pulsed feeder cells or anti-CD3. The membranes show distinct differences in cytokine production between CD4$^+$CD28^{null} T cells of HC and patients suffering from MS, and between CD4$^+$CD28^{null} T cells stimulated by MPB or anti-CD3.
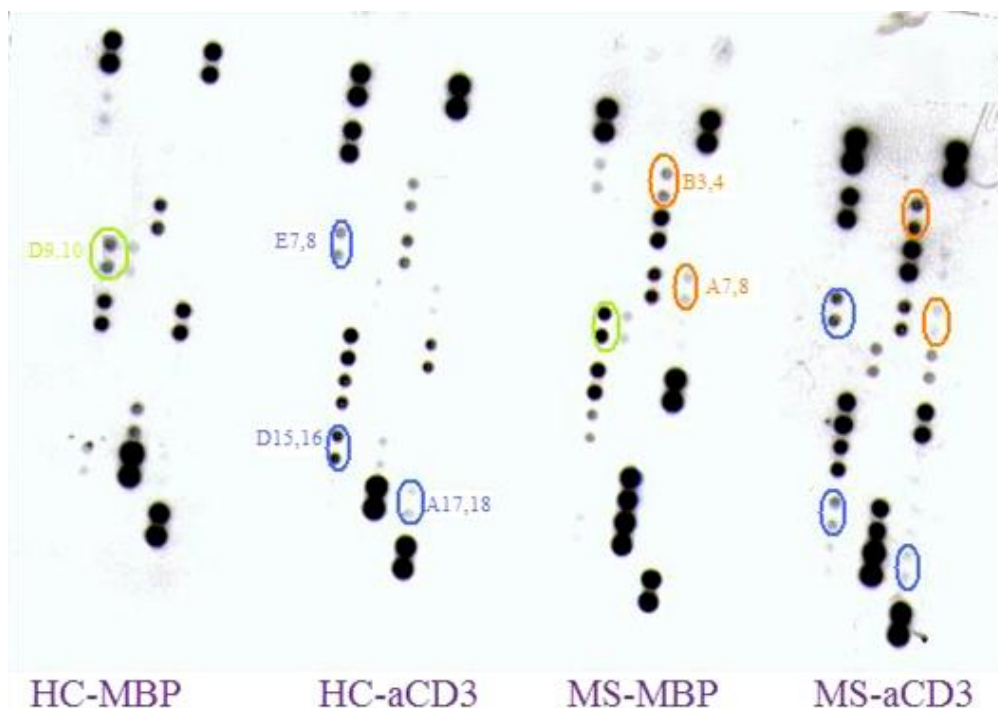

Figure 5 : Photograph of cytokine membranes. Four conditions were plotted, CD4$^+$CD28^{null} T cells of HC and MS patients stimulated by MBP or anti-CD3. The dots encircled in red represent the cytokines differentially expressed by CD4$^+$CD28^{null} T cells of MS patients, the dots encircled in green represent those for MBP stimulated cells and those in blue for anti-CD3 stimulated cells.

The dots encircled in red represent the cytokines that are only produced by CD4$^+$CD28^{null} T cells of MS patients. These cytokines were identified as IL-1α (B3,4) and G-CSF (A7,8). The dots encircled in blue show cytokines which were only produced by cells stimulated with anti-

28

CD3. CD4$^+$CD28^{null} T cells produce TNF-α (E7,8), CCL4 (D15,16) and IFN-γ (A17,18) after stimulation with anti-CD3. Dots D9 and D10, encircled in green, represent CCL2, which was only produced by cells stimulated with MBP. Appendix B shows the full results of the differentially produced cytokines. Only the cytokines visible on the membranes were plotted. By comparing the coordinates of the dots with those of the cytokines listed in the manufactures guide, a cytokine could by placed with every dot on the membrane. Relative production levels were calculated by comparing the dots of the cytokines with those of the positive controls.

Results of 4 major cytokines detected in the cytokine array were validated with a cytometric bead assay. Figure 6a shows the detection of cytokines IL-1α, IL-1β, IL-6 and MCP-1 (CCL2) by the cytokine array. All of these cytokines were also detected by the cytometric bead assay, represented in figure 6b. This assay has one major advantage over the cytokine array, which is quantification. As shown in figure 6b, there was a large inter-donor variability. However, this assay clearly showed that IL-1α, IL-1β and IL-6 were mainly produced by cells of MS patients, while MCP-1 production mostly occurred by cells stimulated by MBP.

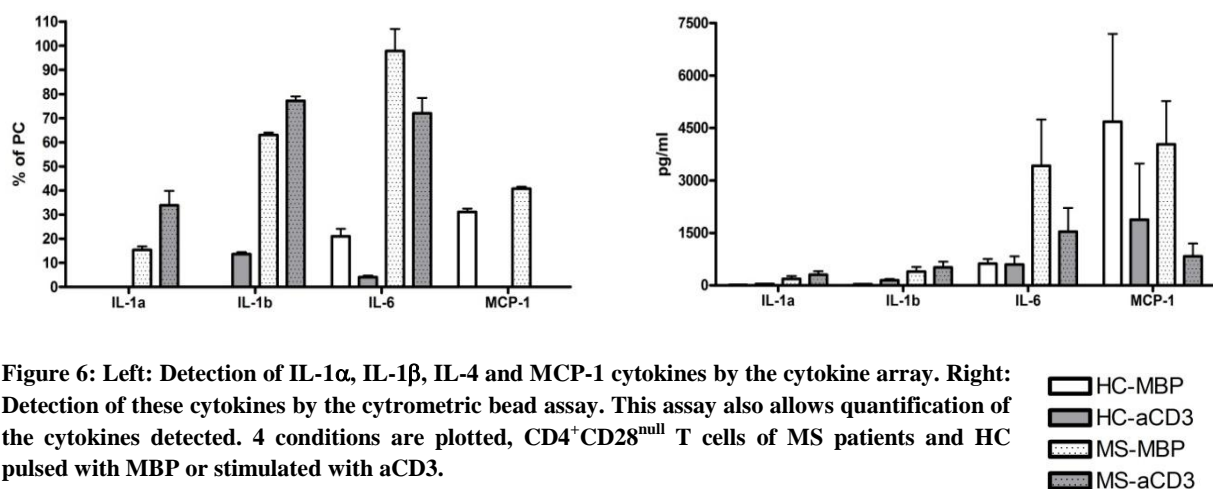**Figure 6: Left: Detection of IL-1α, IL-1β, IL-4 and MCP-1 cytokines by the cytokine array. Right: Detection of these cytokines by the cytrometric bead assay. This assay also allows quantification of the cytokines detected. 4 conditions are plotted, CD4$^+$CD28^{null} T cells of MS patients and HC pulsed with MBP or stimulated with aCD3.**

HC-MBP
HC-aCD3
MS-MBP
MS-aCD3

3. *Effect of IL-12/IFN-β on the expression of CD28*

Previous research indicated that IL-12 has an indirect effect on the de novo transcription and translation of the CD28 gene.[40] Other studies also demonstrated that IFN-β decreases the inflammation[41,42] and suppresses the proliferation of CD4$^+$CD28^{null} T cell populations in MS patients.[8] To confirm these findings, we examined the expression of CD28 after adding IL-12 and IFN-β to PBMC in different concentrations. For IL-12, a dose-response experiment was set up, using a range from 0.1 to 100 ng/ml. Results showed that the highest concentration of

IL-12 resulted in a decline in total expression of CD28 within the CD4 population, represented by the mean fluorescence intensity (MFI). For the lowest concentrations (0.1 and 1 ng/ml), CD28 MFI increased with a peak at day 5. When gated on $CD4^+CD28^{null}$ T cells, CD28 expression did not change significantly.

For IFN-β, a similar experiment was conducted, using a concentration range from 1 to 1000 U/ml. The total CD28 MFI within the $CD4^+$ population decreased in all concentrations when IFN-β was added, the MFI of $CD4^+CD28^{null}$ T cells did not change. (Full results, see appendix A)

4. *Effect of IL-15 on the proliferation and phenotype of $CD4^+CD28^{null}$ T cells*

Increased expression of IL-15 has been suggested to contribute to the immunopathology of several inflammatory diseases, although the precise role of IL-15 has not yet been elucidated.[39] NK cells express the IL-15 receptor complex, and IL-15 secretion by macrophages during inflammation results in an increased proliferation, survival, cytolytic activity and cytokine secretion by these cells[43]. In vitro studies show that IL-15 may be an important regulator of IFN-γ production by NK cells[44]. $CD4^+CD28^{null}$ T cells show several similarities with NK cells, but the effects of IL-15 on this cell population have not been studied so far. It is also not known whether soluble or membrane bound IL-15 have different effects on the function of $CD4^+CD28^{null}$ T cells.

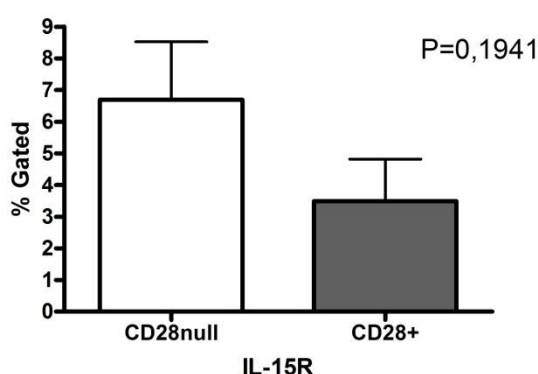### *4.1 Expression of the IL-15 receptor by $CD4^+CD28^{null}$ T cells*

Figure 7: Expression of IL-15R in the $CD4^+$ T cell population.

In order to respond to IL-15, $CD4^+CD28^{null}$ T cells must also express the IL-15 receptor (IL-15R). The presence of IL-15R on both $CD4^+CD28^+$ and $CD4^+CD28^{null}$ T cells was examined using flow cytometry. Results in figure 7 show that both cell populations express IL-15R, with the expression on $CD28^{null}$ T cells being twice as high compared to $CD28^+$ T cells. However, this difference was not statistically significant.

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4.2 Optimization of IL-15 concentration

An optimization was performed to determine the most optimal concentration of IL-15 for further experiments. A dose response experiment was set up using a concentration range from 5 to 100 ng/ml. The expression of NKG2D was monitored, because this has been shown to be influenced by IL-15. The results shown in figure 8 indicate that a concentration of 50 ng/ml IL-15 gives the best result for NKG2D expression. Here, the NKG2D incline is the most stabile.
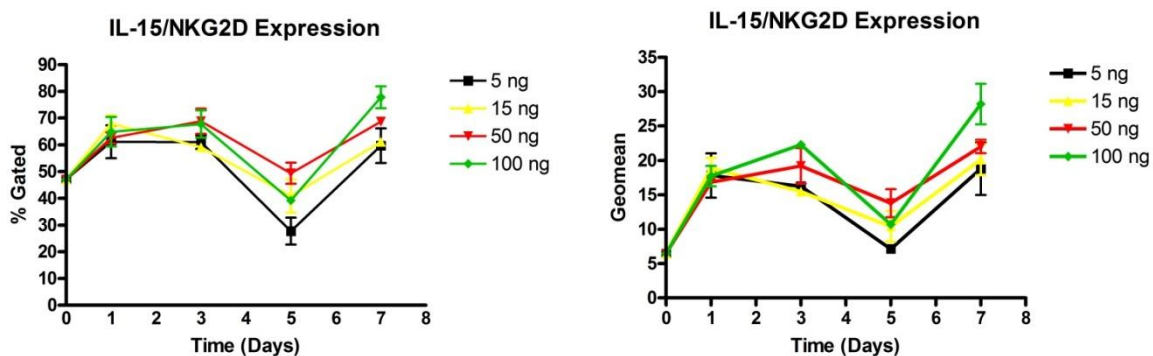

Figure 8 : Optimization of IL-15 through NKG2D expression. The black line shows the expression of NKG2D over a period of 7 days when 5 ng/ml IL-15 is added. The yellow, red and green lines represent the same for 15, 50 and 100 ng/ ml IL-15 respectively. The graph on the left shows the percentage cells gated that express NKG2D, the graph on the right show the mean expression of NKG2D in the entire cell population.

4.3 Effect of IL-15 on proliferation of $CD4^+CD28^{null}$ T cells

Little is known about the proliferation of $CD4^+CD28^{null}$ T cells, while it was shown that proliferation of NK cells increased when stimulated with IL-15. To investigate whether this effect could also be found for $CD4^+CD28^{null}$ T cells, a CFSE assay was conducted.

Figure 9 a and b show the percentages of divided $CD4^+CD28^+$ and $CD4^+CD28^{null}$ T cells after a 5 day incubation of PBMC (HC) in 6 different culture medium conditions. Remarkably, $CD4^+CD28^{null}$ T cells show an overall higher proliferative capacity, compared to $CD4^+CD28^+$ T cells. When both cell populations were stimulated with aCD3, a significant proliferation occurred ($P<0.01$). When aCD3 was supplemented with IL-2, the percentage of dividing cells was similar to aCD3 alone. However, when IL-15 was used in combination with aCD3, there was a significantly higher proliferation rate of both cell populations, compared to aCD3 alone ($P<0.05$). IL-15 alone was also able to induce proliferation in both cell populations, although this only reached statistical significance for $CD4^+CD28^{null}$ T cells ($P<0.05$).
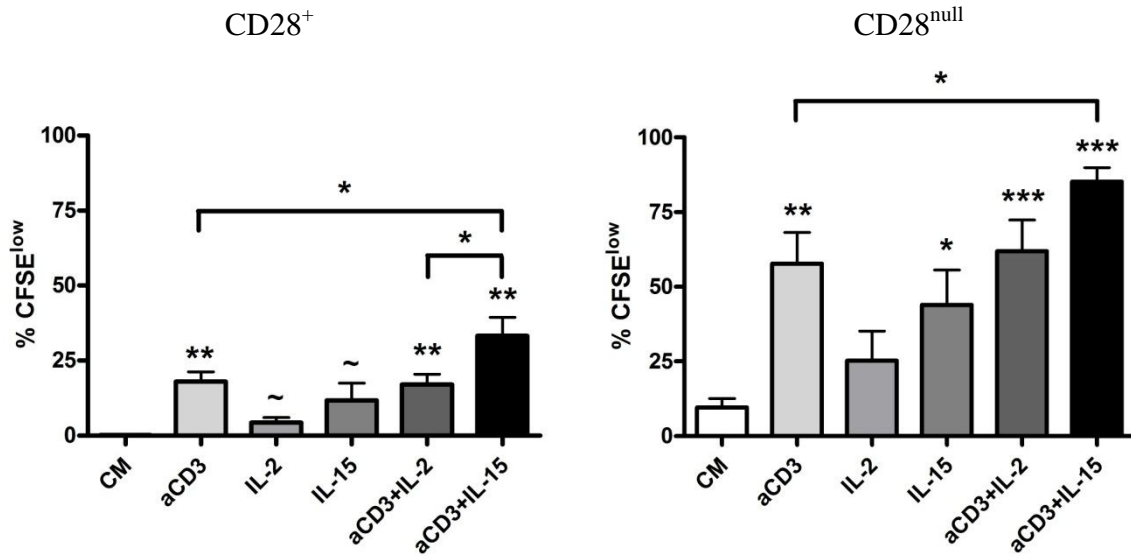
CD28$^+$ CD28^{null}

Figure 9: Percentage of divided CD4$^+$CD28$^+$ T cells (a) and CD4$^+$CD28^{null} T cells (b) after incubation with CFSE in different culture medium conditions: plain culture medium, culture medium supplemented with aCD3, IL-2 or IL-15 and culture medium supplemented with aCD3 and IL-2 or aCD3 and IL-15. Significant differences between the percentage gated cells in plain culture medium and other culture medium conditions are represented with * (P-value <0.05), ** (P<0.01), * (P<0.001) and trends with ~ (P-value between 0.1 and 0.05).**

In the control condition (figure 10), only a peak of undivided cells can be seen, however in the stimulated conditions, (figure 11) smaller peaks of cell division are apparent. When we gated on the CFSE^{hi} cell population in the aCD3 + IL-15 condition, we see that they exist of 95.39% CD28$^+$ T cells, and 4.61% CD28^{null} T cells, which is comparable to the percentages seen ex vivo in the peripheral blood (figure 12a). However, when gated on the CFSE^{lo} population, this balance has shifted to 70.64% CD28$^+$ T cells and 26.36% CD28^{null} T cells, indicating a specific effect on the proliferation of CD28null T cells (figure 12b)
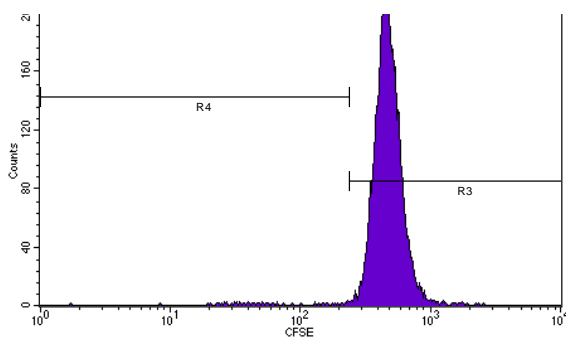

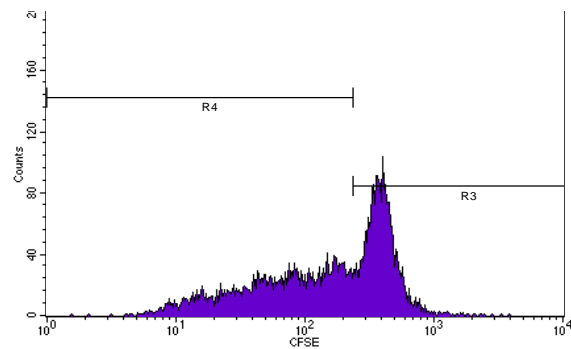**Figure 10: Percentage of CFSE positive cells in CM.**   **Figure 11: Percentage of CFSE positive cells after stimulation.**
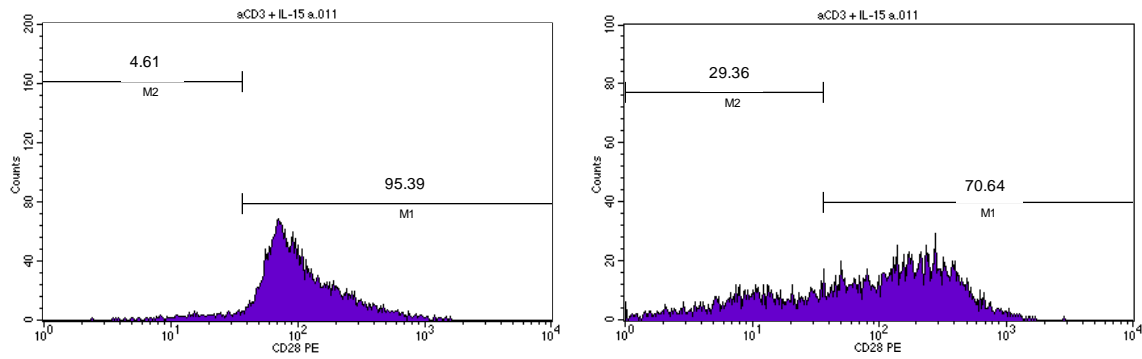
Figure 12: Percentage CD28^{null} and CD28$^+$ T cells in the CFSEhi (a) and CFSElo (b) cell populations after stimulation with aCD3 and IL-15.

4.4 Effect of IL-15 on cytokine production

IL-15 secretion by macrophages is known to increase cytokine production[43], and regulate IFN-γ production by NK cells. Because CD4$^+$CD28^{null} T cells are very similar to NK cells, we examined the effects of IL-15 on cytokine production by these cells. Results, represented in figure 13, confirm that there was a significant increase ($P<0.01$) in IFN-γ production by CD4$^+$CD28^{null} T cells when culture medium is supplemented with IL-15, as opposed to the production of IFN-γ in

Figure 13: Effect of IL-15 on cytokine production of CD4$^+$CD28^{null} T cells. There is a significant increase in IFN-γ and IL-17 when IL-15 is added to the culture medium, with and without activation of the cells with PMA and CaI.

plain culture medium. The same is true for the production of IL-17 ($P<0.001$). When cells were first activated with PMA and CaI, there was also a significant increase in production of IFN-γ and IL-17 in the culture medium containing IL-15 compared to that in plain culture medium.
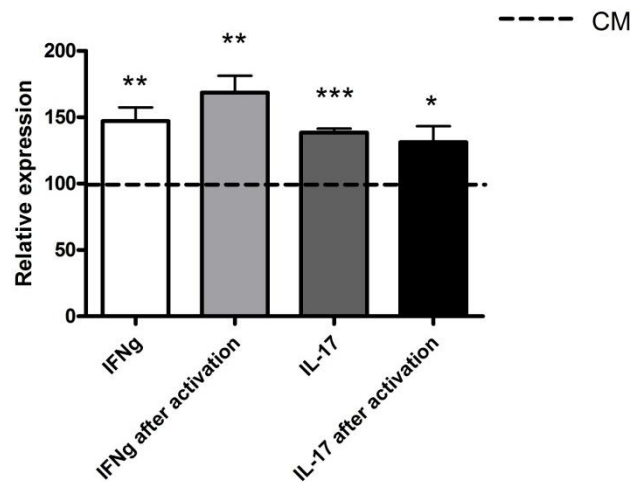
4.5 Effect of IL-15 on cytotoxicity

CD4$^+$CD28^{null} T cells show several similarities with NK cells, including proinflammatory functions and expression of cytolytic molecules such as granzyme B and perforin. These T cells are considered cytotoxic, and are thought to contribute to disease related immune
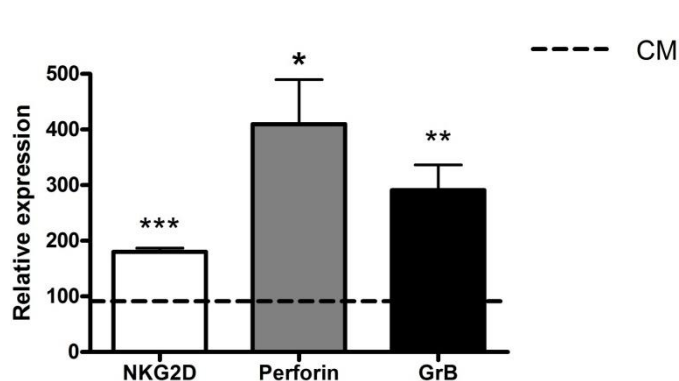
Figure 14: Effect of IL-15 on the cytotoxicity of CD4+CD28null T cells. There is a significant increase in production of perforin, NKG2D and granzyme B upon stimulation with IL-15.

dysfunctions[24,27]. IL-15 influences the cytokine production of NK cells, and as confirmed in the previous result, also of CD4+CD28null T cells. We therefore investigated the effect of IL-15 on the cytotoxicity of CD4+CD28null T cells. Intracellular flow cytometric analysis shows that there is a significant increase ($P < 0.001$) in the expression of the activating NK receptor NKG2D after IL-15 stimulation. Moreover, the production of perforin ($P < 0.05$) and granzyme B ($P < 0.01$) by CD4+CD28null T cells was significantly upregulated after stimulation with IL-15 (figure 14).

4.6 Expression of IL-15 on monocytes

IL-15 can be delivered to the cells in two ways: when it is released from one cell and captured by another, or when it is still bound to the membrane of the first cell. Monocytes/macrophages
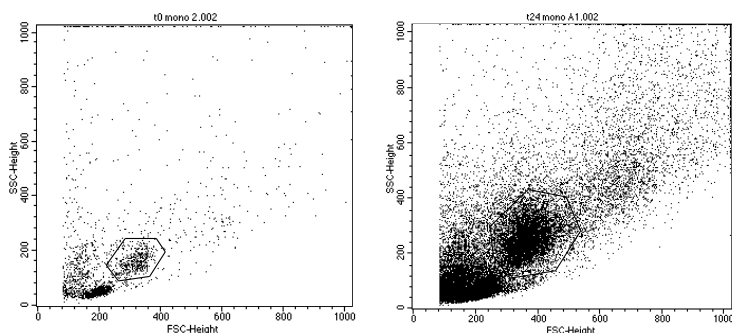

Figure 15: Presence of monocytes in the sample before (a) and after (b) stimulation. There is a strong increase after 24 hours.

have been reported as the main source of this cytokine.[38] Here, the expression of IL-15 on LPS-stimulated monocytes was examined. Using the activation markers CD80 and CD86, we found that LPS induced a significant activation and proliferation of monocytes in culture (figure 15). Moreover, an increase in the expression of membrane-bound IL-15 could be seen (figure 16). In conclusion, LPS-stimulated monocytes can be used as a potent source of membrane-bound IL-15.
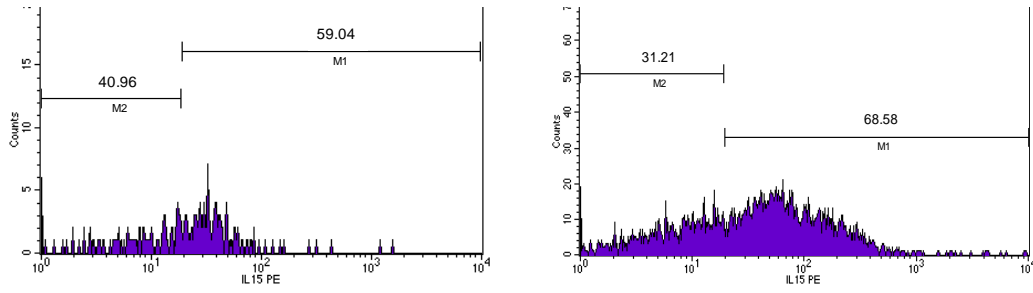
Figure 16: Expression of IL-15 on monocytes before and after stimulation. There is a slight increase after 24 hours incubation.

5. *Cytotoxicity of CD4$^+$CD28^{null} T cells after CMV stimulation*

Cytomegalovirus (CMV) is a β-herpesvirus that continuously activates the immune system. Maintaining protective immunity against CMV is essential, but may have a significant impact on the overall adaptive immunity due to the repeated stimulation of T cells.[4,5] CD4$^+$CD28^{null} T cells exist only in some individuals, and the expansion of this cell population is mainly correlated with CMV infection. In this experiment, we examined the the cytotoxicity of CD4$^+$CD28^{null} T cells in response to CMV by looking at their capacity for degranulation.
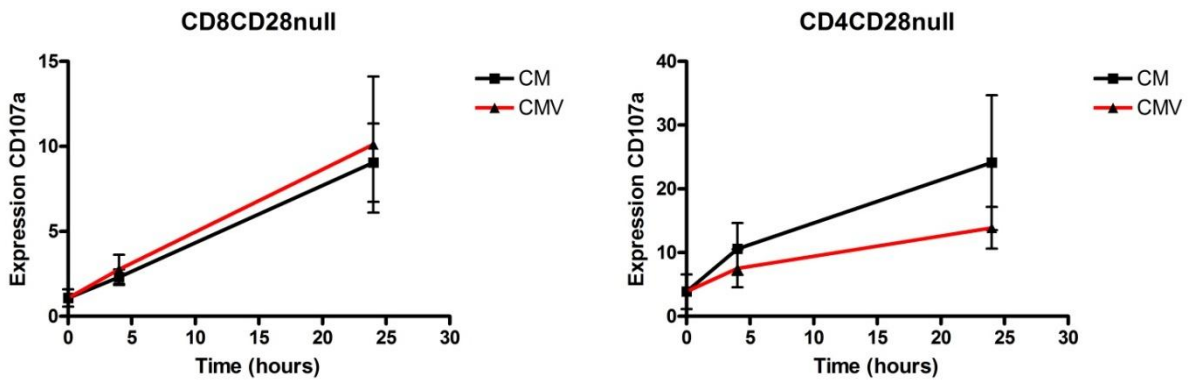

Figure 17: Degranulation of CD8$^+$CD28^{null} and CD4$^+$CD28^{null} T cells after 24 hours in plain CM (black line) and after stimulation with CMV (red line).

Results in figure 17 show that there is no statistical significant difference between the degranulation of both CD8$^+$CD28^{null} and CD4$^+$CD28^{null} T cells in plain CM and after stimulation with CMV.

6. *Presence of CD4$^+$CD28^{null} T cells in brain tissue*

The presence of CD4$^+$CD28^{null} T cells was already shown in brain tissue of patients suffering from MS, particularly in active lesions[34]. For this reason, stainings were performed on brain tissue of HC and AD to check the presence of CD4$^+$CD28^{null} T cells in these control tissues.

This was done by performing a staining for CD4 in combination with CX_3CR1, which has been shown to be expressed on $CD4^+CD28^{null}$ T cells[34].
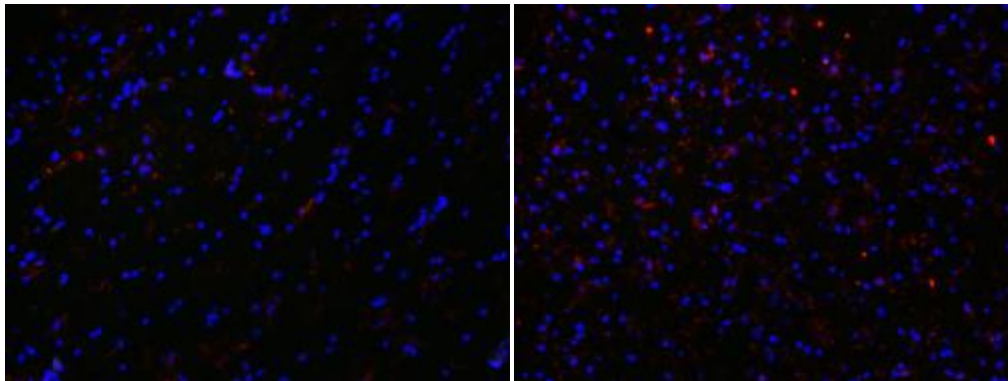

Figure 18 and 19 : Double staining for $CD4^+CD28^{null}$ T cells and CX_3CR1 in brain material of patient with Alzheimer's Disease (left) and HC (right). CX3CR1 is stained in red, $CD4^+CD28^{null}$ T cells in green.

Figure 18 and 19 show a double staining for $CD4^+CX_3CR1$ in frozen brain material of respectively a patient suffering from Alzheimer's disease and a non-demented control (NDC). CX_3CR1 is colored in red, $CD4^+CD28^{null}$ T cells in green. There were no $CD4^+$ cells present in the brains of Alzheimer's disease patient, nor in those of the NDC. However, the fractalkine receptor was expressed in both brains.

7. _Characteristics of $CD8^+CD28^{null}$ T cells_

Contrary to $CD4^+CD28^+$ T cells, $CD8^+CD28^+$ T cells are already considered cytolytic. While the presence of $CD4^+CD28^{null}$ T cells is considered detrimental for the host, the presence of $CD8^+CD28^{null}$ T cells has been shown to also have some beneficial effects e.a. in organ transplantation due to their immunosuppressive action.

7.1 Frequency of $CD8^+CD28^{null}$ T cells

Figure 20: Positive correlation between $CD4^+CD28^{null}$ and $CD8^+CD28^{null}$ T cells in HC. (n=16)

As shown in figure 20, there is a strong positive correlation between the presence of $CD4^+CD28^{null}$ T cells, and $CD8^+CD28^{null}$ T cells in the peripheral blood. The more $CD4^+CD28^{null}$ T cells are present, the more $CD8^+CD28^{null}$ T cells seem to appear.
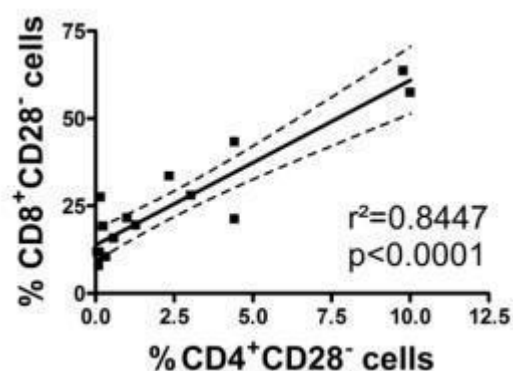
36

7.2 Expression of CX_3CR1

In a previous study, CX_3CR1 was found to be expressed on the majority of $CD4^+CD28^{null}$ T cells while almost being absent on $CD4^+CD28^+$ T cells.[34] For this reason, we studied the expression of CX_3CR1 on $CD8^+$ T cells. Here, expression of the fractalkine receptor CX_3CR1 is correlated with loss of CD28 and CMV infection, as shown on figure 21. There is a significantly higher expression of CX_3CR1 on $CD8^+CD28^{null}$ T cells as compared to $CD8^+CD28^+$ T cells, in both CMV positive and negative blood samples. Overall, there is also a significantly higher expression of CX_3CR1 on the total $CD8^+$ T cell population in CMV infected people. However, the CX_3CR1 expression is not as strictly correlated to $CD28^{null}$ cells, as was the case for $CD4^+$ cells.
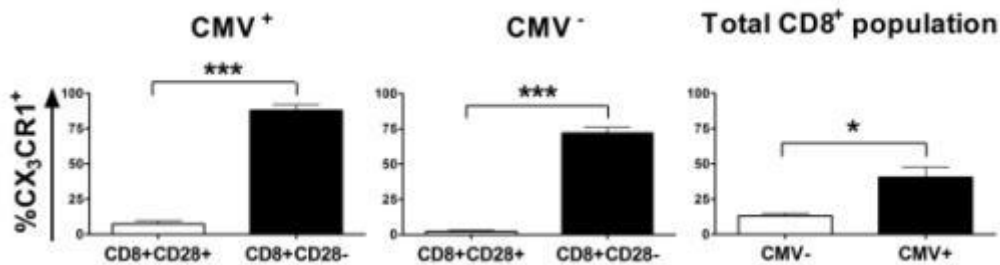

Figure 21: Expression CX_3CR1 on CD8 T cells and correlation with CMV infection in HC. CMV^-: Significant more expression of CX_3CR1 in the $CD8^+CD28^{null}$ T cell population. CMV^+: Significant more expression of CX_3CR1 in the $CD8^+CD28^{null}$ T cell population, and more expression of CX_3CR1 then in CMV^- samples. Total $CD8^+$ population: Overall significant more expression of the fractalkine receptor in CMV positive people.

7.3 Chemotactic capacity of $CD8^+CD28^{null}$ T cells
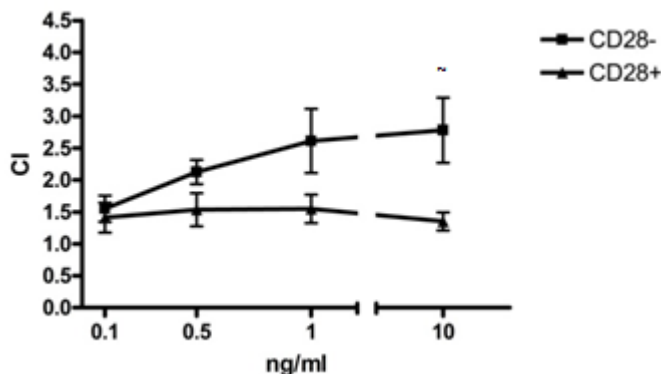
Figure 22: Chemotactic index of CD8 T cells for HC. A value above 1 means the cells are more able to migrate to medium containing fractalkine as compared to plain culture medium. There is no significant difference in migration between the two conditions, but there is a trend (~ = P value between 0.1 and 0.05) for the highest concentration of fractalkine. (10 ng/ml)

The ability of a cell to migrate towards a certain substance is represented by the chemotactic index. This is the difference in migration toward culture medium and culture medium containing that substance. The chemotactic index of $CD4^+CD28^{null}$ T cells for fractalkine, which is upregulated in the brain of MS patients, has already been situated around a value of $4^{[34]}$. Figure 22 shows

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the chemotactic index for $CD8^+CD28^+$ and $CD8^+CD28^{null}$ T cells towards fractalkine. When the chemotactic index is higher than one, there is a greater migration toward fractalkine. Experiments showed a difference in migration between $CD8^+CD28^+$, situated around 1.5; and $CD8^+CD28^{null}$ T cells, situated around 2.5. This difference was not significant. There is, however, a trend noticeable for the highest concentration of fractalkine.

7.4 Presence of $CD8^+CD28^{null}$ T cells in brain tissue

The presence of $CD4^+CD28^{null}$ T cells in the brain of MS patients has already been established[34]. To assess the presence of $CD8^+CD28^{null}$ T cells in the brain, a double staining for CD8 and CX_3CR1 was performed on active lesion brain tissue of MS patients. Results are shown in figure 23, CD8 was stained in green (a) and CX_3CR1 in red (b). The same was done for chronic active and chronic inactive brain tissue of MS patients (pictures not shown). There were no $CD8^+$ T cells present in these tissues which were also positive for the fractalkine receptor CX_3CR1.
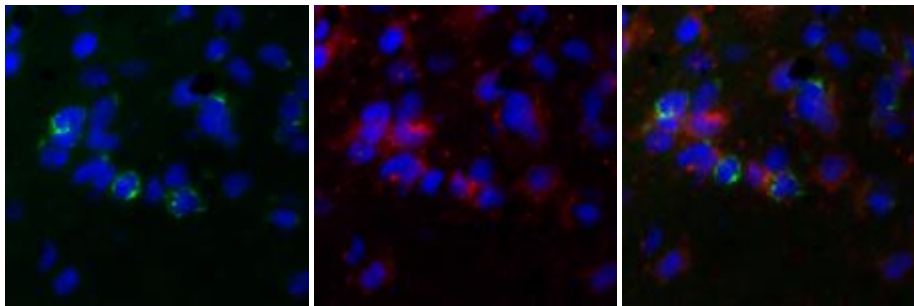
Figure 23 : Active CD8 CX_3CR1: a) Staining for CD8 (green); b) Staining for CX_3CR1 (red); c) Double staining for both CD8 and CD_3CR1

A double staining for CD8 and CX_3CR1 was also performed on control brain tissue of NDC and normal appearing white matter (NAWM). Results are shown in figures 24 and 25. There were no $CD8^+$ T cells present in the brains of NDC and NAWM.

Figure 24: Double staining for CD8 (green) and CX3CR1 (red) in brain tissue of NDC. There are no CD8 T cells present.

Figure 25: Double staining for CD8 (green) and CX_3CR1 (red) in brain tissue of NAWM. There are no CD8 T cells present.

Control staining was performed by omitting the primary antibodies. As shown in pictures 26 and 27, there is little to no background staining for these secondary antibodies.

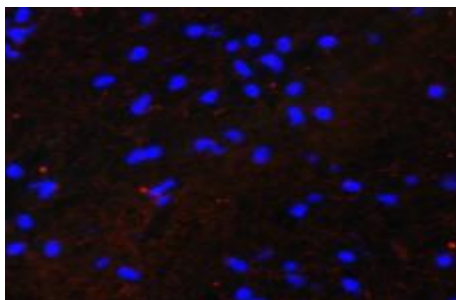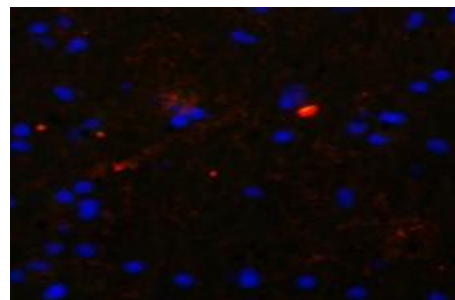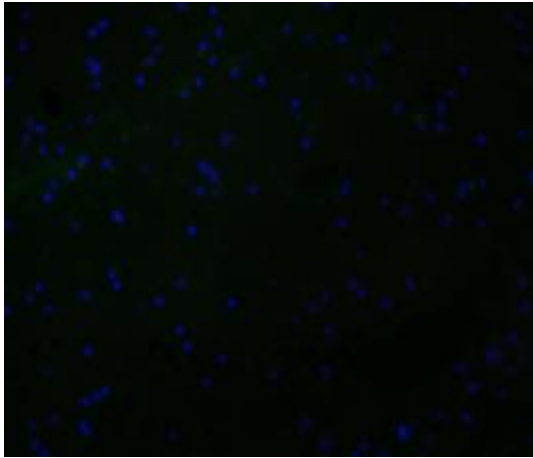**Figure 26: Control staining for DaM Alexa 488. Little to no background staining.**
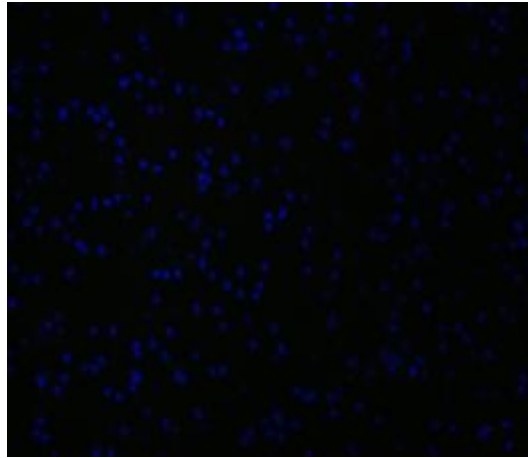
Figure 27: Control staining for GaR Alexa 555. Little to no background staining.

8. _Expression of CX$_3$CR1 on brain cells_

In vitro analyses on brain cell cultures have indicated that fractalkine is mainly expressed on neurons and astrocytes, while its receptor is present on neurons and microglia.[48] It has also been demonstrated that neurons, microglia and oligodendrocytes express CX$_3$CR1 in MS lesions.[34] We studied the expression of fractalkine on neurons, microglia, oligodendrocytes and astrocytes in the brains of AD patients and NDC.

For the identification of CX$_3$CR1$^+$ cells in brain tissue of an AD patient, double stainings were performed for CX$_3$CR1 in combination with Iba1 (microglia), CC-1 (oligodendrocytes), GFAP (astrocytes) and NeuN (neurons), represented by figure 28. There is no overlap in stainings for astrocytes and neurons, meaning that only microglia and oligodendrocytes express the fractalkine receptor in the brains of AD patients.

Figure 28: Staining for CX₃CR1⁺ cells (red) in brain tissue of an AD patient. Microglia (Iba1) and oligodendrocytes (CC-1) but not astrocytes (GFAP) and neurons (NeuN) express CX₃CR1.

For the identification of CX_3CR1^+ cells in brain tissue of NDC, the same double stainings were performed. Results are shown in figure 29. The fractalkine receptor is again only expressed by microglia and oligodendrocytes in the brains of NDC.

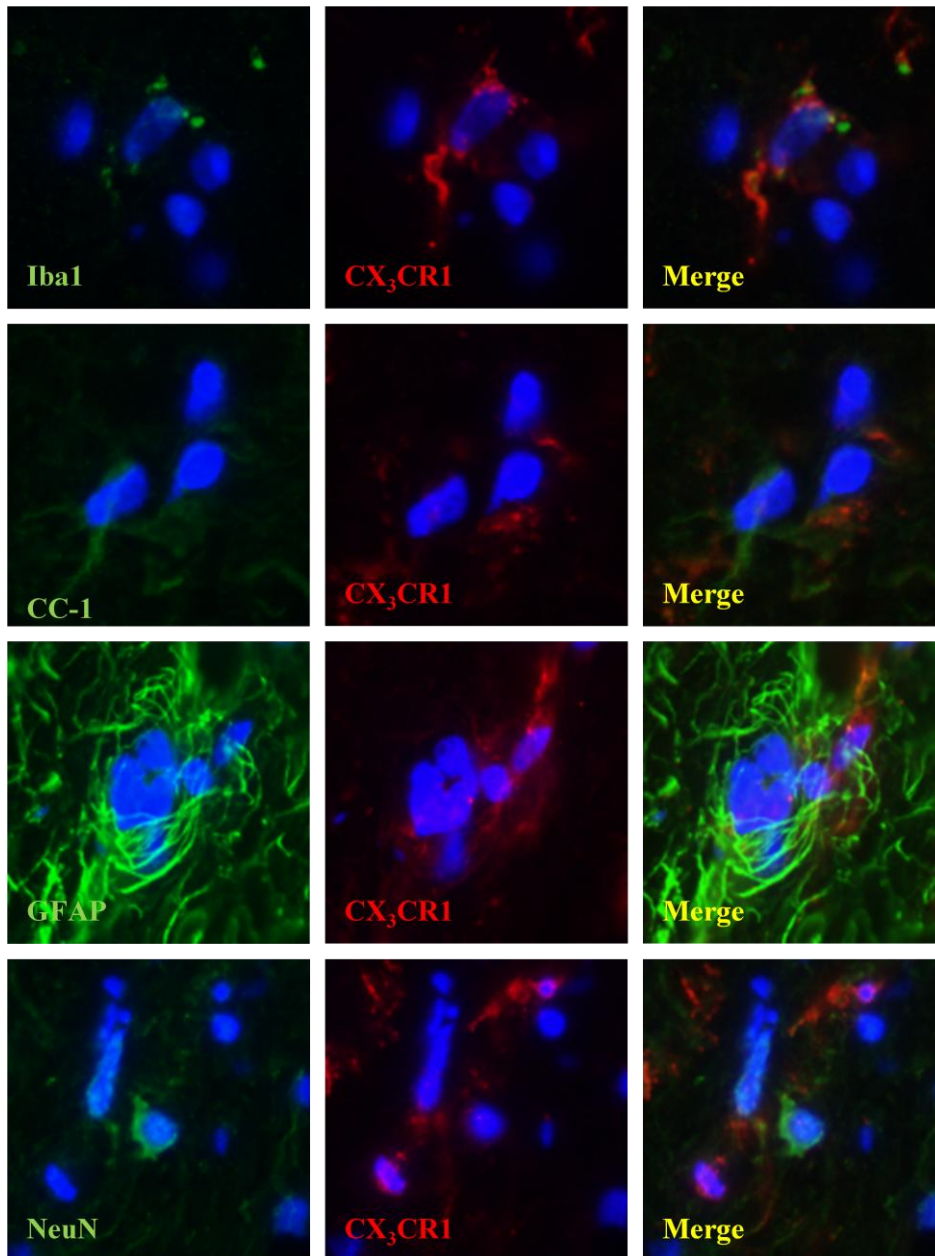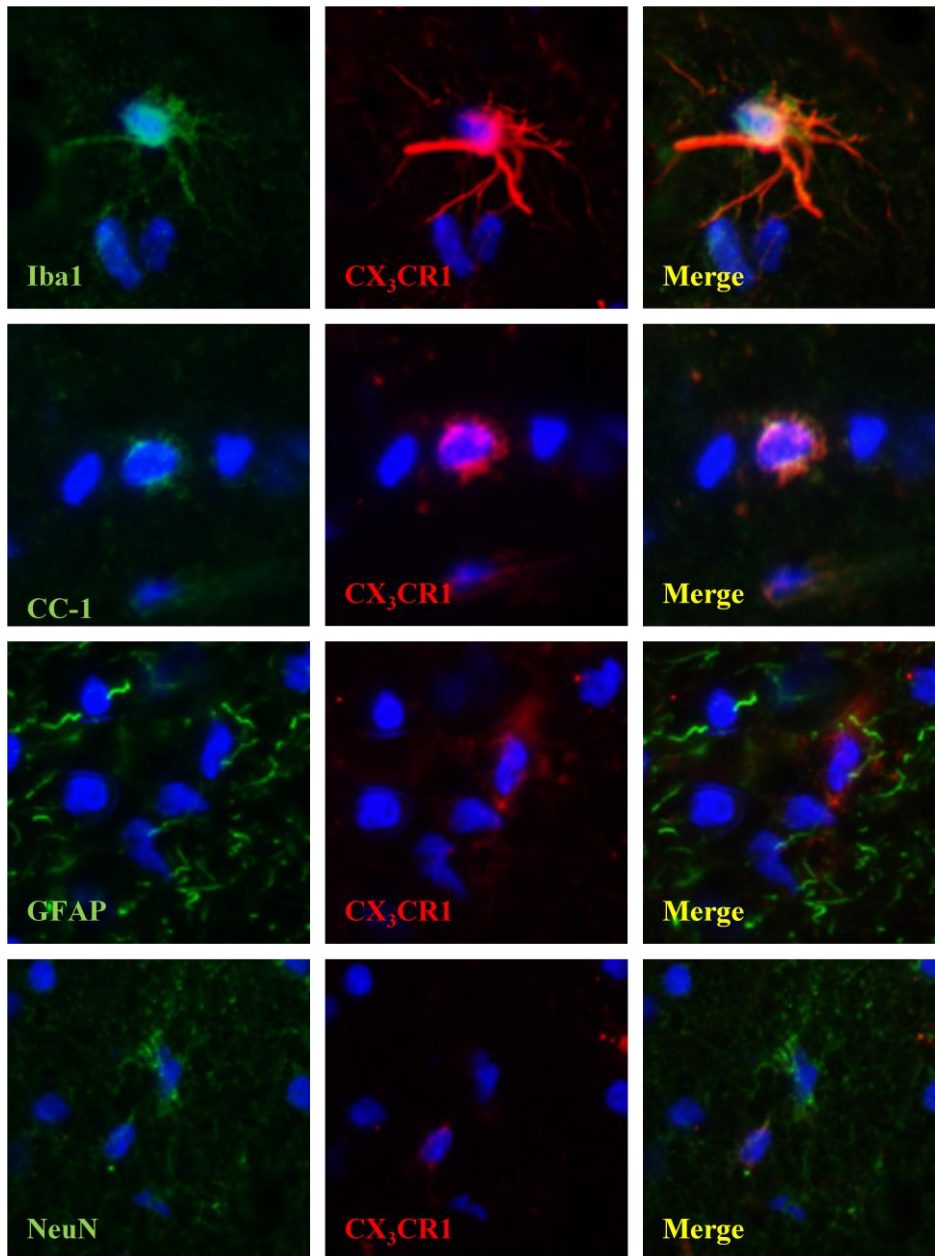**Figure 29: Staining for CX₃CR1⁺ cells (red) in brain tissue of an NDC. Microglia (Iba1) and oligodendrocytes (CC-1) but not astrocytes (GFAP) and neurons (NeuN) express CX₃CR1.**

Discussion

The vast majority of peripheral blood CD4$^+$ T cells express the CD28 molecule, while a subset of CD4$^+$CD28^{null} T cells exists only in some individuals, mainly correlated with CMV infection. On the other hand, CD8$^+$CD28^{null} T cells are more common and show increased frequencies with chronic infections as well as during normal aging[12]. The presence of CD4$^+$CD28^{null} T cells has a negative effect on the host. These cells perpetuate after infection, thereby causing a more severe inflammation. The distinction between CD8$^+$CD28$^+$ T cells and CD8$^+$CD28^{null} T cells is not so clear. CD8$^+$CD28$^+$ T cells are already considered cytolytic, and the presence of CD8$^+$CD28^{null} T cells may also have some beneficial effects on the host e.a. in organ transplantation due to their immunosuppressive action.

CD4$^+$CD28^{null} T cells are enriched in a subset of patients suffering from a multitude of diseases. Many studies have been performed to associate these cells with diseases such as MS[7,8,34], coronary artery disease[10,24], rheumatoid arthritis[15,16,17,21,33,40], Wegener's granulomatosis[20], dermatomyositis and polymyositis[14], and several others. The differentiation and functional properties of CD28^{null} T cells, both CD4 and CD8 T cells, as well as their characterization and stimulatory pathways have been extensively studied.[4,6,9,11,12] Their true functional role in disease pathogenesis and immunosenescence has, however, remained unclear. We aimed to further elucidate the role of both CD4$^+$CD28^{null} T cells and CD8$^+$CD28^{null} T cells in MS through a series of experiments on HC and patients. In this study, we found that there is a strong positive correlation between the presence of CD4$^+$CD28^{null} and CD8$^+$CD28^{null} T cells in HC. The pool of CD4$^+$CD28^{null} T cells seems to increase with an expansion in CD8$^+$CD28^{null} T cells and vice versa.

Previous studies already demonstrated that the fractalkine receptor CX$_3$CR1 is expressed on the vast majority of senescent CD4$^+$CD28^{null} T cells, while it is almost absent on CD4$^+$CD28$^+$ T cells.[34] We therefore studied the expression of CX$_3$CR1 on CD8$^+$ T cells in HC, and found that it was correlated with loss of CD28 and CMV infection in both the CD8$^+$CD28^{null} and CD8$^+$CD28$^+$ T cell populations. Expression of CX$_3$CR1 was significantly elevated in both CD8$^+$CD28$^+$ and CD8$^+$CD28^{null} T cell populations upon CMV infection. We also found a significantly higher expression of CX$_3$CR1 in the total CD8$^+$ T cell population after CMV infection. CMV continuously activates the immune system, leading to repeated stimulation of T cells and eventually the loss of CD28 through replicative senescence.[4,5] These results show that expression of CX$_3$CR1 on CD8$^+$ T cells is both correlated to CMV infection and thus loss

of CD28. The most striking difference with the CD4$^+$ T cell population, is that CX$_3$CR1 is also expressed on CD8$^+$CD28$^+$ T cells, while this is not the case for CD4$^+$CD28$^+$ T cells. The distinction in characterization and function between CD8$^+$CD28$^+$ T cells and CD8$^+$CD28^{null} T cells is therefore not as clear as that between CD4$^+$CD28$^+$ T cells and CD4$^+$CD28^{null} T cells. This leads to the hypothesis that CD8$^+$CD28$^+$ T cells, as well as CD8$^+$CD28^{null} T cells, can migrate towards an increased fractalkine gradient. This has already been shown for CD4$^+$CD28^{null} T cells, which are able to migrate towards a fractalkine gradient in vitro.[34] In this study, we found that both populations showed an increased migratory capacity towards the medium containing fractalkine, with a slightly higher chemotactic index for the CD8$^+$CD28^{null} T cells population in comparison to the CD8$^+$CD28$^+$ T cells. The difference in chemotactic index between both cell populations was not significant, because the CD8$^+$CD28$^+$ T cell population is also capable of migration to an increased fractalkine gradient through the expression of CX$_3$CR1.

In MS patients, fractalkine has been shown to be upregulated in the CSF and serum, and several groups reported an abundant expression of fractalkine and its receptor in the brain.[45-47] In vitro analyses on brain cell cultures have indicated that fractalkine is mainly expressed on neurons and astrocytes, while its receptor is present on neurons and microglia.[48] It has also been demonstrated that neurons, microglia and oligodendrocytes express CX$_3$CR1 in MS lesions.[34] We examined the expression of CX$_3$CR1 on these brain cells in the brains of NDC and AD patients. Results showed that the fractalkine receptor is strongly expressed by microglia in both NDC and AD patients, while there is only a limited expression of CX$_3$CR1 by oligodendrocytes in both conditions. In contrast with MS patients, neurons do not express CX$_3$CR1 in the brains of AD patients and NDC. The expression of CX$_3$CR1 in the brains of MS patients is presumably induced by inflammation of the brain. The brains of AD patients and NDC are not inflamed, explaining why CX$_3$CR1 is not expressed on these neurons.

Because of the identification of CX$_3$CR1 on CD8$^+$ T cells, we were able to perform double stainings on brain material from MS patients. We found that there are CD8$^+$ T cells present in the MS brain, mainly in active lesions, but they do not express CX$_3$CR1. This is contradictory to the chemotaxis assay, which indicated that CD8$^+$ T cells do express CX$_3$CR1 and that they are able to migrate to an increasing fractalkine gradient[34]. However, when we compare previous research on CD4$^+$CD28^{null} T cells with this study, CD8$^+$ T cells are found to have a lower chemotactic capacity. This, and the fact that the in vitro assay can never fully mimic the in vivo situation, might explain why we did not find CD8$^+$CX$_3$CR1$^+$ T cells in the brains of

MS patients. Under healthy conditions, the blood brain barrier is closed, and does not allow migration of T cells to the parenchyma of the brain. Also, fractalkine is only upregulated in the brains of MS patients, compared to HC. This explains why no $CD8^+$ T cells and $CD4^+CD28^{null}$ T cells were found in the brains of NDC and AD patients.

We subsequently analyzed whether $CD4^+CD28^{null}$ T cells can contribute to the disease process of MS, once they have migrated towards the inflamed tissue. We therefore investigated what types of cytokines and chemokines they produce after being activated. The inflammatory response is characterized by the production of various molecules that initiate the recruitment of immune cells to the lesion sites, including the brain. Prolonged and sustained inflammation may have cytotoxic effects, aggravating the incidence and severity of the disease. Chemokines which are involved in the chemoattraction of cells are very important inflammatory factors. They are involved in the recruitment of the main resident immune cell types of the brain and of infiltrating monocytes from the bloodstream across the blood brain barrier (BBB) into the CNS. They can be produced by glial and neuronal cells, which both also express chemokine receptors. [48].

By performing a cytokine array on $CD4^+CD28^{null}$ T cells, our study found that these cells produce a large amount of cytokines and chemokines after activation. Among these, G-CSF and IL-1α were only produced by cells of MS patients, and CCL2 was specifically produced by MBP-stimulated cells. This difference in expression might reveal a disease-specific mechanism. Previous studies already showed that chemokines and chemokine receptors are involved in the pathogenesis of MS. Different pairs of chemokines seem to play a pathogenic role in MS, such as CCL1, CCL2, CCL3, CCL4, CCL5, CXCL9 and CXCL10. The influx of autoreactive T cells and macrophages across the BBB into the CNS is a crucial step in the development of MS. The migration of inflammatory cells is facilitated by the upregulation of adhesion molecules and matrix metalloproteinases which degrade components of the basement membrane. Chemokines can then attract leukocytes by forming concentration gradients over the BBB, especially CCL4 is known mainly for its activity as a chemotactic cytokine[49]. CCL2, previously known as monocyte chemoattractant protein-1, acts on monocytes, activated T cells, NK cells and microglia. CCL2 is known to be produced by astrocytes, microglia, endothelial cells and macrophages. A critical role for CCL2 has been established in animal models for MS. Several studies have demonstrated that CCL2 is not only expressed in neuroinflammatory conditions, but are also constitutively present in the brain in glial cells and neurons. This indicates that CCL2 may act as a modulator of neuronal

functions. It is established that CCL2 plays an important role as mediator in many neuroinflammatory diseases characterized by neuronal degeneration, and that the expression of CCL2 in the brain is increased in autoimmune encephalomyelitis (EAE), an animal model for MS. [48]. Previous research found that CCL2 was expressed by astrocytes and macrophages within acute and chronic MS lesions in humans, and its receptor was detected on macrophages and activated microglia in active MS lesions[49]. [48]. Other studies demonstrated that CCL2 deficient mice were resistant to the induction of EAE and showed a significant reduction in macrophage recruitment to the CNS. Other studies indicated that induction of leukocyte adhesion to the brain microvasculature is an important mechanism by which mainly CCL2 and CCL5 participate in the pathophysiology of MS[50].

Two other chemokines which were found to be produced by $CD4^+CD28^{null}$ T cells are CCL3 and CCL5. Significantly higher levels of these chemokines were found in the CSF of patients with MS in relapses. Treatment with anti-CCL3 antibodies inhibits the onset of EAE, and reduces the accumulation of mononuclear cells in the CNS. Patients with MS also have elevated percentages of CCL5 receptor expressing T cells compared to HC, indicating that the CCL5 receptor plays a crucial role in the recruitment of T cells in MS[49]. Previous research found that the receptors for CCL3 and CCL5 are also expressed on macrophages and activated microglia in the active MS lesions, and their ligands were detected in MS plaques[49].

Our study demonstrated that $CD4^+CD28^{null}$ T cells produce various cytokines, including IL-1α, G-CSF, TNF-α and IFN-γ. Previously, several studies evaluated the involvement of various cytokines in the pathogenesis of multiple sclerosis. Many cytokines participate in nervous tissue damage by promoting demyelization and oligodendrocyte injury or by enhancing local immune response. In addition, several authors reported increased levels of some cytokines in serum and cerebrospinal fluid of patients with multiple sclerosis. These findings suggest that cytokines can play a significant role in the immunopathogenesis of the disease[51]. Different research groups have also extensively studied the associations of cytokine gene polymorphisms in different diseases. The role of cytokine gene polymorphisms in MS has been previously reported in various populations. Several cytokine single gene nucleotide polymorphisms were determined, including IL-1α, IL-1β, IL-1R and TNF-α[52]. IL-1α is a pro-inflammatory cytokine and possesses a wide spectrum of metabolic, physiological and haematopoietic activities[53]. Previous research found that IL-1α plays a major role in autoimmune and inflammatory diseases, and is expressed at elevated levels in

tissue and fluid samples isolated from patients with many chronic inflammatory diseases, such as RA and MS[54].

Another cytokine produced by CD4$^+$CD28^{null} T cells in MS patients, is granulocyte colony-stimulating factor (G-CSF). G-CSF is a potent hematopoietic factor produced by mesothelial cells, fibroblasts, and endothelial cells. Receptors for G-CSF are present on precursors and mature neutrophilic granulocytes, monocytes, platelets, and endothelial cells. Expression of G-CSF is induced by ischemia, where after G-CSF can pass the intact BBB. G-CSF is an endogenous ligand in the CNS that has a dual activity; beneficial both in counteracting acute neuronal degeneration and contributing to long-term plasticity after cerebral ischemia. At the myeloid progenitor cell level, G-CSF stimulates the growth of neutrophil granulocyte precursors and crucially regulates survival of mature neutrophils by inhibition of apoptosis. G-CSF signaling appears to be a novel protective system in the brain that is involved in counteracting acute neurodegeneration and regulating the formation of new neurons[55]. In other studies, however, G-CSF administration resulted in cases of worsened clinical MS status. G-CSF enhanced MS autoreactive T cell line adhesion to the extracellular matrix (ECM) proteins as effectively as the proinflammatory IFN-γ and TNF-α, known to exacerbate MS symptoms. This indicated a link between clinical worsening of MS symptoms induced by G-CSF and the hyper-stimulation of T cell adhesion to ECM elicited by G-CSF[56]. The production of G-CSF by CD4$^+$CD28^{null} T cells in MS patients may thus play a dual role in the neuroprotection on the one hand, and in the increase of the clinical syndromes of MS on the other hand.

Other proinflammatory cytokines such as TNF-α and IFN-γ, trigger a chain of events that result in the formation of demyelinated plaques and damage to axons[49]. The primary role of TNF-α is the regulation of immune cells. TNF-α shows a wide spectrum of biological activities. It causes cytolysis of many tumor cell lines in vitro. The factor also enhances phagocytosis and cytotoxicity in neutrophilic granulocytes and modulates the expression of many other proteins, including IL-1 and IL-6. IL-1 and IL-6 in turn are important factors in Th17 differentiation. In the presence of IL-2, TNF-α promotes the proliferation and differentiation of B cells. Although TNF-α is required for normal immune responses, its overexpression has severe pathological consequences. TNF-α is able to induce apoptotic cell death and inflammation, and inhibit tumorigenesis and viral replication. Dysregulation of TNF-α production has been implicated in a variety of human diseases, including major depression, Alzheimer's disease and cancer. TNF-α is found predominantly on monocytes and

T cells after cell activation. In vivo TNF-α in combination with IL-1 is responsible for many alterations of the endothelium, and is also a potent chemoattractant for neutrophils. TNF-α promotes the proliferation of astroglial cells and microglial cells and therefore may be involved in pathological processes such as astrogliosis and demyelination[57].

IFN-γ is a remarkable cytokine that orchestrates many distinct cellular programs through transcriptional control over large numbers of genes. In most situations, macrophages are activated to acquire microbiocidal effector functions and secrete proinflammatory cytokines, resulting in inflammation and recruitment of immune cells and subsequent elimination of the microbe by phagocytosis or release of toxic metabolites. The most important activating factor for macrophages is IFN-γ, which induces direct antimicrobial and antitumor mechanisms as well as upregulating antigen processing and presentation pathways. IFN-γ directs growth, maturation and differentiation of many cell types. It also enhances NK cell activity and regulates B functions such as immunoglobulin production. IFN-γ initiates cell-mediated immunity via the activation of NK cell effector functions, specific cytotoxic immunity trough T cells, and macrophage activation. IFN-γ also orchestrates the trafficking of immune cells to sites of inflammation trough upregulating expression of adhesion molecules and chemokines[58].

The chemokines and cytokines detected in our study are all known to be produced in response to inflammation. They are involved in the chemoattraction of specific immune cells into the brains of MS patients, display several proinflammatory functions, and thus play a crucial role in the immunopathogenesis of MS.

Another important part of this study focused on the effect of IL-15 on the phenotype and function of $CD4^+CD28^{null}$ T cells. IL-15 secretion by macrophages is known to increase cytokine secretion[43], and regulate IFN-γ production by NK cells. Because $CD4^+CD28^{null}$ T cells are very similar to NK cells, we first examined the effects of IL-15 on cytokine production by these cells. We established that $CD4^+CD28^{null}$ T cells express the IL-15 receptor. When cells were stimulated with soluble IL-15, there was a significant increase in IFN-γ and IL-17 production by $CD4^+CD28^{null}$ T. IL-17 is a potent inflammatory cytokine which induces the expression of pro-inflammatory genes and chemokines, neutrophil recruitment and inflammation. The IL-17 receptor is expressed in different organs such as lung, liver and spleen. Cells capable of responding to IL-17 include dendritic cells, macrophages, lymphocytes, epithelial cells and fibroblasts[59]. We did not detect production of

IL-17 using the cytokine array, for which we used different stimulatory agents to activate the cells. This difference in activation might explain why IL-17 was only detected in the IL-15 assay, and not in the cytokine array. After adding IL-15 to the CD4$^+$CD28^{null} T cells, there was also a significant increase in the production of cytotoxic molecules NKG2D, perforin and granzyme B. Both CD4$^+$ and CD8$^+$ T cells had already been found to be perforin positive in previous research[14]. Our results thus prove that senescent CD4$^+$ T cells become cytotoxic after the loss of CD28. Moreover, the effect of IL-15 on NK cells was found to be present in CD4$^+$CD28^{null} T cells as well, implying a common mechanism or pathway.

IL-15 is also known to increase the proliferation of NK cells. We evaluated whether IL-15 has the same effect on CD4$^+$CD28^{null} T cells by performing a CFSE assay. Remarkably, CD4$^+$CD28^{null} T cells show an overall higher proliferative capacity, compared to CD4$^+$CD28$^+$ T cells. When IL-15 was used in combination with aCD3, there was a significantly higher proliferation rate of both cell populations, compared to aCD3 alone. IL-15 alone was also able to induce proliferation in both cell populations, although this only reached statistical significance for CD4$^+$CD28^{null} T cells. These findings confirm that IL-15 is also able to induce proliferation in CD4$^+$CD28^{null} T cells, as it does for NK cells. This again shows the range of similarities between NK cells and CD4$^+$CD28^{null} T cells, proving these senescent T cells are also cytotoxic.

To evaluate whether CD4$^+$CD28^{null} and CD8$^+$CD28^{null} T cells are responsive to CMV, we determined their capacity to degranulate after stimulation with CMV viral peptides. We found that there is no significant difference in degranulation of both CD4$^+$CD28^{null} and CD8$^+$CD28^{null} T cells after activation with CMV, as compared to their capacity to degranulate in plain CM. This might be due to the fact there was a great deal of background visible for the CM conditions, and the experimental setup has yet to be optimized. Previous research did find that most CD4$^+$CD28^{null} T cells degranulated significantly when activated with aCD3. This response could already be seen after 4 hours of activation in both HC and MS[34]. The cytotoxic capacities of senescent CD4$^+$ T cells have been investigated by several studies. These cells were found to express several NK cell receptors, such as NKG2D. Large intracellular stores of IFN-γ, perforin and granzymes A and B were found to be present in CD4$^+$CD28^{null} T cells, and the majority of these cells were found to be CMV specific[19,60,61].

Our study confirms the cytotoxic potential of CD4$^+$CD28^{null} T cells, through the expression of cytotoxic molecules and their ability to respond to IL-15 stimulation.

Conclusion

In this study, we found that there is a strong positive correlation between the presence of $CD4^+CD28^{null}$ and $CD8^+CD28^{null}$ T cells in HC. Both cell types express the chemokine receptor CX_3CR1, and are able to migrate towards an increasing fractalkine gradient. The expression of CX_3CR1 in the $CD8^+$ T cell population is correlated with loss of CD28 and CMV infection in both the $CD8^+CD28^{null}$ and $CD8^+CD28^+$ T cell subsets. The most striking difference with the $CD4^+$ T cell population, is $CD8^+CD28^+$ T cells also express a functional fractalkine receptor CX_3CR1, and also show an increased migration capacity towards medium containing fractalkine while this is not the case for $CD4^+CD28^+$ T cells. The distinction in characterization and function between $CD8^+CD28^+$ T cells and $CD8^+CD28^{null}$ T cells is therefore not as clear as that between $CD4^+CD28^+$ T cells and $CD4^+CD28^{null}$ T cells. $CD8^+CD28^+$ T cells are already considered cytolytic, and the presence of $CD8^+CD28^{null}$ T cells may also have some beneficial effects on the host e.a. in organ transplantation due to their immunosuppressive action. $CD8^+CD28^{null}$ T cells are more common than $CD4^+CD28^{null}$ T cells and show increased frequencies with chronic infections as well as during normal aging[12].

CX_3CR1 is expressed by microglia and oligodendrocytes in the brains of NDC and AD patients, but not by neurons as in the brains of MS patients. This is due to the lack of inflammation in the brains of non-MS patients, that induces the expression of fractalkine in the brains of MS patients. $CD8^+$ and $CD4^+$ T cells are also exclusively present in the brains of MS patients, mainly in active lesions. However, the $CD8^+$ T cells do not express CX_3CR1.

Six major cytokines were found to be produced by $CD4^+CD28^{null}$ T cells: IL-1α, G-CSF, TNF-α, IFN-γ, CCL2 and CCL4, and four cytokines were validated in another experiment: IL-1α, IL-1β, IL-6 and CCL2. These cytokines all show proinflammatory properties, although G-CSF is also believed to have some neuroprotective abilities. TNF-α and IFN-β induce capillary endothelial cells to express adhesion molecules and chemokines to facilitate the trafficking of immune cells to sites of inflammation. One of those chemotactic cytokines is CCL4. TNF-α also contributes to the pathological process of demyelination by promoting the proliferation of astroglial and microglial cells[57], while IFN-β enhances NK cell activity and activates macrophages[58]. IL-1α and CCL2 both play a major role in autoimmune and inflammatory diseases, and are expressed at elevated levels in tissue and fluid samples

isolated from patients with many chronic inflammatory diseases[54] and the presence of CCL2 in neurons throughout the brain indicates that it might participate in the neurodegenerative processes that take place in these pathologies[48]. More recently, transcriptional profiling with gene microarrays and large-scale sequencing of transcripts from MS lesions have identified a number of genes that are involved in the pathogenesis of acute disease. These include IL-6 which plays a role in the transition from relapsing-remitting to chronic MS[62]. After IL-15 stimulation, $CD4^+CD28^{null}$ T cells are also found to upregulate the production of proinflammatory cytokines IFN-γ and IL-17, and of cytotoxic molecules NKG2D, perforin and granzym B. Through production of these cytokines, $CD4^+CD28^{null}$ T cells may actively contribute to the immunopatholgy of MS.

In this study, we confirmed the role of $CD4^+CD28^{null}$ T cells in disease manifestation of MS. In general, these cells share many similarities with NK cells and are considered cytotoxic and inflammation-seeking cells. They can infiltrate the brains of MS patients through the expression of CX_3CR1 and release an arsenal of cytoxic and proinflammatory cytokines. $CD8^+CD28^{null}$ T cells also have in vitro capacity to infiltrate into the brains of MS patients, through the expression of CX_3CR1, although this has not been proven. Their presence is strongly correlated to that of $CD4^+CD28^{null}$ T cells and they are considered cytotoxic as well. Their contribution to the immunopathology of inflammatory diseases such as MS is, however, not as clear as that of $CD4^+CD28^{null}$ T cells. This is mainly due to the fact that there is no clear distinction between $CD8^+CD28^{null}$ and $CD8^+CD28^+$ T cells, which are already considered cytolytic.

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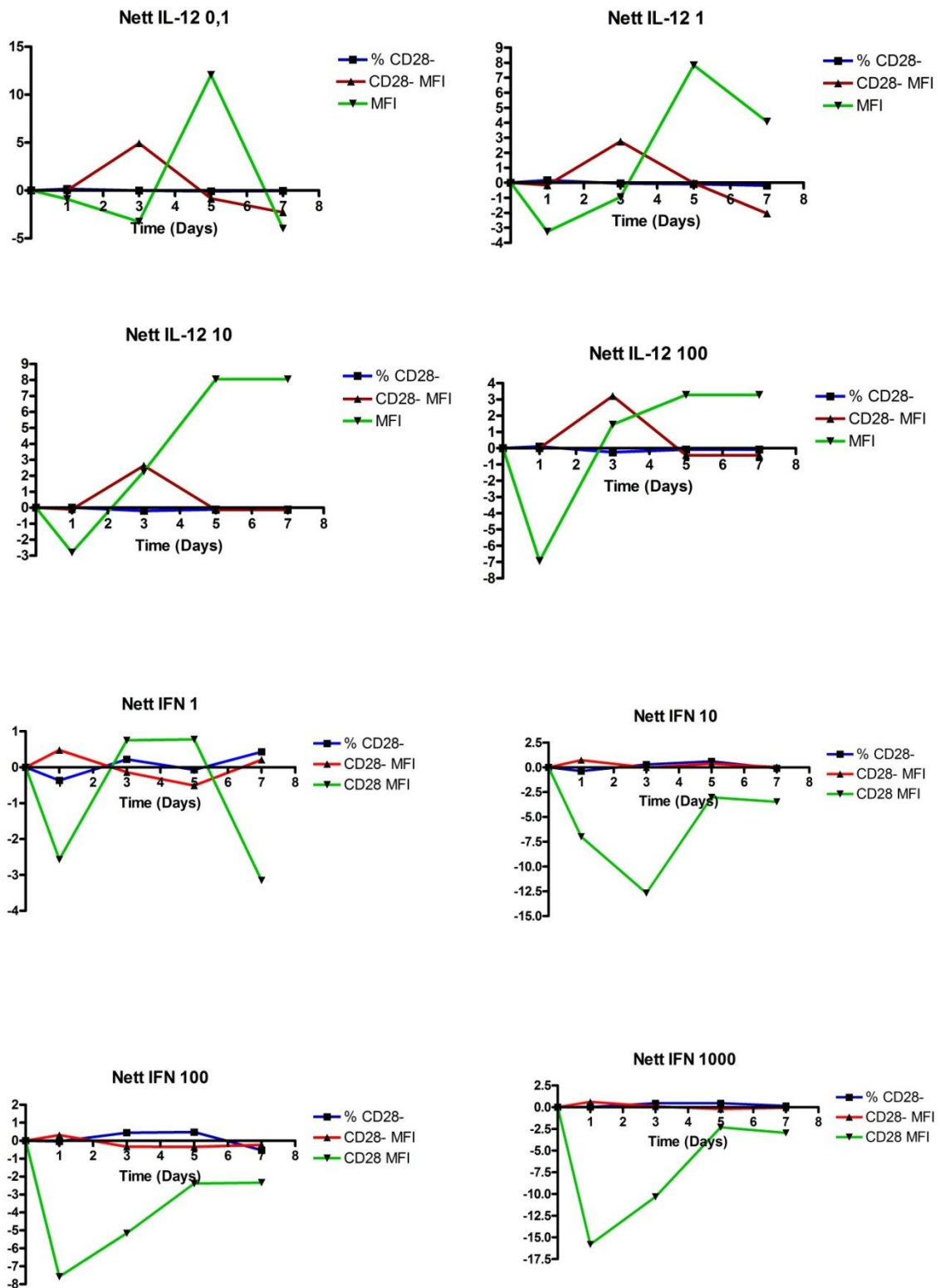
Figure 1: Effects of IL-12 and IFN-β on mean expression of CD28. Expression of CD28 on CD4$^+$CD28^{null} T cells is represented by CD28$^-$ MFI, expression of CD28 on the total cell population is represented by CD28 MFI. One of two experiments is shown.
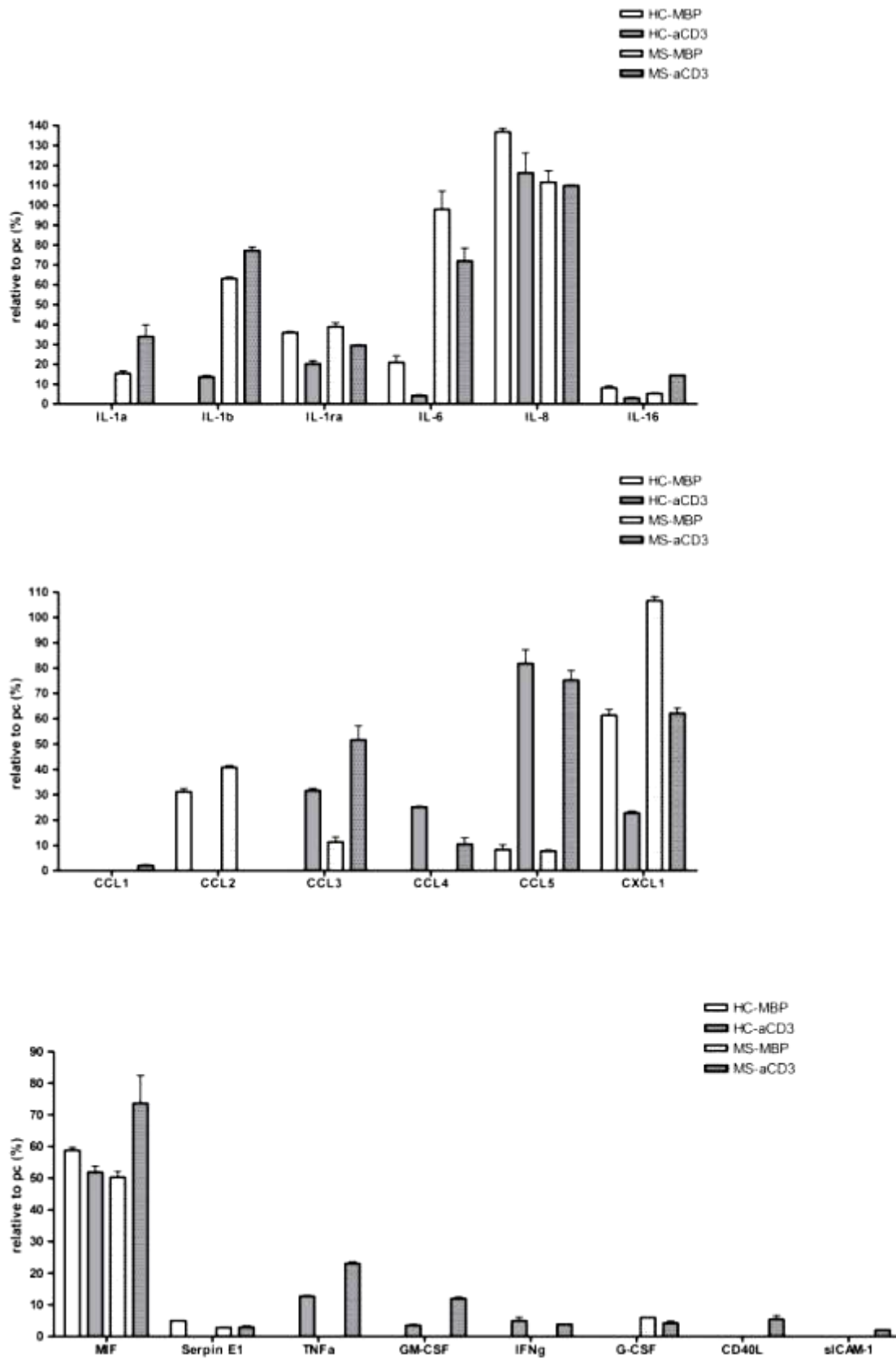
Appendix B: Results Cytokine Array

Figure 2: Cytokines produced by CD4^{+}CD28^{null} T cells in MS patients and HC, and detected with the a cytokine array when pulsed with MBP or stimulated with aCD3.

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Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
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