The role of CD4+CD28NULL T cells in the pathogenesis of multiple sclerosis and rheumatoid arthritis

Anke Vanderstraeten

promotor :
Prof. dr. Pieter STINISSEN

co-promotor :
Prof. dr. Niels HELLINGS

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Abbreviations

aCD3	anti-CD3
AICD	activation-induced cell death
BBB	blood brain barrier
BSA	bovine serum albumin
CB	chemotaxis buffer
CCP	cyclic citrullinated peptides
CD	celiac disease; cluster of differentiation
CIS	clinically isolated syndrome
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
CX_3CL1	fractalkine
CX_3CR1	fractalkine receptor
EBV	Epstein Barr virus
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLS	fibroblast-like synovial cells
GD	Graves' disease
HC	healthy control(s)
hCII	human type II collagen
HLA	human leukocyte antigen
IFN	interferon
Ig	immunoglobulin
IS	immunosenescence
KIR	killer cell immunoglobulin-like receptor
MBP	myelin basic protein
MCP	metacarpophalangeal
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MS	multiple sclerosis
NK cell	natural killer cell
PBMC	peripheral blood mononuclear cells
PE	phycoerithrin
Percp	Peridinin-chlorophyll-protein complex
PHA	phytohemaglutinin
PPMS	primary progressive multiple sclerosis
PsA	psoriatic arthritis
RA	rheumatoid arthritis
RRMS	relapsing-remitting multiple sclerosis
RTE	recent thymic emigrants
SLE	systemic lupus erythematosous
SPMS	secondary progressive multiple sclerosis
TNF	tumor necrosis factor

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Abstract

Senescent $CD4^+CD28^{NULL}$ T cells have been shown to be increased in patients suffering from an autoimmune disease. Their appearance along with aberrant characteristics such as the acquisition of killing receptors and the presence of intracellular cytotoxic granules implicate a possible role for these $CD4^+CD28^{NULL}$ T cells in the pathogenesis of autoimmune diseases. This is currently investigated on a phenotypical as well as functional level in healthy controls (HC) as well as MS patients and RA patients. In this research it is shown that the percentage of $CD4^+CD28^{NULL}$ T cells in peripheral blood of RA patients is increased when compared to HC ($p=0.0185$). Based on surface marker expression, these senescent cells appear as memory type effector cells displaying infiltrating and cytotoxic characteristics. Moreover, the surface expression of the chemokine receptor CX_3CR1 can be used as a marker to define $CD4^+CD28^{NULL}$ T cells since this receptor is present on the vast majority of $CD4^+CD28^{NULL}$ T cells while being present on almost none of the $CD4^+CD28^+$ T cells ($p<0.0001$). Although the chemotactic potential of $CD4^+CD28^{NULL}$ T cells via the interaction of this receptor and its chemokine ligand remains inconclusive so far, $CD4^+CD28^{NULL}$ T cells do show a higher general migratory capacity in HC ($p=0.0012$).
Previous research also indicated the ability of $CD4^+CD28^{NULL}$ T cells in RA patients to provide bystander activation to other T cells by means of sustained CD70 signaling. These results are currently confirmed in HC.

In conclusion, for the characteristics investigated here, very clear differences can be seen between the $CD4^+CD28^{NULL}$ T cell population and the $CD4^+CD28^+$ T cell population. However, no obvious differences can be found for $CD4^+CD28^{NULL}$ T cells in healthy controls and MS or RA patients. This indicates that $CD4^+CD28^{NULL}$ T cells may play a possible, yet indirect, role in the pathogenesis of autoimmune diseases or, alternatively, that $CD4^+CD28^{NULL}$ T cells may be differentially regulated in healthy controls and MS or RA patients.

1. Introduction

1.1 Autoimmune diseases

Autoimmune diseases arise when the body's normal tolerance to auto-antigens fails. This failure of self-tolerance results in the appearance of autoantibodies, often accompanied by autoreactive T cells (1). However, their presence in itself does not necessarily indicate an autoimmune disease. Many autoantibodies may have non-pathologic characteristics, as shown by the presence of autoantibodies in healthy individuals (1). On the contrary, if the failure of self-tolerance leads to innocuous auto-antibodies and autoreactive T cells, autoimmune diseases will develop. A broad range of autoimmune diseases has been described to date, among which multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erythematosous (SLE), celiac disease (CD) and Graves' disease (GD) (1). Two disease types, MS and RA, will be discussed in more detail.

1.1.1 Multiple sclerosis

Multiple sclerosis (MS), as reviewed in (2) and (3), is an autoimmune demyelinating disease of the central nervous system (CNS). In persons aged 20 to 40, MS is the most prevalent form of non-traumatic neurological disability. MS affects approximately 1 in 1000 people in Europe and North America, indicating that the affected population is predominantly Caucasian (4). Moreover, MS is more prevalent among females than males in a ratio of at least 1.5:1 (5).

In MS, the insulating myelin sheath surrounding axons in the CNS and the cells producing this sheath are selectively damaged by autoreactive T cells, resulting in the characteristic focal demyelinated cerebral and spinal cord lesions (Figure 1). Additionally, the axons themselves are damaged. Clinically, this results in symptoms involving motor, sensory, visual and autonomic systems. Few symptoms are specific for MS but the disease does have some characteristic symptomatic features, such as L'Hermitte's symptom, a sensation running down the spine or neck flection, and Unthoff phenomenon, a transient deterioration of symptoms when the body's core temperature rises (2). Aside from physical disability, many patients also show cognitive impairment, reviewed by Chiaravalotti et al (6), such as long-term memory problems, difficulties executing tasks and depression.

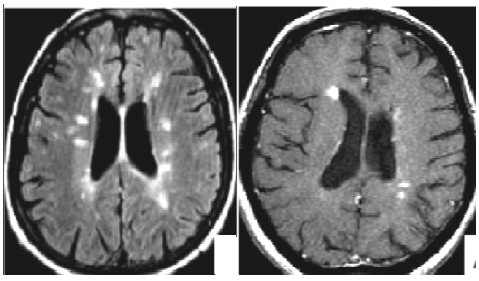

Figure 1: Demyelinated lesions in the brain (7). Cerebral MRI scans (T2-weighted images). Several bright areas of demyelinated tissue surrounding the ventricles are visible.

MS arises when autologous peripheral myelin reactive T cells become activated, which allows them to migrate across the blood brain barrier (BBB). Once migrated to the brain parenchyma, autoreactive T cells are reactivated by antigen-presenting cells residing in the CNS. This in turn elicits an immune response resulting in myelin, oligodendrocyte and axonal damage (Figure 2) (8). These processes consequently cause damage to the myelin sheath, resulting in failure of the conduction of electrical signals across axons. This in turn leads to the aforementioned symptoms, characteristic of MS.

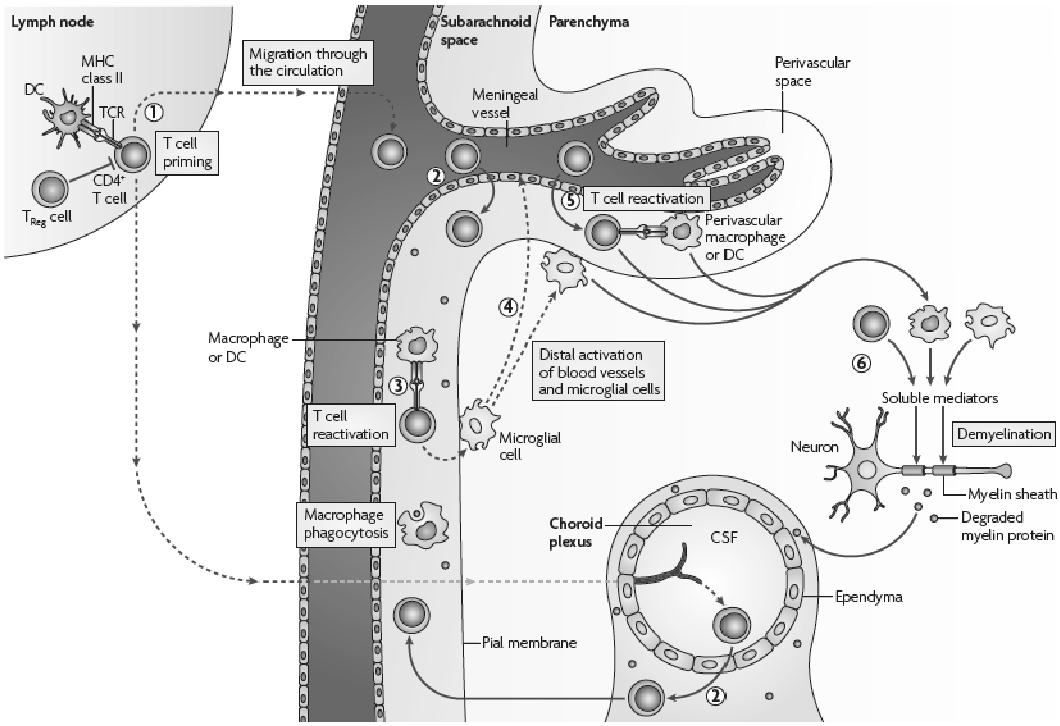

Figure 2: Activation of autoreactive T cells in MS (8). 1. T cells are primed to myelin antigens by dendritic cells in the periphery. 2. Activated T cells subsequently cross the blood-CSF barrier, either via the choroid plexus or the meningeal venules, and enter the subarachnoid space. 3. Within the subarachnoid space, these autoreactive T cells are reactivated by MHC class II expressing macrophages and dendritic cells. 4. Activated T cells trigger microglia resulting in the activation of distal microglia and blood vessels. 5. Activated T cells cross the activated BBB into the perivascular space where they are reactiveatd by dendritic cells and macrophages. 6. Reactivated T cells enter the brain parenchyma after which an immune response arises, ultimately resulting in demyelination.

Although the main focus has always been on the involvement of $CD4^+$ T cells in the MS pathogenesis, recent evidence suggests a role for $CD8^+$ T cells as well, reviewed by Goverman (8). $CD8^+$ T cells outnumber $CD4^+$ T cells in MS lesions and clonal expansions are more frequently detected in the $CD8^+$ population than the $CD4^+$ population isolated from MS lesions. Enrichment of $CD8^+$ memory T cells over $CD4^+$ memory T cells is seen in the cerebrospinal fluid (CSF) and blood of patients suffering from MS. Also this memory population shows evidence of clonal expansion, indicating antigen recognition. Additionally, $CD8^+$ T cells specific for CNS antigen-specific peptides are present in higher numbers in MS patients compared to healthy controls (HC).

One specific cause leading to the activation of autologous myelin-reactive T cells has never been pinpointed. MS is most likely a multifactorial disease in which environmental as well as genetic factors are involved. Globally, the incidence of MS increases with increasing distance from the

equator. Aside from this distribution, there are several areas in which the prevalence is much higher than elsewhere, such as for example areas populated by northern Europeans (2). Genetically, there are several candidates that are associated with MS. The most well-known genetic factor that is associated with the occurrence of MS is the major histocompatibility (MHC) region, especially the human leukocyte antigen (HLA)-DR2 haplotype (9). Three candidate risk genes of the HLA-DR2 haplotype are almost invariably co-inherited. Next to polymorphisms in the MHC region, polymorphisms in several non-HLA related genes were identified in genomic studies, the most significant of which are polymorphisms in the gene encoding for the interleukin-7 receptor (IL-7R) (9-11) and in the gene encoding for the interleukin-2 receptor (IL-2R) (9). Strikingly, most non-HLA genes which have been found to be associated with the disease are immunologically relevant, supporting the involvement of T cells in the MS pathogenesis (12).

The vast majority of MS patients presents with an acute disease episode, known as a clinically isolated syndrome (CIS), possibly affecting multiple sites. After one episode, some patients, not all, evolve to clinically defined MS (3). The majority of MS patients initially suffers from relapsing-remitting MS (RRMS). In RRMS, the neurological deficits are recurrent and reversible. The relapses generally last no longer than a few months and the patient usually regains full neurological function, due to resolution of the inflammation and remyelination which help to restore the axonal conduction capacity. The occurrence rate of relapses does however create a bias concerning disease progression. New inflammatory lesions can remain clinically insignificant; relapses are thus subjective measurements of disease, since their severity depends on the cerebral area in which the new lesions originate.
MS patients may over time progress from the RRMS stage to the secondary progressive MS (SPMS) stage. In this stage relapses no longer occur. The neurological decline is continuous and irreversible.
A minority of MS patients suffers from primary progressive MS (PPMS). Relapses are non-existent in this disease type. The symptoms and clinical disability deteriorate from disease onset (3, 13).

The mechanisms of tissue injury in MS are quite diverse. They are heterogeneous between patients and stage-dependent within patients (14). Four different types of lesions have been described. In classical actively demyelinating lesions, mainly present in RRMS patients, components of the innate immune system as well as autoantibodies and cytotoxic T cells can be found. Which components are found can differ among patients. In some patients lesions are dominated only by T cells and macrophages, while in others complement factors and autoantibodies accumulate or a mild inflammatory infiltrate is present (14). The lesions in progressive MS on the other hand are slowly expanding lesions. In contrast to what can be seen in the active forms, macrophages are rarely found in these lesions. In this type of lesions activated microglia, some containing myelin degradation products, are present at the lesion edge. The demyelination process occurs in close contact with these microglia. T cells, if present, are mainly located perivascularly (14). Besides white matter lesions, cortical lesions also occur in MS. These lesions are mainly located in the cortical sulci. The lesions, which are mainly subpial, appear as large band-like structures. These lesions are most abundant in the deep brain indentations (14).

The inflammatory process in MS results in focal demyelinated plaques in the white matter of both the brain and the spinal cord. The plaques result from the damage done directly to myelin or to oligodendrocytes, the cells which produce the myelin sheaths in the CNS (2). In active lesions the inflammatory process is accompagnied by disturbance of the BBB and axonal damage or even axonal loss (14). Besides the characteristic demyelinated plaques in the white matter, remarkable changes in the normal appearing white matter also take place. These changes involve a diffuse inflammatory process. Inflammatory infiltrates, consisting of T cells (mainly $CD8^+$), are present in the perivascular space and dispersed throughout the white matter (14).

Next to the white matter, the grey matter is also affected by the disease, (3, 15). Demyelination can be found in several grey matter regions, among which the neocortex, the (hippo)thalamus, the basal ganglia, the hippocampus, the cerebellum and the spinal cord. Three different types of grey matter lesions have been described. Type I lesions are areas of demyelination in both the cortex and the subcortical grey matter. Type II lesions are described as small perivascular demyelinated regions and type III lesions are characterized by long demyelinated strips that originate at the pial surface of the cortex, going down the third or fourth cortical layer, across several gyri. Grey matter pathology differs from white matter pathology in that an inflammatory infiltrate and BBB disruption have not been detected so far. On the contrary, transected neurites and an increased number of apoptotic cells are present in grey matter lesions (15).

1.1.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an inflammatory affliction, predominantly affecting joints, resulting in non-proliferative synovitis that often progresses to damaging of cartilage and bone, leading to diverging forms of disability. Extra-articular tissues may also be affected, including skin, heart, blood vessels and lungs (1).

RA primarily affects the small joints of hands and feet, as well as larger joints, such as ankles, knees and shoulders. Some joints are rarely affected, like the joints in the cervical spine and hip joints. Morphologically, the joints show chronic synovitis characterized by inflammatory infiltrates, synovial cell hyperplasia, increased angiogenesis, fibrin aggregates on the synovial membrane and increased osteoclast activity, eventually leading to bone erosion (Figure 3). Damage done to other articular structures - tendons, the joint capsule and ligaments - leads to the characteristic joint deformities (1)

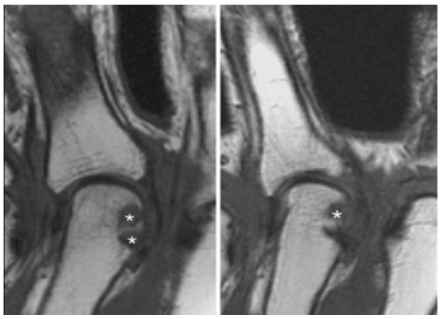

Figure 3: Articular damage in RA. MR images (T1-weighted images) of a metacarpophalangeal (MCP) joint. Areas of erosion can be clearly seen (white stars) (16).

As in MS, there is no single, well-defined cause of RA (1). It is suggested that, in a genetically predisposed person, the initial event in the development of RA is the activation of T helper cells by some kind of arthritogenic agent. This will lead to the activation of macrophages, producing degradative substances, and B cells, producing autoreactive antibodies. These autoreactive antibodies, also known as rheumatoid factor (present in approximately 80% of patients), are directed against autologous immunoglobulin (Ig) G. Besides rheumatoid factor, antibodies directed against autologous cyclic citrullinated peptides (CCP) are also present (17, 18). These antibodies show reactivity against citrullinated vimentin present in synovial tissue and synovial fluid (17).

Besides leading to the activation of other components of the immune system, T cells also drive the secondary activation of endothelium, facilitating the recruitment of inflammatory cells. Additionally, activated T cells present in RA lesions express high amounts of RANK ligand. This ligand stimulates osteoclast differentiation and activation and may thus play a crucial role in the bone resorption seen in RA patients (1).

The importance of genetic predisposition in the development of RA is suggested by a higher prevalence of RA between first-degree relatives and a high concordance rate in monozygotic twins. Additionally, the occurrence of RA is strongly associated with the presence of HLA-D4 and/or HLA-D1 (1).

At disease onset RA appears to be fairly innocent with aching and stiffness of the joints, specifically at awakening. With disease progression, the joints become swollen and enlarged, joint motion is limited and as time progresses total ankylosis, resulting in rigid and deformed joints, may occur. The exact clinical course differs between patients individually. In a minority of patients, the disease may stabilize or diminish over time. However, in the vast majority of patients the disease course will follow a chronic relapsing-remitting course (1).

1.2 The immune system in autoimmune diseases

Under normal circumstances, the immune system acquires tolerance to self-antigens via two distinct mechanisms, central and peripheral tolerance (1). In central tolerance, self-reactive B and T cells are deleted during their maturation in the central lymphoid organs, via a process known as negative selection. Negative selection results in a cell pool that is depleted of self-reactive cells. However this system isn't failure-free. Some cells may slip past the control mechanisms. For T cells, some additional peripheral control mechanisms have been identified (1); anergy, which indicates the irreversible inactivation of self-reactive T cells when the cells encounter their antigen without receiving the necessary costimulatory signal; activation-induced cell death (AICD), involving the apoptosis of activated T cells via Fas-Fas ligand interaction and finally, suppression by regulatory T cells. Regulatory T cells can suppress activated T cells via either the secretion of inhibitory cytokines such as IL-10 or by direct cell-cell contact (1).

1.2.1 *T cell development*

Autoimmune diseases arise when self-tolerance is broken down. This may occur at both the central and peripheral level. Autoreactive T cells may escape the central negative selection. Under normal

circumstances, the peripheral tolerance mechanisms now come into play. However, if these systems fail, autoreactive T cells will circulate in the body. Failure of peripheral tolerance mechanisms may occur due to defects somewhere in the Fas-Fas ligand pathway or because T cell anergy is circumvented. This may occur if cells that normally do not express costimulatory molecules necessary for T cell activation start expressing these molecules. Consequently, autoreactive T cells receive both the T cell receptor signal and the costimulatory signal, preventing T cell anergy. Lastly, it is quite possible that autoreactive T cells end up remaining in the circulation, due to diminished suppression by regulatory T cells (1).

1.2.2 *T cell activation*

There are several possible mechanisms by which T cells can become activated and lead to the onset of an autoimmune disease (1, 4). First, T cells can become activated by a process called molecular mimicry. Some pathogens' epitopes are similar to the epitopes of self-antigens. Consequently, due to the resemblance, the immune system cannot distinguish the pathogen from host tissue and the immune response will be directed against both. Secondly, sequestering of self-antigen during the development of T cells may later on cause an autoimmune reaction (1, 4). During their development, as stated above, T cells that are reactive against self-antigens are deleted. If, however, for some reason certain self-antigens are sequestered, those T cells which are reactive to these antigens will not be deleted. If the sequestered antigens are exposed later on, this may lead to the induction of an autoimmune response. Besides the physical sequestering of antigens, so-called cryptic antigens may become exposed and elicit an immune response. Every antigen has several epitopes. However, not all of these epitopes are effectively processed. During T cell development, cells reactive against the dominantly presented epitopes are normally deleted or rendered anergic. In contrast, T cells that respond to cryptic epitopes that are not expressed are disregarded. If these epitopes were presented to the immune system, due to for example tissue damage, this could elicit an immune response if the epitopes are immunogenic. This process is also known as "epitope spreading" indicating that the immune response spreads to antigens that were initially not recognized. Lastly, anergic autoreactive T cells may nonetheless be activated by superantigens (1).

1.2.3 *T cell homeostasis*

T cell homeostasis in the periphery is maintained by a process known as homeostatic proliferation. This mechanism is meant to ensure adequate numbers of all T cell types in the circulation. This built-in system is designed to ensure that the T cell diversity is such that the host is capable of responding to various antigenic challenges while maintaining memory of previously encountered pathogens (19). However, in certain situations homeostatic proliferation may also pose a threat to the host. During homeostatic proliferation all T cell types proliferate aspecifically in order to ensure adequate population numbers. However, autoreactive T cells that have escaped the central tolerance mechanisms could also start expanding, possibly resulting in the development of immune responses against host tissue (19).

1.3 Immunosenescence

Natural aging is associated with diverse changes in different physiological functions, as well as in immune function. This aging of the immune system is termed immunosenescence (IS). Both the innate and the adaptive immune system are affected by IS and some cell populations are more prone to changes than others, as reviewed by Ginaldi et al (20).

1.3.1 *Innate immune system*

Macrophages and granulocytes and their phagocytic function play a pivotal role in the primary defense against infections. The function of these cell types, however, is impaired in the elderly, rendering them more susceptible to bacterial and viral infections. Once an infection has been established, the immune system cannot adequately prevent its spread (20). Besides macrophages, neutrophils have also been reported to be subject to an overall functional decrease (21). Their phagocytic and chemotactic capacities as well as their intracellular killing capacities are diminished. On the contrary, natural killer (NK) cell function is preserved in the elderly. An age-related increase in the proportion of NK cells is observed and their cytotoxic capacities are maintained (21).

In general, the innate immune system is affected, however not heavily deteriorated, in aged individuals.

1.3.2 *Adaptive immune system: humoral immunity*

Besides the changes in the innate immune system, several changes can also be seen in the adaptive immune system. With regard to humoral immunity, several changes can be seen in the B cell compartment. For example, the serum levels of IgG and IgA are significantly increased in healthy elderly (20, 22). Additionally, a higher frequency of autoreactive antibodies can be detected (22). However, despite the increase in Ig levels, a decrease in the total number of B cells was found in healthy elderly subjects (23). This remarkable increase can be explained in several ways, such as an increase in the number of B cells and plasma cells in organs other than peripheral blood, an increase in the life-span of B cells and plasma cells in germinal centers and augmented Ig production by each B cell individually (22).

1.3.3 *Adaptive immune system: cellular immunity*

During normal aging, thymic involution occurs. On the contrary to previous assumptions, the thymus maintains functional throughout life, albeit at lower levels, as deduced from studies performed with centenarians (24). The thymic area responsible for the production of new naive T cells declines, resulting in a decreased output of these cells. This decreased thymic output possibly results from an altered thymic microenvironment and altered bone-marrow-derived thymocyte progenitors. The thymocyte progenitors change due to a reduced proliferative capacity along with a reduced interaction between the developing thymocytes and the thymal stroma (25). The decreased thymic function is possibly compensated for by other tissues. Increased numbers of mature T cells in the bone marrow during aging indicates that the bone marrow can act as an alternative site of T cell production (26). Consequently to thymic involution and its concomitant

decrease in output, T cells in the periphery will start proliferating in order to maintain adequate levels of T cells by homeostatic proliferation, as mentioned earlier. However, repeated cell proliferation leads to telomere shortening. Eventually, after several cell divisions, this and other mechanisms will lead to replicative senescence of T cells.

Globally, the T cell population in the elderly has undergone a shift from a predominantly naive phenotype to a non-proliferating memory phenotype (24, 25, 27). Additional to this quantitative change in the memory population, several qualitative changes in the memory cell population are also found in aged people (28).

1.3.4 *$CD28^{NULL}$ T lymphocytes*

The most remarkable qualitative change is the appearance of $CD28^{NULL}$ cells. These senescent cells have three main characteristics: altered function, further elaborated on below, shortened telomeres resulting in proliferative arrest and an increased resistance to apoptotic stimuli (29, 30).

CD28 is a costimulatory molecule expressed on T cells, serving to amplify the T cell receptor signal, which will in turn lead to the proliferation of T cells and the release of several immune mediators, such as interleukin-2 (IL-2) and interferon γ (IFN-γ). Hence, CD28 is crucial for the induction and the maintenance of T cell-mediated immune responses (31, 32). Loss of CD28 occurs on both $CD4^+$ and $CD8^+$ T lymphocytes and can be caused by several mechanisms. $CD28^{NULL}$ T cells can appear as a consequence of the aforementioned thymic involution and its concomitant homeostatic proliferation. CD28 expression is also lost due to clonal expansions as a result of persistent infections by pathogens such as Epstein Barr virus (EBV) or cytomegalovirus (CMV) (31, 33). Clonally expanded T cell populations may persist and accumulate for years, resulting in contraction of the T cell repertoire. Additionally, clonally expanded T cells compete with other cells for growth factors and space in such a way that the growth and activation of naive T cells can be inhibited (31).

Molecular studies have shown that the loss of CD28 is due to transcriptional silencing of its encoding gene (34, 35). Specifically, the transcription ceases due to lack of a functional complex between two proteins, nucleolin and ribonucleoprotein D0, necessary for the transactivation of the initiator region of the CD28 gene promoter (36). Remarkably, CD28 downregulation occurs faster in the $CD8^+$ population than in the $CD4^+$ population. This differential loss is associated with inactivation of telomerase causing the $CD8^+$ population to reach replicative senescence faster than their $CD4^+$ counterparts (37).

Part of the CD28 loss may reflect the microenvironment the cells reside in, like for example the cytokine environment. Warrington et al (38) have shown that when $CD4^+CD28^{NULL}$ T cells are exposed to IL-12 they are able to rejuvenate and regain the expression of CD28 associated with their ability to upregulate CD40L.

Concomitant with the CD28 downregulation, $CD4^+$ as well as $CD8^+$ T lymphocytes gain several new aberrant functions which contribute to immune abnormalities often related to disease. First of all, the cells acquire large intracellular stores of IFN-γ (39) and express several surface molecules that

(in)directly play a role in tissue invasion. Additionally, $CD28^{NULL}$ T cells express a variety of killer cell immunoglobulin-like receptors (KIR) which are normally present on NK cells (38). Finally, intracellular stores of granzyme and perforin are present, pointing towards cytotoxic capabilities (40, 41). Interestingly, $CD8^+CD28^{NULL}$ T cells gain suppressive capacities comparable to regulatory T cells in abrogating or reducing ongoing immune responses. However, $CD8^+CD28^{NULL}$ T cells, unlike regulatory T cells, do not exert their suppressive effects by producing suppressive cytokines, but by inhibiting the function of antigen-presenting cells by means of a downregulation of accessory molecules such as CD40 and CD86 (42).
Frequencies of $CD28^{NULL}$ T cells are not equal among different age groups. In younger individuals, $CD4^+$ $CD28^{NULL}$ T cells are rarely found. On the contrary, their frequency may rise up to 70 % in people in their fifties. For the $CD8^+$ compartment, the situation is quite different. In young individuals, 20-30% of $CD8^+$ cells has already lost CD28 and their frequency can increase to 95% in the elderly (43). Clonal populations of $CD28^{NULL}$ T cells are maintained for a long time, due to these cells' increased resistance to apoptosis, as demonstrated by resistance to AICD (44, 45) as opposed to their $CD28^+$ counterparts (46).

1.4 $CD28^{NULL}$ cells in correlation with autoimmune disease: cause or consequence?

Several explanations can be made for the increased presence of autoimmune diseases in the elderly in general, reviewed by Hasler and Zouali (47). First, an increased exposure of the immune system to higher than normal concentrations of autoantigens, due to defective clearance, may lead to an overactivation of lymphocytes (47). Additionally, ongoing inflammatory reactions may break down or alter anatomical barriers, releasing previously sequested self-antigen.

Several studies show that the proportion of $CD4^+CD28^{NULL}$ T cells is age-inappropriately increased in patients with several autoimmune diseases, such as MS or RA (48-50). This indicates that the naturally occurring process of immune aging is accelerated in patients suffering from these diseases. Additionally, in RA, the occurrence of this cell population is correlated with disease severity, concerning joint involvement and extra-articular disease manifestations (51). Research aimed to determine whether these $CD4^+CD28^{NULL}$ T cells are in fact autoreactive and could thus play a role in the pathogenesis of autoimmune diseases yielded contradictory results. Studies performed by Schmidt et al. (52) indicate that $CD4^+CD28^{NULL}$ T cells in RA patients are consistently autoreactive. In this study, $CD4^+CD28^{NULL}$ T cells are shown to be reactive against autologous antigen-presenting cells, suggesting a reactivity towards self-antigens. However, a more recent study performed by Thewissen et al. (53), regarding both MS and RA, shows that $CD4^+CD28^{NULL}$ T cells do not show reactivity to auto-antigens like myelin basic protein (MBP) and human type II collagen (hCII) in specific, this being in contradiction to results obtained by Markovic-Plese et al (48) which show that MBP-specific T cells were present in the $CD4^+CD28^{NULL}$ T cell population of all tested MS patients.

Despite contradictory results concerning their autoreactivity, $CD4^+CD28^{NULL}$ T cells display several characteristics which implicate a possible role in autoimmune diseases. The general phenotype of $CD4^+CD28^{NULL}$ T cells is altered compared to $CD4^+CD28^+$ T cells. Previously performed research has

shown that the expression of several surface markers differs between the two populations. In comparison to $CD4^+CD28^+$ T cells, the amount of $CD4^+CD28^{NULL}$ T cells expressing integrin α4, important for the interaction with the extracellular matrix, as well as the expression level of integrin α4 on this cell subset is increased. Additionally, a smaller number of $CD4^+CD28^{NULL}$ T cells expresses L-selectin, important in the homing process to the lymph nodes, in HC as well as MS and RA patients (53). Surface characterization experiments performed by Fasth et al (54) show an increased surface expression of both complement receptor type 3 and CD57, an NK cell receptor, on $CD4^+CD28^{NULL}$ T cells in comparison to $CD4^+CD28^+$ T cells in RA patients. This study also shows the presence of both CD45RA and CD45RO positive cells within the $CD4^+CD28^{NULL}$ T cell population. Studies performed by Myazaki et al. (49) indicate that $CD45RO^+$ cells are increased in the $CD4^+CD28^{NULL}$ T cell population in both HC and MS patients when compared to the $CD4^+CD28^+$ T cell population. Also they appear to be recently activated, as shown by a relatively low expression of CD69, a marker indicative for early activation (54). This was corroborated by research performed in MS patients by Markovic-Plese et al. (48) which shows that activation markers, like CD25 and CD69 are not constitutively expressed in the $CD4^+CD28^{NULL}$ T cell population. These phenotypical characteristics, along with a vigorous proliferative boost in response to anti-CD3 (aCD3) activation, independent of costimulatory factors, indicate that $CD4^+CD28^{NULL}$ T cells are memory-type effector T cells (54).

As mentioned earlier, $CD4^+CD28^{NULL}$ T cells obtain several aberrant characteristics, one of which is the acquisition of killing receptors (38, 55). $CD4^+CD28^{NULL}$ T cells present in RA patients have been shown to express a variety of these receptors, with KIR2DS2, a stimulatory receptor, being particularly prominent (55). Moreover, the expression of KIR2DS2 has been shown to be a risk factor for extra-articular manifestations in RA (55). Besides its presence in patients suffering from RA, the presence of $CD4^+CD28^{NULL}$ T cells expressing KIR2DS2 is also correlated with disease severity in patients affected with coronary artery diseases, such as unstable angina. The presence of $CD4^+CD28^{NULL}$ T cells expressing KIR2DS2 in these patients is often accompanied by the absence of its inhibitory counterparts, KIR2DL2 and KIR2DL3 (56).

Crosslinking of KIR2DS2 can boost the proliferation of $CD4^+CD28^{NULL}$ cells and enhances IFN-γ production, pointing towards a costimulatory function for KIR2DS2 (30). $CD4^+CD28^{NULL}$ cells in RA patients, in contrast to $CD4^+CD28^{NULL}$ cells in MS patients (49), have also been shown to express NKG2D, a stimulatory receptor normally only present on NK cells, $CD8^+$ T cells and γδ-T cells (57). The expression of NKG2D can stimulate autoreactivity, as shown by Groh et al. (57). The selective expression of stimulatory KIRs, like NKG2D and KIR2DS2, can also provide costimulatory function to $CD4^+CD28^{NULL}$ T cells (57, 58).

Next to the expression of killing receptors, $CD4^+CD28^{NULL}$ T cells in RA patients also express the fractalkine receptor (CX_3CR1) (59), its expression usually being restricted to NK cells and $CD8^+$ T cells. Its chemokine ligand is expressed in the CSF and blood. In inflammatory neurological conditions, fractalkine (CX_3CL1) levels are markedly increased in the CSF. In MS, serum CX_3CL1 levels are also increased (60). Next to this increase in soluble CX_3CL1 levels, the membrane-bound form of CX_3CL1 is also increased in inflammatory neurological conditions (60). In vitro studies performed by Fraticelli et al. (61) indicated that CX_3CL1 can be expressed and/or shedded from

cerebral endothelial cells in response to inflammatory mediators secreted during CNS inflammation, such as IFN-γ and TNF-α.

It is generally thought that CX_3CL1, due to its two separate forms, plays a dual role. In its soluble form, it serves as a chemoattractant for cells expressing its compatible receptor, while in its membrane-bound form it has an adhesion function. Recent work performed in RA patients by Sawai et al. (62) points to additional functions for this chemokine. In RA synovium, the main source for CX_3CL1 are fibroblast-like synovial cells (FLS). Moreover, $CD4^+CD28^{NULL}$ T cells expressing CX_3CR1 have been shown to be present in the synovium. These T cells strongly adhere to membrane-bound CX_3CL1 present on FLS, resulting in the amplification of activation signals and subsequent T cell proliferation, IFN-γ production and granule expulsion, indicating a costimulatory role for CX_3CR1. Synoviocytes expressing CX_3CL1 also provide survival signals for $CX_3CR1^+CD4^+$ T cells recruited to the synovium (63).
Additional to affecting T cell function, the CX_3CR1- CX_3CL1 interaction also affects the synovium itself. The binding of $CD4^+CD28^{NULL}$ T cells to FLS activates a growth promoting loop, leading to the proliferation of FLS.

Next to being a chemoattractant, soluble CX_3CL1 may also have an antagonistic function (63). If soluble CX_3CL1 binds its receptor on $CD4^+CD28^{NULL}$ T cells before they reach the membrane-bound CX_3CL1 on FLS, the receptor's antigen binding site will be occupied when the cells reach the FLS. This way, interactions between the T cells and the synovium are prevented and the costimulatory and growth-promoting effects of CX_3CL1 can no longer take place.

Next to receptor acquisition, intracellular stores of cytotoxic substances such as perforin and granzyme have been found in $CD4^+CD28^{NULL}$ T cells (30, 41, 49). Additionally, upon activation, $CD4^+CD28^{NULL}$ T cells present in MS patients produce large amounts of IFN-γ (49). Together, these findings indicate that these cells may be capable of eliciting tissue damage. Indeed, research has shown that $CD4^+CD28^{NULL}$ T cells are capable of killing endothelial cells in acute coronary syndromes (40). Additionally, preliminary data obtained by Thewissen et al (53) indicate that $CD4^+CD28^{NULL}$ T cells are capable of killing synovial fibroblasts and oligodendroglial cells, target cells in RA and MS respectively.

Another finding giving a possible indication for the involvement of $CD4^+CD28^{NULL}$ T cells in autoimmune diseases is the increased expression of costimulatory CD70 on $CD4^+CD28^{NULL}$ T cells in HC and RA patients and its sustained post-activational overexpression on $CD4^+CD28^{NULL}$ T cells. This is in contrast to $CD4^+CD28^+$ T cells, in which the initial upregulation of CD70 expression after activation is reverted some days after activation (64). The continuous post-activational expression of CD70 on $CD4^+CD28^{NULL}$ T cells is unrelated to promoter demethylation patterns nor does the (re)methylation pattern provide a clue as to why the CD70 expression on $CD4^+CD28^{NULL}$ T cells fails to return to its original state (64).

CD70 is responsible for starting the primary T cell response. An overexpression of CD70 lowers the activation threshold of immune responses which possibly leads to the activation of previously quiescent autoreactive T cells (64). Evidence for the capability of $CD4^+CD28^{NULL}$ T cells to provide

bystander activation via CD70 was also provided by Lee et al. (64). TCR-stimulated PBMC were cocultured with $CD4^+CD28^{NULL}$ T cells of the same donor, after which increased proliferation of the activated PBMC could be observed, which was completely abrogated when an antibody against CD70 was added to the culture.

Taken together, these findings implicate a possible role for $CD4^+CD28^{NULL}$ T cells in the pathogenesis of autoimmune diseases such as MS or RA. This role may be direct via cytotoxic processes or indirect, by for example bystander activation of previously quiescent autoreactive T cells.

1.5 Hypothesis and research goal

This research focuses on senescent $CD4^+CD28^{NULL}$ T cells. The function of $CD4^+CD28^{NULL}$ T cells and their role in the pathogenesis of autoimmune diseases are not fully understood so far. Based on the findings and conclusions of previously performed research, discussed above, it is hypothesized that $CD4^+CD28^{NULL}$ T cells present in patients with autoimmune diseases such as MS or RA are capable of migrating towards chemokines expressed in the CNS at sites of inflammation and that the $CD4^+CD28^{NULL}$ T cells are able to cause damage to the residing cell populations, possibly through bystander effects.

To assess this hypothesis, both phenotypical and functional characteristics of $CD4^+CD28^{NULL}$ T cells will be investigated in HC as well as MS and RA patients and compared to their $CD4^+CD28^+$ counterparts.

The phenotype of $CD4^+CD28^{NULL}$ T cells will be assessed by determining the expression of several surface markers on $CD4^+CD28^{NULL}$ T cells. Functionally, both their chemotactic capabilities and their potential to elicit bystander activation will be investigated. The chemotactic capability, providing an indication for the ability of $CD4^+CD28^{NULL}$ T cells to migrate into sites of inflammation, will be determined by performing transwell chemotaxis assays. The potential of $CD4^+CD28^{NULL}$ T cells to provide bystander activation to other, possibly autoreactive, T cells will be assessed by monitoring the expression pattern of costimulatory CD70 after in vitro activation of $CD4^+CD28^{NULL}$ T cells.

This study will provide novel insight into the role of $CD4^+CD28^{NULL}$ T cells in the pathogenesis of autoimmune diseases, such as MS and RA.

2. Material and methods

2.1 Patient and control populations

Blood samples were collected from 24 healthy subjects, 127 RA patients, and 27 MS patients. Specific data from all groups are summarized in table 1. Blood samples were also obtained from 1 SLE patient and 1 PsA (psoriatic arthritis) patient. Informed consent was provided by all patients.

Table 1: General donor data.

	HC	MS	RA
Blood samples	31	28	127
Males	12	11	90
Females	19	17	37
female/male ratio	1.6	1.6	2.4
Mean age	27.6±7.4	47.5±12.0	60.2±11.8
Mean age males	27.9±10.5	48.4±16.5	60.2±10.5
mean age females	24.9	47±9.3	60.2±12.2

Disease status: MS patients: 2 CIS, 1 likely CIS, 3 RRMS, 10 SPMS, 14 unknown. RA patients: unknown.
Treatment status: MS patients: 2 untreated, 11 treated, 15 unknown. RA patients: unknown.
SLE and SPA patient: data unknown.
*Cut-off value: 1.5 % (or clearly delineated cell population).

2.2 Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood using Ficoll Histopaque (Sigma-Aldrich, Belgium) density centrifugation. After extensive washing in RPMI 1640 medium (GIBCO (Invitrogen) Belgium), cells were maintained in culture medium (RPMI 1640 supplemented with 10 % fetal calf serum (FCS; Hyclone, Belgium), 1% non-essential amino acids (GIBCO), 1% sodium pyruvate (GIBCO) and 0.5% penicillin/streptomycin (GIBCO)) prior to performing experiments.

2.3 Cell sorting

For some experiments, separate populations of $CD4^+CD28^+$ and $CD4^+CD28^{NULL}$ T cells were used. For these experiments, $CD4^+$ T cells were enriched from whole peripheral blood using RosetteSep® (StemCell Technologies, Germany), according to manufacturer's protocol. After isolation, cells were sorted into the two aforementioned populations, using the FACSAria high speed cell sorter (BD Biosciences, Belgium).

2.5 Flow cytometry

Flow cytometry was performed for screening of patients for $CD4^+CD28^{NULL}$ T cells, characterization of these cells, analysis of CD70 kinetics and chemotaxis assays. After washing away all culture

medium remnants with FACS buffer (phosphate buffered saline containing 2% FCS, and Na-azide) cells were stained with either CD4-PerCP and CD28-PE, CD4-PerCP and CD28-FITC or CD4-FITC and CD28-PE (all BD Biosciences, Belgium). In addition cells were stained with CD70-FITC (BD Biosciences) for CD70 kinetics, and CD25-FITC, CD103-FITC, CD31-FITC, CD45RB-FITC, CD49d-FITC, CD62L-FITC, CD44-FITC (all Immunotools Germany) CD11a-FTIC, TCR-α/β-FITC, (both BD Biosciences), CD127-FITC, CD80-FITC (both eBioscience, USA), CD45RO-PE, CD16-PE, TCR-γ/δ-PE, CD56-PE, NKG2D-PE (all BD Biosciences), CD54-PE (eBioscience), CD69-PE (Immunotools), IL-23R-PE, NKG2A-PE, NKG2C-PE (all R&D Systems, Belgium) or CX_3CR1-PE (MBL, Japan) for characterization of $CD4^+CD28^{NULL}$ T cells.

Marker expression was detected using the FACSCalibur (BD Biosciences) and subsequently analysed using the Cellquest software.

2.5 CD70 kinetics

For kinetics experiments, FACS-sorted $CD4^+CD28^+$ or $CD4^+CD28^{NULL}$ T cells were maintained in culture medium in the presence of inactivated feeder cells (PBMC; ratio 1:10) and 5 U/ml interleukin 2 (IL-2, Roche, Belgium) with or without 2 µg/ml anti-CD3 (aCD3, clone 2G3, BIOMED, Belgium) or 5 µg/ml phytohemaglutinin (PHA; Sigma, Belgium) for 14 days. At different time points during the 14-day culture, $CD4^+CD28^+$ and $CD4^+CD28^{NULL}$ T cells were monitored for CD70 expression. Cells from both cell populations were stained with CD4-PerCP, CD28-PE and CD70-FITC (all BD Biosciences) and analysed by flow cytometry.

2.7 Characterization of $CD28^{NULL}$ cells

To further characterize $CD4^+CD28^{NULL}$ T cells, the expression of several surface markers was assessed. PBMC were stained with CD25, CD103, CD31, CD45RB, CD49d, CD62L, CD44, CD11a, TCR-α/β, CD127, CD80, all FITC-labeled, in combination with CD4-PerCP and CD28-PE. CD45RA, CD45RO, CD16, TCR-γ/δ, CD56, NKG2D, CD54, CD69, IL-23R, NKG2A, NKG2C and CX_3CR1, all PE labeled, were tested in combination with CD4-PerCP and CD28-FITC. A detailed list of all markers and their biological function is depicted in Table 2.

Table 2: Markers used for characterization of $CD4^+CD28^{NULL}$ T cells

Marker	Function	Marker	Function
IL-2R α chain (CD25)	Growth, differentiation	Hermes antigen (CD44)	Adhesion, homing
Fc-receptor (CD16)	Antibody binding	B7.1(CD80)	Costimulation
AIM (CD69)	Lymphocyte proliferation	Integrin αE(CD103)	Lymphocyte-endothelium interaction
LFA-1 (CD11a)	Cellular adhesion	Integrin α4 (CD49d)	Lymphocyte-endothelium interaction
IL-7R (CD127)	Lymphocyte maturation	PECAM-1 (CD31)	Adhesion, RTE marker
NKG2C (CD159c)	NK cell receptor	IL-23R	Receptor for IL-23
NKG2A (CD159a)	NK cell receptor	L-selectin (CD62L)	Lymphocyte homing
Ki-24 (CD70)	Costimulation	CD45RA	Marker naïve T cells
CD45RO	Marker mature T cells	CD45RB	CD45 isoform
NCAM (CD56)	Cell-cell adhesion	ICAM-1 (CD54)	Adhesion molecule
TCR-αβ	Antigen recognition	TCR-γδ	Antigen recognition
NKG2D (CD314)	NK cell receptor		

2.8 Chemotaxis assay

To determine the chemotactic potential of $CD4^+CD28^{NULL}$ T cells, a transwell chemotaxis assay was performed. PBMC ($1x10^6$ cells) were seeded in the upper chamber of a 5.0 µm-pore polycarbonate transwell (Costar, the Netherlands). Chemotaxis buffer (RPMI 1640 containing 0.5 % bovine serum albumin (BSA; USBiological, USA)) with 1 ng/ml CX_3CL1 (Peprotech, USA) was added in the bottom well. Migration proceeded for 3h at 37°C, 5 % CO_2.

At the end of the chemotaxis assay, the migrated cells, present in the lower well of the transwell system, were counted using a Bürker counting chamber. Also, both the migrated cells in the lower well and the non-migrated cells in the upper chambre were stained with CD4-PerCP and CD28-PE and analysed using flow cytometry.

2.9 Statistical analyses

All statistical analyses were performed using GraphPad Prism 4.0. For all experiments, unpaired t-tests were applied. When required, Welch's correction was incorporated in the analysis. Differences were considered significant when $p<0.05$.

3. Results

To investigate the hypothesis that $CD4^+CD28^{NULL}$ T cells possible play a role in the pathogenesis of autoimmune diseases, phenotypical as well as functional characteristics of $CD4^+CD28^{NULL}$ T cells were assessed. Specifically, after determining the percentage of $CD4^+CD28^{NULL}$ T cells in peripheral blood of the currently investigated donor populations, the expression of several surface markers and the expression of the chemokine receptor CX_3CR1 was assessed as well as the chemotactic capacity of $CD4^+CD28^{NULL}$ T cells and their ability to provide bystander activation to other T cells. The results which were obtained in the current study are presented.

3.1 $CD4^+CD28^{NULL}$ T cells in peripheral blood

As several groups have previously shown, the senescent $CD4^+CD28^{NULL}$ T cells are enriched in patients with several autoimmune diseases (48-50). To determine the frequency of $CD4^+CD28^{NULL}$ T cells in the peripheral blood of the persons in our study group (31 healthy controls (HC), 28 MS patients, 127 RA patients, 1 PsA patient and 1 SLE patient), PBMC were isolated and screened for the presence of $CD4^+CD28^{NULL}$ T cells by a flow cytometric approach. The percentage of $CD4^+CD28^{NULL}$ T cells within the $CD4^+$ T cell population was subsequently determined (Figure 4). Of all screened samples, 9 HC, 8 MS patients, 42 RA patients and 1 SLE patient possess $CD4^+CD28^{NULL}$ T cells (Table 3).

Table 3: Donors with $CD4^+CD28^{NULL}$ T cells. Data for donors with $CD4^+CD28^{NULL}$ T cell percentages above cut-off are presented.

	HC	MS	RA
Samples with $CD4^+CD28^{NULL}$ T cells*			
Absolute number	9	10	42
Males	4	4	14
Females	5	6	28
% of total blood samples	29	35.71	33
Mean % $CD4^+CD28^{NULL}$ T cells	7.4 ± 3.4	5.7 ± 6.5	9.9 ± 9.7
Mean % males	4.27 ± 1.6	6 ± 9	6.9 ± 4.1
Mean % females	9.4 ± 4.4	5.4 ± 5.1	11.2 ± 11.2

*Cut-off value: 1.5 % (or clearly delineated cell population).

In this study, it can be confirmed that the percentage of $CD4^+CD28^{NULL}$ T cells is significantly increased in RA patients in comparison to HC ($p=0.0167$) (Figure 4). For all further experiments, only donors with a $CD4^+CD28^{NULL}$ T cell population above 1.5 % of the total $CD4^+$ T cell population (Table 3) are considered. When the $CD4^+CD28^{NULL}$ T cell percentage is below this cut off value, but still a clearly defined $CD4^+CD28^{NULL}$ T cell population is present (Figure 5), donors are also included.

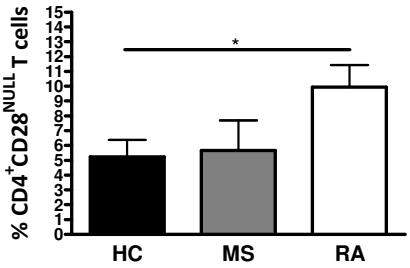

Figure 4: CD4+CD28NULL T cells are increased in patients suffering from rheumatoid arthritis. Mean percentage CD4+CD28NULL T cells for HC (n=9), MS patients (n=10) and RA patients (n=25) is shown. *: p<0.05

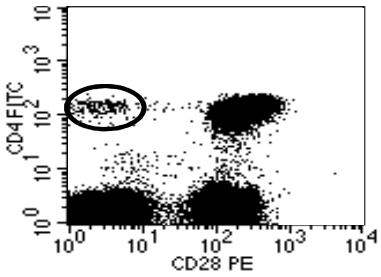

Figure 5: Clearly defined CD4+CD28NULL T cell population. Dot plot showing a clearly defined CD4+CD28NULL T cell population (circle) of 1 MS patient with a CD4+CD28NULL T cell percentage below the cut-off value.

3.2 Phenotypical characterization of CD4+CD28NULL T cells

Several groups have previously shown that several surface markers differ in their expression level on CD4+CD28NULL and CD4+CD28+ T cells (48, 49, 53, 54). This, however, has never been extensively determined for MS patients and HC. Therefore, to further characterize CD4+CD28NULL T cells in HC, RA and MS patients, the expression of several surface markers was assessed via flow cytometry, as described in material and methods (Table 2). Both the percentage of CD4+CD28NULL T cells expressing these surface markers and their expression level were determined.

3.2.1 *Surface marker expression*

Initially, the expression of 23 different surface markers on CD4+CD28NULL T cells of HC (n=4) was determined, as described in material and methods (Table 2). Of these 23, the 12 most relevant markers were chosen to expand the study population to MS and RA patients. In total, cells of 9 HC, 10 MS patients and 25 RA patients were phenotyped using these 12 markers.

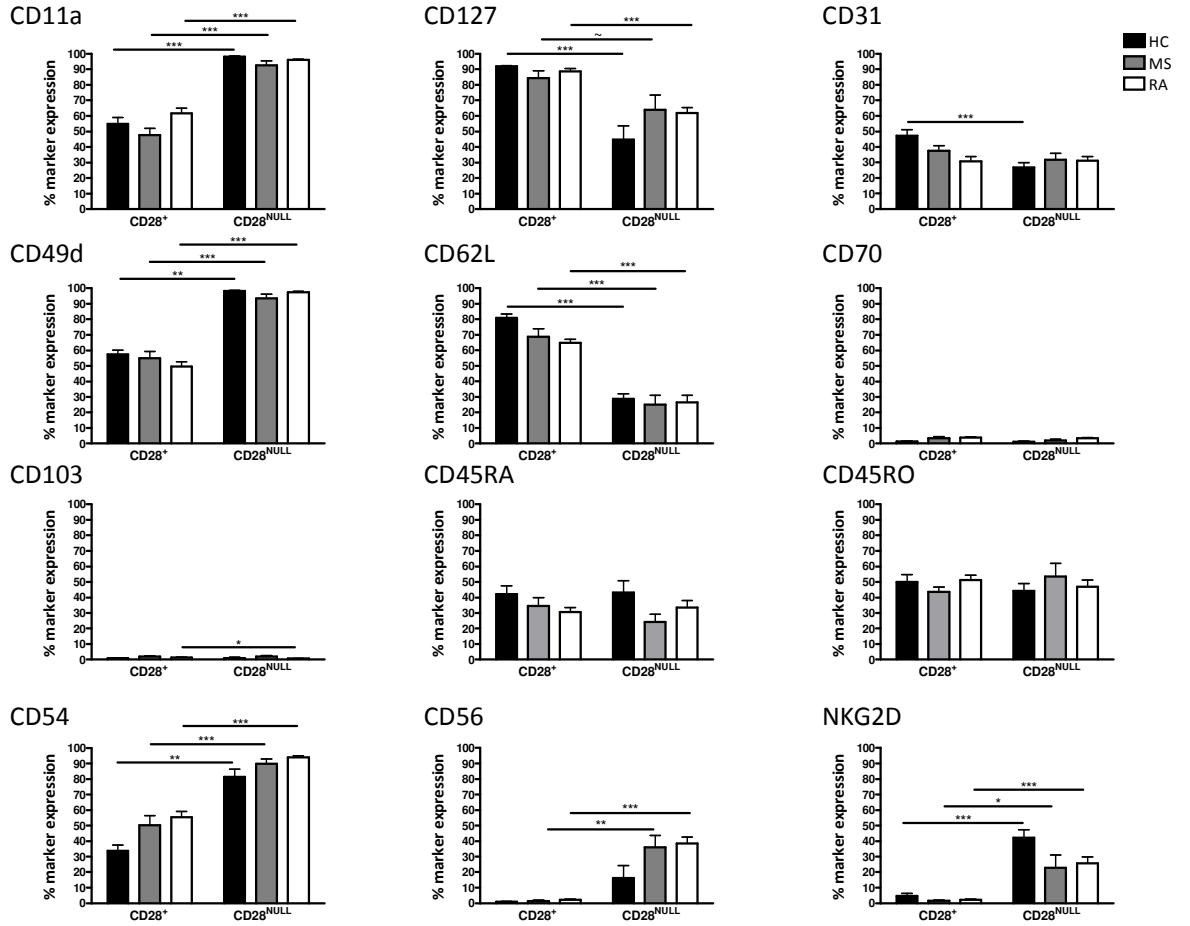

Figure 6: Adhesion molecules are present on a larger population of CD4^{+}CD28^{NULL} T cells. Results are shown for HC (n=9), MS patients (n=10) and RA patients (n=25). *:p<0.05, **: p<0.01, ***: p<0.001, ~: 0.05<p<0.1 (trend towards significance).

As shown in Figure 6, the CD4^{+}CD28^{NULL} T cell fraction expressing several adhesion molecules is significantly increased in comparison to the CD4^{+}CD28^{+} T cell fraction. Respresentative dot plots for those markers with the clearest difference in expression between the two cell populations are shown in Figure 7. Specifically, in all 3 donor groups, the CD4^{+}CD28^{NULL} T cell fraction is increased for the expression of LFA-1 (CD11a, p<0.0001), α4 integrin (CD49d, p<0.0001; for HC: p=0.0015) and ICAM-1 (CD54, p<0.0001; for HC: p=0.0073). Additionally, the CD4^{+}CD28^{NULL} T cell fraction expressing NCAM (CD56) is significantly increased in MS patients (p=0.0100) and RA patients (p<0.0001). A significant decrease is seen in all three donor groups for L-selectin (CD62L, p<0.0001). In addition, in HC, the CD4^{+}CD28^{NULL} T cell fraction expressing PECAM-1 (CD31) is significantly decreased (p=0.0006) and in RA patients, the CD4^{+}CD28^{NULL} T cell fraction expressing αE integrin (CD103) is significantly decreased (p=0.0276). A decreased CD4^{+}CD28^{NULL} T cell fraction expressing the IL-7R (CD127) can also be observed in HC (p=0.0007) and RA patients (p<0.0001).

Additional to differences in surface markers generally found to be expressed in the T cell population, an increased fraction of CD4^{+}CD28^{NULL} T cells expresses NKG2D, an NK cell receptor, in HC (p=0.0001), RA patients (p<0.0001) and MS patients (p=0.0317).

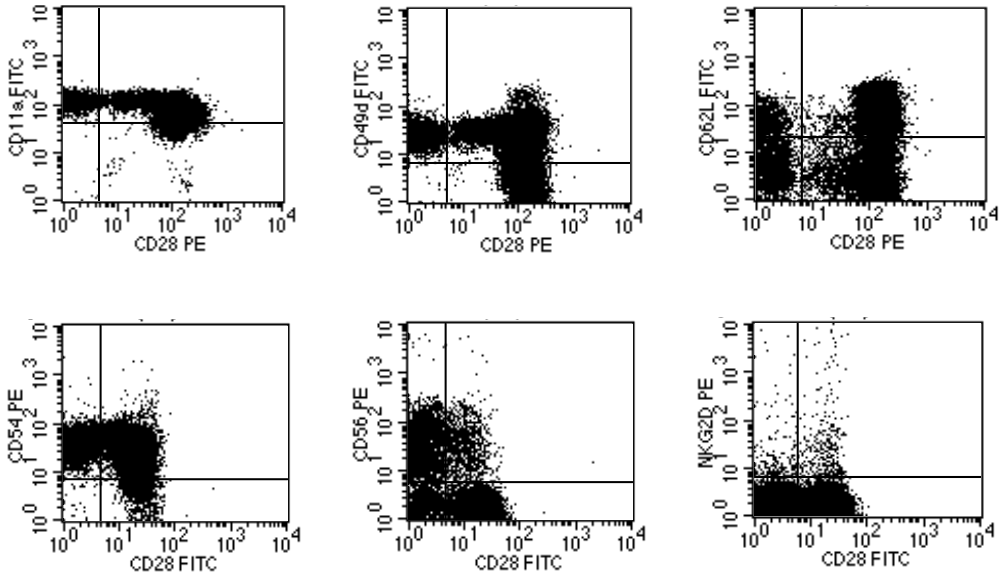

Figure 7: Significantly different surface markers. Representative dot plots are shown for the most significantly differing markers. The expression of the indicated markers on $CD4^+$ T cells is shown for 1 MS patient.

Next to the differences observed in the $CD4^+CD28^{NULL}$ and $CD4^+CD28^+$ T cell population, differences are also found when comparing these two cell populations in HC on the one hand and MS patients (Figure 8) or RA patients (Figure 9) on the other hand.

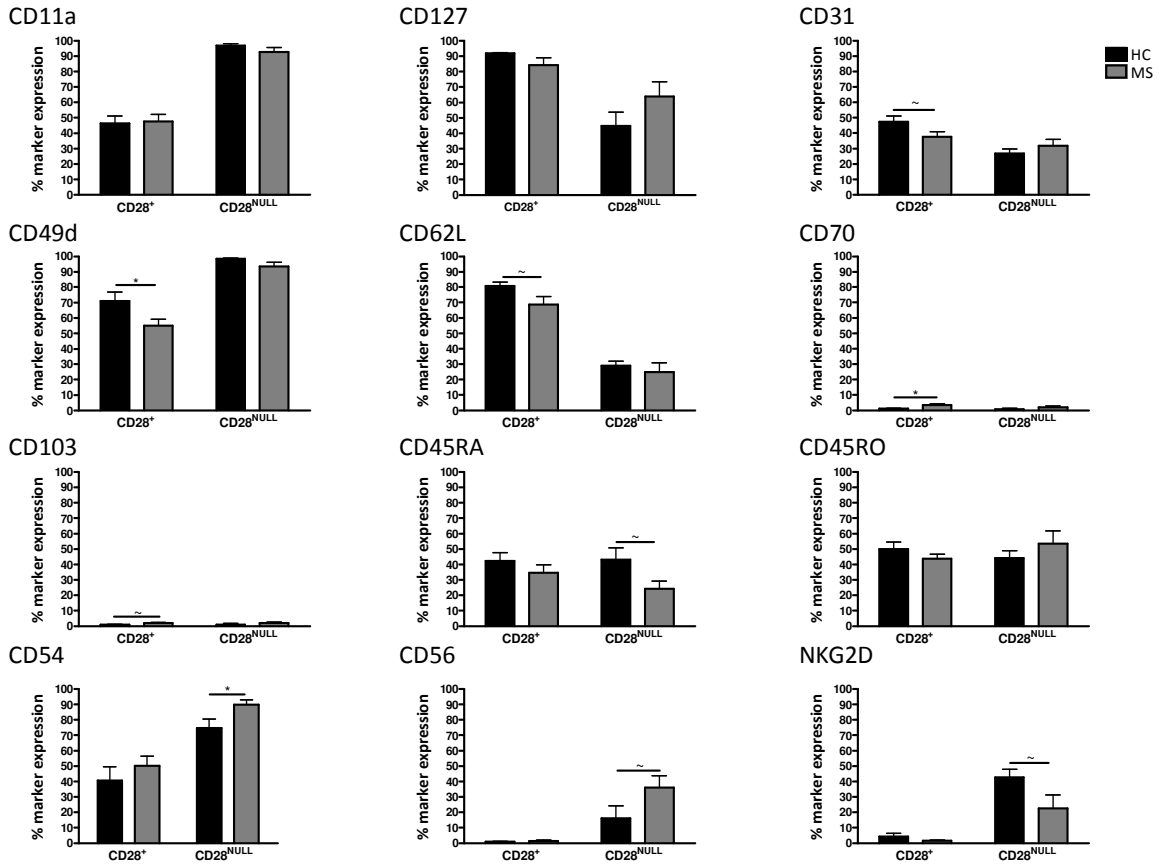

Figure 8: General surface marker expression on $CD4^+CD28^{NULL}$ T cells does not differ between HC and MS patients. The fraction of $CD4^+CD28^{NULL}$ and $CD4^+CD28^+$ T cells expressing the indicated surface markers in HC (n=9) compared to MS patients (n=10) is presented. *: $p<0.05$, ~: $0.05<p<0.1$ (trend towards significance).

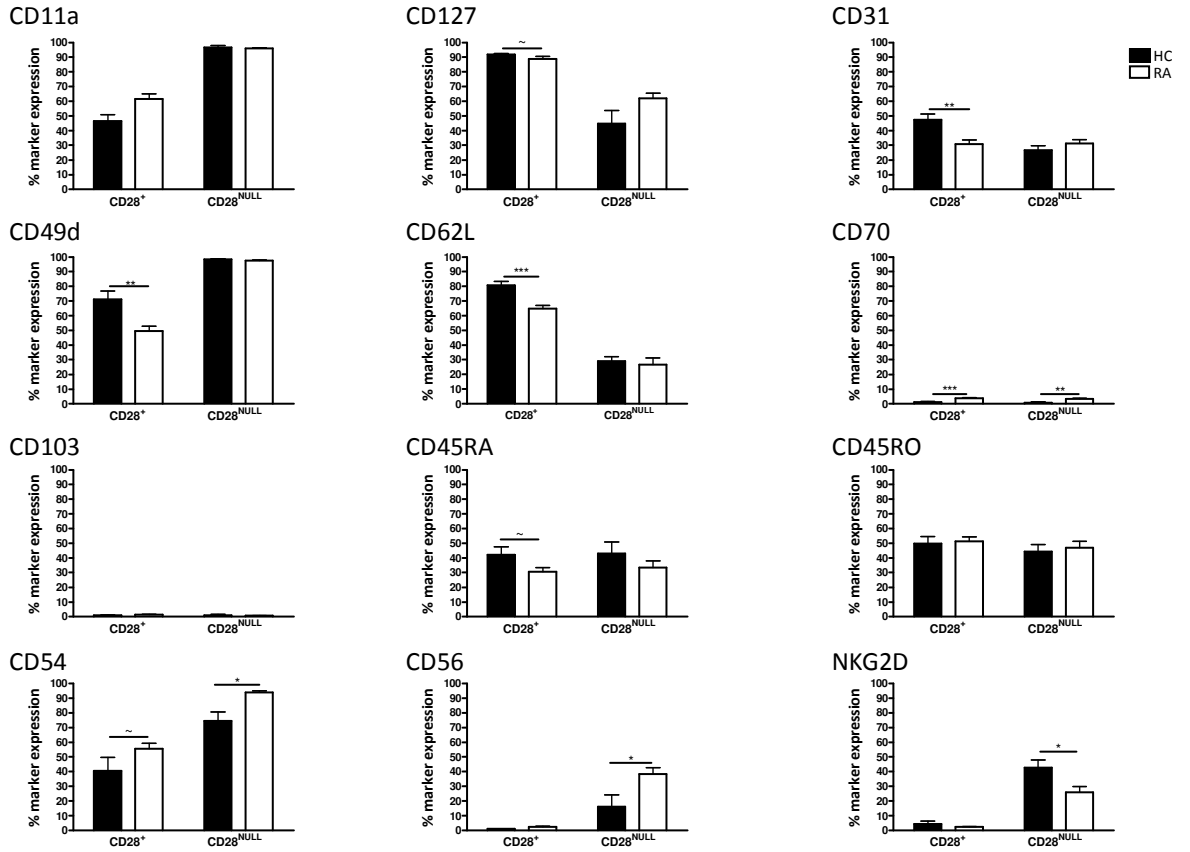

Figure 9: Several surface markers are differentially expressed on CD4+CD28NULL T cells in HC and RA patients. The fraction of CD4+CD28NULL and CD4+CD28+ T cells expressing the indicated surface markers in HC (n=9) compared to RA patients (n=25) is presented. *: p<0.05, **: p<0.01, *** p<0.001, ~: 0.05<p<0.1 (trend towards significance).

In general, when comparing the CD4+CD28NULL T cell population in HC and MS patients (Figure 7), few significant differences can be found for the markers studied here. Only the CD4+CD28NULL T cell fraction expressing ICAM-1 (CD54) is significantly increased in MS patients (p= 0.0290). When considering the CD4+CD28+ T cell population for these two study populations, the cell fraction expressing α4 integrin (CD49d), is significantly decreased in MS patients (p=0.0378) and the cell fraction expressing Ki-24 (CD70) is significantly increased in MS patients (p=0.0396).

When comparing the CD4+CD28NULL T cell population in HC and RA patients (Figure 8), the cell fraction expressing ICAM-1 (CD54, p=0.0153), NCAM (CD56, p=0.0123) and Ki-24 (CD70, p=0.0041) is significantly increased in RA patients. Additionally, the cell fraction expressing NKG2D is significantly decreased (p=0.0359). Looking at the CD4+CD28+ T cell population, a decreased cell fraction expressing PECAM-1 (CD31, p=0.0052), α4 integrin (CD49d, p=0.0015) and L-selectin (CD62L, p=0.0004) and an increased cell fraction expressing Ki-24 (CD70, p=0.0001) is observed.

3.2.1 *Surface marker expression level*

Aside from the percentage of cells expressing the studied markers, the expression level of these markers was also determined using the mean fluorescence intensity (MFI) of the markers.

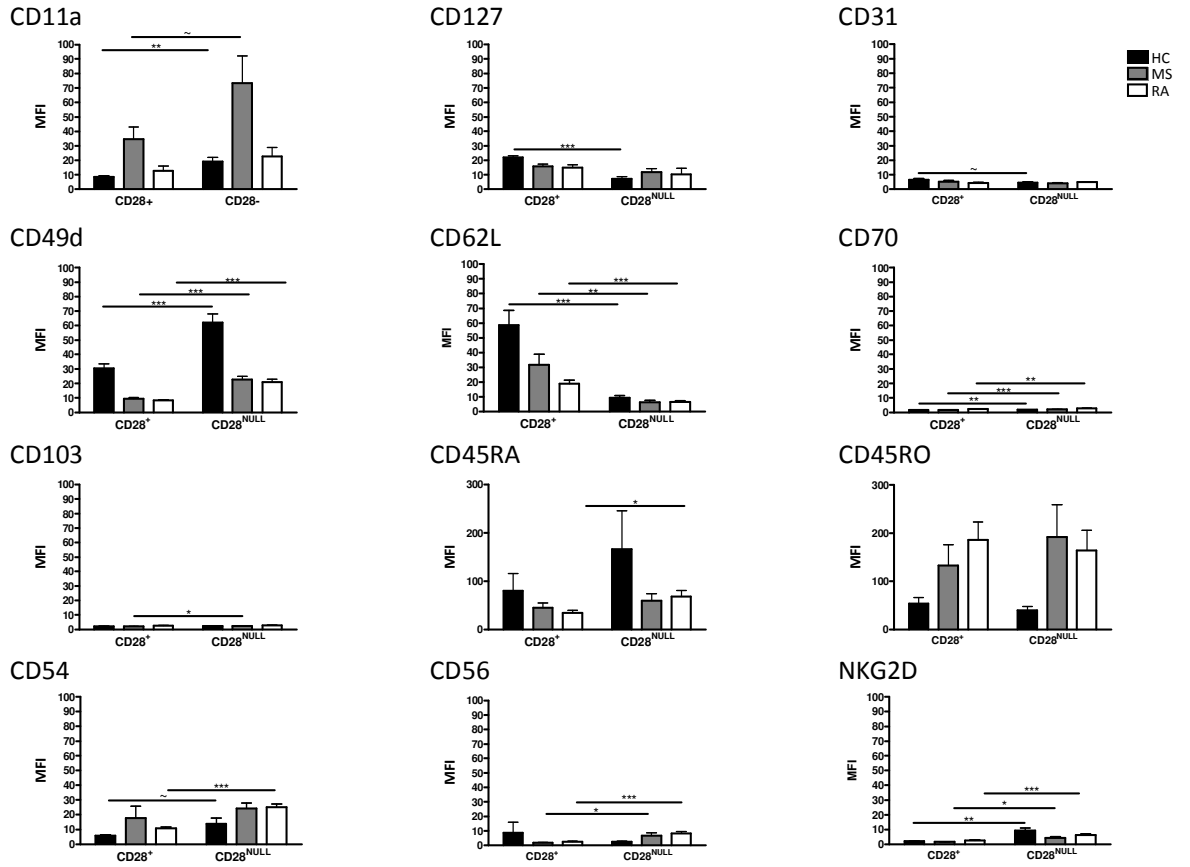

Figure 10: The expression levels of several surface markers is increased on CD4^{+}CD28^{NULL} T cells. The expression level of the indicated surface markers on CD4^{+}CD28^{NULL} and CD4^{+}CD28^{+} T cells in HC (n=9), MS patients (n=10) and RA patients (n=25) is presented. *: p<0.05, **: p<0.01, *** p<0.001, ~: 0.05<p<0.1 (trend towards significance).

In all three donor groups, the expression level of α4 integrin (CD49d; for HC: p=0.0007, for MS patients: p=0.0003, for RA patients: p<0.0001), Ki-24 (CD70; for HC: p=0.0092, for MS patients: p=0.0005, for RA patients: p=0.0077) and NKG2D (for HC: p=0.0022, for MS patients: p=0.0128, for RA patients: p<0.0001) is increased in the CD4^{+}CD28^{NULL} T cell population and the expression level of L-selectin (CD62L; for HC:p=0.0002, for MS patients: p=0.0060, for RA patients: p<0.0001) is decreased (Figure 9). In HC only, the expression of LFA-1 (CD11a, p=0.0032) is increased and the IL-7R (CD127, p<0.0001) expression is decreased in the CD4^{+}CD28^{NULL} T cell population. In MS patients only, the expression of αE integrin (CD103, p=0.0275) is significantly increased in the CD4^{+}CD28^{NULL} T cell population, while the expression of CD45RA (p=0.0180) and ICAM-1 (CD54, p<0.0001) is significantly increased solely in RA patients. Additionally, the expression level of NCAM (CD56) is significantly increased in RA patients (p<0.0001) and MS patients (p=0.0293).

In addition to differences in between the two CD4^{+} T cell subpopulations, several differences are also found between the HC population and the patient populations (Figure 11, Figure 12).

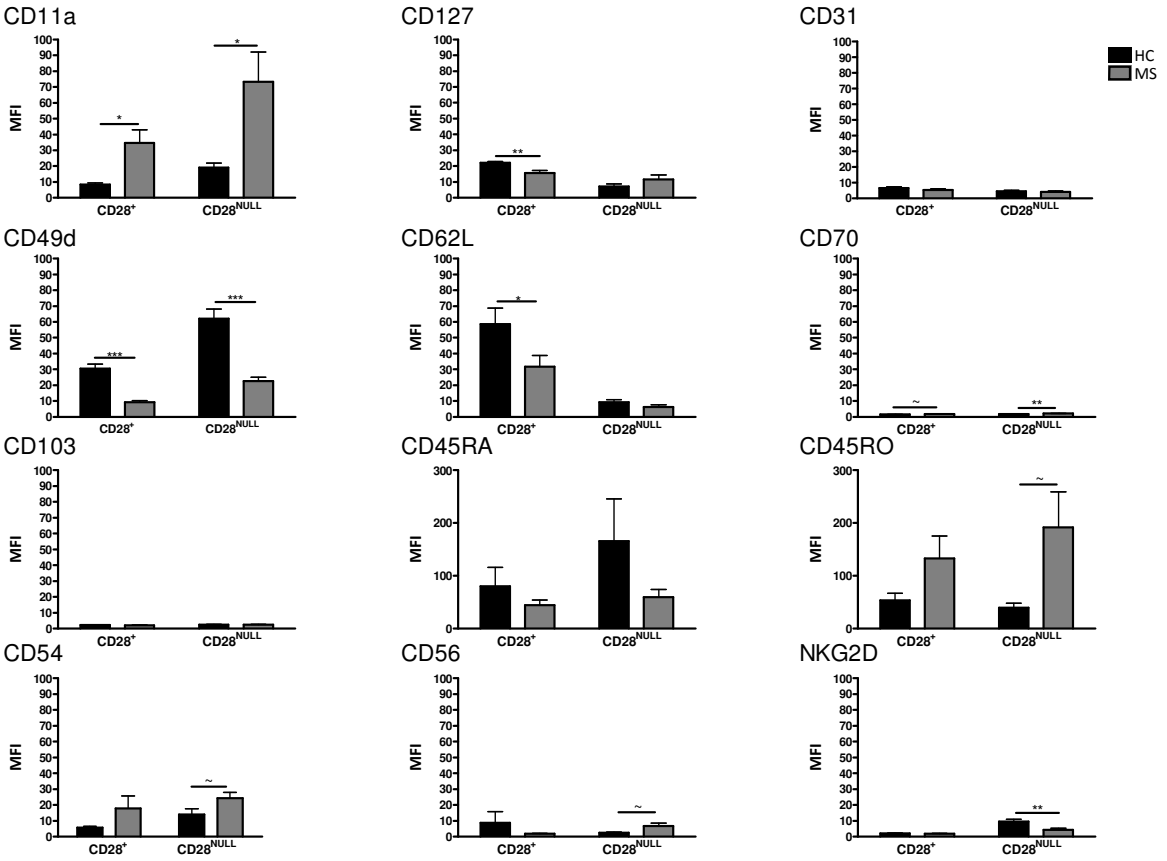

Figure 11: The expression level of several surface markers differs on CD4^{+}CD28^{NULL} T cells in HC and MS patients. The expression levels of the indicated surface markers on CD4^{+}CD28^{NULL} and CD4^{+}CD28^{+} T cells in HC (n=9) compared to MS patients (n=10) is presented. *: p<0.05, **: p<0.01, *** p<0.001, ~: 0.05<p<0.1 (trend towards significance).

As seen in Figure 11, the expression level of LFA-1 (CD11a, p=0.0191) and Ki-24 (CD70, p=0.0044) is significantly higher in the CD4^{+}CD28^{NULL} T cell population of MS patients. On the contrary, the expression of α4 integrin (CD49d, p=0.0001) is significantly decreased in this cell population in MS patients, as well as the expression of NKG2D (p=0.0068).

Significant differences can also be seen when the CD4^{+}CD28^{+} T cell population is considered. CD4^{+}CD28^{+} T cells of MS patients express significantly higher levels of LFA-1 (CD11a, p=0.0110). In contrast, this cell population expresses lower levels of the IL-7R (CD127, p=0.0060) and L-selectin (CD62L, p=0.0377).

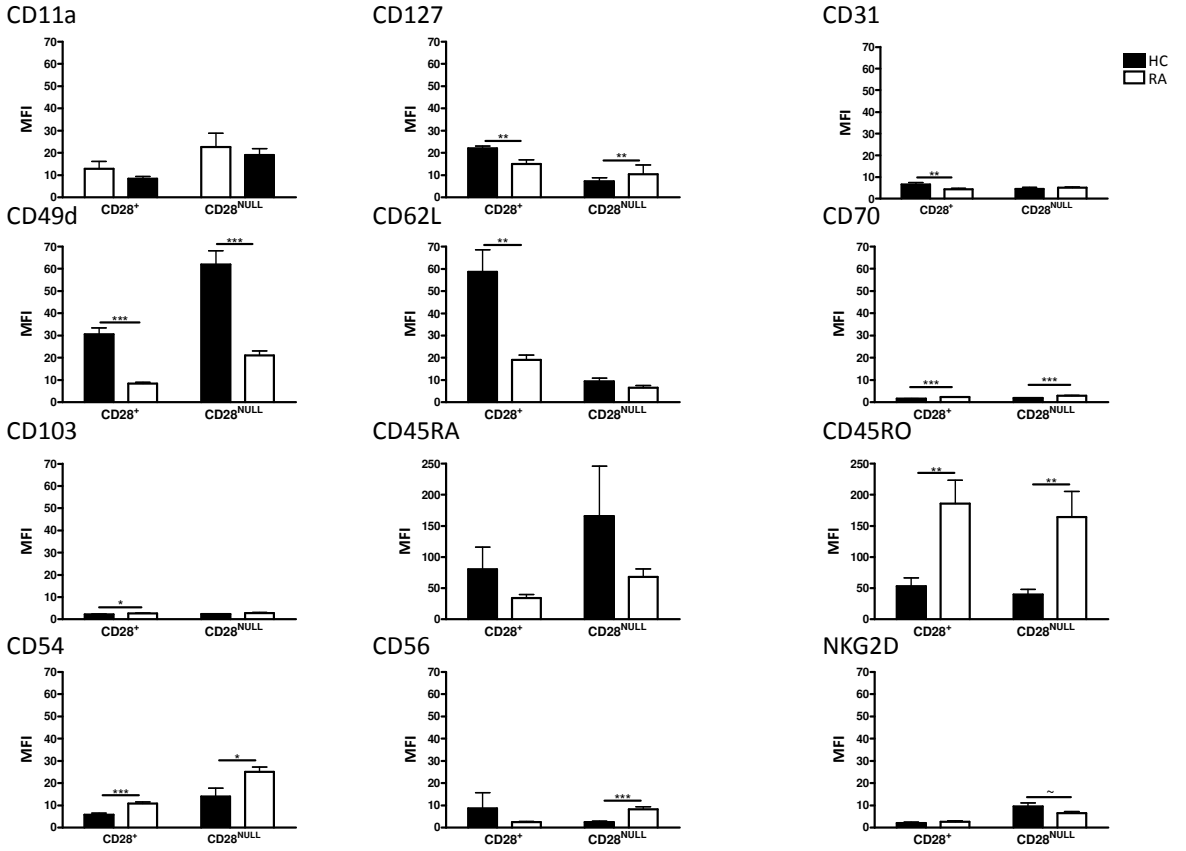

Figure 12: The expression level of several surface markers differs on CD4^{+}CD28^{NULL} T cells in HC and RA patients. The expression levels of the indicated surface markers on CD4^{+}CD28^{NULL} and CD4^{+}CD28^{+} T cells in HC (n=9) compared to RA patients (n=25) is shown. *: p<0.05, **: p<0.01, *** p<0.001, ~: 0.05<p<0.1 (trend towards significance).

As with MS patients, significant differences in the expression level of the studied surface markers can also be found between HC and RA patients (Figure 12). For CD4^{+}CD28^{NULL} T cells, the expression levels of the IL-7R (CD127, p=0.0044), Ki-24 (CD70, p<0.0001), ICAM-1 (CD54, p=0.0112), NCAM (CD56, p<0.0001) and CD45RO (p=0.0075) are significantly increased in RA patients. A decreased expression level can be observed for α4 integrin (CD49d, p=0.0001).

CD4^{+}CD28^{+} T cells in MS patients show an increased expression of ICAM-1 (CD54, p=0.0007), CD45RO (p=0.0024), Ki-24 (CD70, p=0.0003) and αE integrin (CD103, p=0.0455). The expression level of α4 integrin (CD49d, p<0.0001), the IL-7R (CD127, p=0.0025) and L-selectin (CD62L, 0.0045) is decreased on the surface of CD4^{+}CD28^{+} T cells in MS patients.

As mentioned above, several other surface markers have also been tested, though only in HC (Figure 13).

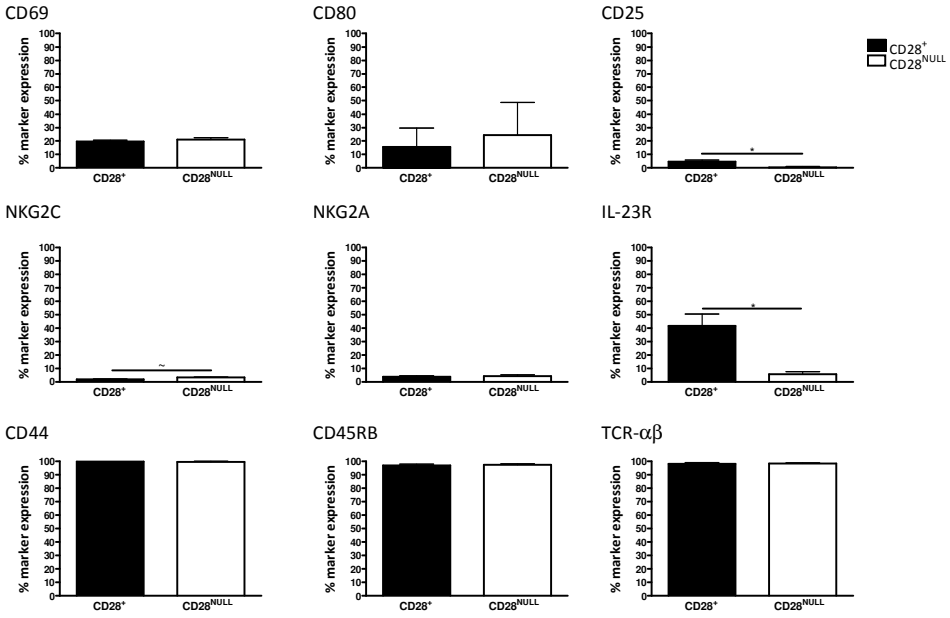

Figure 13: Additional studied surface markers. Data are shown for HC. Not all markers were tested for the same number of HC. For CD44, CD25, NKG2A, NKG2C: n=3. For the remainder of the markers shown: n=4. *: $p<0.05$, ~: $0.05<p<0.1$ (trend towards significance).

The Fc-receptor (CD16) as well as TCR-γδ are virtually absent on both CD4^{+} T cell subpopulations (data not shown). As shown in Figure 13, there is no significant difference in the cell fractions expressing of all shown markers, with the exception of IL-2Rα (CD25, p=0.0311) and IL-23R (p=0.0288), which are significantly increased on CD4^{+}CD28^{+} T cells. As for the expression level of these markers on both subpopulations (Figure 14), a significant decrease in the expression level of IL-2Rα (CD25) can be observed for CD4^{+}CD28^{NULL} T cells (p=0.0330).

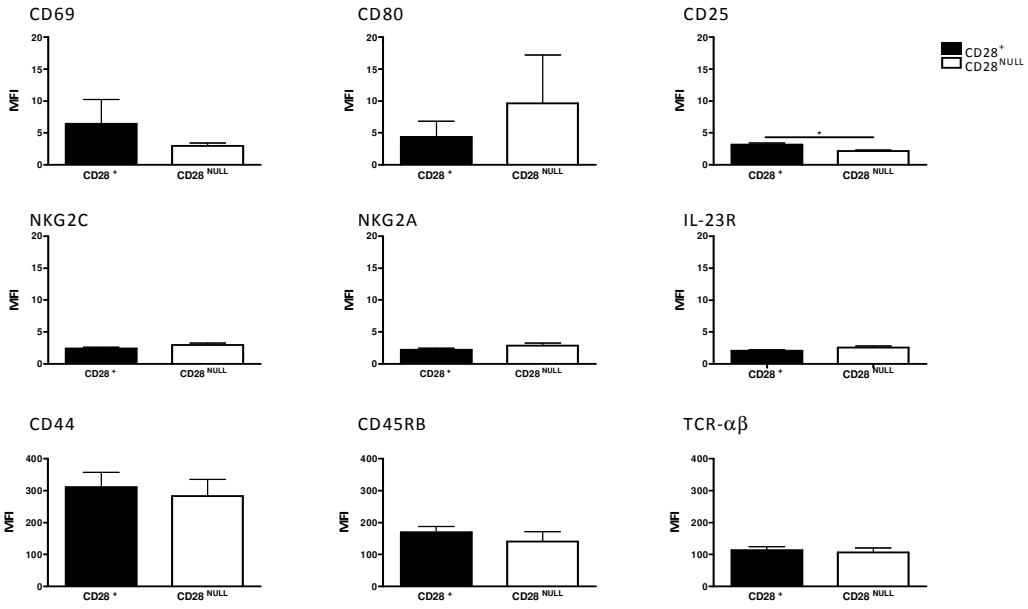

Figure 14: Expression level of additionally studied surface markers. Data are shown for HC. Not all markers were tested for the same number of HC. For CD44, CD25, NKG2A, NKG2C n=3. For the remainder of the markers shown n=4.

From these experiments, some general conclusions can be made. Based on what is seen for L-selectin, PECAM-1 the IL-7R, it appears that CD4$^+$CD28^{NULL} T cells are memory-type effector cells. Additionally, these cells display an adhesive phenotype directed towards tissue infiltration, as shown by the obtained results for surface markers like ICAM-1, NCAM, α4-integrin and LFA-1. Additionally, these cells can have cytotoxic characteristics, evidenced by the expression of NKG2D.

When comparing the HC population to the studied patient populations, it can be observed that the CD4$^+$ T cell population in general appears more mature in the patient populations. Additionally, CD4$^+$ T cells in these populations show a more adhesive phenotype with less infiltrative capacities.

3.3.3 *Chemokine receptor expression*

In RA patients, it has been shown that the receptor for the chemokine fractalkine (CX_3CR1) is upregulated on CD4$^+$CD28^{NULL} T cells (63). However, this observation has never been made for senescent cells in MS patients. Therefore, the expression of the chemokine receptor CX_3CR1 on CD4$^+$CD28^{NULL} T cells was studied in the current study population by flow cytometric analysis.

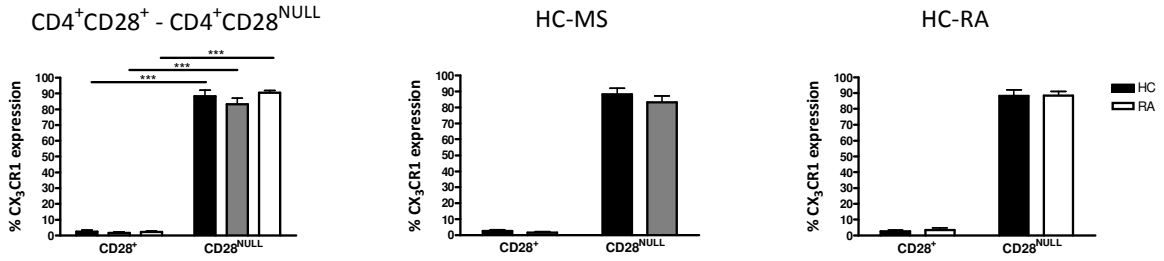

Figure 15: CX_3CR1 expression is significantly increased in CD4$^+$CD28^{NULL} T cells. *Panel A:* CD4$^+$CD28^{NULL} T cells compared to CD4$^+$CD28$^+$ T cells. *Panel B:* both cell populations compared in HC and MS patients. *Panel C:* both cell populations compared in HC and RA patients. Results are shown for HC (n=9), MS patients (n=10) and RA patients (n=25). ***: p<0.0001.

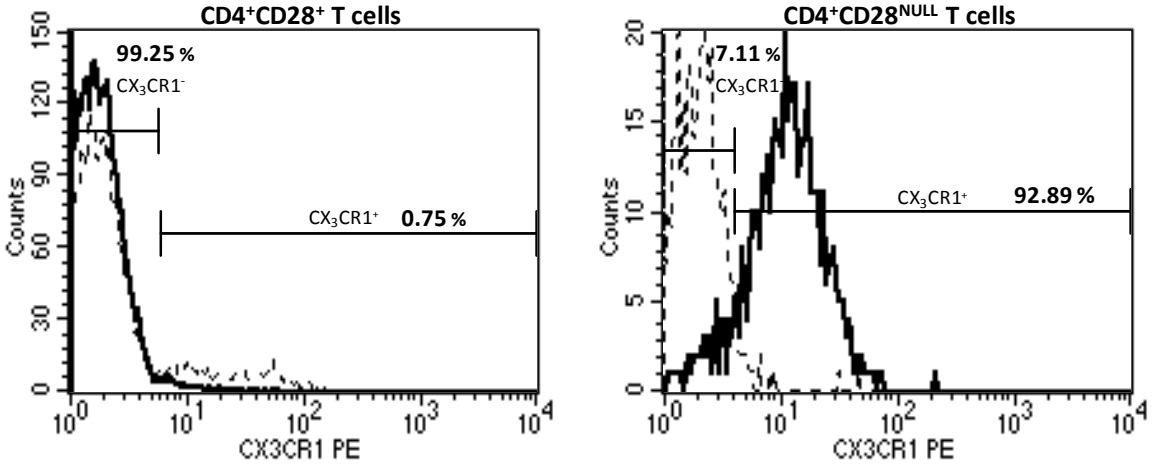

Figure 46: CD4$^+$CD28^{NULL} T cells highly express CX_3CR1. *Left panel:* FACS histogram showing the expression of CX_3CR1 on CD4$^+$CD28$^+$ T cells. *Right panel:* FACS histogram showing the expression of CX_3CR1 on CD4$^+$CD28^{NULL} T cells. Results are shown for 1 RA patient. Bold line: CX_3CR1 expression, dashed line: isotype control.

The fraction of $CD4^+CD28^{NULL}$ T cells expressing CX_3CR1 is substantially higher in all studied groups in comparison to $CD4^+CD28^+$ T cells (Figure 15). In fact, almost all $CD4^+CD28^{NULL}$ T cells express the receptor, in contrast to almost no $CD4^+CD28^+$ T cells (Figure 15, Figure 16)

3.3 Chemotactic potential of $CD4^+CD28^{NULL}$ T cells

Because a remarkable difference was found between $CD4^+CD28^+$ and $CD4^+CD28^{NULL}$ T cells regarding the fractalkine receptor, the chemotactic capabilities of the senescent $CD4^+$ T cells, mediated via CX_3CR1-CX_3CL1 interaction, were investigated using a transwell chemotaxis assay. Several conditions, the migration time, the optimal chemokine concentration, the ideal cell population and chemotaxis buffer, were evaluated separately to determine optimal migration circumstances. All results are expressed as the percentage migrated cells. This is defined as the percentage of cells from the entire subpopulation ($CD4^+CD28^{NULL}$ T cells or $CD4^+CD28^+$ T cells) that has migrated.

3.3.1 *CX_3CL1 concentration and migration time*

To determine the optimal chemokine concentration, several CX_3CL1 concentrations were tested (Figure 17): 10 ng/ml, 100 ng/ml and 500 ng/ml, dissolved in chemotaxis buffer (RPMI 1640 supplemented with 0.5 % BSA). Additional to these chemokine concentrations, several migration times were also included in this setup. Migration patterns of $CD4^+$ T cells were analysed after 1.5 h, 3 h and 5 h.

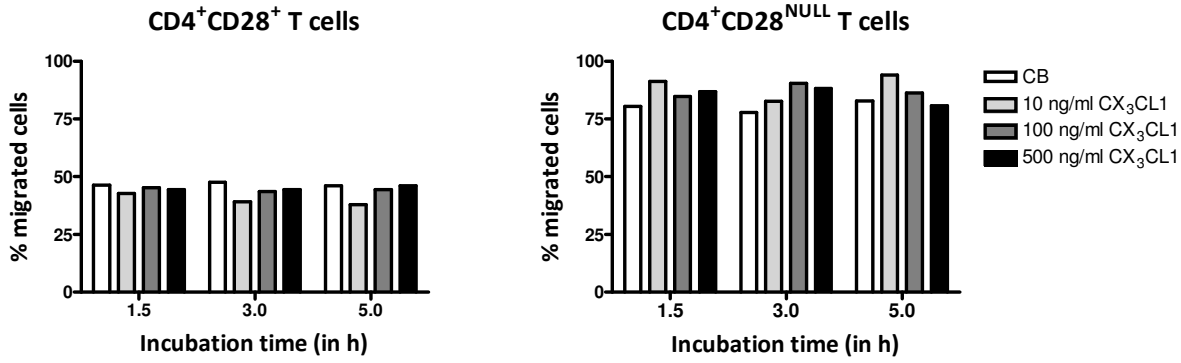

Figure 17: Optimization of CX_3CL1 concentration and migration time. *Left:* $CD4^+CD28^+$ T cell migration pattern. *Right:* $CD4^+CD28^{NULL}$ T cell migration pattern. (CB=chemotaxis buffer). Several CX_3CL1 concentrations were tested along with different migration times. Migration took place at 37°C, 5% CO_2.

As shown in Figure 17, the CX_3CL1 concentration of 10 ng/ml and the migration time of 5 h yields a fairly good migration result for both cell populations. The chemokine-induced migration of the $CD4^+CD28^{NULL}$ T cell population is the highest in this condition while being the lowest in the $CD4^+CD28^+$ T cell population. The spontaneous cell migration (white bars), however, is the highest in this condition.

When migration proceeded for only 1.5 h, spontaneous migration is lower for the $CD4^+CD28^{NULL}$ T cell population, while the chemokine-induced migration is comparable to the 5 h condition, looking

at a chemokine concentration of 10 ng/ml. However, this condition does not yield a good result for the $CD4^+CD28^+$ T cell population.

When migration proceeded for 3 h, the spontaneous migration is low in the $CD4^+CD28^{NULL}$ T cell population and the chemokine-induced migration is higher. Also, the migration pattern is low for the $CD4^+CD28^+$ T cell population. However, the chemokine induced is not as good as seen in the 5 h condition.

Because of the chemokine-induced migration pattern in the 5 hour condition and the spontaneous migration pattern in the 3 h condition, it was opted to perform chemotaxis assays in which migration proceeded for approximately 4 hours, in an attempt to maximize the chemokine-induced migration, while suppressing the spontaneous migration as much as possible.

With these optimal conditions, $CD4^+$ T cells of 5 HC were allowed to migrate for 4 hours in response to a 10 ng/ml CX_3CL1 stimulus.

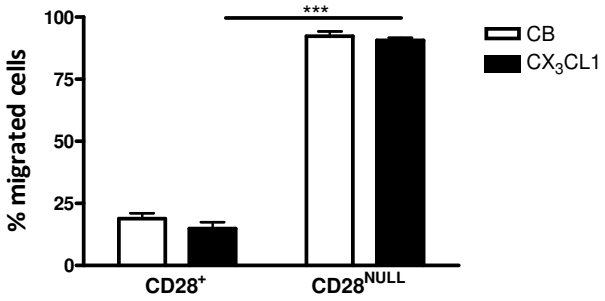

Figure 18: Migration capacity is significantly higher in the $CD4^+CD28^{NULL}$ T cell population. $CD4^+$ T cells migrated to a 10 ng/ml CX_3CL1 stimulus for 4 hours at 37°C, 5 % CO_2 The migration pattern is shown for HC (n=5).

Under the conditions applied in the current setup (Figure 18), the chemokine-induced migration of $CD4^+CD28^{NULL}$ T cells is significantly higher than the chemokine-induced migration of $CD4^+CD28^+$ T cells ($p<0.001$). No difference can be seen between spontaneous and chemokine-induced migration in either cell population.

3.3.2 *Cell population*

For all previous experiments, enriched $CD4^+$ T cells were used. This enrichment was achieved using RosetteSep®. However, this procedure depletes PBMC of $CD56^+$ cells, which are mostly NK cells. As shown in Figure 19, a part of the $CD4^+CD28^{NULL}$ T cells from our donors expresses CD56 (NCAM, upper left quadrant). Consequently, the subpopulation of $CD4^+CD28^{NULL}CD56^+$ T cells, which may have better migratory capacities, is not included when applying this isolation procedure.

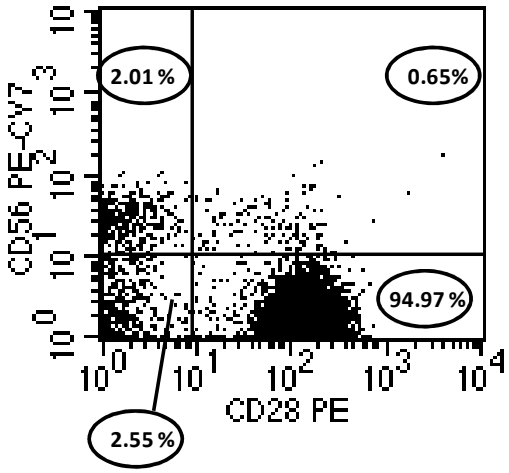

Figure 19: CD4+CD28NULL T cells express CD56 (NCAM). Dot plot showing CD56 expression on CD4+ T cells of 1 HC.

To circumvent the loss of CD4+CD56+ T cells, PBMC were isolated and set up in a chemotaxis assay instead of enriched CD4+ T cells.

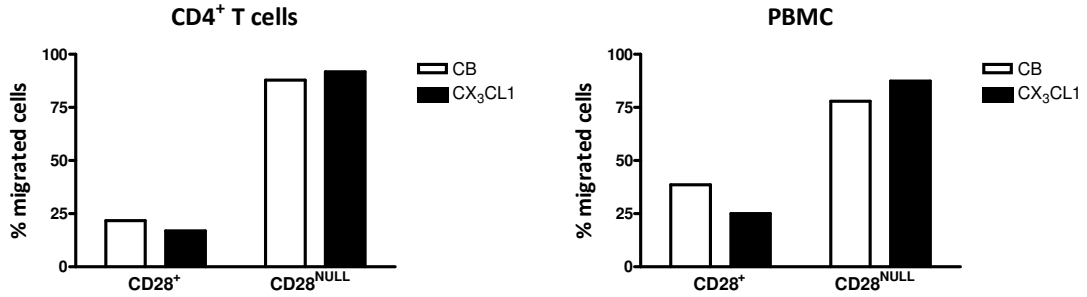

Figure 20: PBMC are an alternative cell population for chemotaxis assays. *Left:* migration pattern of CD4+ T cells of HC (n=1). *Right:* migration pattern of PBMC from the same donor. Migration proceeded for 4 h at 37°C, 5% CO_2 in response to 10 ng/ml CX_3CL1.

When PBMC are used, a clear difference can be seen in the migration pattern of CD4+CD28NULL T cells and CD4+CD28+ T cells (Figure 20, right panel), as is the case when CD4+ T cells are used (Figure 20, left panel). Additionally, for this donor, a clear difference can be seen between the spontaneous and chemokine-induced migration pattern. Because only one donor was used for this experiment, the significance of the differences could not be determined. However, under these conditions the spontaneous cell migration is still quite extensive.

3.3.3 *Chemotaxis buffer*

Next to adjusting the CX_3CL1 concentration, the migration time and the used cell population, the effect of several chemotaxis buffers was assessed. In this setup, 4 different buffers were tested: RPMI without any additions, RPMI with 0.5 % BSA, RPMI with 10 % FCS and RPMI with 20 % FCS.

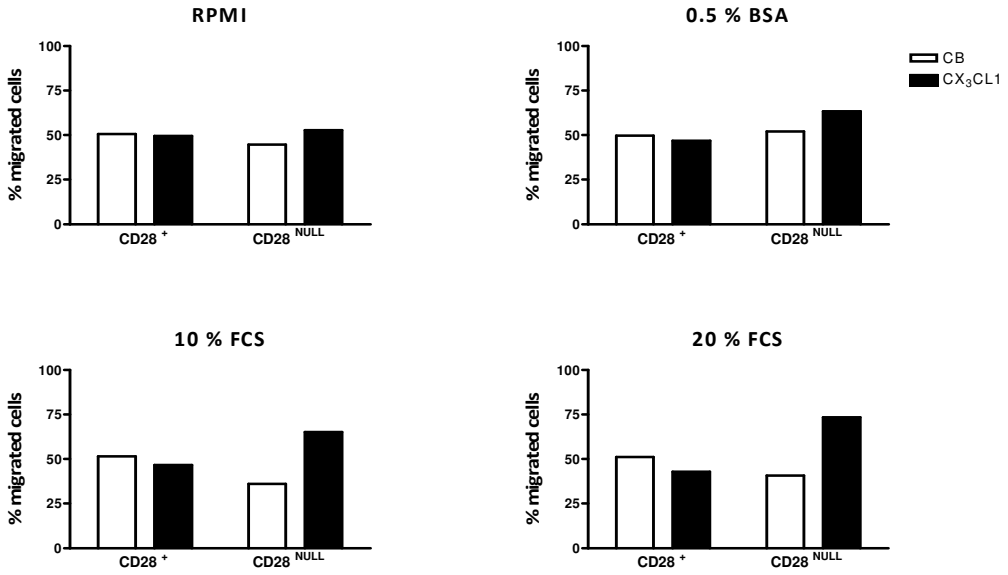

Figure 21: 20 % FCS is the optimal buffer for chemotaxis assays. Results are presented for HC (n=1). Migration patterns are shown for 4 different chemotaxis buffers: RPMI (left upper panel), 0.5 % BSA (right upper panel), 10 % FCS (left bottom panel) and 20 % FCS (right bottom panel). Migration of PBMC proceeded for 4 h at 37°C, 5% CO_2 in response to 10 ng/ml CX_3CL1.

As shown in Figure 21, the optimal outcome is obtained when using 20 % FCS as buffer. Both the difference in spontaneous and chemokine-induced migration pattern and the difference in migration pattern of $CD4^+CD28^+$ T cells and $CD4^+CD28^{NULL}$ T cells are the clearest in this setup.

A chemotaxis assay was performed for multiple HC, using 20 % FCS as buffer. For comparison, 0.5 % BSA was also included in this setup.

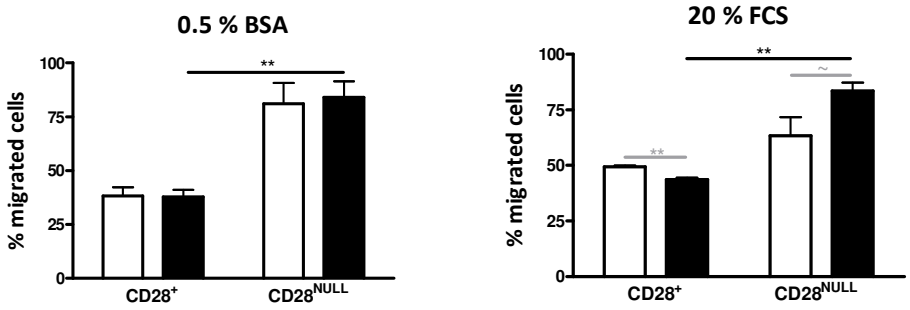

Figure 22: 20 % FCS is the optimal chemotaxis buffer. *Left:* migration pattern for HC (n=4) with 0.5 % BSA as chemotaxis buffer. *Right:* migration pattern for the same donors with 20 % FCS as chemotaxis buffer. Migration proceeded for 4 h at 37°C, 5% CO_2 in response to 10 ng/ml CX_3CL1.

When using 20 % FCS, a significant difference in chemokine-induced migration can be seen between the $CD4^+CD28^{NULL}$ T cells and $CD4^+CD28^+$ T cells (p=0.0020) (Figure 22). Additionally, a significant difference can be seen in spontaneous migration and chemokine-induced migration in the $CD4^+CD28^+$ T cells (p=0.0015). However, the overall migration pattern is still very high for this cell population.

3.3.4 *Reduction of spontaneous cell migration*

Because of the high spontaneous migration seen when serum is present in the buffer, it was speculated that the FCS might contain some chemokines to which the cells migrate. To further reduce this spontaneous cell migration and reduce the migration of the $CD4^+CD28^+$ T cells, several different conditions were tested. The migration time was reduced to 3 h and a lower CX_3CL1 concentration (1 ng/ml) was tested in combination with two different chemotaxis buffers, 0.5 % BSA and 10 % FCS.

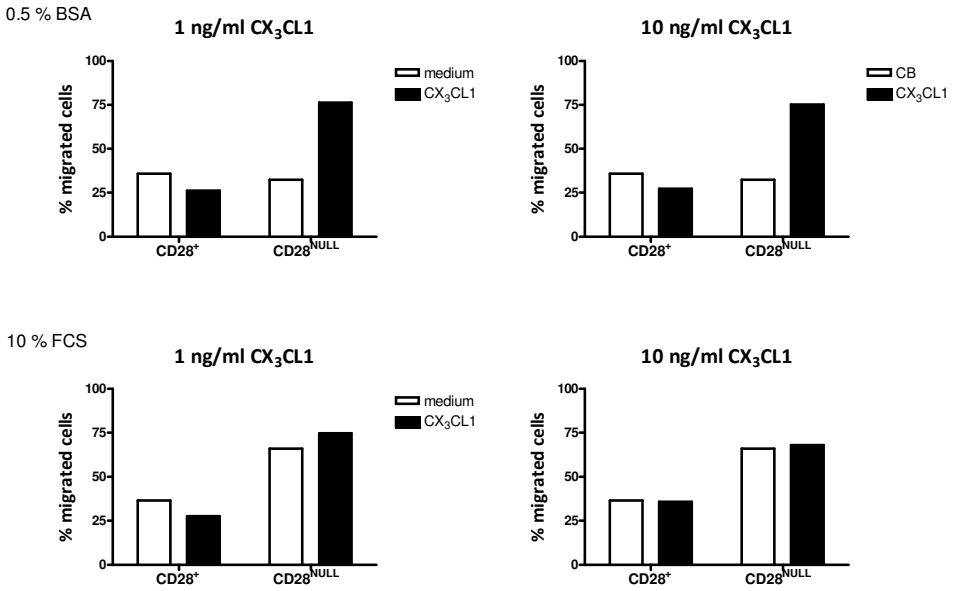

Figure 253: Lower chemokine concentration and 0.5% BSA give the best migration pattern. Next to the 10 ng/ml CX_3CL1, a lower concentration of 1 ng/ml CX_3CL1 was tested (left panels) in combination with 10 % FCS chemotaxis buffer (lower panels) or 0.5 % BSA (upper panels). Migration proceeded for 4 h at 37°C, 5% CO_2. Results are shown for 1 RA patient.

The results indicate that both the spontaneous cell migration and the $CD4^+CD28^+$ T cell migration pattern are strongly reduced when 1 ng/ml chemokine was used in 0.5 % BSA chemotaxis buffer (Figure 23). Additionally, when applying this condition, a good migration pattern can be seen in the $CD4^+CD28^{NULL}$ T cell population. However, because the experiment was set up with only one donor, no statistics could be applied.

From these optimization experiments, the optimal protocol can be deducted. Therefore, experiments should be set up using freshly isolated PBMC; the chemotaxis buffer should be 0.5 % BSA/RPMI; the concentration of the chemokine should be 1 ng/ml; and the migration time should be 3 h.

3.4 Ability of $CD4^+CD28^{NULL}$ T cells to provide bystander activation

Previously, Lee et al. (64) have shown that $CD4^+CD28^{NULL}$ T cells of RA patients are able to provide bystander activation to possibly autoreactive T cells, through the prolonged upregulation of the

costimulatory molecule CD70 on the senescent T cells. However, this potential has not yet been studied in HC. To investigate the ability of CD4$^+$CD28^{NULL} T cells of HC to provide bystander activation, sorted CD4$^+$CD28^{NULL} T cells or CD4$^+$CD28$^+$ T cells were separately cultured in the presence of feeders, IL-2 and an activator (aCD3 or PHA). Over a two-week period, CD70 expression was monitored by flow cytometry (Figure 24).

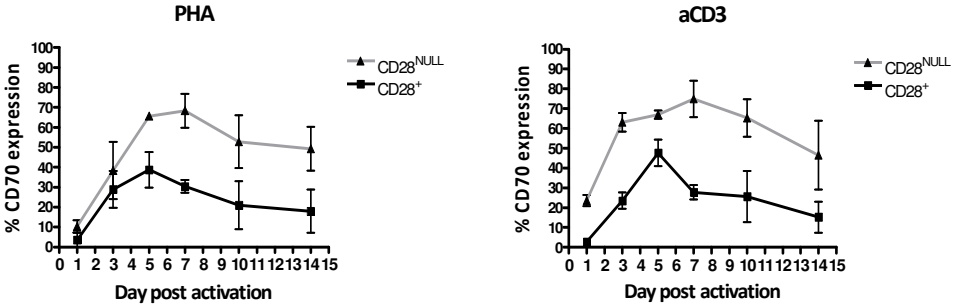

Figure 24: CD70 expression is continuously upregulated on CD4$^+$CD28^{NULL} T cells. *Left panel:* CD70 expression for both cell populations after activation with PHA. *Right panel:* CD70 expression for both subpopulations after activation with aCD3. Results are presented for HC (n=2). Data are corrected for expression in culture medium condition.

The activation effect of PHA and aCD3 yield comparable kinetics. The first 5 days post activation, the CD70 expression is upregulated on both the CD4$^+$CD28$^+$ and the CD4$^+$CD28^{NULL} T cell population. After 5 days however, the CD70 expression decreases again in the CD4$^+$CD28$^+$ T cell population, while remaining high in the CD4$^+$CD28^{NULL} T cell population.

For practical reasons, it was also attempted to perform these kinetics without sorting the two separate cell populations. In this setup, CD4$^+$ T cells were enriched from peripheral blood and subsequently cultured in the presence of IL-2 and an activator (aCD3 or PHA). Over a two-week period, CD70 expression was monitored by flow cytometry.

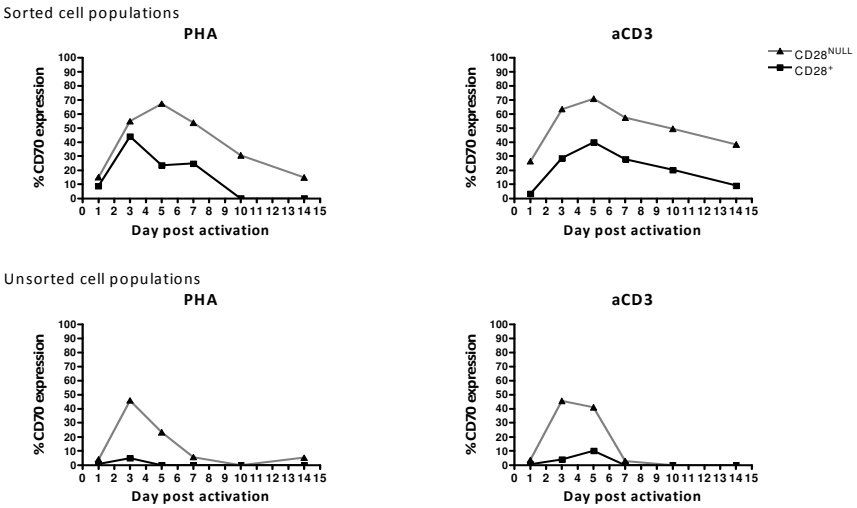

Figure25: Kinetics should be performed after sorting of cell populations. *Upper panels:* kinetics with sorted cell populations. *Lower panels;* kinetics with unsorted cell populations. Results are shown for 1 HC. Both experiments were set up using cells from an identical donor. Data are corrected for expression in culture medium condition.

As shown in Figure 25, the kinetics with unsorted cell populations does not yield a good result. A difference in CD70 expression between the $CD4^+CD28^+$ and the $CD4^+CD28^{NULL}$ T cell population can no longer be observed throughout the 14-day culture. When kinetics are performed with sorted cell populations, kinetics are more successful. Hence, kinetics experiments are best performed using the latter setup.

4. Discussion

In this research, the possible role of senescent $CD4^+CD28^{NULL}$ T cells in the pathogenesis of autoimmune diseases like MS and RA was investigated. Phenotypical as well as functional aspects of $CD4^+CD28^{NULL}$ T cells were assessed in HC, MS patients and RA patients. Phenotypically, the expression of several surface markers was determined, as well as the expression of the chemokine receptor CX_3CR1. Functionally, the chemotactic capacities of $CD4^+CD28^{NULL}$ T cells and their ability to provide bystander activation, mediated via CD70 signaling, were examined. The obtained results show very clear differences between the $CD4^+CD28^+$ T cell population and the $CD4^+CD28^{NULL}$ T cell population. However, no obvious differences were observed, for the characteristics studied here, between the studied HC and patient populations.

In the currently studied donor population, the percentage of $CD4^+CD28^{NULL}$ T cells is significantly increased in RA patients when compared to HC. This result is in concordance with previously performed research (50). Confirming their observations, no significant increase was found in the $CD4^+CD28^{NULL}$ T cell percentage between MS patients and HC.
After determining which donors possess a sufficient amount of $CD4^+CD28^{NULL}$ T cells, both phenotypical and functional characteristics were investigated. $CD4^+CD28^{NULL}$ T cells have been characterized to some extent by other research groups (48, 49, 53, 54). Most of this research has focused on $CD4^+CD28^{NULL}$ T cells in RA patients. Therefore, it was aimed to further characterize $CD4^+CD28^{NULL}$ T cells in RA patients, as well as in HC and MS patients. From the currently obtained results several conclusions can be drawn. First of all, $CD4^+CD28^{NULL}$ T cells are memory type effector cells. This can be concluded from the expression of several markers, like the expression of PECAM-1 (or CD31). Aside from being an important adhesion molecule, CD31 can also be used as a marker for the so-called recent thymic emigrants (RTE), T cells that have very recently migrated from the thymus. As shown by Stockinger et al. (65), CD31 is present on the majority of naive T cells ($CD45RA^+$ T cells), while being present on only a small portion of memory T cells ($CD45RO^+$ T cells). The current observations show that CD31 is present on significantly less $CD4^+CD28^{NULL}$ T cells when compared to their $CD4^+CD28^+$ counterparts, indicating that, following the observations of Stockinger et al., $CD4^+CD28^{NULL}$ T cells are of a more memory phenotype. Corroborating this statement, both the proportion of $CD4^+CD28^{NULL}$ T cells expressing L-selectin, confirming results obtained by Thewissen et al. (53), as well as the expression level of L-selectin on this cell population are decreased in all three study populations. L-selectin is a crucial mediator in the homing process of T cells to the lymph nodes. Effector T cells, however, do not express L-selectin. The decreased L-selectin expression in the $CD4^+CD28^{NULL}$ T cell population thus additionally points to a memory effector phenotype. Lastly, the expression of the IL-7R is significantly decreased on $CD4^+CD28^{NULL}$ T cells of HC as well as RA patients. The IL-7R is an important factor in the development to mature T cells. Its decreased expression implicates that $CD4^+CD28^{NULL}$ T cells have already differentiated to their mature phenotype. Moreover, in the currently studied populations, both $CD45RA^+$ and $CD45RO^+$ $CD4^+CD28^{NULL}$ T cells are present, as was also observed by Fasth et al.(54). However no significant differences between the $CD4^+CD28^{NULL}$ T cell population and the $CD4^+CD28^+$ T cell population can be seen for either marker. The latter observation is in contrast to

data published by Myazaki et al. (49), who were able to show an increased expression of $CD45RO^+$ cells in the $CD4^+CD28^{NULL}$ T cell population of HC and MS patients. However, they only included patients suffering from RRMS in their study.

Further characterization indicates that senescent $CD4^+CD28^{NULL}$ T cells have a more adhesive and migratory phenotype than their normal counterparts. The percentage of $CD4^+CD28^{NULL}$ T cells expressing LFA-1, important for the adhesion of T cells to their target cells, is significantly increased in all three donor groups, as well as the expression level in HC. This observation is in contrast to what was seen by Thewissen et al. (53). No difference between the two cell populations was found here. Additionally, in concert with their research, the $CD4^+CD28^{NULL}$ T cell fraction expressing integrin α4, necessary for transendothelial migration, as well as the expression level of integrin α4 is significantly increased in HC, MS patients and RA patients. A significant increase of ICAM-1, the ligand for LFA-1, can also be found for $CD4^+CD28^{NULL}$ T cells in HC, MS patients and RA patients.

Besides significant differences in surface markers pointing to $CD4^+CD28^{NULL}$ T cells as being effector memory-type T cells with adhesive and migratory capabilities, significant differences in the expression of NK cell markers is also observed. NCAM, expressed both on NK cells and activated T cells, is significantly increased in the $CD4^+CD28^{NULL}$ T cell population of MS patients and RA patients. Additionally, NKG2D, a stimulatory NK cell receptor, is significantly increased in the $CD4^+CD28^{NULL}$ T cell population of both HC and RA patients. Normally, the stimulating effect of NKG2D is downmodulated by MICA, if present in the environment the cells expressing NKG2D reside in. Aberrant expression of MICA in the synovium of RA patients has been described (57). Normally, this would lead to a downmodulation of NKG2D on $CD4^+$ T cells present in the synovium. However, IL-15 is also present in the synovium of RA patients. This cytokine is capable of upregulating NKG2D expression on $CD4^+$ T cells in such a way that it overrides the downmodulating effects of MICA. Hence the cytotoxic properties of NKG2D are maintained in the synovium of RA patients.

Taken together, these results indicate that $CD4^+CD28^{NULL}$ T cells appear to be effector memory-type T cells with an adhesive and migratory phenotype, possibly possessing cytotoxic properties.

When comparing the studied HC population to the MS and RA populations, several significant differences are found for the markers currently investigated for both cell populations. From these differences, one can conclude that when compared to HC, the $CD4^+$ T cells in general of both patient groups have a more mature phenotype and that they are more directed towards adhesion. Remarkably, in comparison to HC, $CD4^+$ T cells of MS patients and RA patients seem less inclined to infiltrate peripheral tissues and they appear less cytotoxic. This could be explained by the origin of the cells that were under investigation here, that is, all cells were isolated from peripheral blood. It may however be the case that those cells that have the highest infiltration capacity and the highest cytotoxic potency have already migrated from the blood stream to peripheral target tissue; the synovium in case of RA patients and the central nervous system in case of MS patients. Consequently, these cells no longer reside in the peripheral blood and could not be included in the current analyses.

Additional phenotypical characterizations should still be performed for more MS patients, since no clear-cut differences can be seen between HC and MS patients for the number of donors tested so far, but nonetheless several near significant differences are obtained.

Besides surface marker expression, the expression of the chemokine receptor CX_3CR1 on $CD4^+CD28^{NULL}$ T cells was also examined. Research by Pingiotti et al. (59), has shown that in RA patients, the vast majority of $CD4^+CD28^{NULL}$ T cells expresses CX_3CR1, while in HC the receptor is only present on a minority of $CD4^+CD28^{NULL}$ T cells. In the current research, the expression of CX_3CR1 on $CD4^+CD28^{NULL}$ T cells was evaluated in HC, as well as in RA patients and MS patients. It is found that in all three investigated donor populations, the expression of CX_3CR1 is expressed on the vast majority of $CD4^+CD28^{NULL}$ T cells while being expressed on only a very small fraction of $CD4^+CD28^+$ T cells. This implies that CX_3CR1 can be used as an additional marker to identify $CD28^{NULL}$ T cells, only within the $CD4^+$ T cell population. The contradictory results obtained for HC in this research and the results of Pingiotti et al. are possibly explained by the percentage of $CD4^+CD28^{NULL}$ T cells in the studied HC population. The mean percentage of $CD4^+CD28^{NULL}$ T cells in the currently studied HC population is 7.4% of the total $CD4^+$ T cell population. For the HC population in the research of Pingiotti et al. this percentage is a mere 0.7 %. This implies a very low $CD4^+CD28^{NULL}$ T cell number with which analyses could be done. Consequently, possibly only a very low fluorescent signal could be acquired, leading to the detection of a fairly low percentage of cells expressing CX_3CR1.

Several groups have previously shown that the ligand of CX_3CR1, fractalkine (or CX_3CL1) is upregulated in the synovium of RA patients (61-63) and also in inflammatory conditions of the central nervous system (60). Considering the currently obtained results and those of Pingiotti et al (59), it was speculated that, through the interaction of CX_3CL1 and CX_3CR1, $CD4^+CD28^{NULL}$ T cells are capable of migrating from the blood stream to target tissues, like the synovium and the central nervous system. To investigate this hypothesis, chemotaxis assays were performed. Several separate experimental conditions were assessed in order to obtain the optimal circumstances for the cell migration to proceed. After various attempts, the optimal chemokine concentration, migration time, chemotaxis buffer and cell population to be used was determined. Using this protocol, it can be observed that the overall migration in the $CD4^+CD28^{NULL}$ T cell population is significantly increased in comparison to the $CD4^+CD28^+$ T cell population. This is in concert with what is shown in the phenotypical characterization, previously discussed. The observed migration is possibly due to the receptor-ligand interaction. However, this cannot unambiguously be established. Based on what can be seen for the expression of CX_3CR1, it is to be expected that only a very small fraction of the $CD4^+CD28^+$ T cell population would migrate in response to CX_3CL1. This was however not the case. A bit less than half the $CD4^+CD28^+$ T cell population migrates through the transwell. Additionally, if the migration is in fact due to the CX_3CL1-CX_3CR1 interaction, the chemokine induced migration should be significantly increased in comparison to the spontaneous migration. However the obtained results show no significant difference between the chemokine-induced migration and the spontaneous migration for either cell population. When using a buffer containing FCS, the chemokine-induced migration does appear higher than the spontaneous migration. However, it cannot be excluded that this migration is under the influence of additional

chemokines possibly present in the serum, which may attract T cells via interaction with additional chemokine receptors present on T cells. In addition to being inconclusive concerning the receptor-ligand interaction, no unambiguous results can be obtained applying the current setup. Consequently, further optimization is required. Therefore, several experimental conditions can be adjusted. The transwell membrane can for example be coated with fibronectin or endothelial cells (for example by using the HUVEC cell line), in order to create a more strict migration barrier so that only those cells with greater migration capacity are capable of migrating across the membrane. Additionally, the starting cell population can be depleted from certain cell types, like monocytes or NK cells. When keeping the same population size to start the migration with, this will increase the number of $CD4^+CD28^{NULL}$ T cells present in the starting population and additionally these cells will have better access to the membrane, since less cells are present that are possibly blocking the $CD4^+CD28^{NULL}$ T cells. Alternative to the depletion of certain cell types, it can be opted to sort $CD4^+CD28^{NULL}$ T cells and $CD4^+CD28^+$ T cells and subsequently perform a chemotaxis assay with these two cell populations separately. Lastly, aside from optimizing the experimental conditions, it may be useful to verify the functionality of the chemokine itself. This can be achieved by for example examining the migration pattern of monocytes or $CD8^+$ T cells, since these cell types also express CX_3CR1. When optimized, the chemotactic capabilities of $CD4^+CD28^{NULL}$ T cells in HC as well as MS patients and RA patients can be addressed.

Lastly, the ability of $CD4^+CD28^{NULL}$ T cells to provide bystander activation was assessed in HC by monitoring the expression of costimulatory CD70 (Ki-24) over time. Lee et al. (64) have shown that in RA patients, CD70 is continuously overexpressed in the $CD4^+CD28^{NULL}$ T cell population after in vitro activation. Since CD70 is a costimulatory molecule crucial for the initiation of an immune response, a continuous overexpression will lead to a continuous activation of the immune system, which may cause the activation of previously quiescent autoreactive T cells. Post-activational CD70 overexpression on $CD4^+CD28^{NULL}$ T cells has however never been verified before in HC. The results for these experiments show that in both $CD4^+CD28^{NULL}$ T cells and $CD4^+CD28^+$ T cells, the CD70 expression is initially upregulated after in vitro activation. Some days after activation, the CD70 expression decreases again in the $CD4^+CD28^+$ T cell population. In the $CD4^+CD28^{NULL}$ T cell population, on the contrary, CD70 is continuously overexpressed, providing a clue that $CD4^+CD28^{NULL}$ T cells in HC are capable of providing bystander activation. These experiments should be repeated for a larger number of HC and should be extended to MS patients and RA patients.
Additional experiments performed by Lee et al. have shown that $CD4^+CD28^{NULL}$ T cells are in fact capable of eliciting bystander activation. TCR-stimulated PBMC were cocultured with $CD4^+CD28^{NULL}$ T cells of the same donor, after which clear proliferation of the activated PBMC could be observed. When $CD4^+CD28^+$ T cells or a blocking antibody against CD70 was added, the effect on proliferation was not seen. Therefore, the authors suggest that the induction of proliferation was completely due to the prolonged CD70 expression on $CD4^+CD28^{NULL}$ T cells. To verify this observation, comparable experiments may be performed using $CD4^+CD28^{NULL}$ T cells of donors in the currently investigated study population, and this phenomenon should also be investigated in MS patients.

Aside from additional experiments for the characteristics investigated in the current study, other properties of $CD4^+CD28^{NULL}$ T cells that may implicate a role for these cells in the pathogenesis of autoimmune diseases can also be taken into account. The cytotoxic properties of $CD4^+CD28^{NULL}$ T cells toward target cells in MS or RA, for example can be investigated or the antigen-reactivity of $CD4^+CD28^{NULL}$ T cells can be verified in more detail.

In conclusion, although numerous differences can be found between the two $CD4^+$ T cell subpopulations, no obvious differences can be found when comparing the $CD4^+CD28^{NULL}$ T cell population in HC and patients. This can point to a number of things. First, confounding factors are possibly present in the studied population. A large age difference between all three donor groups is present. The healthy control group is substantially younger than both patient groups. In addition, a large difference in age is present between both patient groups. To be fully comparable, the mean age of all donor groups should be similar. The age category within which the donors are present is also fairly limited. In order for the donor population to be representative, donors from a wider age window should be included in the study and all age categories should be fairly equally represented. The disease state of the patient population may also be a confounding factor. For example, as indicated in previous research (51), for RA, the presence of $CD4^+CD28^{NULL}$ T cells is correlated with a severe disease state involving joint destruction and extra-articular manifestations. In order to overcome any bias concerning disease state, patients in different disease stages, for MS as well as RA, should be taken into account. The current results could indicate that $CD4^+CD28^{NULL}$ T cells in fact do not play a role in the pathogenesis of autoimmune diseases, like MS and RA, but rather arise as a side-effect of the presence of an autoimmune disease. More specifically, the ongoing inflammation in patients with autoimmune diseases may cause T cells to become senescent through persisting antigen challenge. Second, it could also mean that $CD4^+CD28^{NULL}$ T cells do play a role in the development of autoimmune diseases, yet in an indirect way. Possibly a yet unknown factor may be co-acting with $CD4^+CD28^{NULL}$ T cells to give rise to an autoimmune disease. Also, $CD4^+CD28^{NULL}$ T cells can be differentially regulated between HC and patients. Research by Thewissen et al. (53), has shown that the regulation of $CD4^+CD28^{NULL}$ T cells by regulatory T cells (Tregs) is diminished in comparison to $CD4^+CD28^+$ T cells. Their results show that Tregs are capable of suppressing the aCD3-induced proliferation of $CD4^+CD28^+$ T cells, but not of $CD4^+CD28^{NULL}$ T cells. The cytokine production, on the contrary can be suppressed by Tregs, however, this suppression is more effective for $CD4^+CD28^+$ T cells. Autoreactive $CD4^+CD28^{NULL}$ T cells in RA patients or MS patients could also encounter certain cytokines or other immune cells that are necessary for their activation or that may alter their phenotype to a more destructive kind. Lastly, the treatment that some of the patients included in this study have received may also bias the obtained results.

Nonetheless, $CD4^+CD28^{NULL}$ T cells comprise a peculiar subpopulation of T cells, which should be investigated in more detail in patients with various autoimmune diseases.

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