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ROLE OF EOMES^{+} TYPE 1 REGULATORY T-CELLS IN MULTIPLE SCLEROSIS

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ACRONYMS AND ABBREVIATIONS

Ab	Antiboy
ACT	Adoptive cell therapy
Ag	Antigen
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
ATP	Adenosine tri-phosphate
BBR	Blood-brain barrier
BCR	B-cell receptor
BM	Bone marrow
CD	Cluster of differentiation
CDMS	Clinically definite MS
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T-cells
CTLA-4	Cytotoxic T lymphocyte associated antigen
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DMF	Dimethyl fumarate
DMT	Desease-modifying treatments
DIS	Dissemination in space
DIT	Dissemination in time
EAE	Experimental autoimmune encephalomyelitis
EBNA1	Anti-EBV nuclear antigen 1
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay

EP	Evoked potentials
GA	Glatimer acetate
GWAS	Genome-wide association studies
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Gzm	Granzyme
HC	Healthy control
HD	Healthy donor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem cells transplant
INF-γ	Interferon-gamma
Ig	Immunoglobulin
IgG	G-class Immunoglobulin
IL	Interleukin
IL-2RA	IL-2 receptor α-chain
IM	Infectious mononucleosis
IMSGC	International multiple sclerosis genetics consortium
IRT	Immune reconstitution therapy
ITIM	Immunoreceptor Tyrosine–based Inhibitory Motif
JCV	John Cunningam Virus
MALT	Mucous-membranes associated lymphoid tissues
mAb	monoclonal antibodies
MET	Mainteneance/escalation therapy
MBP	Myelin basic protein
MHC	Major Histocompatibility Complex

MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MRI	Magnetic resonance imaging
MSIF	Multiple Sclerosis International Federation
NEDA	no evidence of disease activity
NIRT	not selective IRT
NK	natural killer
NKT	natural killer T lymphocytes
NMSS	National multiple sclerosis society
Nrf-2	Nuclear factor erythroid 2–related factor 2
NTZ	Natalizumab
OCBs	Oligoclonal bands
OCR	Ocrelizumab
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral mononuclear blood cells
PD-1	Programmed death-1
PML	Progressive multifocal leukoencephalopathy
PNS	Peripheral nervous system
PPMS	Primary progressive multiple sclerosis
PRR	Pattern Recognition Receptors
RRMS	Relapsing-remitting multiple sclerosis
RIS	Radiologically isolated syndrome
ROS	Reactive oxygen products
S1P	Sphingosine1-phosphate
SEB	Staphylococcal enterotoxin B
SIRT	Selective IRT

SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphisms
SPMS	Secondary progressive multiple sclerosis
TCM	Central memory T-cell
TCR	T-cell receptor
TEM	Effector memory T-cell
TEMRA	Effector memory re-expressing CD45RA T-cell
TFH	Follicular T-helper
TGF	Transforming growth factor
Th	Helper T-cell
Th3	TGF-β secreting type 3 helper
TIGIT	T-cell immunoglobulin and ITIM domain
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TNF	Tumor necrosis factor
Treg	Regulatory T-cells
Tr1	Type 1 regulatory T-cells
UVB	Ultraviolet B light
vD	Vitamin D
VCAM-1	Vascular cell adhesion molecule
VLA-4	Very late antigen-4
WHO	World Health Organization

ABSTRACT

Multiple sclerosis (MS) is a degenerative autoimmune disease of the central nervous system (CNS), where autoreactive $CD4^+$T-cells are believed to attack the myelin sheath of neurons causing CNS damage. MS is also associated with viral infections, in particular with Epstein–Barr virus (EBV), but the role of viruses in MS progression is debated.

Auto-reactive and overshooting anti-viral T-cell responses are controlled by regulatory T-cell subsets, namely $FOXP3^+$Treg and IL-10-producing type 1 regulatory cells (Tr1) cells. Both subsets were proposed to be involved in MS, but the role of Tr1 cells *in vivo* in MS remains unclear. Eomesodermin (Eomes), a putative lineage-defining transcription factor of Tr1 cells that controls directly the expression of Granzyme (Gzm)K, allows their analysis *ex vivo*. Notably, in order to suppress immune responses efficiently, regulatory T-cells have to be activated by antigens, and their antigen specificity is a key feature. Cell-therapy with regulatory T-cells was established in other immune-mediated diseases, but the subset that efficiently suppresses pathogenic T-cells in MS needs first to be identified.

The aim of this thesis is to understand the role of Tr1-cells in MS, in particular, to analyze their CNS-homing capacities and their specificity for self- or viral-antigens, in order to identify subsets that are suited for MS cell-therapy. Therefore, in this project I monitored a cohort of relapsing-remitting MS patient that were either untreated or treated with natalizumab – the anti-α4 integrin antibody that block the CNS-homing of lymphocytes – by multidimensional cytometric analysis.

I found that GzmK$^+$Tr1 cells – and not FOXP3$^+$Treg or GzmB$^+$CTL (cytotoxic lymphocytes) – are strongly and selectively enriched in the cerebrospinal fluid (CSF) of active MS patients, suggesting a role in relapses. Moreover, Tr1 cells were reduced in the blood of MS patients and were highly proliferating *in vivo*, suggesting that Tr1 cells are recruited and activated in the CNS of these patients. Consistently, natalizumab-treated MS patients showed normal Tr1 frequencies and proliferation rates. Conversely, MS patients had strikingly higher frequencies of Tregs and a reduced *in vivo* turnover, while CTL were unaltered.

To assess *ex vivo* the antigen specificity by flow cytometry, a new assay was successfully established. Tr1 and their putative precursors cells responded strongly and selectively to the EBV latency-associated antigen EBNA1 in MS patients, and not with lytic ones, but responded only weakly in healthy individuals. They also failed to respond to myelin antigens or to the John Cunningham Virus. Interestingly, natalizumab-treated patients had significantly higher levels of EBV-specific Tr1 cells, suggesting that these cells are recruited to and/or generated from precursors in the CNS. Tr1 cells have enhanced anti-inflammatory properties in MS patients, secreting higher levels of IL-10 in response to polyclonal stimulation. Moreover, we have preliminary evidences that Tr1 cells produce also considerable amounts of IL-10 in the CSF and even in response to EBV/EBNA1 in the blood of MS patients.

Overall, our results are consistent with the notion that there is a dysregulated immune response against EBV in the CNS of MS patients,

and suggest a dual role for Eomes^{+}Tr1 cells regulating EBV-specific and not myelin-reactive T-cells. A key finding for this project is that Tr1 cells may have a beneficial role in relapses since they are present in the CNS and produce the anti-inflammatory cytokine IL-10. But at the same time, the specificity for EBV in the latent phase could be at the basis of the inefficient response to the virus and therefore of MS progression. In the future a better understanding of Tr1 cell role in MS could lead to novel therapeutic approaches, although further investigations on Tr1 cells are needed to understand their suppressive abilities, the genes involved and their role in progressive MS.

Chapter 1. General introduction

1.1. THE IMMUNE SYSTEM

The immune system is a set of highly specialized organs, tissues and cells with the primary task of preserving the functional state of the healthy individual's body. The immune system defends the body by eliminating possible foreign substances and external pathogenic agents (non-self) such as toxins and microorganisms (i.e. parasites, bacteria, viruses and fungi); but also it has the function to defend the body against components of the host itself (self) that have lost their normal function (i.e. impaired), such as cells infected with viruses or damaged-dead cells and tumorigenic cells. This requires a precise discrimination between self and non-self, and this essential feature of the immune system has evolved to a high level of complexity in higher vertebrates.

Specifically, the immune system consists of:

- lymphatic system: primary (bone marrow and thymus) and secondary lymphatic orjgans (lymph nodes and spleen), and tertiary lymphoid organs/tissues (mucous-membranes associated lymphoid tissues MALT in tonsils, appendix and Peyer's intestinal plaques), are interconnected by lymphatic and blood vessels;
- leukocytes or "white blood cells": specialized cells capable of circulating in the blood, lymph and tissues. These cells originate from progenitors in the bone marrow, pluripotent hematopoietic stem cells (HSC) that differentiate into granulocytes (neutrophils,

basophils, eosinophils, and mast cells), monocytes (macrophages and dendritic cells, DC) and lymphocytes;

- cytokines: proteins acting as chemical mediators that orchestrate the different immune responses, exchanging signals between them in order to control the functioning of the different organs and lymphatic tissues. Common cytokines include interleukins (IL), chemotaxis-promoting chemokines and interferons (IFN).

The body is permanently exposed to external agents (i.e. through contact, ingestion, inhalation), therefore the immune system is constantly on alert and reacts immediately when harmful pathogens enter the body, acting through a multiple levels defence of increasing specificity. The first level consists of natural mechanical (skin, mucous membranes, saliva, tears), chemical (acid pH, antimicrobic enzymes) and biological barriers (gut commensal flora). However, if the foreign agent manages to break through these physical barriers, an internal defensive process is triggered, which in most cases results in the elimination of the pathogen. Therefore, the innate immune system provides an immediate, but unspecific response. It is a general defense mechanism, present from birth, which acts rapidly (minutes or hours) and indiscriminately against any external agent, by secreting molecules that initiate inflammation and cell recruitment. The innate immune system is found in all plants and animals and does not confer long lasting immunity against a pathogen. Finally, if pathogens successfully evade also the innate response, vertebrates possess a third level of protection, the specific or adaptive immune system, which is activated by the innate response. The adaptive response

develops slowly (over a few days) and improves itself after the first encounter with a specific pathogen. This specific improved response is then maintained after the pathogen clearance, in the form of an immunological memory, thus allowing the adaptive immune system to respond faster and more effectively each time it encounters this pathogen again. (Bedoui, Gebhardt, Gasteiger, & Kastenmuller, 2016; St, 1997)

However, at the end of the immune response, the action of the innate and adaptive systems is blocked by immunosuppressive mechanisms to avoid the effects of excessive or too prolonged reactions. Generally, the failure in the regulation of immune system responses, but also in their genesis, can result in lethal or recurrent infections, in chronic inflammation, tumors, allergies, degenerative diseases, autoimmunity, and many other disorders (Bedoui et al., 2016; Cools, Ponsaerts, Van Tendeloo, & Berneman, 2007b).

1.1.1. THE INNATE IMMUNE SYSTEM

The innate immune system (aspecifc or natural) mainly consists of several leukocytes which include phagocytes (monocytes, macrophages and neutrophils), dendritic cells, mast cells, eosinophils, basophils and natural killer (NK) lymphocytes. These “sentinel” cells reside in the tissues or travel throughout the body patrolling it, in order to identify and eliminate pathogens. Usually, the innate system is aspecifically activated by pattern recognition receptors (PRR) on pre-existing cells, able to recognize both the components of large group of

microorganisms and of any impaired cells (i.e. infected, damaged, stressed, tumoral), which are named pathogen-(PAMP) and damage-associated molecular patterns (DAMP) respectively (Li & Wu, 2021). Activated immune cells, such as eosinophils and phagocytes, attack the pathogens through contact or by engulfing them, while cytotoxic NK cells destroy impaired cells. These systems respond in a generic way, sending alarm signals to other cells and thus initiating the inflammatory process. Eicosanoids (such as prostaglandins and leukotrienes) and certain cytokines are pro-inflammatory soluble mediators, that induce changes in blood flow, increasing permeability of blood vessels, and then the migration of fluids, proteins and leukocytes from the circulation to the site of tissue damage. During the inflammatory event, growth factors and cytotoxic factors can also be released. These cytokines and other chemicals, after the pathogens are removed, can also promote healing of any damaged tissue (Medzhitov, 2007).

For example, macrophages are immune cells highly differentiated in the various tissues of the organism (ex. osteoclasts in the bone, microglia in the central nervous system, Kupffer cells in the liver), where they play the role of "scavengers" of the human body. Macrophages are concentrated where there is a need to eliminate a microorganism or residuals, such as tissue-breakdown products, or a damaged cell. In addition to the ability to phagocytize pathogens, macrophages are able also to secrete eicosanoids, chemokines and other cytokines such as tumor necrosis factor (TNF)-α, and interleukin(IL)-1, IL-6, IL-8, and IL-12. The release of these mediators

allows the amplification of the protective response, thus inducing the synthesis of further inflammatory mediators, such as interferons (type II IFN- γ), the extracellular release of oxidizing compounds (i.e. reactive oxygen products, ROS), the activation of the vascular endothelium to facilitate the local recruitment of other immune cells (neutrophils, monocytes and lymphocytes), the maturation and migration of dendritic cells to the lymph nodes (Arango Duque & Descoteaux, 2014). Macrophages are also important mediators in the development of lymphoid organs and in the activation of the adaptive immune system, mainly by the presentation of pathogens to lymphocytes (Bedoui et al., 2016).

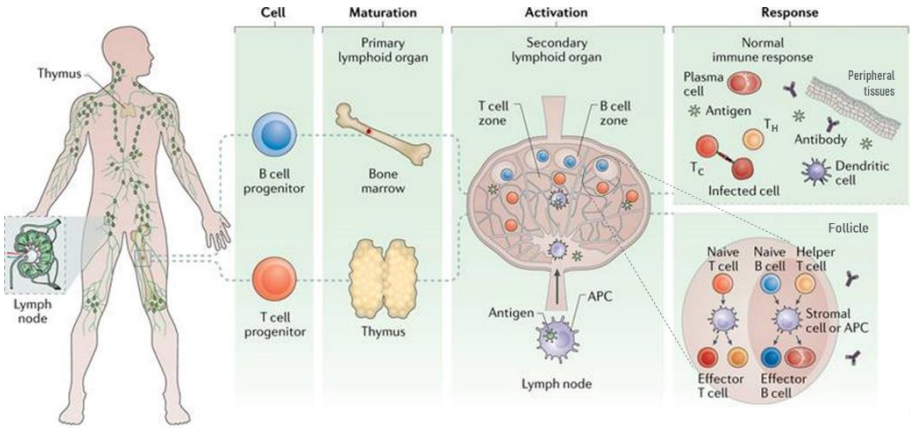
In addition, in peripheral tissues pathogens are sensed and captured by activated DC, which then migrate to secondary lymphoid organs where they instruct and activate an appropriate adaptive immune cell response (Steinman, 1991) (Figure 1.1, activation).

1.1.2. THE ADAPTIVE IMMUNE SYSTEM

The adaptive (specific or adoptive) immune system is critical for clearance and long-term protection against pathogens and tumors, but it also underlies autoimmunity and allergy. The basic strategy was to generate a vast repertoire of cells, named lymphocytes, each with a unique specificity, able to activate a slightly different response for each pathogen (maturation), and to functionally inactivate or bring to death those reactive against self, without altering the maturation of non-self-reactive cells (immunological tolerance) (Bedoui et al., 2016;

Romagnani, 2006). Therefore, each lymphocyte is equipped with a specific receptor for a single molecule, named antigen (Ag), resulting from a variable gene rearrangement. Lymphocytes derive from HSC precursors in the bone marrow and originally they differ according to the primary lymphoid organ where they will complete their development: B-cells in the bone marrow (BM), while T-cells in the thymus (Figure 1.1, maturation). Mature cells enter the circulation and assume a specific migration pattern for the recirculation among different secondary lymphoid organs, such as spleen and lymph nodes (Bedoui et al., 2016). Here lymphocytes enter in the follicles and localize in specific B-cell and T-cell zones. In these zones, B- and T-cells encounter intact antigens or processed antigens presented on APCs (Figure 1.1, activation). The recognition of the antigen leads to activation, proliferation and functional differentiation of lymphocytes into effector cells, which then migrate to the periphery at sites of inflammation and infection. Furthermore, B- and T-cells give rise to two different types of responses: the humoral (i.e. mediated by soluble molecules) and the cell-mediated immunity respectively (Figure 1.1, response).

Figure 1.1 - The different levels of the adaptive immune response.
Lymphocytes undergo genesis, maturation and activation before performing their effector functions. APCs, such as dendritic cells, encounter antigens in the periphery at the infection site and present it to naïve lymphocytes in the lymph node to induce strong and sustained responses. T_C, cytotoxic T-cell; T_H, helper T-cell. *Adapted from Kim, S. et al. (2019) Nat. Rev. Mater. doi:10.1038/s41578-019-0100-9*



However, before being able to perform its effector function, the lymphocyte must undergo mitosis many times, generating clones of daughter cells (clonal expansion) with the same receptor, this clonal division can take several days. For this reason, the primary adaptive response to an unknown pathogen is slow, but it is central in the development of immunological memory. In fact, as result of the selection made by the antigen, the lymphocytes that are able to respond become significantly more abundant and remain as long-lived memory cells. Therefore, the activation of memory lymphocytes allows a faster and improved secondary response towards previously recognized antigens (St, 1997).

1.1.2.1. HUMORAL IMMUNITY AND B-CELLS

B lymphocytes are responsible for the humoral (from *humor*, body fluid) immune response through training in the generation of specific receptors during maturation and their secretion in the soluble form of antibodies, at the effector stage. Mature B lymphocytes, move from the BM and continuously recirculate throughout the body in search of the antigen, entering the follicles of secondary lymphoid organs.

Mature B-cell express the B-cell receptor (BCR), a membrane immunoglobulin (Ig) capable of specifically recognize complex structures on extracellular antigens, of which mediate internalization. Upon contact with the antigen, the BCR forms clusters and activates the B-cell – with the help of T-cells –, inducing its proliferation and differentiation into plasma cells. These are effector cells capable of synthesizing and secreting an impressive number of antibodies (Ab), the soluble form of Ig (sometimes called gamma (Y)-globulins due to their unique Y conformation). The antibodies are then disseminated in the blood and other body fluids, such as saliva, mucus or breast milk. There are five classes of Ig (IgG, IgA, IgM, IgD e IgE), distinguished on the basis of their structure, distribution and function. The antibodies do not directly destroy the foreign pathogen or the impaired cells but, after binding, they neutralize and make them visible to other immune cells, such as cytotoxic cells but also macrophages (antibody-dependent cell-mediated cytotoxicity) or induce the activation of an enzymatic cascade that eliminate the target (complement-dependent cytotoxicity). Another important function of B-cell is to internalize the

antigen for subsequent fragmentation (i.e. processing) and presentation of resulting peptides to T-cells, thus playing the role of APCs (Avalos & Ploegh, 2014).

Furthermore, part of B lymphocytes eventually evolve into memory cells, which continue to circulate for much longer periods than short-lived plasma cells and in the case of a new exposure to the pathogen they quickly reactivate and proliferate (St, 1997).

1.1.2.2. CELL-MEDIATED IMMUNITY AND T-CELLS

T lymphocytes interact with the infected or altered cells of our body and are responsible for cell-mediated immunity. They contribute to the elimination of intracellular pathogens or impaired cells: both directly by the killer activity of cytotoxic T lymphocytes (CTL), and indirectly by activating other immune cells thanks to helper T (Th) cells.

The T-cell receptor (TCR), produced and exposed on the membrane after maturation, is unique to each T lymphocyte and is able to recognize a specific antigen based on its chemical structure. In particular, the TCR recognizes small fragments of peptides, only if they are presented by particular proteins belonging to the so-called "major histocompatibility complex" (MHC). The major histocompatibility complex – originally discovered because its involvement in organ-transplant rejection – belongs to a group of highly polymorphic genes (i.e. there are numerous alternative variants of the same gene, called alleles, with a frequency > 1% in the population), which in humans is

called human leukocyte antigen (HLA) system. There are two main classes of genes, encoding Class I (HLA-A, B, C) and Class II (HLA-DR, DP, DQ) molecules, which present antigenic peptides to T lymphocytes. These proteins have the ability to bind peptides inside the cell and expose them on the outer membrane. MHC of class I molecules are expressed on all nucleated cells, while class II MHC molecules are expressed on professional APCs, such as DC, macrophages and B-cells. MHC of class I is associated with peptides derived from endogenous antigens, such as microorganisms or altered proteins present in the cytoplasm, while the peptides restricted for class II MHC molecules derive from internalized antigens, such as phagocytosed extracellular pathogens. Importantly, due to retrograde transport of antigenic peptides from vesicles to cytosol, the presentation of extracellular ones is also enabled on class I MHC (i.e. cross-presentation), and is particularly important for the activation of cytotoxic T-cells (Neefjes, Jongsma, Paul, & Bakke, 2011).

T-cells use their TCR to recognize the peptide-loaded MHC presented by APCs, and this interaction leads to their activation, through a characteristic signal transduction chain. TCR is part of a molecular membrane complex, which includes the cluster of differentiation (CD)3 and a co-receptor, such as CD8 or CD4, expressed on the respective lymphocyte. In fact, these two specific molecules on the membrane allows to distinguish between $CD8^+$ and $CD4^+$ T-cells, which bind to class I and II MHC molecules respectively. This first interaction, initially induce the synthesis of IFN-γ, interleukins (IL-4, IL-2) and their receptors, such as IL-2R, which mediate an important autocrine

proliferative stimulus (Lanzavecchia et al. 2001). However, in addition to the antigen-specific input (named signal 1), other signals are required to properly activate naïve T-cells.

In addition, costimulatory molecules, such as CD40, CD80 (B7-1) and CD86 (B7-2) expressed by professional APCs, interact with induced CD154 (CD40L) and CD28 expressed on T-cells (Davis et al. 2003). The binding of B7 family molecules to CD28 (signal 2) provides the lymphocyte with anti-apoptotic and proliferative signals. Instead, the engagement of CD40 on APCs by CD40L induces these cells to produce cytokines (signal 3), which in turn have a pivotal role in the differentiation and polarization of T-cell effector functions and their future characteristics (Curtsinger et al., 1999). Noteworthy, effector T lymphocytes do not depend on costimulatory signals for the performance of the effector functions, thus they can rapidly respond to a subsequent encounter with the antigen (Croft, 1994).

Anyway, after the phase of intense proliferation induced by IL-2, activated $CD4^+$ and $CD8^+$ lymphocytes differentiate into Th and CTL, respectively, then migrate to infected peripheral tissues to control infection. After pathogen clearance, effector T-cells substantially contract, but a small fraction of antigen-experienced effetctor T-cells convert into memory T-cells (Figure 1.2). At different stages of differentiation, the expression of certain molecules changes. For example, after the thymic development, mature naïve T-cells (TN) are characterized by the expression of CD45RA, CD62L and CCR7. Due to the expression of CD62L and CCR7, they recirculate throughout secondary peripheral lymphoid organs, until they are activated by

APCs. Instead, circulating memory T-cells express CD45RO and low level of CD25 (IL-2 receptor α-chain, IL-2RA), and based on the differential expression of CD45RA and CCR7, can be divided into several subsets: $CD45RA^{-}$ $CCR7^{+}$ central memory (T_{CM}), which are long-lived and reside in secondary lymphoid organs, $CD45RA^{-}$ $CCR7^{-}$ effector memory (T_{EM}), and $CD45RA^{+}$ $CCR7^{-}$ effector memory re-expressing CD45RA (T_{EMRA}) T-cells, which circulate between lymphoid organs and peripheral tissues (Sallusto, Langenkamp, Geginat, & Lanzavecchia, 2000; Sallusto, Geginat, & Lanzavecchia, 2004).

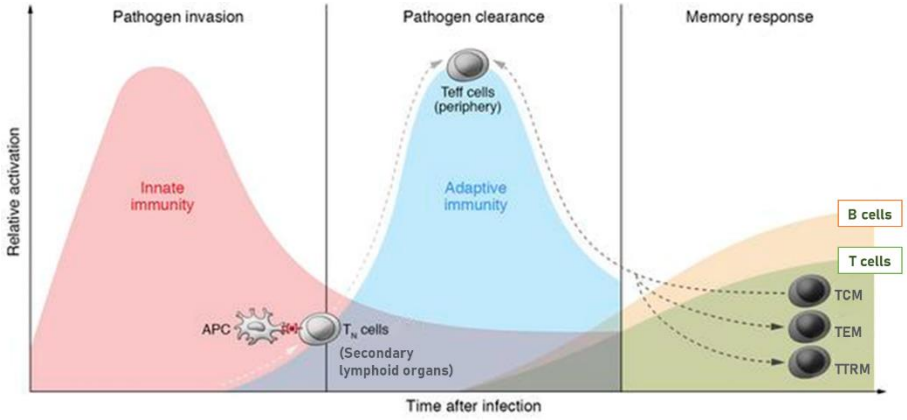


Figure 1.2 - Induction of a long-term memory response.
Sequential activation of innate and adaptive immunity during infection, and origination of memory response. In secondary lymphoid organs, APCs present antigen to naïve T (T_N) cells and convert them to effector T (Teff) cells. These Teff cells then migrate to infected peripheral tissues at infection site. After pathogen clearance, a small fraction of antigen-experienced Teff cells convert to: central memory T (T_{CM}) cells; effector memory T (T_{EM}) cells; tissue-resident memory T (T_{TRM}) cells, residing in peripheral tissues. *Adapted from Khader, S. A. et al. 2019 J. Clin. Invest. doi:10.1172/JCI128877*

Cytotoxic T-cells

Mainly $CD8^+$T-cells differentiate into effector cytotoxic T lymphocytes (CTLs), but also some $CD4^+$T-cells and a distinct subpopulation of lymphocytes, named natural killer T-cells (NKT), can exert similar killer functions and contribute to the cytotoxic responses.

$CD8^+$ CTL perform their action secreting cytokines and primarily eliminating specific target cells (infected cells and tumor cells), that expose the same MHC class I-peptide complex. The principal mechanisms of contact-dependent cell killing is through the interaction between the pro-apoptotic molecule CD95L (FasL) with the death receptor FAS (CD95) on the target cell (a process known as activation-induced cell death) and the release of toxic granules, stored in vesicles and containing perforin and granzymes (degranulation) (Bedoui et al., 2016). In particular, perforin forms pores in the plasma membrane and mediate the entry of granzymes into the cytoplasm, thus inducing apoptosis or simply osmotic lysis.

Granzymes are a family of highly conserved serine-type proteases (enzymes that cleave proteins after aspartate residues), located in granules of both CTLs and NK cells, that cause cell death through apoptotic induction by proteolysis of specific cell substrates, mainly caspases. There are 5 isoforms of granzymes expressed in humans (A, B, H, K, and M). Granzyme (Gzm)B acts both in a caspase-dependent or -independent manner, it is abundant and for this reason it has also been the most characterized (Bots & Medema, 2006). In contrast, GzmK has been less studied and its function is not yet well defined.

However, its known that GzmK acts through caspase-independent mechanisms, and induce cell death by cell lysis, mitochondrial and DNA damage. However, recent evidences have also highlighted non-cytotoxic functions of granzymes, included GzmK, which has a role inhibition of viral replication and modulation of inflammation (i.e. endothelial activation and immune cytokine response) (Bouwman, van Daalen, Crnko, Ten Broeke, & Bovenschen, 2021).

In addition, class I MHC-restricted $CD8^+$ CTL play a fundamental role in the control of viral infections and tumors also thanks to cross-presentation on APC of extracellular antigens by class I MHC molecules – usually presented by class II ones –, since in both cases there are mechanisms to avoid the presentation of endogenous peptides by the MHC Class I in target cells. In this case CTLs act indirectly through the secretion of cytokines, such as IFN-γ and TNF, inducing the suicide of target cells (RW.ERROR - Unable to find reference:567).

Helper T-cells

The main feature of $CD4^+$ helper T (Th) cells is their ability to secrete cytokines following antigen-specific TCR activation, and thus to coordinate all immune responses by helping other cells in their functions: they induce differentiation of B-cells into plasma cells, regulate the activity of killer cells (both NKT and CTL), and increase the phagocytic activity of macrophages (Bedoui et al., 2016; Geginat, J. et al., 2013).

Depending on the disease process, the naïve T-cells differentiation is influenced by a different cytokine microenvironment, and their effector functions can vary to meet distinct challenges in immunological defense (Martinez-Sanchez, Huerta, Alvarez-Buylla, & Villarreal Lujan, 2018). Once polarized, distinct Th cell subsets upregulate peculiar master transcription factors and express distinct cytokine secretion patterns (Geginat, J. et al., 2013) (summarized in Figure 1.3).

For a long time, effector T-cells were divided into two main types, named as $CD4^+$ Th type 1 (Th1) and type 2 (Th2), derived from the exposure of an uncommitted $CD4^+$T-cell to IL-12 or IL-4 respectively. Th1 cells produce IL-2, IFN-γ and TNF-α, activating macrophages and killer cells to eradicate viral and intracellular pathogens. IFN-γ induces activation of the transcription factor T-bet, which in turn establishes a positive feedback loop. IL-2 induces proliferation, acting both in an autocrine and paracrine way, for example on pre-activated $CD8^+$ cells. In contrast, Th2 cells produce different cytokines, such as IL-4, IL-5, IL-10 and IL-13, but not Th1 cytokines, and direct mast cell, eosinophils and basophils mainly against large extracellular pathogens, such as helminths, and are involved in allergies. IL-4 induces the master regulator GATA-3 in Th2 cells. Furthermore, IL-4 and IL-13 stimulate the production of neutralizing IgE antibodies by B-cells. Effectively, Th1 and Th2 cells are not two distinct subsets of $CD4^+$T-cells, but are considered as two highly polarized forms of $CD4^+$ Th cells that mediate the adaptive immune response (Annunziato & Romagnani, 2009; Mosmann & Coffman, 1989).

Subsequently, another type of effector $CD4^+$T-cells was characterized in both mice and humans. These cells have been named Th17 because the main cytokine they produce is IL-17 and orchestrate the neutrophil attack on extracellular pathogens, such as fungi and bacteria. Th17 are also involved in the pathogenesis of inflammatory and autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and autoimmune encephalitis. Th17 differentiation is induced by a combination of cytokines, such as IL-6, transforming growth factor (TGF-)β, but also IL-1β, IL-21 and IL-23, and in absence of IFN-γ and IL-4 (Figure 1.3). The master transcription factor upregulated is RORγt (Bettelli, Korn, & Kuchroo, 2007). In particular, Th1/Th17 cells that co-express RORγt and T-bet and co-produce IFN-γ and IL-17, are particularly pathogenic. These cells can be generated by IL-23 and are present in lesions of patients with MS (Geginat, J. et al., 2013; Paroni et al., 2017).

Moreover, Th cell subsets require specific migratory behaviours and can also be discriminated by expression of a particular chemokine receptor profile, which enable a different homing potential to infected or inflamed tissues. Th1 cells express the chemokine receptors CXCR3 and CCR5, while Th2 are mostly $CCR4^+$. Instead, CCR4 and CCR6 are marker of Th17 cell subset (Geginat, J. et al., 2013) (Figure 1.3). Although nearly all $CD4^+$T-cells express CXCR5 rapidly after activation, only a subset of these cells retains high level of CXCR5 and develops into the follicular “B helper” T-cells (Tfh) that are found in the follicle of secondary lymphoid organs (Hughes & Nibbs, 2018)

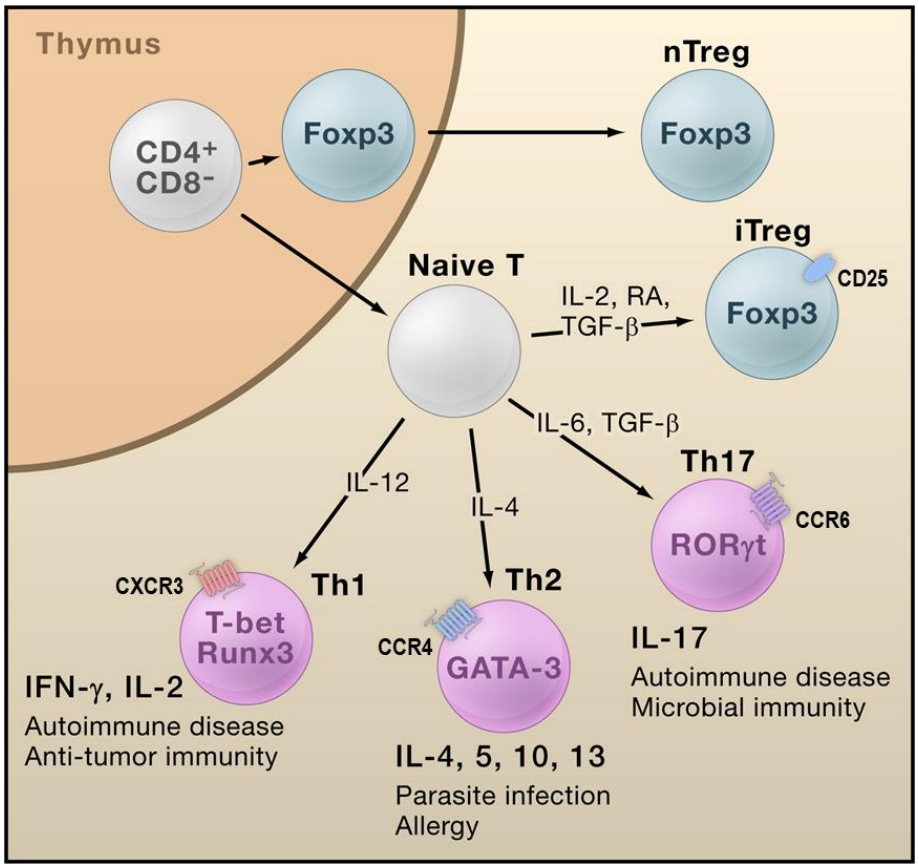


Figure 1.3 - Differentiation of naïve CD4⁺ T-cells into effector or regulatory T-cells.

Cytokines promote the differentiation of naïve T-cells into the various indicated types of CD4⁺ T-cells. Lineage-defining transcription factors induce the production of characteristic effector cytokine and the expression of specific surface surface markers. nTreg, natural Treg; iTreg, induced Treg; RA, retinoic acid. *Adapted from Sakaguchi, S. et al. (2008) Cell. doi:10.1016/j.cell.2008.05.009*

1.1.3. IMMUNOSUPPRESSION AND REGULATORY T-CELLS

After the immune reaction has started, the immune system activates immunosuppression, which, in general, should be considered as a self-regulating mechanism of the immune system, to avoid or limit the damage that can derive from excessive and/or prolonged activation. Immunosuppression also play an important role in inducing and maintaining tolerance to self or harmless antigens (Romagnani, 2006; Sakaguchi, Wing, & Yamaguchi, 2009). The immunosuppressive and immunoregolatory mechanisms are numerous and include: the induction of inactivation (i.e. anergy) by secretion of immunomodulatory cytokines, such as IL-10 and TGF-β, or of cytolisis, via the perforin- and granzyme-dependent pathway. Other proposed mechanisms include the interruption of energy metabolism and the subtraction of cytokines, such as IL-2, essential for the survival of their target cells, and lastly indirect suppression by the negative modulation of APC functions through inhibitory molecules such as cytotoxic lymphocyte-associated (CTLA-4), programmed death (PD)-1 and lymphocyte-activation gene (LAG-)3 (Pandiyan, Zheng, & Lenardo, 2011; Sakaguchi et al., 2009) (summarized in Figure 1.4).

CTLA-4 (also referred to as CD152) and PD-1 (CD279) are inhibitor molecules belonging to the CD28 family of co-receptor. Both molecules are found mainly on the surface of T-lymphocytes, but their expression has been also reported in B-cells, DC and NK cells, and perform their inhibitory function when activated by other cells of the immune system. The CTLA-4 molecule is a high affinity homologue of

the activating co-receptor CD28 with which strongly competes for binding to the same B7 family ligands on APCs or other presenting cells. CTLA-4 induces anergy, reduce proliferation and survival, activation threshold and IL-2 production (but also of IFN-γ and TNF), both providing directly a negative signal and interfering with the positive signal given by CD28. This happens after stimulation as a mechanism to downregulate the immune response. In fact, the CTLA-4 molecule is externalized at increasing levels on activated lymphocytes and ultimately blocks the continuation of the T-cells response (Cools, Ponsaerts, Van Tendeloo, & Berneman, 2007a; Romo-Tena, Gomez-Martin, & Alcocer-Varela, 2013). At the molecular level, anergy is characterized by a lower expression of TCR and a block of signal transduction and ctokines production, and by the induction of the expression of CTLA-4 and PD-1 (Tai et al., 2012). However, CTLA-4 also has indirect suppressive functions through the negative modulation of APCs (i.e. induction of IDO, downregulation of B7 ligands and CD40) (Figure 1.4d). CTLA-4 acts as an inhibitor of T lymphocytes while they are still in the lymph nodes, while PD-1 acts similarly later, and inhibits previously-activated T lymphocytes throughout the body, since its ligands PD-L1 and PD-L2 are mainly expressed on cells in peripheral tissues (Buchbinder & Desai, 2016).

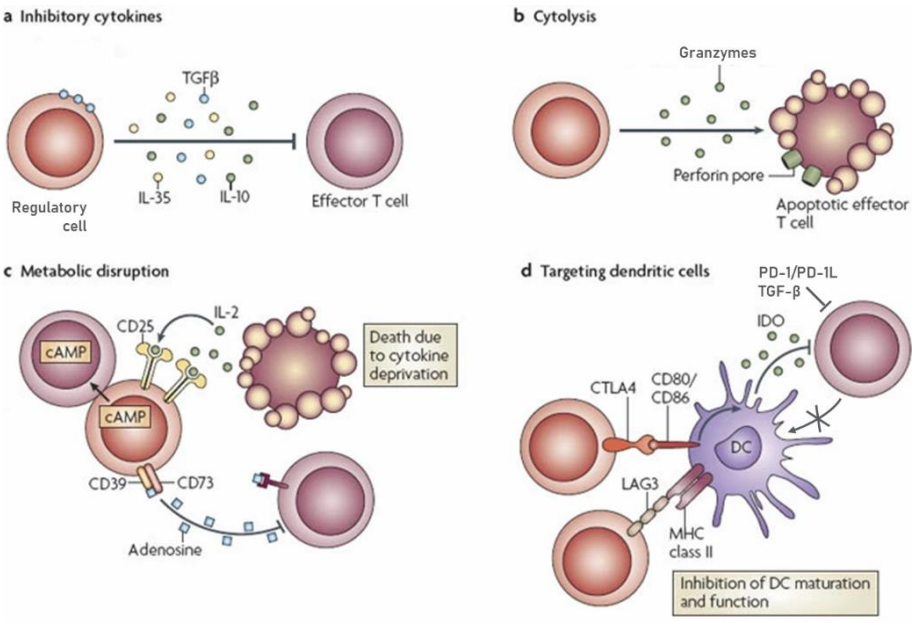


Figure 1.4 - Putative mechanisms used by regulatory T-cells.

a. Production of inhibitory cytokines – including IL-10, TGF-β and IL-35; **b.** Cytolysis – direct cytotoxic effect hrough the production of granzymes and perforin; **c.** Metabolic disruption – includes cytokine deprivation, cyclicAMP-mediated and adenosine-mediated immunosuppression; **d.** Targeting DCs – includes mechanisms that modulate APC activity and that disrupt effector-cell engagement with APCs, such as lymphocyte-activation gene 3 (LAG3/CD223)-MHC-class-II-mediated suppression of DC maturation, and cytotoxic T-lymphocyte antigen-4 (CTLA4)–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs; CD80/CD86 engagement on the DC surface by regulatory T-cells also leads to weak or abrogated signals to naïve/effector T-cells. *Adapted from Vignali, D. et al. (2008) Nat. Rev. Immunol. doi:10.1038/nri2343*

Other important immunosuppressive mediators are TGF-β and IL-10, cytokines capable of blocking the proliferation and functions of effector T lymphocytes, inhibiting the production of cytokines and also promoting the differentiation of cells with immunosuppressive phenotype. TGF performs another important function inhibiting the activation of macrophages and modulating the repair process (Sanjabi, Oh, & Li, 2017). Moreover, IL-10 can be produced by almost all immune cells and has long been known primarily for its tolerogenic and anti-inflammatory properties. This cytokine is in fact capable of blocking the production of pro-inflammatory cytokines, of inhibiting the T-cell stimulatory capacity of DC and macrophages and the proliferation of $CD4^+$T-cells (Ng et al., 2013). IL-10 plays a protective role mainly in the intestine in maintaining homeostasis and tolerance to the commensal flora, in fact genetic defects of IL-10 in mice and in humans leads to the development of excessive inflammation and chronic colitis (Izcue, Coombes, & Powrie, 2009; Uhlig et al., 2006). It also appears to play a similar role in the central nervous system and in MS as well (Bettelli et al., 1998; Martinez-Forero et al., 2008). However, IL-10 has a dual role, because it is also known for its stimulatory effects on the humoral response and its contribution in the pathogenesis of SLE. Indeed, this cytokine is also a potent B-cell grow factor, promoting its survival, activation, action and differentiation (reviewed by (Geginat, J. et al., 2016; Geginat, Jens, 2019).

Immunosuppression and immunoregulation are operated by $CD4^+$ T cells, but also with the participation of $CD8^+$ and NKT lymphocytes, which act by eliminating or making anergic other T lymphocytes

involved in a specific immune reaction. Once activated, regulatory T-cells exert their suppressive functions on a wide range of adaptive immunity cells, such as naïve and effectors T-cells, B-cells, NKT lymphocytes, and innate cells such as macrophages, DCs and NK cells. In addition, immunosuppression is generally specific, although the underlying molecular mechanisms are unclear, but it can inhibit the response of other cells even regardless of their specificity, in a bystander mode. However, *in vitro* studies have first of all shown that suppression requires cell-cell contact, although the nature of this interaction has not yet been clarified (Sercarz & Krzych, 1991).

FOXP3$^+$ regulatory T-cells

The best characterized regulatory cells are CD25$^+$ FOXP3$^+$ Treg. These cells are CD4$^+$T-cells produced through a process of thymic selection and that – unlike the majority of thymus-produced naïve T-cells – are already "effectors" functionally suppressive (natural Treg, or nTreg). Alternatively, they can also be induced in peripheral tissues by naïve T-cells in presence of particular factors, such as TGF-β and IL-2 (induced Treg, or iTreg) (Figure 1.3). Tregs specifically express the master transcription factor FOXP3 (forkhead box P3), which regulates their development and functionality. FOXP3 directly induces the expression of CD25 (IL-2RA) and CTLA-4, but at the same time downregulates the CD127 (IL-7RA), IL-2, IFN-γ, and IL-4 (Fontenot & Rudensky, 2005). Genetic defects in the expression of FOXP3 and its controlled genes, such as CD25, result in the manifestation of severe

immune pathologies in both humans and mouse models (Bennett et al., 2001; Sakaguchi et al., 2020). It is therefore clear that Tregs play a crucial role in maintaining tolerance and homeostasis. In fact, their absence not only leads to autoimmunity, but determines an increase in the reactivity towards non-self and tumors (Sakaguchi, Yamaguchi, Nomura, & Ono, 2008; Sakaguchi et al., 2020).

The survival and activity of Tregs depend on IL-2 produced by other cells, such as activated T lymphocytes. Therefore, a negative feedback is established in which the IL-2 produced to stimulate the immune response is subtracted from the microenvironment (Figura 1.4c) and at the same time favours the regulatory T-cells, which in turn suppress the non-regulatory ones. For this reason, CD25, the α-subunit of the high affinity IL-2 receptor (IL-2RA), is another critical molecule for Tregs (Sakaguchi et al., 2008). In contrast, Tregs express low levels of CD127 (IL-7RA), the α-chain of the IL-7 receptor, which is an important cytokine for the homeostasis of various populations of T-cells, in particular helper T-cells which express high levels of CD127/IL-7RA (Carrette & Surh, 2012).

Tregs express chemokine receptor patterns for homing that are usually similar to those of effector T-cells. They migrate not only to regional lymph nodes where tissue-specific or microbial antigens are presented, but also to non-lymphoid tissues, such as sites of inflammation and infection or tumors, where they are activated (Belkaid & Oldenhove, 2008; Duhen, Duhen, Lanzavecchia, Sallusto, & Campbell, 2012)**.** TCRs of Tregs are highly self-reactive, therefore they induce activation even in the presence of low antigen concentrations.

Tregs perform their immunosuppressive functions both through cell-to-cell contact and with the secretion of TGF and IL-10 (Sakaguchi et al., 2009) (Figure 1.4a). Another function could be performed by CD39 an ecto-enzyme that cleaves the extracellular ATP (adenosine tri-phosphate), an important substrate for cellular energy metabolism. ATP is removed from the activated cells and releases adenosine, that has anti-inflammatory properties (Borsellino et al., 2007) (Figura 1.4c).

Type 1 regulatory T-cells

Besides to traditional well-established FOXP3$^+$ Treg, other cells play an important immune regulatory role such as the TGF-β secreting type 3 helper (Th3) cells and the IL-10 producing type 1 regulatory (Tr1) cells, whose long-term stability is still under discussion. It has been proposed that Tr1 cells derive from naïve T-cells differentiated in the presence of IL-10 and IL-27, suggesting them as an additional CD4$^+$T-cell lineage, or that just represent a transitory state. In any case, a main feature of Tr1s is the production of immunosuppressive cytokine IL-10 and also IFN-γ, which can have paradoxical functions *in vivo*, inducing apoptosis or inhibiting T-cell activation and proliferation (Geginat, J. et al., 2013; Wood & Sawitzki, 2006) (Figure 1.5).

However, the production of IL-10 alone is not a sufficient parameter to define this population, because iL-10 is also produced by FOXP3$^+$ Tregs and Th cells (Ng et al., 2013). Gagliani et al in 2013 proposed LAG-3 and CD49b/α4-integrin as marker to enrich for Tr1 cells. However, LAG-3 is generally expressed on activated cells, therefore it

would exclude non-activated ones and precursors (Gagliani et al., 2013). In addition, Tr1s also express CTLA-4, PD-1, TIM-3 (T-cell immunoglobulin and mucin-domain containing-3), TIGIT (T-cell immunoreceptor with immunoglobulin and ITIM domain), ICOS (Inducible T-cell COStimulator) and CCR5 (Alfen et al., 2018). In any case, none of these markers is exclusive of Tr1 and the absence of a master gene regulator makes it difficult to identify these cells (Brockmann et al., 2018; Roncarolo, Gregori, Bacchetta, Battaglia, & Gagliani, 2018; White & Wraith, 2016). Recently, our laboratory has identified a possible lineage-defining transcription factor, named Eomesodermin (Eomes) for the *in vivo* occurring Tr1 cells in humans (Cossarizza et al., 2021; De Simone et al., 2021; Gruarin et al., 2019). Eomes is characteristic for cytotoxic T lymphocytes (CTL) and controls IFN-γ production, Granzyme K expression and cytotoxic functions (Zhang et al., 2017). However, *ex vivo* gene expression and miRNA profiling of $Eomes^{+}$Tr1-like cells provide additional evidence that are a unique T-cell subset, with an intermediate phenotype between Th1 cells and cytotoxic $CD4^{+}$T-cells (De Simone et al., 2021) (see Chapter 2). Interestingly, a single nucleotide polymorphism of EOMES was recently confirmed to be strongly associated with multiple sclerosis in genome-wide association studies (GWAS) (International Multiple Sclerosis Genetics Consortium, 2019; Parnell et al., 2014)

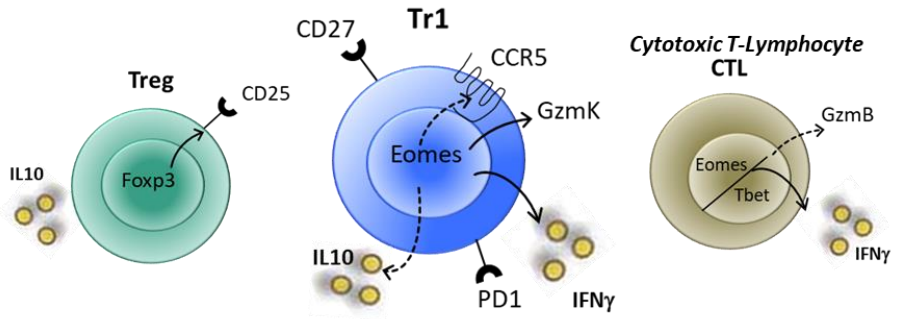


Figure 1.5 - Tr1 are a unique regulatory T-cell subsets.
Tr1 cells differently from Treg are IL-7R^{-}FOXP3^{-}CD25^{-} and coproduce IFN-γ with IL-10. They express the transcription factor eomesodermin (Eomes) and, differently from CD4^{+}CTL, secrete Granzyme (Gzm)K and express CD27, PD-1 and CCR5.

1.1.4. IMMUNOLOGICAL TOLERANCE

A healthy individual is tolerant towards his own antigens, a phenomenon known as immunological tolerance, which is a fundamental property of the immune system. The failure or breakdown of self-tolerance mechanisms leads to autoimmunity and autoimmune diseases. Therefore, it is necessary that the immune system, during the development and maturation of lymphocytes, learns to recognize and to coexist with its self-antigens, and at the same time preserve, expand and remember the ability to react against non-self-antigens or corrupted ones. When T lymphocytes interact with antigens specific for their receptor (MHC-peptide complex complementary to their TCR), depending on the signal strength and the involved co-stimuli, they can be induced to survive or to die: this is

how tolerance is implemented. Immunological tolerance can be induced by central tolerance and peripheral tolerance (Xing & Hogquist, 2012).

Central tolerance develops during lymphopoiesis in the BM or in the thymus, where immature B- and T-cells, respectively, with high affinity receptors meet the self-antigen. Autoreactive cells undergo a process of programmed death, named negative selection. In addition, there is also a positive selection in which the lymphocytes receive survival stimuli through the receptors with a correct affinity for the respective ligands. Consequently, there are no more cells capable of recognizing the self-antigens present in the primary lymphoid organs (Kamradt & Mitchison, 2001). However, it must be emphasized that numerous clones of mature T lymphocytes escape this selection process, because their development occurs mainly during fetal life, when not all human antigens are yet available at the thymic level to operate the negative selection. In this case the potentially autoreactive clones leave the thymus and reach the periphery, where they must be controlled by peripheral tolerance (Xing & Hogquist, 2012).

Peripheral tolerance is mainly important to maintain tolerance towards those autoantigens that are expressed in secondary peripheral lymphoid tissues. In particular, peripheral tolerance can be induced in autoreactive lymphocytes with three main mechanisms (Mueller, 2010; Xing & Hogquist, 2012):

- clonal anergy: consists in a prolonged or irreversible state of functional non-responsiveness that occurs in the absence of the costimulatory signal or as a consequence of a lack of activation of

innate immunity. In some conditions, the correct activation of T-cells cannot occur because professional APCs or other cells (epithelial, endothelial cells, etc), present antigens by class II MHC molecules to the T lymphocyte in the absence of the costimulatory B7 family molecules (such as CD80 and CD86) on their surface (Cools et al., 2007a). In addition, the state of clonal anergy is also observed in presence of higher levels of the inhibitory molecule CTLA-4 that competes with the CD28 present on T-cells, subtracting its B7 counterpart on presenting cells (Romo-Tena et al., 2013).

- clonal deletion: is death that occurs in absence of inflammation and/or upon repeated stimulation. This type of apoptosis is called activation-induced death, and is induced both by a death-receptor pathway (i.e. FAS/FasL interaction) or by a mitochondrial pathway (Krammer, 2000).
- Immunosuppression mediated by regulatory lymphocytes.

1.1.5. AUTOIMMUNITY AND AUTOIMMUNE DISEASES

Autoimmunity is defined as a disorder of the immune system that no longer recognizes its own cellular and tissue structures and activates humoral- and cellular-type response, and is therefore capable of altering the integrity and function of cells and target organs.

The autoimmunization process takes place both by activating the expression of costimulatory molecules and cytokines in the inflammatory areas by tissue APCs otherwise in the resting phase, both

by favouring the transformation of self-antigens into reactive neo-antigens, and by causing the release of self-antigens normally "sequestered" and inaccessible to the immune system (Kamradt & Mitchison, 2001). Additionally, some infectious agents can share antigens that have cross-reactivity with autoantigens, such as to induce an immune response which also affects the self (molecular mimicry) (Berger, 2015). The physiological processes of induction and maintenance of tolerance can be altered both as a result of environmental factors, such as trauma, infections and hormonal conditions and due to genetic anomalies, especially in genes involved in the antigen presentation to lymphocytes, such as the class II MHC/HLA (Simmonds & Gough, 2005). Defects in regulatory T-cell development, maintenance, and function also lead to autoimmunity (Qiu et al., 2020; Rosenblum, Remedios, & Abbas, 2015)

Overall, autoimmune diseases are classifiable in two general groups: organ-specific diseases, characterized by the auto-reactivity against one or more antigens belonging to a specific organ (such as Hashimoto thyroiditis, coeliac disease in the gastrointestinal tract, type I diabetes in the liver, multiple sclerosis in the central nervous system) and non-organ specific, or systemic, diseases in which several organs are affected and the autoimmune response is directed against self-molecules widely distributed in the body (in particular intracellular molecules involved in DNA transcription or components of the connective tissue), such as SLE and rheumatoid arthritis. Frequently, some of these diseases are classified as immune-mediated

inflammatory diseases to underline the pathogenic role of chronic inflammation (Qiu et al., 2020; Rosenblum et al., 2015).

1.2. NEUROIMMUNOLOGY

1.2.1. THE CENTRAL NERVOUS SYSTEM

The nervous system is composed by the brain, spinal cord, sense organs and all the nerves that connect these organs with the rest of the body. It is possible to distinguish a central nervous system and a peripheral nervous system (PNS), which in turn can be divided into several components, each with a specific function. In particular, in this thesis, I will focus on central nervous system (CNS).

The CNS is a very well organized structure that include the brain and spinal cord, which have the function to process information from and to the entire body. Both structures are constituted by different cell types: an adult brain contains about a hundred billion nerve cells – best known as neurons – and an even greater number of cells that perform support functions, collectively named glia or neuroglia, and consisting of microglia, astrocytes, and oligodendrocyte. The brain, which is located inside the cranium, is formed by two hemispheres joined together and it is directly connected with the spinal cord, which instead is a cylindrical structure that resides inside the vertebral column (Figure 1.6). Both the structures are surrounded by a series of protective membranes, named meninges. The brain and spinal cord "float" in the cerebrospinal fluid (CSF, also called *liquor*) and are permeated by this brain-born liquid with a primarily protective

function, which fills the larger spaces within the CNS and flows between the two meninges, *pia mater* and arachnoid (Figure 1.6a). Nerves branch off to other parts of the body (Thau, Reddy, & Singh, 2021).

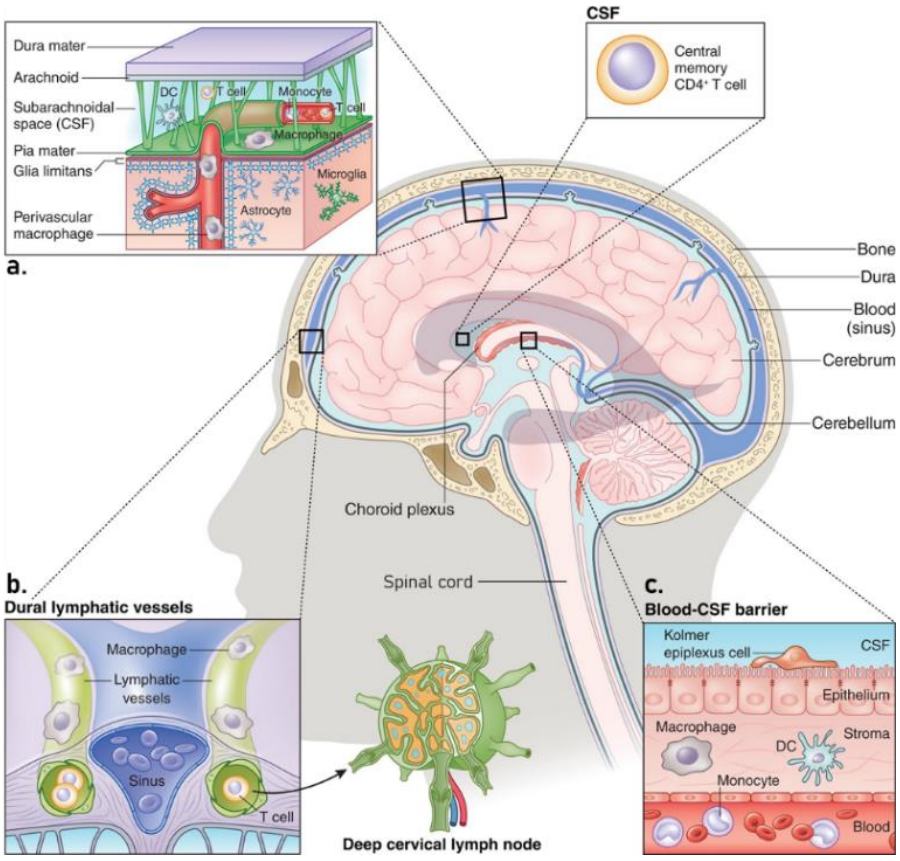
1.2.2. THE MYELIN

Myelin is a gelatinous whitish multilamellar structure consisting mainly of lipids (79%) and proteins (18%), which abundantly constitutes the "white matter". In anatomy, the white matter is composed by the axons of neurons, grouped in bundles that connect the brain and the spinal cord. The bundles appear white due to the myelin coating. In contrast, gray matter is the name used to refer to any area of the CNS with a high concentration of neuronal bodies. In detail, neurons are the functional units of the nervous system. Structurally, they include: the soma, which contains the cell nucleus, the dendrites, which are short and thin extensions, and the axon, which corresponds to a body-extension and is required to spread nervous information. The axon can be myelinated or unmyelinated (Stadelmann, Timmler, Barrantes-Freer, & Simons, 2019).

The myelin sheath is the plasma membrane of the glia: in the CNS there are the oligodendrocytes, while the Schwann cells in the PNS. This sheath consists of extremely thin double-membrane flaps of these cells, repeatedly wrapped around the axon of neurons. In the CNS, a single oligodendrocyte can envelope and myelinize multiple adjacent axons (up to 60), thus forming and maintaining the nerve fiber.

Through their axons, neurons transmit electrical signals, called “action potentials”, responsible for the transmission of information between cells. In all of this, the important role of the myelin is to isolate the axon, as well as the covering of electrical wires, thus allowing a faster propagation of the electrical signal (up to 100-150 m/s) than unmyelinated axons (5 m/s) (Stadelmann et al., 2019).

Figure 1.6 - The CNS immune system during homeostasis.
Scheme of the nondiseased brain, depicting anatomical structures and cells involved in ensuring tissue integrity. **a.** Layers of connective tissue sheets, the dura mater and leptomeninges (arachnoid mater and pia mater), are located beneath the skull. CSF is produced in the choroid plexus (which has a blood-CSF barrier), bathes the brain, contains T cells and flows both in the parenchyma and in the subarachnoid area, which comprises arteries and the perivascular space. Whereas the CSF drains back to blood circulation, **b.** immune cells and proteins in CSF may be drained primarily through meningeal lymphatic structures to reach deep cervical lymph nodes, prototypical secondary lymphoid organs. **c.** Leukocytes (granulocytes, T and B-cells) stay within the blood vessels and usually do not enter healthy brain tissue. Thus, the only endogenous immune cells within the CNS are parenchymal (microglia) and nonparenchymal macrophages (perivascular, meningeal and Kolmer epiplexus cells), and some DCs and central-memory T-cells in the CSF. *Adapted from Prinz, M., & Priller, J. (2017) Nat. neurosci. doi:10.1038/nn.4475*



1.2.3. THE CNS IMMUNE SYSTEM

The CNS, along with a few other anatomical areas of the body, is historically considered an immune-privileged site: an area where, under normal conditions, the immune surveillance mechanisms to foreign antigens are limited and the adaptive immune response to CNS antigens does not take place. This was thought to be due to the lack of classical draining lymphatics, the immune-tolerance conditions and the isolation of CNS by a complex set of barriers that provide not only structural protection but also limit and tightly regulate the entry of

cells and soluble mediators into the parenchyma. These CNS barriers include the meninges and the blood–brain barrier (BBB) (Figure 1.6a), the blood-CSF barrier of the choroid plexus (Figure 1.6c), and even a CSF-interstitial fluid barrier at the interface with the CNS parenchyma (Buckley & McGavern, 2022; Prinz & Priller, 2017).

Actually, the CNS borders are more permissive to immune reactions than previously thought and the old concept of an immune-privileged site has evolved towards the idea of a tissue with an exclusive and dynamic immune-regulation that depends on physiological processes, cellular interactions and anatomical compartments (Papadopoulos, Herz, & Kipnis, 2020). Recently, this idea has been strengthened from the discovery of a meningeal lymphatic drainage system within the brain (Eide, Vatnehol, Emblem, & Ringstad, 2018; Louveau et al., 2015), that, therefore, like any other tissue it is connected to the peripheral immune system. In fact, functional lymphatic vessels in the complex area of the "dural sinuses" – that is the cavity in the bones of the face connecting with the nasal cavities – are able to carry both the fluid and the immune cells coming from the CSF, and are also connected to the deep cervical lymph nodes (Figure 1.6b) (Buckley & McGavern, 2022; Prinz & Priller, 2017). Thus, lymphatic collection of Ags from the CNS is an active part of its homeostasis in the exclusive immune surveillance of CNS (Papadopoulos et al., 2020) (summarized in Figure 1.6)**.**

1.3. MULTIPLE SCLEROSIS

1.3.1. GENERAL FEATURES

Multiple Sclerosis (MS), also known as disseminated sclerosis, is a neurodegenerative disease of the CNS. MS is characterized by a chronic inflammatory demyelinating condition, with unclear etiology and autoimmune pathogenesis, in which the immune system selectively attacks and gradually destroys the healthy myelin sheath of nerve fiber, interfering with signaling pathways throughout the brain and the body, and causing neuronal loss (Filippi et al., 2018). It is therefore evident that in MS patients, due to myelin loss and severe axonal damage, the nerve fibers are no longer able to effectively transmit signals, resulting in a relative slowdown or interruption of the transmission of nerve impulses. In MS this process can evolve from an early acute inflammatory phase to a chronic one, in which these demyelinated areas acquire scar-like characteristics, hence the term "sclerosis". For these characteristics, in 1868, the French neurologist Jean-Martin Charcot (1825-1893) named it "*Sclérose en plaques*". He was the first to recognize MS as a peculiar disease (Zalc, 2018). Furthermore, these sclerotic plaques can appear repeatedly over time and everywhere in the CNS. Due to this multifocal lesion distribution it is also defined as "multiple" or "disseminated" (Reich, Lucchinetti, & Calabresi, 2018).

The symptoms of MS vary widely between people**,** depending on the location and extention of inflammation and neuronal injury, and there are no exclusive symptoms of the disease. This heterogeneity causes a

broad spectrum of potential signs and symptoms involving sensitive perception, cognitive ability and motor coordination. The most frequent symptoms, especially at onset, include blurred or double vision, loss of thermal and tactile sensitivity, limb tingling, dizziness, gradual weakness togheter with fatigue. These latter symptoms can worsen until unabling walking, standing and even causing complete paralysis (Filippi et al., 2018; Reich et al., 2018). MS is a chronic disease that makes life unpredictable, often leading to severe disability. Patients with MS have different symptoms variously associated, some may recur more frequently, others may appear, change and worsen to different extent over the disease progression.

1.3.2. **DIAGNOSIS**

MS is a very complex disease and it is difficult to diagnose. Many symptoms are common to other diseases or conditions so that diagnosis unfortunately can be delayed. Early MS usually occurs with an acute episode of neurological deficits named “attack”, in which typical symptoms of the desease appear depending on the location of the demyelinating lesion in the CNS and the extent of inflammation. In clinical practice MS is often diagnosed only after numerous attacks, as the initial symptoms are not very specific and can be traced back to other diseases. Nowadays, there is still no single laboratory test or medical exam that can confirm MS diagnosis which is mainly based on three elements: the symptoms reported by the patient, neurological

physical examination and instrumental and biological tests (Dobson & Giovannoni, 2019; Thompson et al., 2018).

First, the patient's clinical history (anamnesis) provides the neurologist with information about rencent symptoms, previous disorders, family diseases and other useful details to get an overall general overview. Second, a careful neurological examination is carried out with the aim of evaluating different neurological functions of the organism (i.e. movement, language, coordination, sensitivity, balance, reflexes). Third, in case of “suspected MS”, the doctor may consider some specific tests to exclude other pathologies with similar symptoms, which can lead to the final diagnosis.

Magnetic resonance imaging (MRI) is the instrumental test of choice to detect MS since it provides solid evidence of demyelinated plaques and inflammation. This allows to visualize them and thus to locate and number alterations, also discriminating active lesions with recent onset of myelin loss. In fact, it is possible that the lesions are not only spread in different areas of the CNS (dissemination in space, DIS) but have also occurred at different times (dissemination in time, DIT).

In some cases, to increase the diagnostic accuracy of MRI, it is useful the examination of the cerebrospinal fluid (CSF). The lumbar puncture (rachicentesis) allows sampling of this fluid. The CSF examination can highlight the abnormal presence of leucocytes cells and increased levels of proteins and antibodies, reflecting the "activation" of the immune system. A high concentration of similar antibodies, called G-class Immunoglobulin (IgG), is detected by a particular laboratory test, which shows the presence of a specific pattern of oligoclonal bands

(OCBs) in the CSF and not in the blood. However, this activation of the immune system, although very frequent, is not specific to MS. Additionally, blood tests are part of the initial testing for suspected MS, to exclude other pathologies such as HIV/AIDS, SLE, rare hereditary disorders. Moreover, the examination of the evoked potentials (EP) can be useful to enstablish DIS, allowing to identify even asymptomatic mild lesions to nerve fibers that are still latent (Thompson et al., 2018).

After having performed all the tests, if a diagnosis of "possible MS" has been formulated, the person will be asked to repeat, after a few months, some tests, such as MRI, to check for the possible appearance of new lesions and inflammatory processes. Therefore, the set of results and prolonged clinical observation make it possible to confirm or exclude the presence of clinically definite MS (CDMS). Currently, in many case the diagnosis of MS can be made earlier and more accurately, reducing the possibility of error due to a missing or false diagnosis, thanks to a clinical and instrumental protocol developed by an international group of expert neurologists. Since 2001, diagnostic criteria used are the so-called "McDonald's criteria", especially using MRI. In the current version of 2017, revisors highlighted the need to associate the MS diagnosis with the dissemination in space (DIS) and time (DIT) of at least two lesions. Furthermore, OCBs can now be considered as evidence of DIT, if DIS has already been demonstrated. (Thompson et al., 2018).

Among the priorities reported by the experts, there is the importance of accurately differentiate MS from other potentially confusing

diseases, such as neuromyelitis optic. It is also suggested to provide at least a provisional definition of the disease form, specifying whether at that time it is in an active phase or not, and whether it is progressing or not according to its course over the previous year. Finally, the form must be redefined periodically at each subsequent verification (Hartung, Graf, Aktas, Mares, & Barnett, 2019).

1.3.3. MS PHENOTYPES

The evolution of MS is not completely predictable and moreover it varies from person to person. In fact, the clinical course of MS is not always linear, but it is variable since it can be characterized by unpredictable acute episode with new or increasing symptoms (relapses, sometimes called flares or exacerbations), and/or progressive deterioration of neurological functions. Based on the occurrence of symptoms (activity), the evolution over time (progression) and the accumulation of disability (worsening), four main forms can be distinguished: relapsing-remitting MS, secondary progressive MS, primarily progressive MS and clinically isolated syndrome (Lublin et al., 2014).

Multiple Sclerosis is initially suspected with **clinically isolated syndrome** (CIS), which consists of a single mono- or poly- symptomatic attack, lasting at least 24 hours, accompanied by evidence of one or more “active” demyelinated lesions in the CNS (Miller, D., Barkhof, Montalban, Thompson, & Filippi, 2005). Somentimes, concurrently with the occasion of this diagnosis, patients often have evidence of

other plaques in different stages of evolution. Infact, unrecognized demyelinating events preceding the clinical manifestation could be asymptomatic or cause symptoms that are too vague to suggest MS (asymptomatic or prodromal phase). Sometimes, MRI findings suggestive of MS lesions, known as radiologically isolated syndrome (RIS), are detected in healthy individuals when MRI is done for unrelated purpose (i.e. migraine, head trauma or screening), in the absence of any outward typical signs or symptoms (Dobson & Giovannoni, 2019; Okuda et al., 2014). Since clinical evidences are absent and aspecific, RIS is not classified as a subtype, but it highly suggests a greater risk of developing MS in the future (Lublin et al., 2014). Instead, given the clinical similarity and inflammatory-demyelinatin characteristics, CIS can beconsidered as the first clinical presentation of the disease, that has yet to fulfill criteria of dissemination in time. In some cases, it remains a one-time episode with no consequences, instead in the majority, a second event occurs with symptoms or lesions, and the diagnosis of clinically definite MS (CDMS) is confirmed (Lublin et al., 2014; Thompson, Baranzini, Geurts, Hemmer, & Ciccarelli, 2018)**.**

Approximately 85% of people with MS are initially diagnosed with a **relapsing-remitting** form (RRMS) characterized by the succession of acute attacks or relapses, alternating with periods of recovery (remission) in between. Recovery from relapses can be incomplete and disability gradually accumulates over time. On average, if left untreated, people relapse about every 2 years, but the frequency widely varies. The remaining 12-15% have the **primary progressive** MS

(PPMS), which has a slow and gradual increase of the disease activity from onset, in absence of attacks or remissions, and has a steady worsening of symptoms and disability, with occasional plateaus or temporary improvements. The progressive-relapsing course is rare and it also presents few acute episodes with symptoms that partially or do not regress. The diagnostic criteria for the PPMS are less certain than those for the relapsing-remitting form and thus the diagnosis is often made long after the onset of neurological symptoms when the person has already developed a significant disability (Miller, D. H. & Leary, 2007).

After years or decades, almost 15-30% of RRMS cases will gradually switch to a **secondary progressive** course (SPMS), which is similar to the primary one. Typically, after an initial relapsing course, SPMS is characterized by an even lower frequency of acute attacks until their disappearance and by a continuous worsening of functions, even in the periods between relapses (Lorscheider et al., 2016). To date, there are no clear criteria to determine the transition point to SPMS. However, the percentages of these patients are lower than previously reported (65%), and could reflect the effect of new disease modifying treatments (Thompson et al., 2018; Westerlind, Stawiarz, Fink, Hillert, & Manouchehrinia, 2016).

1.3.4. EPIDEMIOLOGY

Multiple sclerosis is one of the most common non-traumatic neurological cause of disability, especially among young people,

between 20 and 40 years. Most patients experience the first clinical symptoms of the disease at an average age of 32, which means that people are living with MS for many decades. This differentiates MS from other neurological conditions which predominantly affect older people (over 65), such as dementia and stroke. However, an MS diagnosis can come at any age. There are very few cases in which MS develops after the age of 40, but also, in 5% of cases, can occur under the age of 18 (Multiple Sclerosis International Federation, 2021; Walton et al., 2020). Multiple Sclerosis is not equally distributed between the two genders: women are twice as likely to have MS (69%) than men (31%), albeit in some countries, such as the Western Pacific and Southeast Asian regions, women are even three times more susceptible (Walton et al., 2020).

While age and gender distribution at diagnosis remain unchanged, the number of people with MS continues to grow: currently a total of 2.8 million of people are living with MS (Browne et al., 2014; Multiple Sclerosis International Federation, 2021; Walton et al., 2020). The total estimation of MS is represented by the global median prevalence of 35.9 cases per 100,000 inhabitants (approximately to 1 in every 3,000) in the world living with the disease. The prevalence has increased in all WHO regions (Europe, Americas, South-East Asia, Eastern Mediterranean, Western Pacific) since 2013, which is consistent with stated increases in national prevalence in some countries over this time, wich have doubled or even tripled their prevalence (Molodecky et al., 2012; Multiple Sclerosis International Federation, 2021).

Although MS is present in every region of the world, it has a heterogeneous distribution and its prevalence undergoes the effect of the latitude gradient, with a progressive increase as we move away from the equator. In tropical regions, such as South-East Asia and Sub-Saharan Africa, the prevalence is around 5 cases per 100,000 inhabitants, while in temperate climate areas it rises up to 200 per 100,000, especially in Northern Europe, the United States, Canada, South Australia and New Zealand (particularly where the population is caucasian). However, MS prevalence varies considerably within regions. For example, in the European region, San Marino (337 per 100,000) and Germany (303) have even the highest prevalence in the world. In contrary, in Eastern Europe countries and in France, Spain and Portugal, prevalence figures even lower than the global average (<35.9). Italy is considered a "high-risk country" with a prevalence of 198 cases per 100,000 inhabitants, and although the prevalence in Sardinia is higher (370), there is no evidence of a latitudinal gradient. As highlighted by the italian multiple sclerosis association (AISM), today in Italy there are an estimated 130,000 people with MS out of the 1.8 million in Europe (https://agenda.aism.it/2021/), with more than 3,400 new cases per year.

Furthermore, it is estimated that 107,000 people per year have been diagnosed with MS worldwide, this means that every day in the world, at least 300 people are diagnosed with MS, which is equivalent to 1 person every 5 minute. In Italy the estimated incidence is between 5.5 and 6 per 100,000 per years, up to 12 for Sardinia. The pooled incidence rate across reporting countries is 2.1 per 100,000 persons

per year. Unfortunately, this rate cannot be applied to the total global population given the relative lack of data especially from low income and lower prevalence countries (Walton et al., 2020). Geographical variations in the incidence and prevalence of MS have resulted in an imbalance of "real-world data" of MS to support research, clinical practice and health policy among different countries (GBD 2016 Multiple Sclerosis Collaborators, 2019).

1.3.5. AETIOLOGY

Despite longstanding and intense scientific efforts, the underlying cause of MS and mechanisms are only partially known, although complex gene-environment interactions almost certainly play a significant role. It is now clear that MS is a multifactorial disease in which several elements can be involved in its onset. Accumulating evidence from genetic and epidemiological studies indicate that genetic predisposition to MS makes an individual more or less sensitive to certain environmental factors (infectious or not), which lead to abnormal immune response and trigger the autoimmune reaction affecting the CNS (Olsson, Barcellos, & Alfredsson, 2017) (Table 1.1).

1.3.5.1. GENETIC FACTORS

The individual's genetic background appears to play an important role in MS. First of all, the prevalence in women is well known. The reasons behind this sexual dimorphism are not yet clear, but genetic and hormonal differences are likely to be involved, as well as the

different social, lifestyle and environmental exposures between the sexes. Several population genetic studies have found a higher frequency of MS in people of Caucasian ethnicity (especially in the North-Europe) and in members of the same family (Harirchian, Fatehi, Sarraf, Honarvar, & Bitarafan, 2018). Although multiple sclerosis is not strictly hereditary, already in the 1960s it had been noted that having a first-degree relative with MS significantly increases the risk of developing the disease, although the incidence is still very low in absolute terms. In fact, children and siblings of people with MS have a risk that is only 3-5% higher than family members of people without MS. Instead, the case of twins better explains the genetic component of MS: in homozygous twins the concordance for the disease risk is about 15-30%, while in heterozygotes the probability drops to 4% (Compston & Sawcer, 2002). These evidences support the hypothesis that the family aggregation of MS is likely due to the inheritance of predisposing genetic variants.

Since the early 1970s, the association with some genes of the major histocompatibility complex (MHC) molecules has been demonstrated (Jersild, Svejgaard, & Fog, 1972). The higly polymorphic MHC/HLA system is located on the short arm of chromosome 6 and includes a dense cluster of genes (>200) encoding a set of proteins expressed on the surface of different cell-types that play central roles in adaptive immune recognition by T lymphocytes, helping the body to distinguish self from the non-self. In particular, certain combinations of gene variants, referred to as multigenic haplotype, and expecially class II HLA genes, due to their close proximity, are not randomly distributed

among individuals in a population (*linkage disequilibrium*), and are usually inherited together in a set of common haplotypes. The main known haplotype predisposing to MS is the HLA-DR15 (DRB1*1501-DQA1*0102-DQB1*0602 allelic variants), which alone accounts up to 60% of the total genetic risk, and has been repeatedly investigated in case-control and family-based studies from countries around the world. It is quite common, expecially in Caucasian populations of Northern European descent, and about a quarter of the healthy population is HLA-DR15 positive (Fogdell, Hillert, Sachs, & Olerup, 1995). In MS, there is a striking association with the class II variant HLA-DRB1*15:01 (odds ratio, OR≈3), while the class I variant HLA-A02 is protective (OR≈0.6). How the DR15 haplotype contributes to MS is not fully understood, but it has been speculated that it depends mainly on the ability and modality to present certain antigens (Martin, Sospedra, Eiermann, & Olsson, 2021).

For many years the DRB1*1501 allele was the only locus to be repeatedly confirmed to be most strongly associated with MS risk, whereas subsequent studies revealed other genes associated with MS, many of which (about 30%) encode for proteins involved in immune response and inflammation. Currently, thanks to genome-wide association studies (GWAS), about 200 genetic risk factors have been identified in total, each of them modestly increase disease susceptibility (small OR = ∼1.1-1.2) (Baranzini & Oksenberg, 2017; Cotsapas & Mitrovic, 2018). Mainly these are single nucleotide polymorphisms (SNP) that lie close to regulatory – rather than coding – regions of genes coding for cytokine pathway proteins (CXCR5,

IL2RA, IL-7R, IL7, IL12RB1, IL22RA2, IL12A, IL12B, IRF8, TNFRSF1A, TNFRSF14, TNFSF14), co-stimulatory (CD37, CD40, CD58, CD80, CD86, CLECL1) and signal transduction proteins. In particular, polymorphisms in genes involved in T-cell activation and proliferation (such as IL2 and IL-7R) are a major feature of the disease, together with polymorphisms in other components of adaptive and innate immunity (such as genes that modulate TNF) (International Multiple Sclerosis Genetics Consortium et al., 2011). Moreover, also SNPs in proximity of the transcription factor EOMES gene are associated with a higher risk of MS (Parnell et al., 2014). These variants potentially modulate its expression mainly in T-cells, and consequently their differentiation and function (International Multiple Sclerosis Genetics Consortium, 2019). It is noteworth that a significant proportion of these genes and particularly class II HLA genes (i.e. DR15 haplotype and its individual alleles), have also been associated with other autoimmune diseases or immune-mediated disorders (such as Crohn's disease, type I diabetes, narcolepsy and SLE) (Martin et al., 2021). Primary progressive MS (PPMS) patients have largely the same genotype as RRMS patients, and only a few genes have been reported to associate exclusively with progressive MS (Martinelli-Boneschi et al., 2012). In contrast, only few genes with a role in axonal neurodegeneration were found (MANBA, GALC, KIF21B) (International Multiple Sclerosis Genetics Consortium et al., 2011; International Multiple Sclerosis Genetics Consortium (IMSGC) et al., 2013). Despite considerable progress, it is not currently possible to use these variants to precisely predict the individual risk of developing the disease. However, this information is of great use to

better understand the pathogenetic mechanisms underlying the disease and to eventually develop new drugs.

1.3.5.2. ENVIRONMENTAL FACTORS

Lifestyle and environmental factors are also relevant in multiple sclerosis, since genetics explains only a fraction of the overall risk. There is no single risk factor that provokes MS, but several nongenetic factors, both of infectious and non-infectious nature, have been proposed as risk factors of developing MS.

The most likely candidates include certain infectious agents, insufficient vitamin D and sun exposure or ultraviolet B light (UVB), obesity and smoking. In particular, it is hypothesized that exposure to certain environmental factors before sexual maturation (puberty) may predispose a genetically susceptible person to develop MS. Further, certain factors interact with HLA risk genes, pointing at a pathogenetic pathway involving adaptive immunity (Alfredsson & Olsson, 2019; Olsson et al., 2017) (Table 1.1).

Several studies suggest that low vitamin D (vD) levels in the blood, caused by insufficient sun exposure and/or dietary intake, lead to a greater risk of developing MS. Poor exposure to sunlight results in low levels of vD, which is thought to support immune function and may help protect against immune-mediated diseases like MS (Sintzel, Rametta, & Reder, 2018). For this reason, some researchers believe that sun exposure may explain the latitude gradient in MS prevalence: people living closer to the equator are exposed to greater amounts of

UVB rays year-round, and therefore have higher levels of naturally-produced vD and a lower MS risk. Also, in already diagnosed MS patient with lower vitamin D levels, symptoms appear more frequently and are worse (Lucas, Byrne, Correale, Ilschner, & Hart, 2015). However, the effect of the latitude gradient on MS risk varies based on where people spend the first 15 years of their life. Where people subsequently live, regardless of the climate, no longer affects the risk. Migration studies consistently indicate that exposure to some environmental factors before puberty may predispose a person to develop MS. For example, people who move – or migrate – before the age of 15 from high-risk areas to an area with a lower risk, assume the risk of their new area. The adult migrants from low risk countries to Europe are at low risk of developing MS, while their children born there are at high risk (Kurtzke, 2013).

Factor	OR	HLA gene interaction	Combined OR (nongenetic factor + HLA allele)	Effect during adolescence	Immune system implied	Level of evidence
Smoking	~1.6	Yes	14	No	Yes	+++
EBV infection (seropositivity)	~3.6	Yes	~15	Yes	Yes	+++
Vitamin D level <50 nM	~1.4	No	NA	Probably	Yes	+++
Adolescent obesity (BMI >27 at age 20 years)	~2	Yes	~15	Yes	Yes	+++
CMV infection (seropositivity)	0.7	No	NA	Unknown	Yes	++
Night work	~1.7	No	NA	Yes	Yes	++
Low sun exposure	~2	No	NA	Probably	Yes	++
Infectious mononucleosis	~2	Yes	7	Yes	Yes	++
Passive smoking	~1.3	Yes	6	No	Yes	+
Organic solvent exposure	~1.5	Unknown	Unknown	Unknown	Unknown	+
Oral tobacco/nicotine	0.5	No	NA	Unknown	Yes	+
Alcohol	~0.6	No	NA	Unknown	Yes	+
Coffee	~0.7	No	NA	Unknown	Yes	+

Table 1.1 - Lifestyle and environmental risk factors for MS.

BMI, body mass index; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HLA, human leukocyte antigen; MS, multiple sclerosis; NA, not applicable; OR, odds ratio; +++, high level of evidence from large prospective studies or a case-control observation that is supported by Mendelian randomization studies; ++, case–control observations that have been replicated and/or supported by independent methods; +, non- replicated observations that require further study. *Adapted from Olsson, T. et al. (2016) Nat. Rev. Neurol. doi:10.1038/nrneurol.2016.187*

Epidemiological studies also suggest links between MS and some previous viral infections agents in prepubertal age. Numerous infectious agents have been investigated over the years as potential factors that can trigger the onset of the disease, starting with the Measles, Rubella and Varicella-Zoster viruses, and other pediatric infections. Hypotheses involving retroviruses or, in general, herpesviruses have also taken hold (Tselis, 2011). Because the relapsing-remitting phase of MS in many ways is analogous to the recurrence of herpesvirus infections, a herpesvirus is an attractive etiologic candidate. In particular, the scientific community has focused on the role of the Epstein-Barr virus, EBV, as the main predisposing factor in MS development, even if a causal role has not been definitively demonstrated (Bjornevik et al., 2022; Tselis, 2012). In most subjects, the EBV infection has a harmless and asymptomatic course, while in some people it causes diseases such as acute infectious mononucleosis (IM). Once a person is infected, the virus establishes an "immunological equilibrium" and normally remains latent in their B lymphocytes (Laderach & Munz, 2021). Numerous epidemiological

data provide evidence for a strong association between MS and EBV infection, particularly with IM before the age of 15 (Endriz, Ho, & Steinman, 2017; Xu et al., 2021). A study has shown that IM and high levels of certain anti-EBV (i.e. EBV nuclear antigen 1, EBNA1) antibodies increase both independently and synergistically the MS risk, while being truly EBV negative is protecting (Hedstrom et al., 2020). Further, IM may synergize with HLA-DR15 increasing the chance of developing MS (Olsson, 2021; Xu et al., 2021) (Table 1.1).

Since several etiological factors cause the development of MS, also acting simultaneously, the possibility of modifying them before the disease onset constitutes a potential opportunity to decrease the risk of development.

1.3.6. IMMUNOPATHOLOGY

Multiple sclerosis is primarily defined as an inflammatory immune-mediated demyelinating disease. The inflammatory process that occurs in the CNS in MS patients plays a leading role in the pathogenesis of the disease, which is responsible for the demyelination and the resulting axonal degeneration. The myelin of the CNS differs antigenically from that of the SNP (the former derives from oligodendroglia, while the other derives from Schwann cells). Therefore, some autoimmune demyelinating diseases attack the CNS (the prototype is MS), while others act on the peripheral nervous system (the prototype is Guillain-Barre syndrome) (Dendrou, Fugger, & Friese, 2015; Filippi et al., 2018).

In particular, MS is a T-cell driven autoimmune disease and the its pathogenesis is strongly associated with the disruption of self-tolerance mechanisms in genetically predisposed individuals (Martin et al., 2021). In addition, symptomatic EBV infection is also believed to be a relevant environmental risk factor, but the numerous studies conducted to date have failed to unravel its role in promoting MS and in breaking tolerance (Laderach & Munz, 2021) (Figure 1.7). Besides EBV, also neurotropic and/or endogenous persistent retroviruses, are involved in MS, whose reactivation in the CNS could be responsible for relapses, through concomitant recruitment of autoreactive and virus-specific T-cell subsets (Ho et al., 2017; Stoner, 1991). As already mentioned, the role of viruses in pathogenesis remains controversial and the antigen(s) that initiate T-cell activation in MS remains unknown. However, the mouse experimental model of EAE demonstrates how the pathology is induced with myelin oligodendrocyte glycoprotein (MOB) derived peptides, in the absence of viral infections (Fletcher, Lalor, Sweeney, Tubridy, & Mills, 2010), while, in the alternative model of neurotropic virus-induced MS, both virus-specific and myelin-reactive effector T-cells are found (Geginat, J. et al., 2017; Tsunoda & Fujinami, 2010).

Immune cells involved are several and include B-cells, $CD8^+$T-cells, $CD4^+$T-cells and regulatory T-cells, which can be found in lesions and CSF of MS patients (Dendrou et al., 2015; Magliozzi et al., 2013). B-cells infected with EBV could function mainly as a vehicle to the CNS through the blood-brain barrier and as APCs for T-cells, as well as being responsible for the production of the oligo-clonal antibodies present

in CSF, which are mainly specific for neurotropic viruses (Magliozzi et al., 2013). On the other hand, there are also $CD8^+$T-cells which are EBV-specific (Lossius et al., 2014) and can attack virus-infected B-cells in the lytic phase (Angelini et al., 2013). Damage in the CNS could be caused not only by the virus itself, but also, following the antiviral response, by virus-specific $CD8^+$ and $CD4^+$ T-cells that cross-react with myelin components (Berger, 2015). In fact, some viral antigens contain peptide that are similar to self-proteins (i.e. molecular mimicry), as in the case of EBNA1 and MBP (Myelin basic protein), respectively, for which are found cross-reactive $CD4^+$ and $CD8^+$ T-cells in MS patients (Berger, 2015; Geginat, J. et al., 2017).

The main pathogenetic actors in MS development are T-cells. Self-reactive $CD4^+$T-cells specific for myelin and peptides restricted for HLA-DRB1*15:01 are enriched in the CSF of MS patients after an attack, while they are found at low frequencies in the blood of both MS patients and healthy donors (Ellmerich et al., 2004; Elong Ngono et al., 2012). However, myelin-reactive T-cells have more pro-inflammatory properties in MS patients than in healthy individuals, and mainly belong to the Th1/Th17 cell subset. Under normal conditions, both Th1/Th17 and Th1 cells, play an antiviral role, contributing to the immune-surveillance of the CNS (Cao et al., 2015; Paroni et al., 2017). In addition, the presence of a degenerated TCR can also be hypothesized, since CSF-derived Th1/Th17 cells react with APCs even in the absence of added peptides (Paroni et al., 2017). Both antiviral subsets stably express the chemokine receptors CXCR3 and CCR5, and mostly belong to the $CCR7^+$ central memory compartment,

suggesting that these cells recirculate between the lymph nodes and the CNS. Other effector T-cell subsets involved in MS are granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting $CD4^+$ and $CD8^+$ T-cells (Rasouli et al., 2015). Interestingly, self-reactive Th1/Th17 produce high levels of GM-CSF and the CNS-homing VLA-4 integrin, thus suggesting their encephalitogenic potential (Paroni et al., 2017).

The pathogenetic model of MS currently proposed (Figure 1.7) (Dendrou et al., 2015; Filippi et al., 2018), sees the activation of autoreactive and/or cross-reactive pro-inflammatory T-cells in periphery. These T-cells migrate and enter the CNS through a VLA-4 (α4β1)-mediated transmigration, binding to the vascular cell adhesion molecule (VCAM)-1 expressed on the activated vascular endothelium of the CNS and crossing the blood brain barrier interruptions. Within the CNS, pathogenic T-cells are reactivated by APCs and attack myelin, triggering inflammatory processes (i.e. production of pro-inflammatory cytokines INF-γ, TNF-α and IL-2) and stimulating the recruiting and activation of effector cells such as macrophages, B-cells and other T-cells. Macrophages (including tissue-resident microglia) and T-cells attack the myelin sheath through cytotoxic mediators. B-cells differentiate into plasma cells that secrete demyelinating antibodies, which amplify myelin damage (McFarland & Martin, 2007). Finally, when the barrier regains its integrity, the T-cells are trapped within the brain tissue.

The characteristic pathological hallmark of MS are inflammatory lesions located around post-capillary venules (i.e. perivenular), leading to demyelinating plaques. MS lesions are characterized by heavy

inflammatory infiltrates consisting of active T-cells (mainly $CD8^+$), B-cells, plasma cells, macrophages and CNS resident cells such as activated microglia and astrocytes. There are also deposits of complement proteins, immunoglobulins and several pro-inflammatory cytokines (Dendrou et al., 2015; Filippi et al., 2018). $CD4^+$T-cells are found mainly in the perivascular spaces and meninges, $CD8^+$T-cells are found in the parenchyma of MS lesions (Machado-Santos et al., 2018; Selter & Hemmer, 2013). The classic "active lesion" is distinctive of

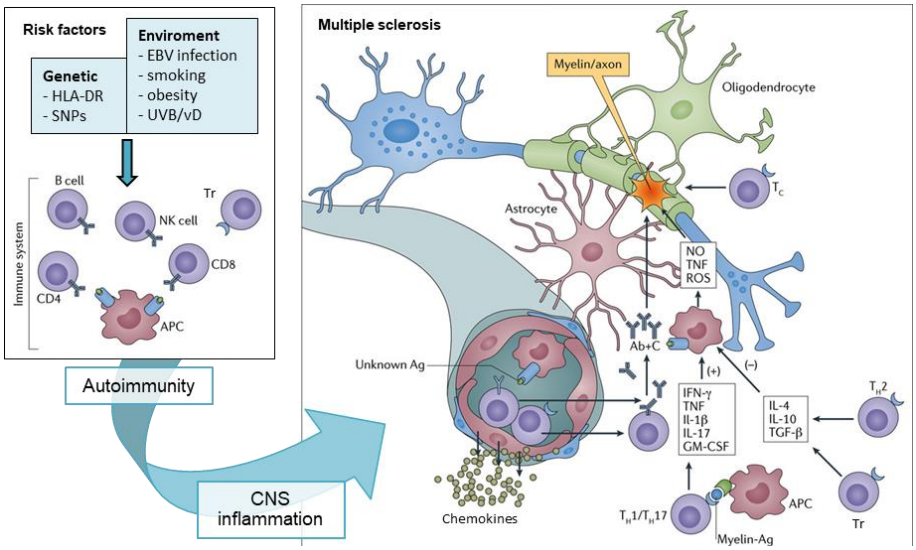


Figure 1.7 - Immune system dysregulation in MS.

Several risk factors can affect immune system function, which can lead to autoimmunity and neuroinflammation in the CNS and eventually trigger and/or perpetuate multiple sclerosis. APC, antigen-presenting cells; Ab, antibody; C, complement; EBV, Epstein–Barr virus; GM-CSF, granulocyte monocyte colony stimulating factor; myelin Ag, myelin antigens; NO, nitric oxide; ROS, reactive oxygen species; TC, cytotoxic T-cell; TGF-β, transforming growth factor β; TH, helper T-cell; TNF, tumour necrosis factor; Tr, regulatory T-cell. *Adapted from Olsson, T. et al. (2016) Nat. Rev. Neurol. doi:10.1038/nrneurol.2016.187.*

RRMS, while in progressive forms inflammation and the cellular component is reduced (although the proportion of B-cells and plasma cells is higher) and tend to have an inactive core surrounded by a narrow rim of activated microglia and macrophages (Mahad, Trapp, & Lassmann, 2015). Lesions usually involve the white matter, but extensive cortical damage to the grey matter is found mainly at onset and in progressive patients. In the first years of the disease, the inflammation often regresses spontaneously and there is, in fact, an improvement or complete remission of symptoms. When relapses increase, or if the inflammation that occurs is very important, the damage to the CNS tissues can become irreversible and, consequently, remissions begin to be less and less complete.

Finally, another mechanism that may explain the pathogenetic role of viruses is bystander activation – nothing excludes that these mechanisms may overlap in some way. Activation of the virus in the CNS results in damage to myelin, and then the activated DCs migrate to the lymph nodes where they present antigens both derived from the virus and from myelin, thus activating T-cell responses (Geginat, J. et al., 2017). Normally, the bystander activation of autoreactive T-cells is suppressed by regulatory T-cell subsets, such as Treg and Tr1 cells (Cervantes-Barragan et al., 2012), but these cells are defective in MS patients and effector T-cells are more suppression-resistant, as suggested by MS-associated genetic polymorphisms (Astier & Hafler, 2007; Kleinewietfeld & Hafler, 2013; Martinez-Forero et al., 2008). However, the results regarding these possible dysfunctions are rather controversial, which is probably due to the lack of a marker that

specifically defines the regulatory CD4 population. Data obtained recently suggest that some alterations in the regulatory population between healthy people and patients can be highlighted by focusing on the composition of Eomes expressing cells (Raveney et al., 2021a).

Ongoing efforts to learn more about the immune-mediated process in MS – what triggers it, and how to slow or stop it – will bring us closer to understanding the cause of MS, better therapies and ultimately a cure.

1.3.7. DISEASE-MODIFYING TREATMENTS

Multiple sclerosis is a chronic, unpredictable and progressively disabling disease. To date, there is no cure for MS, which means that people should live with the disease for many decades.

Until the 1990s, MS therapies were only symptomatic, with no effect on MS long term prognosis. Instead, in the last 25 years, thanks to research, several drugs have been approved that reduce relapse rate and slow the progression of the desease (summarized in Table 1.2), even able to delay the attainment of severe disability for more than 25 years. These disease-modifying treatments (DMT) are mainly immunomodulators and immunosuppressants, wich act by reducing neuroinflammation and consequently also neurodegeneration. Although, MS affects the CNS, there are evidences of a generally impaired immunity even outside, thus targeting peripheral immune system is beneficial to MS patients (Thompson et al., 2018).

The DMT are variously used in clinical practice. There are two different therapeutic approaches available: the escalation strategy and the induction. The maintenance/escalation therapies (MET) is a continuous long-term strategy of gradual increase in intensity, that consists in the initial use of modestly effective (so-called first-line) DMT and, in case of insufficient response to treatment (continuous relapses), the escalation to more effective drugs (second and third-line DMT), but potentially less safe and more expensive (Table 1.2). MET therapies are in turn divided into immunomodulating and immunosuppressive (Dobson & Giovannoni, 2019; Giovannoni, 2018). Instead, the induction therapy is reserved to patients who show an higly active and/or rapidly evolving profile of the disease from the outset. In particular, recently approved new DMT treatments are referred to as immune reconstitution therapies (IRT), as they deplete the defective immune system and consequently allow it to rebuild itself, which does not occur in immunosuppressive treatment, where the immune system is blocked. IRT are administered intermittently for a short period of time, with the early use of higly active drugs to obtain subsequently a persistent (even drug-free) remission or long-term maintenance therapy with less effective DMT (Hauser & Cree, 2020; Karussis & Petrou, 2018; Thompson et al., 2018). Currently, the IRT therapeutic strategy is the closest to a potential cure for MS.

1.3.7.1. MAINTENANCE/ESCALATION THERAPIES

For the treatment of RRMS, the first drugs allowed were immunomodulators: interferons and glatimer acetate (GA), which are well tolerated but modestly reduce the relapse frequency.

Conversely, immunosuppressants include the commonly prescribed oral drugs fingolimod, dimethyl fumarate (DMF), and teriflunomide. Fingolimod (commercially known as Gylenia) affects the signaling system regulated by sphingolipids, that prevents the egress of lymphocytes from peripheral lymphatic tissues (i.e. lymph nodes) and the infiltration of autoreactive cells in the CNS, without suppressing systemic immune responses. DMF (Tecfidera) is the methyl ester of fumaric acid that reduce the release of inflammatory cytokines and the damage induced by macrophages (Giovannoni, 2018; Hauser & Cree, 2020). Lastly, teriflunomide (Aubagio), a pyrimidine synthesis inhibitor, is assumed to act by reducing the proliferation of dividing immune cells, including presumably self-reactive activated lymphocytes (Hauser & Cree, 2020; van Oosten et al., 1997).

There are also numerous monoclonal antibodies (mAb), produced with genetic engineering techniques, which have a specific affinity for a single target to which they bind, neutralizing it. Among these, in 2017, Ocrelizumab (Ocrevus) was the first approved treatment for the progressive forms and also RRMS (Montalban et al., 2017). Ocrelizumab is a humanized antibody that targets the CD20 molecule, a transmembrane protein, expressed on immature and mature cells B-cells, but not on differentiated plasma cells and lymphoid stem cells.

Ocrelizumab selectively depletes circulating CD20-expressing B-cells, preserving the existing humoral immunity and the ability to replenish the B-cell pool (Mulero, Midaglia, & Montalban, 2018).

Instead, natalizumab (NTZ, commercially known as Tysabri) was the first humanized mAb approved by FDA in 2004. It binds to the α4 subunit of both α4β1 and α4β7 integrins expressed on the surface of mononuclear leukocytes (except neutrophils), thus inhibiting the α4-integrins binding to their ligands. Therefore, natalizumab exerts its activitiy by preventing the extravasation and blocking the entry into the CNS of potentially pathogenic lymphocytes (Butzkueven et al., 2020). Unfortunately, although it is well tolerated, due to impaired immune surveillance in the CNS, treatment with natalizumab (but also other drugs) is associated with the progressive multifocal leukoencephalopathy (PML), a rare and mostly fatal viral deseas caused by reactivation of the John Cunningam virus (JCV) or *de novo* infection (Ho et al., 2017). JCV is a very common polyomavirus: about half of the individuals were infected at a young age remaining latent. However, natalizumab is more effective than any other drug in preventing relapse in MS, for this reason the risk of developing PML is

monitored by the presence of anti-JCV antibodies, before and during treatment (Thompson et al., 2018).

1.3.7.2. IMMUNE RECONSTITUTION THERAPY

IRT therapies are divided into selective IRT (SIRT), that exclusively target the adaptive immune system, which includes cladribine, and not selective IRT (NIRT), that affect both the innate and the adaptive immune system, which include alemtuzumab, mitoxantrone, in addition to autologous hematopoietic stem cell transplantation (HSCT) (Karussis & Petrou, 2018).

Drug	Relapse reduction	Mechanisms of action	Indication
IFN-β	Low/moderate	MMPs and proinflammatory profile inhibition	CIS and RRMS
GA	Low/moderate	Shift from a proinflammatory profile to a regulatory profile	RRMS
Mitoxantrone	High	DNA intercalator	RRMS and SPMS
Natalizumab	High	Anti-α4-integrin: inhibition of immune cell entrance to the CNS	RRMS
Alemtuzumab	High	Anti-CD52 mAb: depletion of $CD52^+$ T and B cell populations	RRMS
Daclizumab	High	Anti-CD25 mAb: expansion of CD56 NK cells	RRMS
Rituximab Ocrelizumab Ofatumumab	High	Anti-CD20 mAb: proinflammatory and antigen-presenting function inhibited by B cell depletion	RRMS RRMS and PPMS (ocrelizumab)
Fingolimod	Moderate	Sphingosine 1-phosphate receptor inhibitor	RRMS
Siponimod Ozanimod	Moderate	Sphingosine 1-phosphate receptor modulator	CIS, RRMS, and SPMS
DMF	Moderate	Nrf2 pathway induction	RRMS
Teriflunomide	Low/moderate	Dihydroorotate dehydrogenase inhibitor	RRMS
Cladribine	High	Deoxyadenosine analog	RRMS

Table 1.2 - Approved disease-modifying therapies for MS.

CIS, clinically isolated syndrome; CNS, central nervous system; DMF, dimethyl fumarate; GA, glatiramer acetate; IFN, interferon; mAb, monoclonal antibody; MMP, matrix metalloproteinase; MS, multiple sclerosis; NK, natural killer; Nrf2, nuclear factor (erythroid-derived 2)-like 2; RRMS, relapsing-remitting MS; PPMS, primary progressive MS; SPMS, secondary progressive MS. *Adapted from Bross, M., Hackett, M., & Bernitsas, E. (2020) Int. J. Mol. Sci. doi:10.3390/ijms21124312*

Cladribine (Mavenclad) is an analogue of purine nucleosides, act preferably on highly proliferative lymphocytes as an inhibitor of DNA synthesis, inducing apoptosis. Cladribine gradually causes peripheral T- and B-cell depletion and allows for a progressive reconstitution of peripheral lymphocyte counts over several months (Giovannoni et al., 2010). Cladribine has shown a high level of efficacy in suppression of disease activity in RRMS patients, but also in SPMS, and even a robust efficacy in prevention of the conversion of CIS to clinically definite MS (CDMS) (Karussis & Petrou, 2018). Recent research papers have positively evaluated the efficacy of mitoxantrone (Novantrone) which acts by suppressing DNA replication and blocking cell replication. This drug was authorized also as a treatment for secondary progressive forms (Goodin et al., 2003). Alemtuzumab (Lemtrada) is a recombinant human monoclonal antibody directed against CD52, a glycoprotein widely expressed on all leukocytes, it induces cytotoxicity on CD52-expressing causing their complete depletion. Alemtuzumab is administered intravenously in two cycles just one years apart, it has shown the most impressive results, longlasting after discontinuation of treatment (Coles et al., 2012; Karussis & Petrou, 2018).

Lastly, autologous hematopoietic stem cell transplantation (HSCT) is showing good results in numerous studies, but, due to the risks involved, it is an indicated treatment only for severe cases of MS, which have failed to respond to other DMT. Hematopoietic stem cells (HSC) are the precursors that have the potential to replace all types of blood cells. In HSCT, autologous hematopoietic stem cells derived from bone marrow are stored before depleting the immune system of the

MS patient, using chemotherapy or immune-cell depleting drugs. Then, the HSC are reintroduced into the body and over time replenish the immune system (Karussis & Petrou, 2018). IRT are very promising approach: there are patients treated with alemtuzumab and autologous HSCT who experience no evidence of disease activity (NEDA) for over 10 years (Steingo et al., 2020). However, autologous cell-based therapies and in particular those involving the adoptive transfer of regulatory cells, are currently under study and constitute a valid future for the development of an effective and permanent solution to treat MS (Duffy, Keating, & Moalem-Taylor, 2019).

1.4. SCOPE OF THE THESIS

The immune system has well-regulated mechanisms to control the response to foreign pathogen agents and to the self, ensurig the integrity of the individual. Failure to suppress the immune response against pathogens or breakdown of self-tolerance mechanisms can lead to the development of chronic inflammatory conditions and autoimmunity, as in the case of multiple sclerosis (MS). The genetic predisposition of an individual and the interaction with environmental factors lead to an increased risk of this pathology.

In MS, myelin auto-reactive $CD4^+$T-cells and/or $CD4^+$T-cells cross-reactive to viruses, such as EBV, are thought to be responsible for the development and progression of the disease, although the mechanisms are not yet known. In particular, subsets of regulatory cells such as $FOXP3^+$Treg or type 1 regulatory (Tr1) cells are involved in

MS. However, Tr1s are not yet well characterized and their role in MS is still not clear.

This work aims at the phenotypic and functional characterization of the Tr1 cell subset, the identification of their possible role in MS and setting the basis for the possible development of a cell-based therapy for MS. Thus, my aims were:

I. define the peculiar genetic profile of Eomes^{+} Tr1 cells and identifing factors that regulate the expression of these genes, such as miRNAs;

II. establish a strategy that allows the *ex vivo* study of Tr1 cells by multiparametric cytometry, using Eomes and Granzyme K as markers;

III. understand the role of Tr1-like cells in MS focusing on their homeostasis, their CNS-homing properties and their specificity for MS-relevant self- and viral antigens.

1.5. REFERENCES

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Chapter 2. *Ex vivo* microRNA and gene expression profiling of human Tr1-like cells suggests a role for miR-92a and -125a in the regulation of EOMES and IL-10R.

De Simone M, Chirichella M, Emming S, Mazzara S, Ranzani V, Gruarin P, Moschetti G, Pulvirenti N, Maglie S, Vasco C, Crosti MC, Rossetti G, Pagani M, Abrignani S, Monticelli S, Geginat J.

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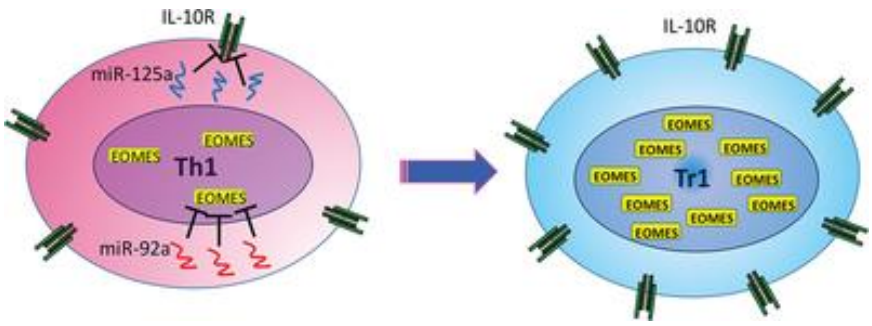
In this publication I contributed elaborating the gating strategy for the identification of the analyzed populations.

2.1. ABSTRACT

Ex vivo gene expression and miRNA profiling of Eomes$^+$ Tr1-like cells suggested that they represent a differentiation stage that is intermediate between Th1-cells and cytotoxic CD4$^+$T-cells. Several microRNAs were downregulated in Eomes$^+$Tr1-like cells that might inhibit Tr1-cell differentiation. In particular, miR-92a targeted Eomes, while miR-125a inhibited IFN-γ and IL-10R expression.



Regulatory T-cells, comprising both FOXP3^{+}Tregs and FOXP3^{-} type-1 regulatory T-cells (Tr1), are required to maintain immune homeostasis. We previously identified a population of human IL-10 and IFN-γ co-producing Tr1-like cells, which are involved in graft-versus-host disease, colitis, autoimmunity, and cancer (Alfen et al., 2018; Bonnal et al., 2021; Facciotti et al., 2016; Gruarin et al., 2019; Haringer, Lozza, Steckel, & Geginat, 2009). They express the transcription factor eomesodermin (EOMES) (Gruarin et al., 2019; Zhang et al., 2017), which is characteristic for cytotoxic T-lymphocytes (CTL) and controls IFN-γ production and cytotoxic functions (Pearce et al., 2003). T-bet expression, differentiation requirements, and clonotype sharing suggests that EOMES^{+} Tr1-like cells are derived from Th1-cells (Bonnal et al., 2021; Gruarin et al., 2019; Zhang et al., 2017). MicroRNAs (miRNAs) regulate gene expression and shape differentiation states, and are required for the functions of FOXP3^{+} Tregs (Rossi et al., 2011). The role of miRNAs in the biology of Tr1-like cells is in contrast largely unknown.

Since different subsets of human CD4^{+}T-cells express EOMES (Gruarin et al., 2019), we asked how they were molecularly related. We purified EOMES-expressing CD4^{+}T-cell subsets, that is, Th1 effector memory cells ($Th1_{EM}$), CD4^{+}CTL, and Tr1-like cells *ex vivo* from peripheral blood of healthy donors according to an established gating strategy (Gruarin et al., 2019) (*Supporting Information Fig. 1A*) [for *Supporting Information* see the online version of this paper] and performed gene expression analysis. Th1 central memory cells

($Th1_{CM}$), which largely lacked EOMES expression (*Supporting Information Fig. 1B*), were analyzed as control. We identified 424 differentially expressed genes ($p < 0.01$, *Supporting Information Table 1*). Hierarchical clustering revealed limited donor-to-donor variability (Fig. 2.1A), suggesting that the analyzed subsets represent conserved differentiation stages. This analysis resulted in one major cluster containing all EOMES-expressing subsets, and a second cluster containing $Eomes^{-}$ $Th1_{CM}$. In the $EOMES^{+}$ subcluster, Tr1-like cells clustered together with CTL. Notably, principal component analysis (PCA) positioned Th1CM and CTLs at opposite sides of the three-dimensional space, and Tr1-like cells were positioned between Th1EM and CTL (Fig. 2.1B), suggesting that they represent an intermediate differentiation state. Indeed, the majority of differentially expressed genes were downregulated in Tr1-like cells as compared to Th1-cells, but upregulated as compared to CTL (*Supporting Information Fig. 1C and Table 2*). Tr1-like cells expressed higher levels of GZMK as compared to $Th1_{CM}$ and CTL, and of IL-10R as compared to Th1-cells. Moreover, they expressed higher levels of EOMES, GZMA, NKG7, CCL5, and HLA-G as compared to $Th1_{CM}$, but had downregulated FOXO1 and LTA. $CD4^{+}$CTL expressed the lowest levels of CCR7, CD27, and LEF1, suggesting that they are terminally differentiated effector cells. Selected differentially expressed genes and relevant controls were then measured by RT-qPCR in independent donors (Supporting Information Fig. 2A). GZMK and EOMES were highly expressed in Tr1-like cells, as expected (Gruarin et al., 2019). IFNG mRNA was constitutively expressed in CTL and Tr1-like cells, whereas IL10 and

GZMB mRNA were largely restricted to Tr1-like cells and $CD4^+$CTL, respectively. miRNA expression in human $CD4^+$T-cell subsets is superior compared to gene expression patterns to map $CD4^+$T-cell differentiation stages (Rossi et al., 2011). We therefore analyzed the expression of 664 miRNAs in the same T-cell subsets. Twelve miRNAs were found to be differentially expressed, as detected by TaqMan miRNA arrays (Fig. 2.1C; *Supporting Information Table 3*). Hierarchical clustering revealed again that Tr1-like cells clustered together with $CD4^+$CTLs. Most of the differentially expressed miRNAs were downregulated in Tr1-like cells and in CTL. Three of these miRNAs were highly expressed in Th1., suggesting that they might be involved in repressing cytotoxic cell fates. Conversely, miR-186, miR-194, and miR-345 were highly expressed, although not uniquely, in Tr1-like cells. Validation of selected miRNAs by RT-qPCR in independent donors confirmed downregulation of miR-150, miR-31, and, most notably, miR-92a and miR-125a in Tr1-like cells (Fig. 1D). Inspection of the putative targets using TargetScan revealed that both miR-125a and miR-92a targeted Tr1-expressed genes. Specifically, the intersection of differentially expressed genes with the top 500 TargetScan predicted targets (irrespective of site conservation) of the miR-125 family and of miR-92a-3p identified genes involved in Tr1-like cell biology.

Thus, putative targets of the miR-125 family included IL10RA, while a putative target of miR-92a was EOMES (Fig. 2A). The 3'-untranslated region (3'UTR) of the EOMES mRNA contains a putative miR-92a responsive element (*Supporting Information Fig. 2B*). We therefore

performed dual luciferase assay to assess whether this region was a target of miR-92a.

Upon transfection in HEK-293T-cells, a synthetic miR-92a mimic oligonucleotide significantly reduced luciferase expression from a reporter plasmid containing the 3'UTR of the human EOMES gene, as compared to a scrambled control oligonucleotide (Fig. 2.2B). To investigate whether miR-92a could affect EOMES protein expression in primary human T-lymphocytes, we isolated $CCR5^{+}CD4^{+}$T-cells, which are enriched for $Eomes^{+}$ cells (*Supporting Information Fig. 2C*). After transfection with either a miR-92a mimic or scrambled control oligonucleotide, the levels of EOMES protein expression were moderately, but consistently, reduced (*Supporting Information Fig.*

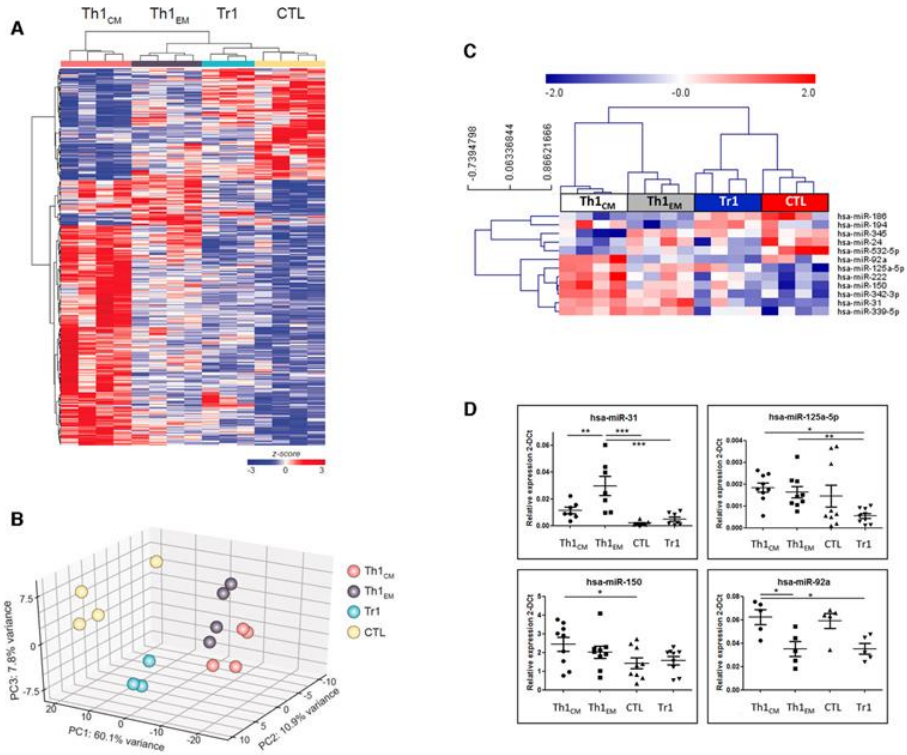


Figure 2.1 - Gene expression and miRNome analysis of human EOMES$^+$CD4$^+$ T-cell subsets.

(**A**) Hierarchical clustering of differentially expressed genes in EOMES$^+$ Tr1-like cells (n = 3), CD4$^+$CTL, Th1$_{CM}$, and Th1$_{EM}$ (n = 4) according to one-way ANOVA ($p < 0.01$). (**B**) Three-dimensional PCA of selectively expressed genes. (**C**) Hierarchical clustering of 12 miRNAs expressed in Th1$_{CM}$, Th1$_{EM}$, CD4$^+$CTL, and Tr1-like subsets, selected by one-way ANOVA ($p < 0.01$). Data, normalized on global mean, are presented as z-scores calculated on ΔCt. (**D**) Differential expression of four selected miRNAs (miR-31 (n = 7), miR-125a-5p (n = 8), miR-150 (n = 9), and miR-92a (n = 5)) in independent donors were analyzed by RT-qPCR (data represented as 2–ΔCt). Statistical analysis was performed using a one-way ANOVA and Tukey post-test between four groups: Th1$_{CM}$, Th1$_{EM}$, CTL, and Tr1 (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

2D), suggesting that this miRNA could indeed suppress EOMES expression in CD4$^+$T-cells. Next, we focused on miR-125a. Notably, its closely related family member miR-125b is expressed exclusively in naïve CD4$^+$T-cells (Rossi et al., 2011) (Fig. 2C). Conversely, miR-125a was also expressed in Th1-cells, but remained low in CTL and Tr1-like cells. The seed sequences (nucleotide 2-to-7 of the miRNAs, responsible for target specificity) of miR-125a and miR-125b are identical, as expected for a miRNA family, suggesting that they possess similar target specificities. Therefore, potential differences in their mRNA targeting are rather due to their different expression patterns. The IL-10R is highly expressed on regulatory T-cells, including Tr1-like cells (Fig. 2.2D; *Supporting Information Fig. 2E*), and it is required to maintain IL-10 production and suppressive capabilities (Brockmann et

al., 2017). Moreover, the IL-10RA gene was shown to be targeted by miR-125b in human $CD4^+$T-cells (Rossi et al., 2011). To assess the ability of miR-125a to regulate the expression of IL10RA, we performed luciferase reporter assay using a plasmid containing the 3'UTR of this gene, either wild-type or mutated in the region complementary to the miR-125 seed sequence (Rossi et al., 2011). Co-transfection of miR-125a strongly and significantly reduced reporter expression from the wild-type, but not from the mutated, 3'UTR (Fig. 2.2E). To assess the role of miR-125a in primary human T-cells, we transfected $CD4^+CD45RA^-$ memory T-cells with either a miR-125a mimic, an antagomir to inhibit miR-125a activity or with scrambled controls. After 2 days, the expression of miR-125a was strongly elevated in miR-125-mimic transfected cells, and diminished upon antagomir transfection (*Supporting Information Fig. 2F*). Under these conditions we monitored the expression of the predicted targets by RT-qPCR and by flow cytometry. Both IFN-γ and IL-10Rα were slightly reduced both at the mRNA and protein level upon transfection with the miR-125a-mimic, and were instead slightly elevated with the miR-125a antagomir (*Supporting Information Fig. 2G*). In conclusion, by performing gene expression and miRNA profiling of *ex vivo* isolated human $EOMES^+$ Tr1-like cells, we provide additional evidence that Tr1-like cells are a unique T-cell subset. Moreover, our data suggests that miR-92a and miR-125a target the expression of Tr1-associated genes like EOMES and IL-10R, and might thus act as inhibitors of Tr1 differentiation.

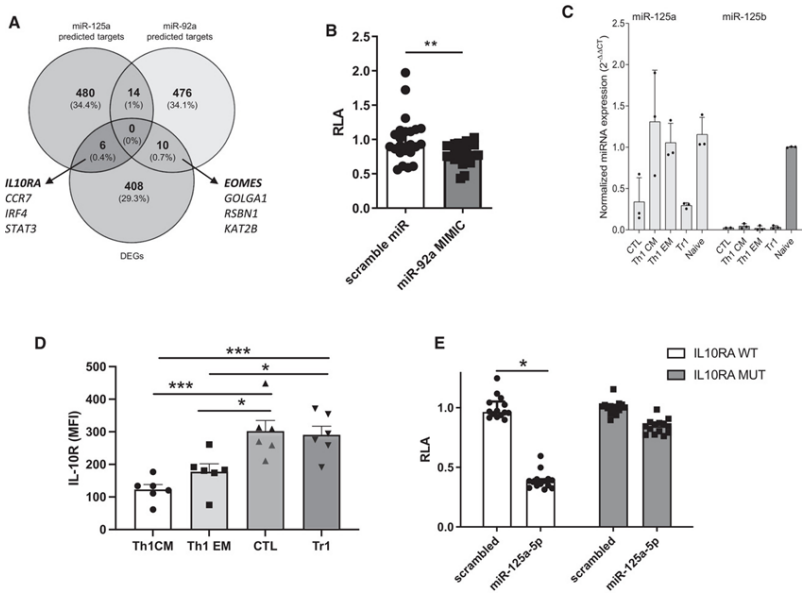


Figure 2.2 - Identification of putative gene targets of miR-92a and miR125a.

(**A**) Venn Diagram showing the overlap between differentially expressed genes and the miRNA targets predicted by TargetScan. (**B**) Dual-luciferase assay in HEK-293T cells transfected with the human EOMES 3'UTR together with miR-92a or a scrambled control. Mean of three independent experiments with six to nine technical replicates. Statistical analysis was performed using a Wilcoxon matched-pairs signed rank test (*$p < 0.05$). Error bars show median and interquartile range. (**C**) Expression of miR-125a and miR-125b was measured by RT-qPCR in the indicated $CD4^+$ T-cell subsets (3 independent donors analyzed in 3 experiments). (**D**) IL-10Rα protein levels in gated $CD4^+$CTL, Tr1-, Th1EM-, and Th1CM-cells and measured by flow cytometry (n = 6, 1 experiment). Shown is the MFI; Fluorescence minus one was used as negative control. the statistical analysis was performed using a one-way ANOVA. (**E**) Dual-luciferase assay in HEK-293T cells transfected with the human IL-10RA 3'UTR together with a miR-125a or scrambled control. Data show four independent experiments with three to four technical replicates. Error bars show median and interquartile range. Statistical analysis was performed using a Kruskal-Wallis test (**$p < 0.005$).

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Chapter 3. **Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition).**

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DOI: 10.1002/EJI.202170126. EPUB 2021 DEC 7. PMID: 34910301.

For this work I optimized the intracellular staining protocols (paragraph 3.3.4) and developed the gating strategy to identify Tr1 cells *ex vivo*, based on the expression of cytotoxic molecules Granzyme K (paragraph 3.2.4). In addition, I contributed to the analysis and elaboration of some of the figures presented.

HUMAN IL-10 PRODUCING REGULATORY T CELLS (TR1 CELLS) [SECTION III, CHAPTER 13]

3.1. OVERVIEW

Regulatory T-cells are a minor fraction of the $CD4^+$T-cell compartment and contain excessive immune reactions. Besides the well-defined $FOXP3^+$ Tregs also other T-cell populations have been reported to possess regulatory functions. Several different types of $FOXP3^-$ regulatory T-cells have been described in humans, but the best characterised ones are those that produce high amounts of the anti-inflammatory cytokine IL-10. These cells are referred to as type 1 regulatory T-cells (Tr1), and have been studied initially exclusively in *in vitro* cultures. However, recent progress allows now to identify cells with Tr1-like characteristics also directly *ex vivo* by flow cytometry in

human tissues. We will explain here some special features to be considered to induce T-cell IL-10 production and to measure IL-10 by flow cytometry, and discuss strategies to track populations of Tr1-like cells by surface markers, transcription factors and cytotoxic molecules in human tissues.

3.2. INTRODUCTION

Regulatory T-cells that express the lineage-defining transcription factor FOXP3 and the high affinity receptor for IL-2, CD25, represent approximately 5-10% of $CD4^+$T-cells in human blood, and are required to suppress multi-organ autoimmune diseases (see Chapter human Tregs). Besides $FOXP3^+$Tregs, several other T-cell populations were reported to exert regulatory functions, but most of these populations are poorly defined and can thus not be easily monitored by flow cytometry. Similar to $CD25^+$Tregs, IL-10 producing regulatory T-cells ("type 1 regulatory T-cells, Tr1") were identified more than 20 years ago (Groux et al., 1997; Sakaguchi, Sakaguchi, Asano, Itoh, & Toda, 1995). However, due to the lack of specific surface markers and transcription factors they remained for a long time an enigmatic population, and in humans they were mainly identified after *in vitro* culture according to IL-10 production (Roncarolo, Gregori, Bacchetta, Battaglia, & Gagliani, 2018). However, a caveat of this approach is that IL-10 is not exclusively produced by regulatory T- cells, and that it can be acquired or lost in culture (Dong et al., 2007; Gerosa et al., 1996; Kapitein et al., 2007). It is therefore critical to track Tr1-like cells

directly *ex vivo*, with no or minimal *in vitro* manipulation. Recent progress in the field have this goal made now feasible also in the human system, and revealed that Tr1-like cells play key roles in several human diseases, including autoimmune diseases (Facciotti et al., 2016; Okamura et al., 2015), IBDs (Alfen et al., 2018; Brockmann et al., 2018), allergy (Akdis et al., 2004), graft-versus-host disease (Groux et al., 1997) and cancer (Bonnal et al., 2021; Song et al., 2018). Clinical trials to treat graft-versus-host-disease and IBDs with *in vitro* generated Tr1-cells have been performed (Desreumaux et al., 2012; Gregori, Bacchetta, Hauben, Battaglia, & Roncarolo, 2005). We discuss here the different strategies that were reported to identify Tr1-cells by flow cytometry.

3.2.1. FLOW CYTOMETRIC DETECTION OF T-CELL IL-10 PRODUCTION

IL-10 is the characteristic cytokine of Tr1-cells, and is often, but not always (Geginat et al., 2019), associated with regulatory functions. Suppression assays are therefore mandatory to confirm that populations of IL-10 producing T-cells contain indeed Tr1-like cells (see Chapter Tregs/functional assays). IL-10 is a difficult cytokine to be measured with standard intracellular staining protocols (see Chapter human $CD4^+$T-cells). Indeed, IL-10 production in human $CD4^+$T-cells has a complex regulation, and may require peculiar stimulation conditions. Naïve helper T-cells are devoid of IL-10 producing capacities, and start to produce IL-10 following TCR stimulation in the

presence of permissive cytokines only after several days (Assenmacher et al., 1998; Kapitein et al., 2007). Conversely, antigen-experienced CD45RO^{+} memory T-cells possess significant IL-10 producing capacities *ex vivo*. The frequencies of IL-10^{+} cells among CD4^{+}T-cells after brief polyclonal standard stimulation, like 4-6 hours with PMA and Ionomycin or anti-CD3 and anti-CD28 antibodies, is however low (approximately 1%) (Haringer, Lozza, Steckel, & Geginat, 2009). Consequently, IL-10 is often measured by ELISA, which does not provide though any information on the frequencies or the characteristics of IL-10 producing T-cells. The large majority of antigen-

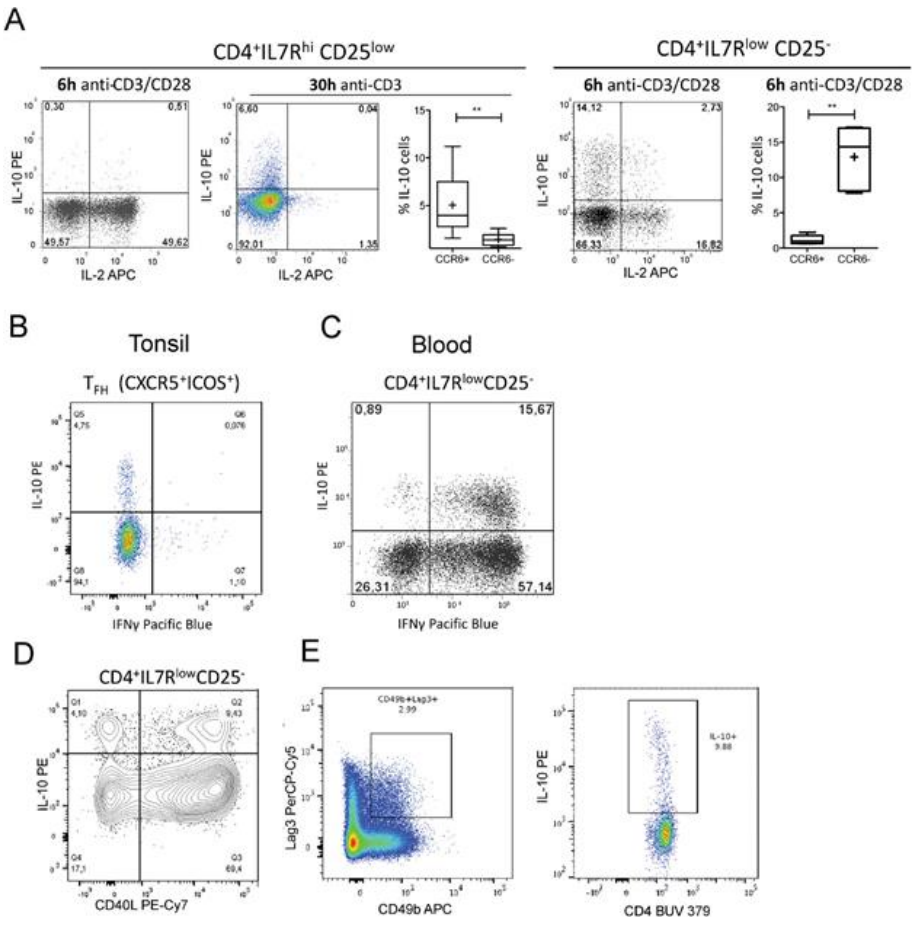

Figure 3.1 - Flow cytometric analysis of IL-10 production by human CD4$^+$T-cell subsets.

(**A**) Conventional human CD4$^+$T-cells (Complete gating strategy see Chapter human CD4$^+$T-cells) were isolated according to IL-7R and CCR6 expression and stimulated for 4 or 30 hours with anti-CD3 Abs in the absence or presence of anti-CD28 Abs or 100 U/ml IL-2. The production of IL-2 and IL-10 is shown. The frequencies of IL-10$^+$ cells are reported (Statistics: paired student's t-test, *n=5*). (**B** and **C**) Representative intracellular IL-10 and IFN-γ stainings of FACS-purified CD4$^+$CXCR5$^+$ICOS$^+$ tonsillar TFH-cells (**B**) or of human blood CD4$^+$IL-7R^{low}CD25$^-$T-cells (**C**) stimulated with PMA and Ionomycin for 4 h. (**D**) Same as in C, but IL- 10 was analyzed with a secretion assay and combined with CD40L surface staining. (**E**) IL-10 secretion of human CD4$^+$T-cells following overnight stimulation of total PBMC with SEB gated on CD4$^+$LAG3$^+$CD49b$^+$Tr1-cells. 2010 Häringer et al. Originally published in J. Exp. Med. https://doi.org/10.1084/jem.20091021 (Fig. 3.1A) and https://doi.org/10.1084/jem.20082238 (Fig. 3.1C).

experienced CD4$^+$T-cells in human peripheral blood are resting memory T-cells. IL-10 production in the latter has delayed kinetics when compared to other cytokines (Rivino et al., 2010), and is consequently hardly detectable in response to brief polyclonal standard stimulation (Figure 3.1A). However, IL-10 production by memory T-cells can be quite efficiently induced by a more sustained TCR stimulation. Thus, stimulation of purified CD4$^+$memory T-cells with anti-CD3 antibodies for 30 hours (Rivino et al., 2010), or super-antigen stimulation overnight induces more robust frequencies of IL-10$^+$ T-cells (approximately 5%). Notably, some memory T-cells can

produce IL-10 in the absence of CD28 co-stimulation. This anti-CD3-induced IL-10 production requires however IL-2, and is largely confined to $CCR6^+$T-cells (Rivino et al., 2010) (Figure 3.1A). *In vitro* or *in vivo* activated T-cells in contrast produce IL-10 after brief polyclonal standard stimulation. Thus, tonsillar TFH-cells, i.e. activated B helper effector cells (see Chapter human CD4), produce IL-10 upon brief PMA and ionomycin stimulation (Figure 3.1B). $CD4^+$effector T-cells in peripheral blood of healthy donors are rare, but are present among $CD25^+$ $IL\text{-}7R^-$ cells and produce rapidly high levels of IL-10 together with IFN-γ (Haringer et al., 2009) (Figure 3.1C). Notably, also $FOXP3^+$ Tregs in human peripheral blood are activated cells, and produce rapidly some IL-10 (<5%) (Haringer et al., 2009). Intracellular staining inevitably kills the analysed cells, but viable IL-10 producing T-cells can be purified with a cytokine secretion assay (Scheffold et al., 1998). The latter allows to isolate T-cells according to the secretion of up to two cytokines, and can thus be exploited to isolate for example IL-10 and IFN-γ co-producing T-cells (Dong et al., 2007). Moreover, it can be combined with surface markers. An efficient approach to isolate IL-10 producing T-cells that can suppress B-cell responses is the combination of IL-10 secretion and the lack of the helper molecule CD40L (Facciotti et al., 2016) (Figure 3.1D). CD40L is up-regulated by virtually all helper T-cells upon activation (Frentsch et al., 2005), but $FOXP3^+$ Tregs and terminally differentiated $IL\text{-}7R^-$ Tr1/effector cells have lost this capacity (Facciotti et al., 2016; Schoenbrunn et al., 2012). IL-10 secreting cells that suppress $CD4^+$T-cell proliferation can also be purified according to Tr1-associated surface markers, like CD49b and

LAG3 (Gagliani et al., 2013), after stimulation of total CD4^{+}T-cells with super-antigen overnight (Brockmann et al., 2018) (Figure 3.1E). In conclusion, IL-10 production can be quantified by flow cytometry, but it is critical to use appropriate stimulation conditions for the population of interest.

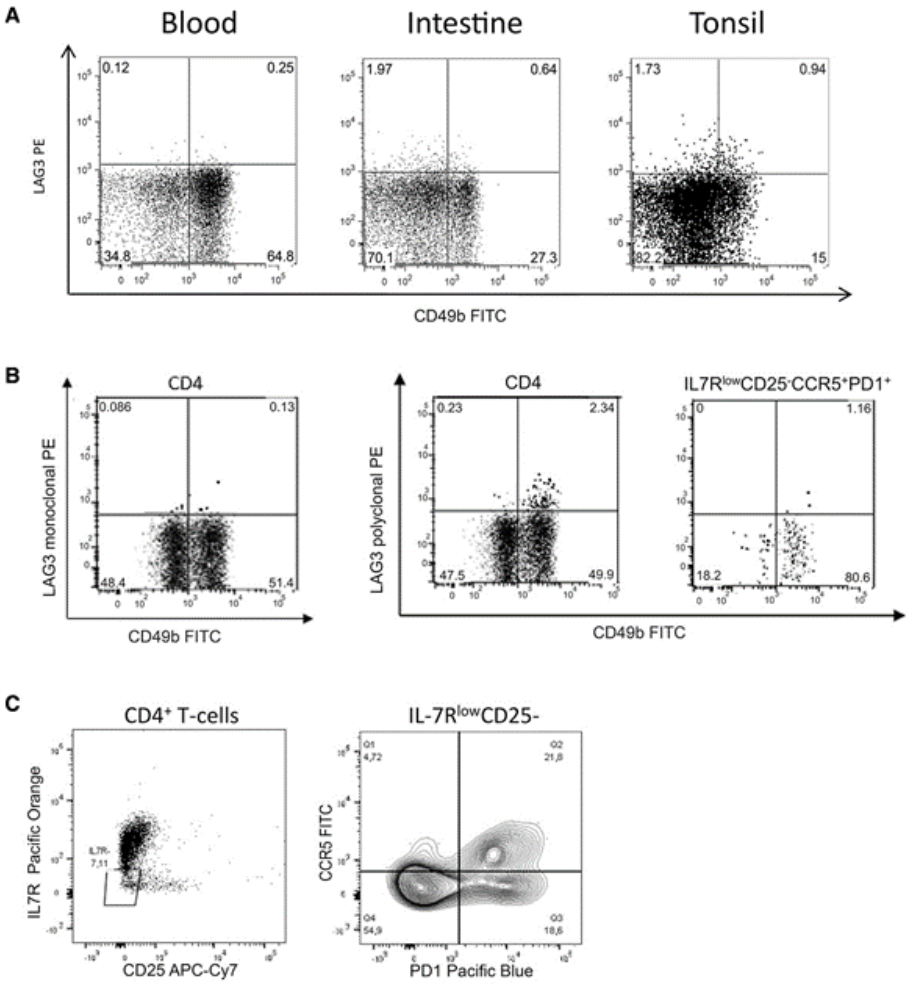

Figure 3.2 - Expression of phenotypic markers associated with human Tr1-cells.

(**A**) LAG3 and CD49b surface stainings of human peripheral blood of a healthy donor, in an inflamed tonsil and in the intestinal lamina propria *ex vivo*. (**B**) *Ex vivo* LAG3 and CD49b surface staining with a polyclonal or a monoclonal anti-LAG3 Ab on total $CD4^+$T-cells, or on gated IL-$7R^-CCR5^+$ Tr1-like cells in human peripheral blood of a healthy donor. (**C**) CCR5 and PD1 co-expression among gated $CD4^+$IL-$7R^{low}CD25^-$T-cells allows to enrich for Tr1-like cells in different tissues. Shown is peripheral blood of a representative healthy donor. *2010, Rivino et al.*

3.2.2. PHENOTYPIC MARKERS TO ENRICH FOR IL-10 PRODUCING TR1-LIKE CELLS IN HUMAN TISSUES AND DISEASES

Phenotypic markers that are associated with IL-10 production allow to enrich for IL-10 producing T-cells with regulatory functions without activating the TCR. The most used surface marker to track and enrich Tr1-cells is LAG3, an activation-induced co-inhibitory receptor, alone or in combination with the integrin CD49b (Gagliani et al., 2013; Okamura et al., 2009). CD49b and/or LAG3 were used to track and purify suppressive Tr1-cells from peripheral blood (Gagliani et al., 2013), tonsils (Sumitomo et al., 2017) and the intestinal lamina propria (Brockmann et al., 2018). $CD49b^+$ $LAG3^+$ T-cells are rare in peripheral blood of healthy donors, but increase in inflamed tissues (Figure 3.2A). Caveats of LAG3 surface staining are the different frequencies of $LAG3^+$ cells that are obtained with the original polyclonal (Gagliani et al., 2013) and the more recent monoclonal antibodies (Figure 3.2B), and

the fact that LAG3 may be cleaved from the surface by proteolysis (Graydon, Mohideen, & Fowke, 2021) (see also Tips and Tricks). An alternative strategy that identifies IFN-γ and IL-10 co-producing Tr1-like cells is to gate "conventional" $CD25^{-}CD4^{+}$T-cells that have down-regulated IL-7R expression and that co-express the Th1-associated chemokine receptor CCR5 and the co-inhibitory receptor PD1 (Alfen et al., 2018; Facciotti et al., 2016) (Figure 3.2C) (see also Tips and Tricks). Tr1-like cells with this phenotype can produce very high levels of IL-10 following brief polyclonal stimulation *ex vivo* (Figure 3.3A), although the frequencies of $IL\text{-}10^{+}$ cells vary strongly in individual donors (Facciotti et al., 2016). Notably, cytotoxic $CD4^{+}$T-cells (CTL) have a very similar phenotype, but can be discriminated since they lack CD28 and CD27 expression (Gruarin et al., 2019) (see also Chapter human $CD4^{+}$T-cells). $IL\text{-}7R^{-}CCR5^{+}$ Tr1-like are rare in peripheral blood of healthy donors and in tonsils (<1%) (Facciotti et al., 2016), but are increased in the circulation of SLE patients (>1%) (Facciotti et al., 2016) and in the intestine (approx. 2%) (Alfen et al., 2018), and can become quite abundant (>5%) in tumours (Bonnal et al., 2021).

The publication of two different strategies to identify human Tr1-cells raises the question if the reported surface markers are largely redundant, since most of these markers reflect chronic and/or recent activation. Indeed, LAG3, CD49b, CCR5 and PD1 are co-expressed on Tr1-cells in a mouse colitis model (Alfen et al., 2018) (see Chapter murine Tr1-cells), and in a relevant fraction of human Tr1-cells identified according to IL-10 secretion following overnight stimulation with super-antigens (Brockmann et al., 2018). In contrast, in

unstimulated peripheral blood of healthy donors the overlap between CD49b^{+}LAG3^{+} and IL-7R^{-}CCR5^{+}PD1^{+} Tr1-cells is surprisingly minimal (Alfen et al., 2018) (Figure 3.2B). In other words, in this experimental standard condition, CD49b^{+}LAG3^{+} and IL-7R^{-}CCR5^{+}PD1^{+} Tr1-cells represent unfortunately two largely distinct populations. Notably, the frequencies of IL-7R^{-}CCR5^{+} T-cells that co-express LAG3 on the cell surface are often increased in inflamed human tissues. Nevertheless, given the heterogeneity of Tr1-cells, the co-expression of Tr1-associated surface markers with IL-10 should first be experimentally determined in order to identify the best strategy for the tracking or purification of Tr1-cells, in particular in tissues or patients where Tr1-cells have not been previously analysed.

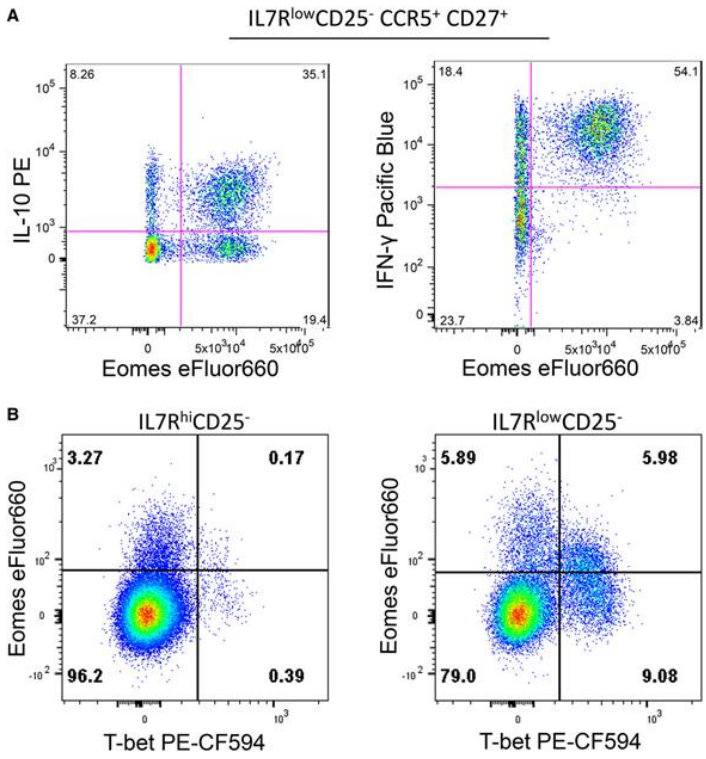

Figure 3.3 - Human Tr1-like cells tracked by intracellular staining for transcription factors.

(**A**) Intracellular Eomes versus IL-10 or IFN-γ expression in FACS-purified human blood $CD4^{+}IL\text{-}7R^{low}CD25^{-}CCR5^{+}CD27^{+}$Tr1-like cells after PMA and Ionomycin stimulation for 4 h. (**B**) *Ex vivo* Eomes versus T-betexpression in PBMC gated on conventional $CD4^{+}$T-cells according to IL-7R expression. Among $IL\text{-}7R^{low}CD25^{-}CD4^{+}$T-cells, Tr1-like cells can be identified as $Eomes^{hi}T\text{-}bet^{low}$, while CTL are $T\text{-}bet^{hi}Eomes^{low}$.

3.2.3. TRANSCRIPTION FACTOR EXPRESSION IN TR1 CELLS

The identification of the lineage-defining transcription factor FOXP3, which is selectively expressed in $CD25^{+}$ Tregs and required for their function, was a milestone in the field of regulatory T-cells (Hori, Nomura, & Sakaguchi, 2003). A Tr1-specific transcription factor with the same characteristics was unfortunately not identified, possibly because Tr1-cells are more heterogeneous. Several transcription factors have been shown to regulate T-cell IL-10 production and could thus in principal be exploited to track Tr1-cells. However, these transcription factors are normally not unique for Tr1-cells, and are often broadly expressed among human $CD4^{+}$T-cells. Thus, c-Maf regulates not only IL-10 production by Tr1-cells, but is also critical for IL-4 production and the generation of TFH-cells (Apetoh et al., 2010; Kim, Ho, Grusby, & Glimcher, 1999; Pot et al., 2009). Similarly, AHR controls not only regulatory T-cell differentiation, but also IL-22 production in Th17/22-cells (Gandhi et al., 2010; Trifari, Kaplan, Tran,

Crellin, & Spits, 2009). Blimp-1 is an effector T-cell-associated transcription factor that is required for IL-10 production by Tr1-cells (Iwasaki et al., 2013; Neumann et al., 2014; Parish et al., 2014), but it is unfortunately difficult to detect by intracellular staining in human $CD4^+$T-cells. In murine GvHD, the T-box transcription factor Eomesodermin (Eomes) was shown to control generation and IL-10 production of Tr1-cells in concert with Blimp-1 (Zhang et al., 2017). Eomes is also highly expressed in human IL-$7R^-$Tr1-like cells, acts as a lineage-defining transcription factor (Gruarin et al., 2019; Intlekofer et al., 2008; Lupar et al., 2015; Pearce et al., 2003; Zhang et al., 2017) and can be easily analysed by flow cytometry (Figure 3.3A). Notably, also $CD4^+$CTL express Eomes, but in contrast to $Eomes^+$Tr1-like cells they express also high levels of T-bet (Gruarin et al., 2019). Thus, the combination of IL-7R, T-bet and Eomes is a simple and powerful strategy to track IFN-γ producing Tr1-like cells (as IL-$7R^{lo}Eomes^{hi}$ T-bet^{lo}, Figure 3.3B) in peripheral blood and in other human tissues (Gruarin et al., 2019). Notably, $Lag3^+$Tr1-cells express only low levels of Eomes *ex vivo* (Gruarin et al., 2019), but they were reported to express the transcription factor Egr-2 in mice and in humans (Okamura et al., 2009). However, a strategy to track human $Lag3^+$Tr1-cells according to transcription factor expression by flow cytometry has to our best knowledge not yet been reported.

3.2.4. CYTOTOXIC MOLECULES EXPRESSED BY TR1-CELLS

Tr1-cells have been consistently reported to possess cytotoxic functions. In particular, they can kill myeloid APC via a perforin-dependent pathway (Magnani et al., 2011), and were reported to express the cytotoxic molecule GzmB, but not GzmA (Grossman et al., 2004). A caveat of these studies is, however, that the analysed Tr1-cells were generated and activated *in vitro*, since GzmB is rapidly induced upon *in vitro* stimulation in human $CD4^+$T-cells (Grossman et al., 2004). In addition, the expression patterns of GzmB might be different in humans and mice. GzmB can be easily stained intracellularly in conventional, $FOXP3^-CD4^+$T-cells from human peripheral blood in the complete absence of *in vitro* stimulation (Figure 3.4A). These in vivo occurring $GzmB^+CD4^+$T-cells correspond to terminally differentiated $CD4^+$CTL that lack CD27 and CD28 expression and express high levels of Granulysin (Bonnal et al., 2021), perforin and GzmA (Cheroutre & Husain, 2013) (see also Chapter human $CD4^+$T-cells). Notably, *ex vivo* isolated $CD4^+GzmB^+$ CTL are largely devoid of IL-10 producing and suppressive capabilities (Gruarin et al., 2019). This is surprising, since $IL\text{-}7R^-$ Tr1-cells express Eomes, which controls cytotoxic lymphocyte functions (Cruz-Guilloty et al., 2009; Pearce et al., 2003) and is involved in the regulation of GzmB expression. Indeed, $Eomes^+$ Tr1-like cells do possess cytotoxic functions, but they lack GzmB *ex vivo* and exhibit instead high levels of GzmK (Figure 3.4A, B) and also of GzmA (Gruarin et al., 2019). The selective expression of GzmK in Tr1-cells and of GzmB in $CD4^+$CTL is conserved in different

human tissues and tumours and can thus be exploited to monitor these 2 cytotoxic T-cell subsets *ex vivo* (Gruarin et al., 2019). GzmB and perforin were also reported to be expressed in FOXP3$^+$ Tregs in mice, in particular in tumour models (Cao et al., 2007), but circulating human FOXP3$^+$ Tregs express only very low levels of GzmB (Ashley & Baecher-Allan, 2009) (Figure 3.4A). Nevertheless, it is recommended to exclude FOXP3$^+$Tregs before analysing "conventional" CD4$^+$T-cells for the expression of GzmK and GzmB (Figure 3.5A). GzmK expression among CD4$^+$T-cells is largely restricted to Eomes$^+$ cells (Figure 3.5C), and largely absent from CD4$^+$CTL. Thus, if the number of parameters that can be analyses simultaneously by flow cytometry is limiting, GzmK is actually the most reliable single marker to track Tr1-like cells among CD4$^+$IL-7R^{low}T-cells. Notably, among total GzmK$^+$CD4$^+$T-cells only a subset corresponds to Eomes$^+$ Tr1-like cells, which can however be easily identified by the low levels of IL-7R surface expression (Figure 3.5D). GzmK$^+$Eomes$^+$Tr1-like cells lack CCR6 (Figure 1A, 3.4D) and CD161 expression, while a relevant fraction of IL-7R$^+$GzmK$^+$cells co-expresses CCR6 (Figure 3.4D) and CD161. This heterogeneity reconciles an apparently conflicting report on the expression of Eomes in human "unconventional" (CCR6$^+$ CD161$^+$) Th1-cells (Mazzoni et al., 2019), which produce GM-CSF, but not IL-10 and possess thus pro-inflammatory properties. In conclusion, intracellular staining for GzmK, alone or in combination with Eomes, among CD4$^+$CD25$^-$ IL-7R^{low} effector T-cells allows to monitor a population of Tr1-like cells *ex vivo*, without the absolute need to induce IL-10 by *in vitro* stimulation. This is particular helpful in tissues and diseases where the number of viable

T-cells is limiting, or where Tr1-cells down-regulate IL-10 production, like the inflamed intestine of IBD patients (Alfen et al., 2018).

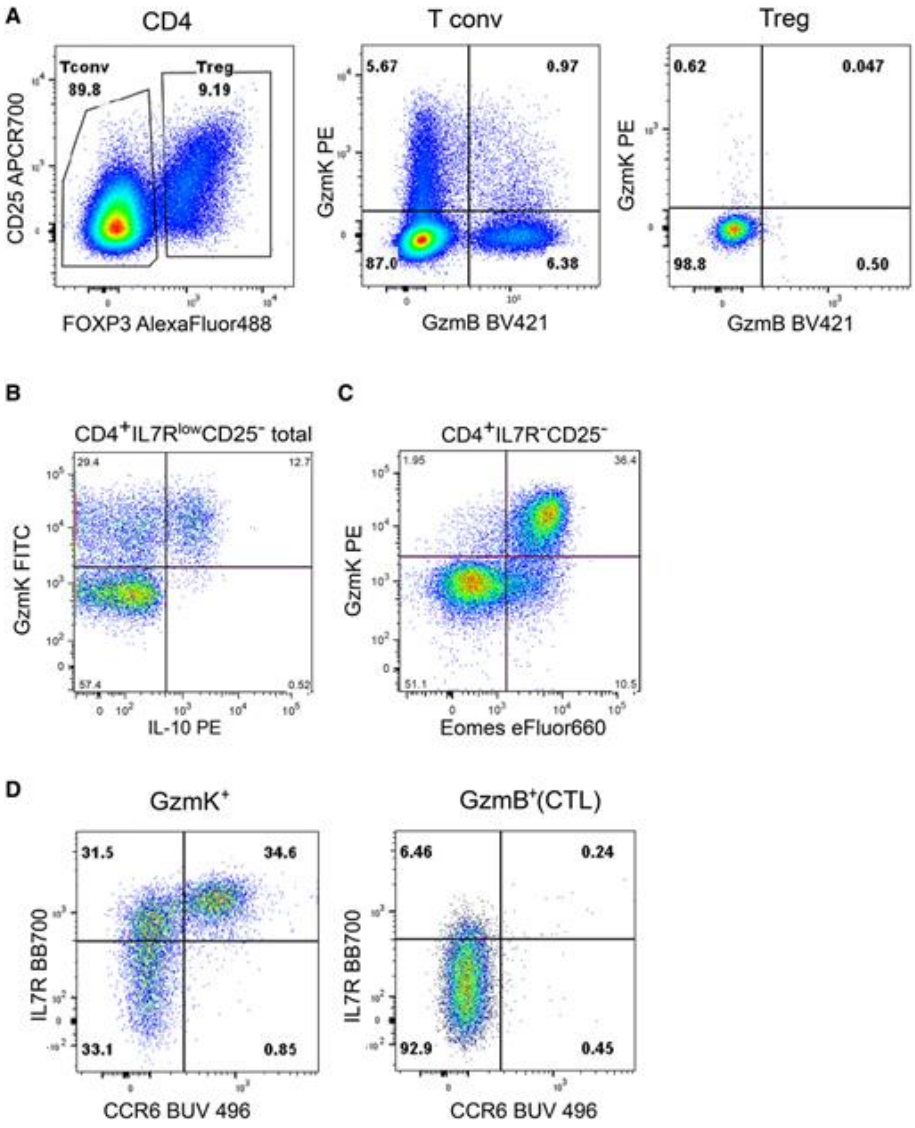

Figure 3.4 - Human Tr1-like cells tracked by intracellular staining for cytotoxic molecules.

(**A**) *Ex vivo* GzmK and GzmB expression in gated conventional $CD4^+$T-cells and $FOXP3^+$Tregs. (**B**) GzmK and IL- 10 co-expression in FACS-purified $CD4^+$IL-$7R^{low}CD25^-$T-cells after 4 hours of stimulation with PMA and Ionomycin. (**C**) Co-expression of GzmK and Eomes in FACS-purified $CD4^+$IL-$7R^{low}CD25^-$T-cells *ex vivo* D: *Ex vivo* IL-7R and CCR6 expression patterns in among conventional $CD4^+$T-cells gated as $GzmK^+$ or $GzmB^+$ (see A).

3.3. STEP BY STEP PROTOCOL

3.3.1. ISOLATING PBMC

1.1. Isolate PBMC from heparinized blood or buffy coat by using Ficoll-Paque according to manufacturer'sprotocol.

1.2. Collect the PBMC ring in 50 mL tubes.

1.3. Add PBS up to 50 mL and centrifuge for 8 min at 515 × g at room temperature (RT).

1.4. Decant supernatant, resuspend pellet up to 50 mL of Phosphate Buffer Saline (PBS) and centrifuge for10 min at 200 × g at RT.

1.5. Count cells and adjust concentration to 2,5-5 × 10^6 cells/mL. Note: continue skip the point 2 for the (ex-vivo) surface phenotype.

***Top tricks*:**

- When possible, use Sodium Citrate or heparin as anticoagulant. EDTA may interfere with cytokineproduction.

- See also Chapter human $CD4^+$T-cells for additional tricks

3.3.2. STIMULATING PBMC FOR THE DETECTION OF CYTOKINES

1.1. Transfer up to 5×10^5 PBMC to a 96-well U bottom plate (3788, Corning) in 200 µL of culture medium (RPMI containing 10% FCS, 1X nonessential aminoacids, 1X Na pyruvate and glutamax).

1.2. Then add Ionomycin (500 ng/mL) and PMA (50 ng/mL) to the correct wells.

1.3. Incubate for 4 h in a cell incubator at 37°C, 5% CO_2.

1.4. After 90 min, add Brefeldin A (BFA; 10 µg/mL) in order to block the secretion pathway and retaincytokines intracellularly.

1.5. At the end of incubation, centrifuge plate for 5 min at 450 × g at RT.

1.6. Decant supernatant, re-suspend cells in 200 µL *PBS* and continue with point 3.2.

3.3.3. SURFACE STAINING

3.1. Transfer up to 1×10^6 PBMC to a 96-well V bottom plate (Greiner BioOne).

3.2. Centrifuge the plate for 5 min at 450 × g at RT.

3.3. Meanwhile prepare surface staining mix (containing a pre-titrated appropriate amount of Ab and including the *live/dead*

exclusion dye) in a total volume of 35 µL *Brilliant Staining Buffer (BSB, BD)* for eachwell (prepare 1× extra every 5).

3.4. Decant supernatant and add 35 µL surface staining Ab-cocktail for each well gently resuspending cellsby pipetting 3 times.

3.5. Incubate for 20 min at RT, protected from light.

3.6. Add 200 µL *PBS* and centrifuge at 450 × g at RT for 5 min.

3.7. Decant supernatant and add 150 µL *PBS* gently resuspending cells by pipetting to analyze by flowcytometry or continue with the intracellular staining protocol.

***Top tricks*:**

- Some surface receptors, like IL-7R or LAG3, may be lost or acquired during the stimulation period. If *ex vivo* expression is critical, cells may be sorted first according to phenotypic markers, and then stimulated.
- *Ex vivo* purification: most IL-7R^{-}CCR5^{+} Tr1-like cells express PD1 *ex vivo* (Figure 3.2C), and therefore PD1 only moderately improves their enrichment. Moreover, anti-PD1 antibodies used for surface staining are often neutralising antibodies, and may thus interfere with suppressive capabilities. In contrast CD27 allows the separation of Tr1-cells from CTL and should always be included. In addition, exclusion of CCR6^{+}T-cells may be helpful to eliminate contaminating pro-inflammatory T-cells.
- LAG3 surface staining works very well with *in vitro* activated T-cells, but is challenging *ex vivo*. Fresh blood may give better results

than buffy-coated blood. The use of a monoclonal antibody is recommended.

- IL-7R staining is critical for the analysis Eomes^{+}Tr1-like cells, so the anti-IL-7R antibody should be tested and titered. Since IL-7R expression is not bimodal, co-staining with CD25 or FOXP3 is helpful to set the gate for IL-7R^{low} T-cells. Notably, the cells that have completely lost IL-7R expression produce the highestlevels of IL-10.
- See also Chapter human CD4^{+}T-cells for additional tricks.

3.3.4. INTRACELLULAR STAININGS OF CYTOPLASMATIC MOLECULES AND TRANSCRIPTION FACTORS.

4.1. After the wash 3.6, decant the supernatant and add 100 µL 1x *Fixation/Permeabilization* buffer.

4.2. Gently resuspend the cells by pipetting up and down 5 times.

4.3. Incubate for 20 min at RT, protected from light.

4.4. Add 100 µL of 1x *Permeabilization/Wash Buffer* directly into the well, without washing the cells.

4.5. Centrifuge for 5 min at 580 × g at RT

4.6. Decant supernatant and resuspend cells by pipetting 3 times in 35 µL of the intracellular staining mix prepared in 1x *Permeabilization/Wash Buffer.*

4.7. Incubate 50 min at 4°C, protected from light.

4.8. Add 150 µL 1x *Permeabilization/Wash Buffer* to each well and centrifuge for 5 min at 580 × g at 4°C.

4.9. Aspirate supernatant and resuspend cells in 150 µL *PBS* and analyze by flow cytometry.

***Top tricks*:**

- After step 4.3 cells can be washed and stored in PBS at +4°C. To continue the protocol is important to resuspend cells in 200 ul of Permeabilization/Wash Buffer for 15 min.
- The use of the eBioscience Set is generally preferable. The BD Pharmingen Set allows a better visualization of transcription factors. Howewer the BD Fixation Buffer contains methanol and it's harmful for some fluorochromes (e.g. PE and APC and relative tandems, BB700) which have been used for the surface staining.
- To see the cytokines only the use of classic protocol of fixation with PFA 2% and permeabilization with Saponin 0.05% is sufficient.

3.3.5. SORTING AND SECRETION ASSAY FOR IL-7R^{-}10^{+} TR1 CELLS

In order to isolate Eomes^{+}Tr1-like cells (IL-7R^{-} IL10^{+}) cells a double round of sorting is recommended (see above).

DAY ONE: SORTING

5.1. Isolate CD4^{+}T cell using human CD4^{+}T Cell Isolation Kit (Miltenyi, cat number 130-096-533) according to manufacturer's instructions.

5.2. Re-suspend pellet up to 50 mL of PBS and centrifuge for 10 min at 200 × g at RT.

5.3. Collect and count cells; stain for surface markers: CD4, CD127, CD25 for 20 minutes at 37°C.

5.4. Wash cells with 10 mL of PBS and centrifuge for 10 min at 200 × g at RT.

5.5. Proceed to the first round of FACS sorting (70 µM nozzle) to isolate $CD4^{+}CD127^{low}CD25^{-}$cells ($CD4^{+}CD127^{+}CD25^{-}$ and $CD127^{lo}CD25^{+}$Tregs may be sorted as control cells).

5.6. Transfer up to 3 × 105 sorted cells to a 96-well U bottom plate (3788, Corning) in 200 µL of culture (RPMI added with 5% Human serum) and let them rest ON at +37°C 5% CO_2.

DAY TWO: SECRETION ASSAY AND SORTING

5.7. The day after stimulate cells with PMA/Ionomycin as in paragraph 2, without blocking the secretion (skippoint 2.4).

5.8. At the end of incubation collect stimulated cells in a 15 mL tube and proceed with secretion assay for IL- 10 (Miltenyi, human IL-10 Secretion Assay-Detection Kit, cat. Number 130-090-434) following the manufacturer's instruction. Be careful and add opportune quantity of anti-CD40-L antibody after the incubation with IL-10 detection primary antibodies.

5.9. Wash cells and proceed to a second round of FACS sorting to isolate $IL10^{+}$ $CD40L^{-}$ Tr1 cells

Alternatively, Tr1 can be isolated from total $CD4^{+}$T-cells according to surface markers, such as Lag3 andCD49b, and IL10

secretion. $CD4^+$T-cells are first purified using CD4-coated beads (Miltenyi).

5.10. $CD4^+$Tcells are re-stimulated overnight in full media ($1x10^6$ cell/mL) supplemented with Staphylococcal enterotoxin B (SEB) 1µg/mL at 37°C, 5%CO_2.

5.11. Perform human Miltenyi secretion assay as stated from point 5.7 to 5.8 counterstaining with Tr1associated surface markers Lag3 and CD49b.

5.12. Wash cells and proceed to FACS sorting to isolate $Lag3^+CD49b$ IL-10^+ Tr1 cells.

Top tricks:

- Secretion assay: work fast and use media at the indicated temperature. Up to two different cytokines can be assessed simultaneously.
- If you do not have rotation device for tubes be sure to gently mix the tubes each 5 minutes for all thetimes of incubation.
- Before sorting pass the cells through a filter (50µ filter siringe type; 340601, cat number 340601) to avoid clumps.
- Very high frequencies of cytokine-secreting cells (>50%) may lead to false-positive events.

3.4. MATERIALS

3.4.1. LIVE/DEAD EXCLUSION DYE

Fixable Viability Stain 780 (FVS780, BD).

3.4.2. ANTIBODIES

These are detailed in Tables 3.1 and 3.2.

Table 3.1 - Suface Stainings

Marker	Fluorochrome	Clone	Brand
CD3	BUV737	UCHT1	BD Biosciences
CD4	BUV563	SK3	BD Biosciences
CD4	BUV395	RPA-T4	BD Biosciences
CD4	PerCP	RPA-T4	BioLegend
CD4	APC-Fire750	RPA-T4	BioLegend
CD8	BUV805	SK1	BD Biosciences
CD25	APC-R700	2A3	BD Biosciences
CD25	APC-Cy7	CD25	BioLegend
CD49b	FITC	AK7	BD Biosciences
CD49b	Alexa Fluor 647	AK7	BD Biosciences
IL-7R	BB700	HIL-7R-M21	BD Biosciences
IL-7R	BV510	AO19D5	BD Biosciences
LAG-3	PerCP-eFluor710	3DS223H	eBioscience
LAG-3	PE	REA351	Miltenyi
LAG-3 PE	PE	polyclonal	R&D
ICOS	eFluor450	ISA-3	eBioscience
PD-1	BV421	EH12.2H7	BioLegend
CXCR5	PE	FAB190P	R&D
CCR5	FITC	2D7	BD Biosciences
CCR6	BUV496	11A9	BD Biosciences
CCR6	PE-Cy7	R6H1	eBioscience

Table 3.2 - Intracellular staining

Marker	Fluorochrome	Clone	Brand
CD40-L	PE-Cy5	24-31	BioLegend
CD40-L	PE-Vio770	5C8	Miltenyi
EOMES	eFluor660	WD1928	eBioscience
FOXP3	Alexa Fluor 488	259D	BioLegend
Granzyme B	BV421	GB11	BD Biosciences
Granzyme B	FITC	GB11	BD Biosciences
Granzyme K	PE	GM6C3	Santa Cruz
IFN-ϒ	FITC	4S.B3	BioLegend
IFN-ϒ	PB	4S.B3	BioLegend
IL-2	APC	MQ1-17H12	eBioscience
IL-10	APC	JES3-9D7	BioLegend
IL-10	PE	JES3-9D7	BioLegend
T-bet	PE-CF594	O4-46	BD Biosciences

3.4.3. FLOW CYTOMETER

Experiments were performed either on a FACS canto or on a BD FACSymphony flow cytometer with a 5 lasers (488 nm, 561 nm, 640 nm and 405 nm and 355 nm), 29 colors (6-5-3-8-7) configuration (BD Bioscience).

Filters(laser):

- 780/60(488) for BB790-p; 750/30 for BB755; 710/50(488) for BB700 or PerCP-eFluor710; 670/30 for BB660; 610/20 for BB630; 530/30(488) for FITC or Alexa Fluor 488.
- 780/60(561) for PE-Cy7; 710/50(561) for PE-Cy5.5; 670/30(561) for PE-Cy5; 610/20(561) for PE-CF594; 586/15(561) for PE.

- 680/60(640) for FVS780 or APC-Cy7 or APC-Fire750; 730/45(640) for APC-R700; 670/30(640) for APC or eFluor 660 or Alexa Fluor 647.
- 780/60(405) for BV786; 750/30(405) for BV750; 710/50(405) for BV711; 677/20(405) for BV650;
- 605/40(405) for BV605; 586/15(405) for BV570; 525/50(405) for BV510 or Pacific Orange;450/50(405) for BV421 or eFluor450 or Pacific Blue.
- 810/40(355) for BUV805; 735/30(355) for BUV737; 670/25 for BUV661; 605/20 for BUV615; 580/30 for BUV563; 515/30 for BUV496; 379/28 for BUV395.

3.4.4. **REAGENTS**

- Ficoll-Hypaque Plus (GE Healthcare, endotoxin tested, cat. no. 17-1440-03)
- FCS (fetal calf serum) batch-tested for low endotoxin
- PBS 1x (Gibco DPBS, no calcium, no magnesium; cat. no. 14190144)
- Culture medium: RPMI 1640

  2 mM glutamine

  1% non-essential amino acids 1 mM sodium pyruvate

  50 µM β-mercaptoethanol

  1% penicillin/streptomycin

  5% human serum (HS) or 10% FCS
- Washing medium:

  RPMI-1640 w/ Hepes (25 mM)

  1% FCS or 0.5% HS

- Flow cytometry buffer (FACS buffer):

  Phosphate buffered saline (PBS 1x)

  2.5% FCS or 1% HS

  0.01% (w/v) sodium azide (to be added in the case of long-term storage)

2mM EDTA pH 8.0 (to prevent clots)

- Stimulation mix:

  Culture medium

  1 µg/mL Ionomycin (Sigma-Aldrich, cat. no. I0634)

  2 x 10^{-7} M PMA (Sigma-Aldrich, cat. no. P8139)

  10 µg/mL BFA (Sigma-Aldrich, cat. no. B7651)

- Cytofix/Cytoperm 1x solution (BD Biosciences; cat. no. 554722)
- 1x Perm/Wash (always prepare freshly before use):

  10% 10× perm/wash (BD Biosciences; cat. no. 554723)

  90% ddH2O

- 1x Fixation Buffer working solution (eBioscience Foxp3 / Transcription Factor Staining Buffer Set; cat. 00-5523-00) for intra-nuclear staining of transcription factors:

  75% Fixation/Permeabilization Diluent (component cat. 00-5223)

  25% 4x Fixation/Permeabilization Concentrate (component cat. 00-5123)

- 1x Permeabilization Buffer working solution (eBioscience Foxp3 / Transcription Factor Staining Buffer Set; cat. 00-5523-00) for intra-nuclear staining of transcription factors:

  10% 10x Permeabilization Buffer (component cat. 00-8333)

  90% ddH2O

- 1x Fixation Buffer working solution (BD Pharmingen Transcription Factor Buffer Set, cat. 562574) for intra-nuclear staining of transcription factors:

  75% TF Fix/Perm Diluent Buffer (component cat. 51-9008101)

  25% 4x TF Fix/Perm Buffer (component cat. 51-9008100)
- 1x Permeabilization Buffer working solution (BD Pharmingen Transcription Factor Buffer Set, cat. 562574) for intra-nuclear staining of transcription factors:

  20% 5x TF Perm/Wash Buffer (component cat. 51-9008102)

  80% ddH_2O.

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Chapter 4. Role of Eomes^{+} type 1 regulatory T-cells in multiple sclerosis

4.1. INTRODUCTION

Multiple sclerosis (MS) is a neurodegenerative autoimmune disease of the central nervous system (CNS) mediated by myelin-specific CD4^{+}helper T (Th) cells. The central role of CD4^{+}Th cells is supported by the strong association of MS susceptibility with MHC/HLA class II alleles (Jersild, Svejgaard, & Fog, 1972; Schmidt, Williamson, & Ashley-Koch, 2007) and the fact that experimental autoimmune encephalomyelitis (EAE) in mice is induced by activation of myelin-specific CD4^{+}T-cells (Bittner, Afzali, Wiendl, & Meuth, 2014; Fletcher, Lalor, Sweeney, Tubridy, & Mills, 2010). Several studies confirm that in MS, as in other autoimmune diseases, the immune-regulatory mechanisms are compromised in genetically predisposed individuals and this could lead to the development of a pathological immune response and chronic inflammation (International Multiple Sclerosis Genetics Consortium et al., 2011; Kleinewietfeld & Hafler, 2013). However, it is still unclear whether this aberrant regulation is the leading cause or only one consequence of the disease. Although recently some researches are highlighting a possible role of B-cells (Franciotta, Salvetti, Lolli, Serafini, & Aloisi, 2008), the various theories mainly focus on the existence of CNS-specific pathogenic Th cells (i.e. inflammatory Th1 and Th1/Th17 cells) and on the insufficient function of regulatory T-cells. In particular, auto-reactive T-cells play a pathogenic role in neuro-inflammation and in MS, whereas normally

T-cells specific for neurotropic viruses, such as EBV or JCV, should be protective in the CNS. Furthermore, different regulatory subsets, namely FOXP3$^+$ Treg and IL-10-producing type 1 regulatory T (Tr1) cells cells), control autoreactive and overshooting antiviral responses (Belkaid & Tarbell, 2009; Sakaguchi, Yamaguchi, Nomura, & Ono, 2008). Both subsets were proposed to be involved in MS, however, human Tr1 cells are poorly defined *in vivo*, and their role in MS remains speculative (Kleinewietfeld & Hafler, 2013; Martinez-Forero et al., 2008). Notably, in order to suppress immune adaptive responses, Tr1 cells need to be activated by an antigen, thus their antigen-specificity is a key feature that needs to be unveiled. Our recently published strategy to identify Tr1 cells by Eomes expression in human tissues allows now their functional and molecular analysis *ex vivo* (Cossarizza et al., 2021; Gruarin et al., 2019) considering all their phenotype carachteristics (i.e. Lag3, GzmK markers).

In this scenario, the present work aims to contribute to deepen a phenotypic and functional characterization of Tr1 cells in humans and to understand their role in MS, focusing on their CNS-homing capacities and their specificity for MS-relevant self- and viral-antigens. These results could unveil a potential beneficial effect in MS, thus allowing regulatory T-cell augmentation or adoptive therapy as a potential treatment strategy. Notably, cell-based therapy with FOXP3$^+$ Tregs and Tr1 cells was established in other immune-mediated diseases (Duffy, Keating, & Moalem-Taylor, 2019), but the subset that efficiently and selectively suppresses pathogenic T-cells in MS needs still to be identified.

The cohorts of MS patients included in this research were relapsing-remitting (RR) patients that were either untreated or treated with natalizumab, the anti-α4 integrin antibody that inhibits lymphocyte homing to the CNS preventing relapses. Since, treatment with natalizumab is known to induce the accumulation of CNS-homing T-cells in the blood (Butzkueven et al., 2020), natalizumab-treated patients are relevant to study CNS-homing T-cells, in particular when compared to untreated patients. Many other MS therapies impact directly or indirectly on lymphocytes proliferation and/or differentiation, so they were considered to be unsuited for our analysis.

4.2. RESULTS

4.2.1. GATING STRATEGY TO IDENTIFY EOMES$^+$ TR1 CELLS

Auto-reactive and overshooting anti-viral T-cell responses are controlled by regulatory T-cell subsets, namely FOXP3$^+$ Treg and IL-10-Tr1 cells. Both subsets were proposed to be involved in MS (Kleinewietfeld & Hafler, 2013; Martinez-Forero et al., 2008), but Tr1 cells are poorly defined. The role of *in vivo* occurring Tr1 cells in MS remains thus to be understood, mainly due to the lack of exclusive markers. Usually, Tr1 cells were tracked following *in vitro* culture by IL-10 production (Brockmann et al., 2018; Haringer, Lozza, Steckel, & Geginat, 2009a). We recently identified Eomesodermin (Eomes) as a putative lineage-defining transcription factor of Tr1 cells, and the

cytotoxic molecule Granzyme (Gzm) K among the most strongly upregulated genes (Gruarin et al., 2019).

CD4$^+$T-cells were gated accordingly to the expression of CD3 and the lack of CD8, among the lymphocytic population, after the exclusion of dead cells and doublets. CD4$^+$T-cells are heterogeneous and contain four major subsets according to the expression of CD25, FOXP3, Eomes, GzmK, GzmB, with largely distinct characteristics and functions. "Conventional" FOXP3$^+$ Tregs were gated as FOXP3$^+$ CD25$^+$, cytotoxic T-lymphocytes (CTL) as FOXP3- GzmB$^+$, and Tr1-enriched cells as FOXP3- GzmK$^+$, while the large majority of CD4$^+$T-cell lacking of GzmB/K and FOXP3 expression were identified as helper T (Th)-cells and analysed as control (Figure 4.1A). Eomes is differently expressed among the distinct cell subsets, with GzmK$^+$ Tr1-enriched cells showing higher levels (47.07±3.97) compared to CTL (29.80±5.02%), while in Th and Treg cells is basically absent (<1%). Subsequently, Eomes-enriched GzmK$^+$ cells were subdivided according to IL-7R and CCR6 expression into IL-7R$^+$ CCR6$^+$ cells (Mazzoni et al., 2019), IL-7R^{low} CCR6$^-$ Tr1 effector cells and IL-7R^{hi} CCR6$^-$ "pre-Tr1" cells, the putative "precursors" of Tr1 (Gruarin et al., 2019) (Figure 4.1). This improved gating strategy to track Tr1 cells *ex vivo* was established and published in the Third Guidelines for Flow Cytometry in the European Journal of Immunology in December 2021, Tr1 chapter (Cossarizza et al., 2021) (see Chapter 3).

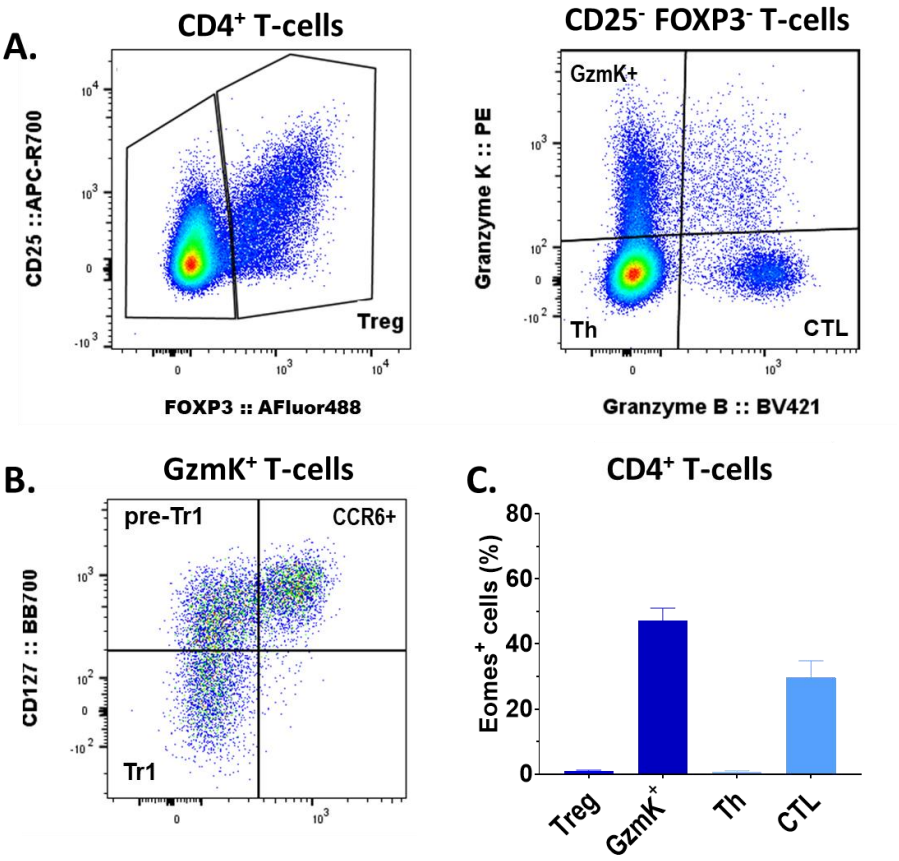


Figure 4.1 - Gating strategy according to the expression of FOXP3, CD25, Granzyme K/ B, Eomes, IL-7R and CCR6 in CD4⁺T-cells.

PBMCs isolated from healthy donor (HD) were analysed by flow-cytometry. Representative stainings show the gating strategy used to distinguish several cell subsets among CD4⁺T lymphocytes in healthy donors: **A.** Treg (CD25⁺ FOXP3⁺), GzmK⁺ Tr1-enriched cells (CD25⁻FOXP3⁻ GzmK⁺), T helper (Th, CD25⁻FOXP3⁻GzmK⁻GzmB⁻) and Cytotoxic T-Lymphocyte (CTL, CD25⁻FOXP3⁻GzmB⁺) cells; **B.** GzmK⁺ cells are further subdivided in IL-7R⁻CCR6⁻ Tr1 cells, IL-7R⁺CCR6⁻ "Tr1 precursors" (pre-Tr1) cells and IL7R⁺CCR6⁺ cells; **C.** Differential expression of Eomes in the main CD4⁺T-cell subsets. Percentages of Eomes in FOXP3⁺ Treg cells, GzmK⁺ Tr1-enriched cells, GzmK⁻/B⁻ Th cells and GzmB⁺CTL in HD (*n=15*). The graph shows means ±SEM.

4.2.2. SELECTIVE REDUCTION OF CIRCULATING TR1 CELLS IN UNTREATED MS PATIENTS

We aimed to monitor the frequencies of different regulatory cell subsets, such as Tr1 cells and Tregs, and other $CD4^+$subsets, in the blood of MS patients compared to healthy controls (HD) and in the CSF of untreated MS patients. We first analysed the samples with 8-colour flow cytometry on a FACS Canto and then we successfully established a multi-dimensional flow cytometry panel by FACS Symphony.

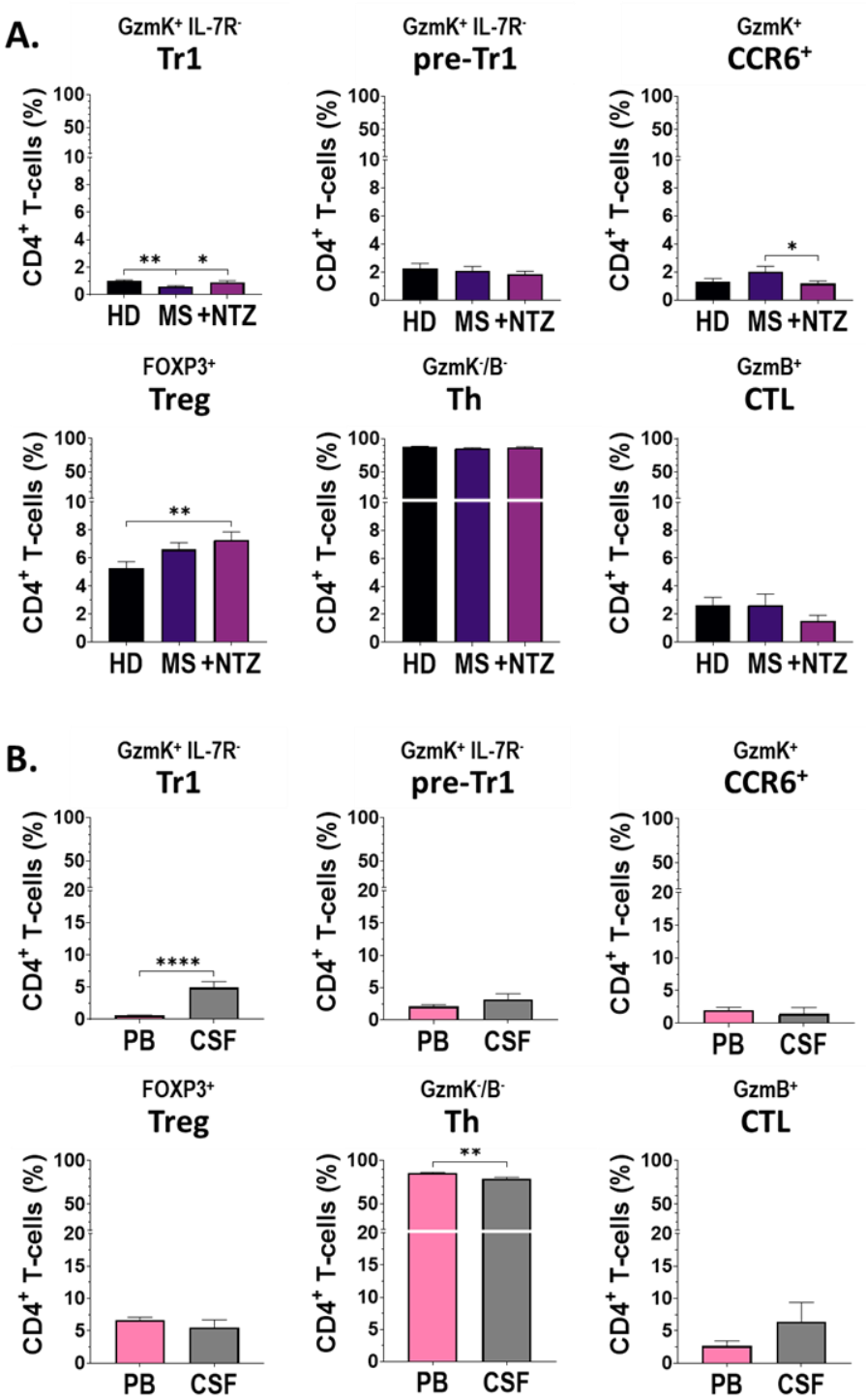
Within the compartment of $CD4^+$lymphocytes, circulating IL-$7R^-$ Tr1 cells are significantly reduced by almost twice in untreated RRMS patients (MS, *n=15*; 0,59±0.07%) as compared to healthy controls (HD, *n=35*; 1,0±0,08%), while the frequencies of the other subsets were almost unaltered or not significantly different, as for Treg cells (p=0,077) (Figure 4.2A). Thus, the observed reduction in RRMS was unique for Tr1 cells. However, in patients treated with the anti-α4-integrin antibody natalizumab (+NTZ, *n=22*), we found restored Tr1 frequencies (0,91±0,11%; Figure 4.2A). $GzmK^+CCR6^+$cells (1,16±0,19%) also return to the values of HD (1,303±0,23%), while Tregs accumulate significantly in the blood of treated MS patients (7,29±0,58%).

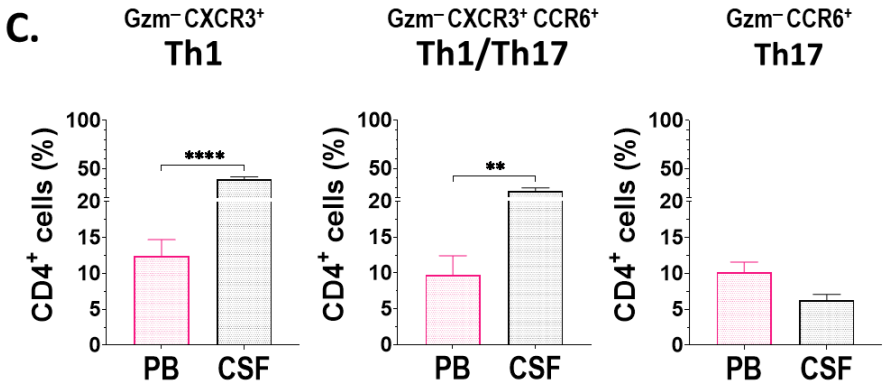
Consistently, there was a strong, selective and highly significant enrichment of Tr1 cells (>10-fold; 4,92±0,93%) in the CSF (*n=8*) of active MS patients, while Tregs and CTL had similar frequencies as compared to the peripheral blood (PB, *n=15*) (Figure 4.2B). On the other hand, helper T-cells undergo a significant reduction in the CSF (78,63±1,81%). However, among the Th cells we confirmed the enrichment of CXCR3-expressing Th1 and Th1/Th17 cells in CSF on

untreated MS patients (Figure 4.2C) (Paroni et al., 2017). The CSF was obtained from untreated MS patients shortly after an attack (1-2 days). The volume of CSF samples was between 3 and 7 ml from which were obtained an average of 18,1 x 10^4 (±33,9 x 10^4 SD, median of 8,7 x 10^4) total cells, therefore, data with <10 events were excluded. Only in few cases we had a paired sample of peripheral blood from the same patients, and therefore an unpaired analysis was performed anyway. Moreover, we tried to collect cells for single cell RNA sequencing of T-cells from CSF and blood of MS patients, but up to now we did not obtain a sufficient number of viable T-cells (>5 x 10^4) from CSF to perform this this cutting-edge analysis.

Figure 4.2 - Frequencies of $CD4^+$T-cells subsets in blood and cerebrospinal fluid of MS patients.

The percentages of Tr1 cells ($GzmK^+IL\text{-}7R^-CCR6^-$), pre-Tr1 cells ($GzmK^+IL\text{-}7R^+CCR6^-$), $CCR6^+$ cells ($GzmK^+IL\text{-}7R^+CCR6^+$), Treg ($CD25^+FOXP3^+$), Th cells ($FOXP3^-GzmK^-GzmB^-$) and CTL ($FOXP3^-GzmB^+$) cells among $CD4^+$T-cells **A.** in peripheral blood of healthy donors (HD, *n=35*), relapsing-remitting (RR-) MS patients either untreated (MS, *n=15*) or natalizumab-treated (+NTZ, *n=22*), and **B.** in the cerebral-spinal fluid (CSF) of active RRMS patients (*n=8*). **C.** Th cells were further subdivided in Th1 ($CXCR3^+CCR6^-$), Th1/Th17 ($CXCR3^+CCR6^+$) and Th17 ($CXCR3^-CCR6^+$) in PB and CSF of active RRMS patients. Statistical significance was calculated by unpaired t-test (P-value: *≤0.0332, **≤0.0021, ***≤0.0002). Graphs show means ±SEM.


A.
GzmK+ IL-7R-
Tr1
pre-Tr1
GzmK+
CCR6+
FOXP3+
Treg
GzmK-/B-
Th
GzmB+
CTL
CD4+ T-cells (%)
HD
MS
+NTZ
B.
PB
CSF

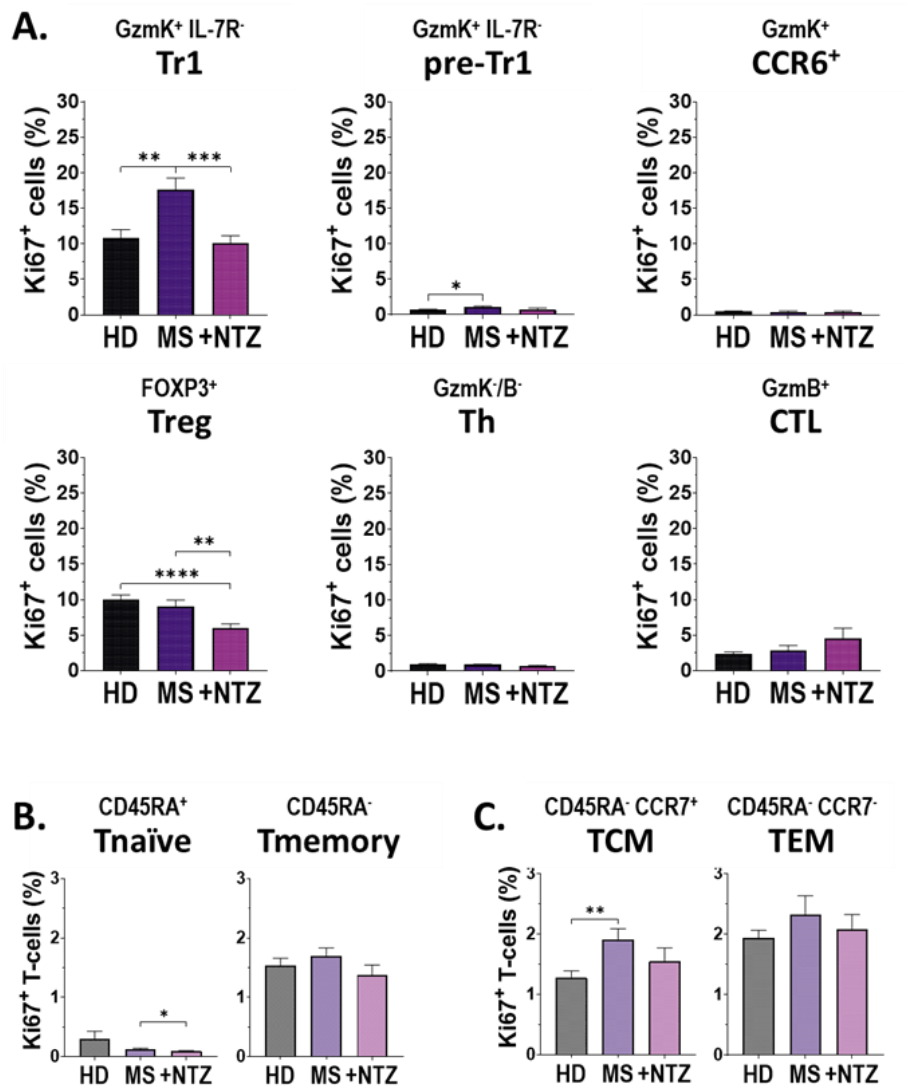


4.2.3. ALTERED HOMEOSTASIS OF TR1 CELLS IN MS PATIENTS IS RESTORED BY NATALIZUMAB THERAPY

The turnover of CD4⁺T-cell subsets was assessed by intracellular staining of the proliferation-associated marker Ki67, which is expressed in T-cells that proliferated *in vivo* in the previous 2-5 days (Shedlock et al., 2010). In healthy individuals, proliferation is known to be higher in FOXP3⁺ Tregs and Tr1 cells (≈10% Ki67⁺) than pre-Tr1 cells and total Th cells (<1% Ki67⁺) (Figure 4.3A) (Geginat, Sallusto, & Lanzavecchia, 2001; Gruarin et al., 2019; Smigiel, Srivastava, Stolley, & Campbell, 2014). However, Th cells can be subdivided in quiescent naïve T-cells (0.29±0.13% Ki67⁺) and memory T-cells that have a very low turnover (1.53±0.12% Ki67⁺) (Figure 4.3B). Moreover, we found that also CD4⁺CTLs have a moderate *in vivo* turnover (approx. 4% Ki67⁺ cells) (Figure 4.3A). Strikingly, Tr1 cells showed a strongly increased *in*

vivo proliferation (17.63±1.61% Ki67$^+$) in untreated MS patients. Since Tr1 precursors also undergo a significant increase in turnover (1.08±1.14% Ki67$^+$), and all other analyzed subsets had largely unaltered proliferation rates, this finding was again unique for GzmK$^+$ Tr1 cells (Figure 4.3A). Although not significant, there seems to be a reduction of CD45RA$^-$ naïve T-cells and an increase in proliferation of CD45RA$^+$ memory T-cells in MS patients (Figure 4.3B). In particular, the increase in the latter appears to be mainly due to the contribution of CCR7$^+$central memory T -cells that have a significantly higher turnover compared to healthy individuals. However, natalizumab treatment is able to restore the normal rates of proliferation (Figure 4.3C). Moreover, Tr1 cells in natalizumab-treated patients had normal proliferation rates (10.14%±0.99% Ki67$^+$), while FOXP3$^+$ Treg (5.99%±0.59% Ki67$^+$) and T naïve (0.08±0.01) were significantly reduced (Figure 4.3A and B).

Figure 4.3 - I*n vivo* turnover of CD4$^+$T-cells subsets.
Intracellular expression of the Ki67 proliferation marker in CD4$^+$T-cells subsets: **A.** Tr1 cells (GzmK$^+$IL-7R$^-$CCR6$^-$), pre-Tr1 (GzmK$^+$IL-7R$^+$CCR6$^-$), CCR6$^+$ cells (GzmK$^+$IL-7R$^+$CCR6$^+$), FOXP3$^+$ Treg (CD25$^+$FOXP3$^+$), helper T-cells (Th, CD25$^-$FOXP3$^-$GzmK$^-$/B$^-$) and Cytotoxic T-Lymphocyte (CTL, CD25$^-$FOXP3$^-$GzmB$^+$). **B.** Th cells were further divided in CD45$^+$ naïve and CD45RA$^-$ memory T-cells, **C.** CCR7$^+$ central memory (T_{CM}) and CCR7$^-$ effector memory (T_{EM}). PBMCs obtained from peripheral blood of healthy donors (HD, *n=35*), relapsing-remitting MS patients either untreated (*n=15*) or natalizumab-treated (+NTZ, *n=22*). Statistical significance was calculated by unpaired t-test (P-value: *≤0.0332, **≤0.0021, ***≤0.0002). Graphs show means ±SEM.


A.
GzmK+ IL-7R-
Tr1
pre-Tr1
GzmK+
CCR6+
FOXP3+
Treg
GzmK-/B-
Th
GzmB+
CTL
Ki67+ cells (%)
HD
MS
+NTZ
B.
CD45RA+
Tnaïve
CD45RA-
Tmemory
Ki67+ T-cells (%)
C.
CD45RA- CCR7+
TCM
CD45RA- CCR7-
TEM

4.2.4. INCREASED IL-10 PRODUCTION BY TR1 CELLS IN MS PATIENTS

In patients with Inflammatory Bowel disease (IBD, a group of immune-mediated disorders characterized by chronic inflammation of the gastrointestinal tract), Tr1 cells down-regulate IL-10 production in response to pro-inflammatory cytokines like IL-23 (Alfen et al., 2018). This cytokine plays a key role not only in IBDs but also in EAE, the principal mouse model of MS. For this reason, we decided to investigate IL-10 production by Tr1 in our cohort of MS patients.

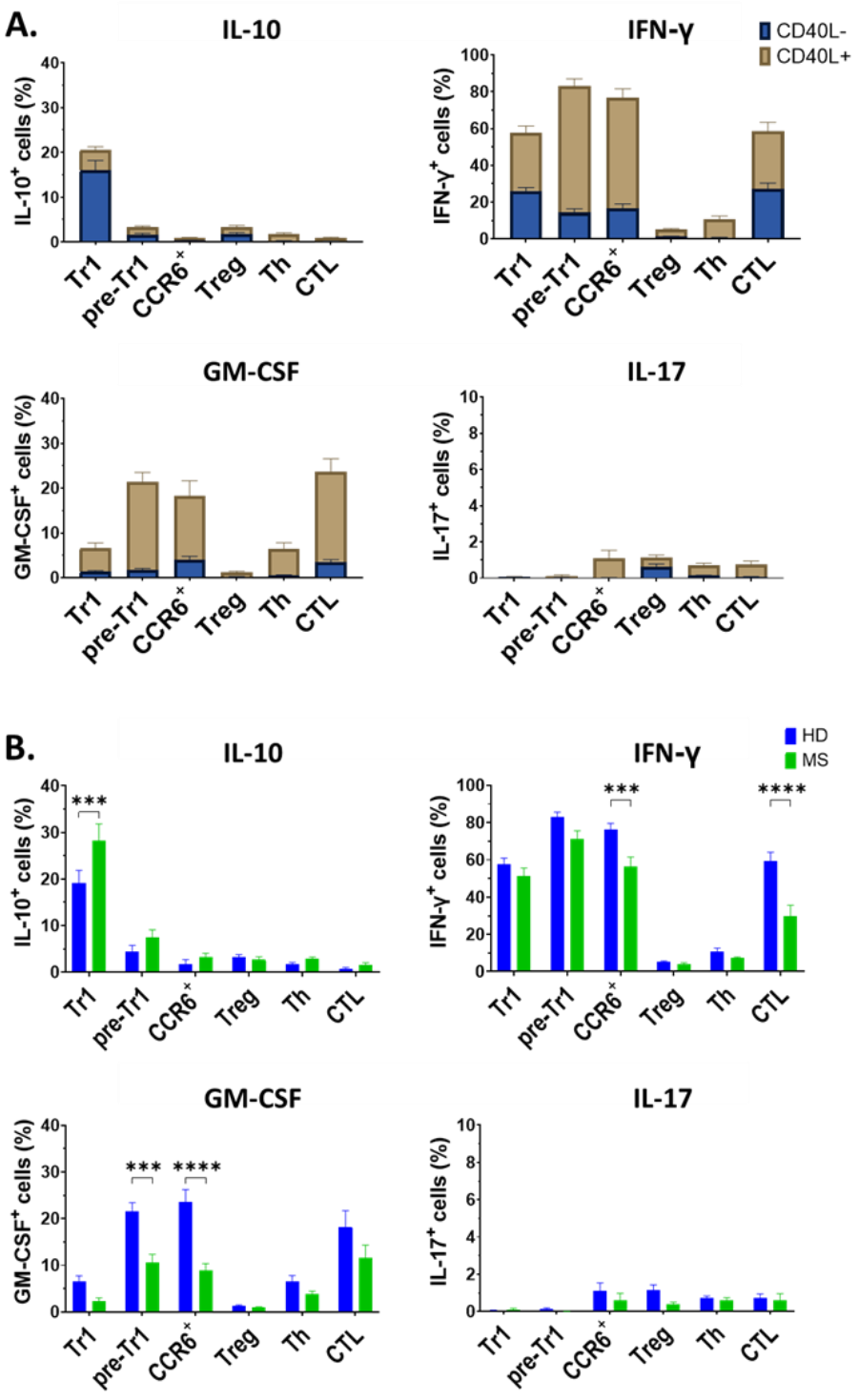
First, we confirmed and extended previous findings in healthy individuals that Tr1 cells produce high levels of IL-10 and IFN-γ, and express low levels of CD40L and GM-CSF (Alfen et al., 2018; Gruarin et al., 2019) (Figure 4.4A), a key pro-inflammatory T-cell effector cytokine found in the CNS of MS patients (Rasouli et al., 2015). Notably, Tr1 subset consistently showed the highest frequencies of IL-10$^+$ cells (19.07±2.79%) as compared to all other T-cell subsets, including Tregs (3.30±0.55%) (Figure 4.4B). Conversely, both IL-7R$^+$GzmK$^+$ T-cell subsets, namely CCR6$^-$pre-Tr1 and CCR6$^+$cells, had high percentages of cells expressing CD40L together with the pro-inflammatory cytokines GM-CSF and IFN-γ (Figure 4.4A). However, while the CCR6$^+$ subset was IL-10$^-$ and showed low percentages of IL-17$^+$ cells (1.08±0.45%), the CCR6$^-$ subset was IL-17$^-$, but a considerable fraction of cells was IL-10$^+$ (4.37±1.41%) (Figure 4.4B). As expected, FOXP3$^+$ Tregs were CD40L$^-$, with moderate percentages of cells positive for the cytokines IFN-γ (5.09±0.72%), GM-CSF and IL-17 (≈1%), and IL-10 (3.26±0.52%). Finally, GzmB$^+$ CTL had low frequencies of CD40L$^+$ cells, showed very low

percentages of IL-10$^+$ and IL-17$^+$ cells (<1%), but high fraction of IFN-γ (59.57±4.53%) and GM-CSF$^+$ cells (18.23±3.49%) (Figure 4.4A and B).

Surprisingly, in natalizumab-treated MS patients we observed a general decrease of pro-inflammatory cytokines. In particular, CCR6$^+$ T-cell showed a significant frequency reduction of both IFN-γ and GM-CSF positive cells. The percentages of IFN-γ$^+$ cells were reduced significantly in CTL, while in pre-Tr1 cells the GM-CSF$^+$ cells. Conversely, the frequencies of IL-10$^+$ cells were generally increased, especially in Tr1 cells, where the increase reaches the statistical significance (Figure 4.4B). Unfortunately, CSF of untreated MS patients is a very rare sample so we need to further improve these findings with a larger cohort of patients. However, we have obtained the proof of principle that, after an attack, in the blood of an untreated MS patient, Tr1 cells had the highest frequencies of IL-10$^+$ cells (9.06%) when compared to other CD4$^+$ subsets, and that this fraction of Tr1 IL-10$^+$ cells is even higher in the CFS (33.3%) (figure 4.5B).

Figure 4.4 - Cytokine profiles of CD4$^+$T-cell subsets upon polyclonal stimulation.

A. The percentage of distinct cytokines combined or not with CD40L up-regulation in healthy donor. **B.** Total cytokine levels. The percentages of cytokines produced by the indicated CD4$^+$T-cell subsets in peripheral blood of healthy donors (HD, *n=15*), and natalizumab-treated relapsing-remitting MS patients (+NTZ, *n=12*) are shown. PBMCs were stimulated with polyclonal PMA/Ionomycin stimuli. Statistical significance was calculated by ANOVA (P-value: *≤0.0332, **≤0.0021, ***≤0.0002, ****≤0.0001). Graphs show means ±SEM.


A.
IL-10
IFN-γ
CD40L-
CD40L+
GM-CSF
IL-17
IL-10+ cells (%)
IFN-γ+ cells (%)
GM-CSF+ cells (%)
IL-17+ cells (%)
Tr1
pre-Tr1
CCR6+
Treg
Th
CTL
B.
HD
MS
***
****

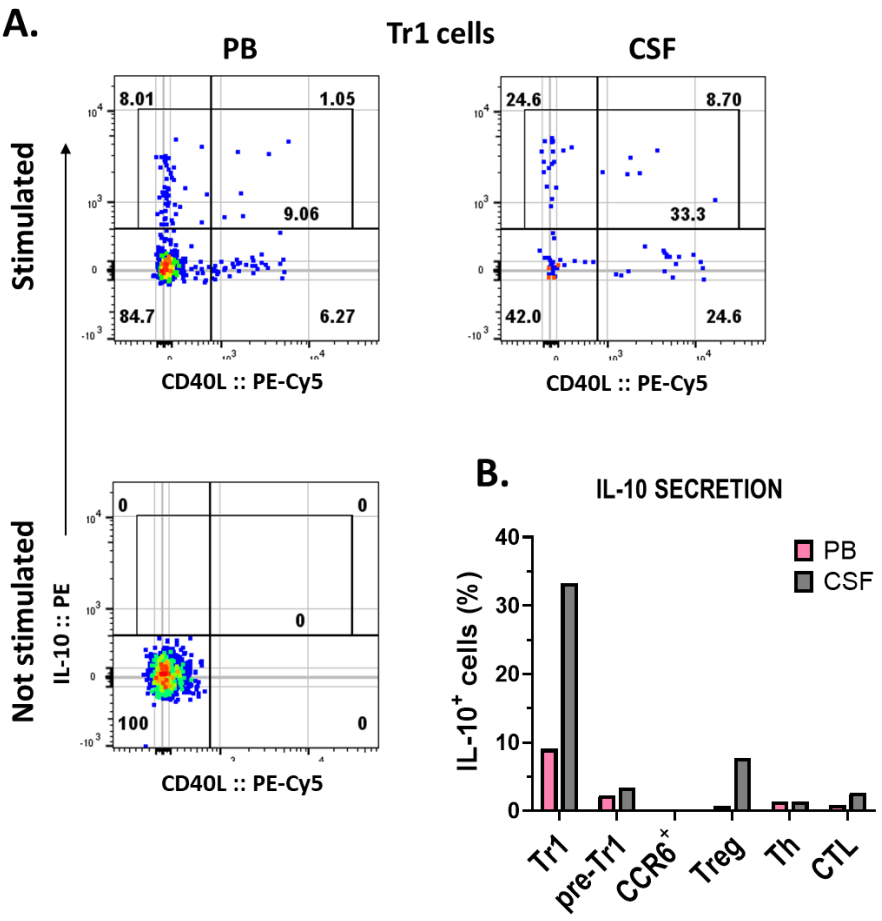


Figure 4.5 - Frequencies of IL-10$^+$ cells in one untreated MS patient.

A. Representative stainings show the production of IL-10 combined or not with CD40L in Tr1 cells from peripheral blood (PB) and cerebrospinal fluid (CSF) of one untreated MS patient after an attack. Mononuclear cells were stimulated or not with polyclonal PMA/Ionomycin stimuli. **B.** Graphs show the percentages of secreted IL-10 among the indicated CD4$^+$T-cell subsets.

4.2.5. SPECIFIC RESPONSE OF TR1 CELLS TO EBNA1, A LATENT EBV-DERIVED ANTIGEN

The results obtained so far suggest that Tr1 cells, once activated, may have a beneficial anti-inflammatory role in MS, since they are selectively and strongly enriched in the CSF after an attack and produce high levels of the anti-inflammatory cytokine IL-10. However, the effector functions of Tr1 cells, such as IL-10 production, require antigen-specific stimulation (Haringer, Lozza, Steckel, & Geginat, 2009b; Roncarolo, Gregori, Bacchetta, Battaglia, & Gagliani, 2018), and the antigenic specificities of Tr1 cells in MS are still unknown. We therefore decided to analyse the antigen-specificities of Tr1 cells in MS patients. This represented the major technical challenge because Tr1 cells are rare and anergic. Furthermore, only a limited amount of blood (10-12 ml) was available from MS patients which is not enough to perform a proliferation assay with FACS-purified Tr1 cells, as it was performed with Tr1 cells obtained from buffy-coated blood (300-500 ml) (Haringer et al., 2009b).

For this reason, I have established a new assay based on multi-dimensional flow cytometry to detect *ex vivo* the antigen specificity of very rare T-cell subsets including anergic/effector cells like Tregs, CTL and Tr1 cells using only 1 milliliter of blood (i.e. containing approximately 1 x 10^6 PBMCs) (Figure 4.6 in material and methods). This assay exploits an early time window where most of the markers that are used to track $CD4^+$T-cell subsets (i.e. CD4, FOXP3, GzmB/K, IL-7R, CCR6) are still stably expressed, while activation-induced markers (up-regulation of CD69, CD40L and various cytokines) are already

efficiently expressed (Figure 4.6). With this approach, I was able to find statistical significant responses to antigenic peptide pools when compared to negative control. The sensitivity of the assay can be appreciated in Figure 4.7 where 1 responding $CD4^+$T-cell in 10.000 is detected (indicated by * on the top of the column of CTL/JCV/HD and $GzmK^+$-Tr1-preTr1/EBV-EBNA1/HD). This amazing sensitivity is comparable to an ELISPOT assay, which however does not provide any information about phenotype of the cytokine-producing cells.

We considered antigen-specific activated Tr1 cells, the cells positive for the activation marker CD69 and the cytokine IFN-γ after incubation with commercially available overlapping peptide pools containing all possible epitopes from potentially antigenic proteins. The presence of this antigen-specific cells were monitored in 21 healthy donors and 26 RRMS patients (13 untreated and 13 natalizumab-treated). Based on our previous studies on relevant antigen specificities of $CD4^+$T helper cells in MS patients (Paroni et al., 2017), I monitored responses to myelin-derived self-antigens (MOG/MBP, Figure 4.7A, first row), and to viral-antigens derived from JCV (VP1/VP2, Figure 4.7A, second row) and EBV (pool of unknown immune-dominant peptides, Figure 4.7A, third row). Stimulation without peptides or with the super-antigen SEB (Staphylococcal enterotoxin B) were considered as negative and positive controls, respectively (Figure 4.6). Besides $GzmK^+$ Tr1-enriched cells, $GzmK^-/B^-$ helper T-cells and $GzmB^+$CTL were also analysed (Figure 4.7A, from left to right). Since the frequencies of $GzmK^+$ and $GzmB^+$ cells are low and variable (see Figure 4.2A), the

percentages of antigen-specific cells are in all cases referred to total $CD4^{+}$T-cells.

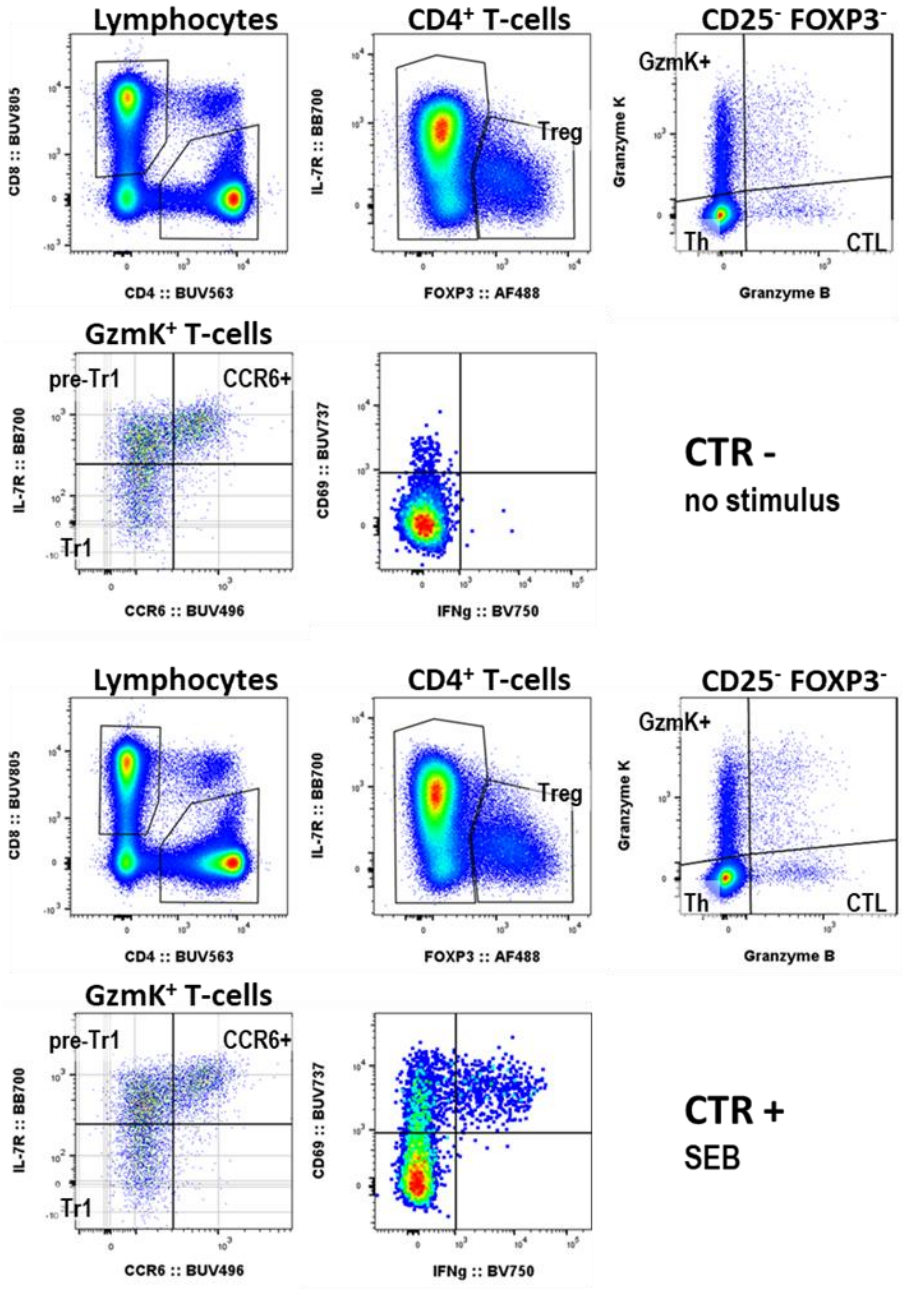


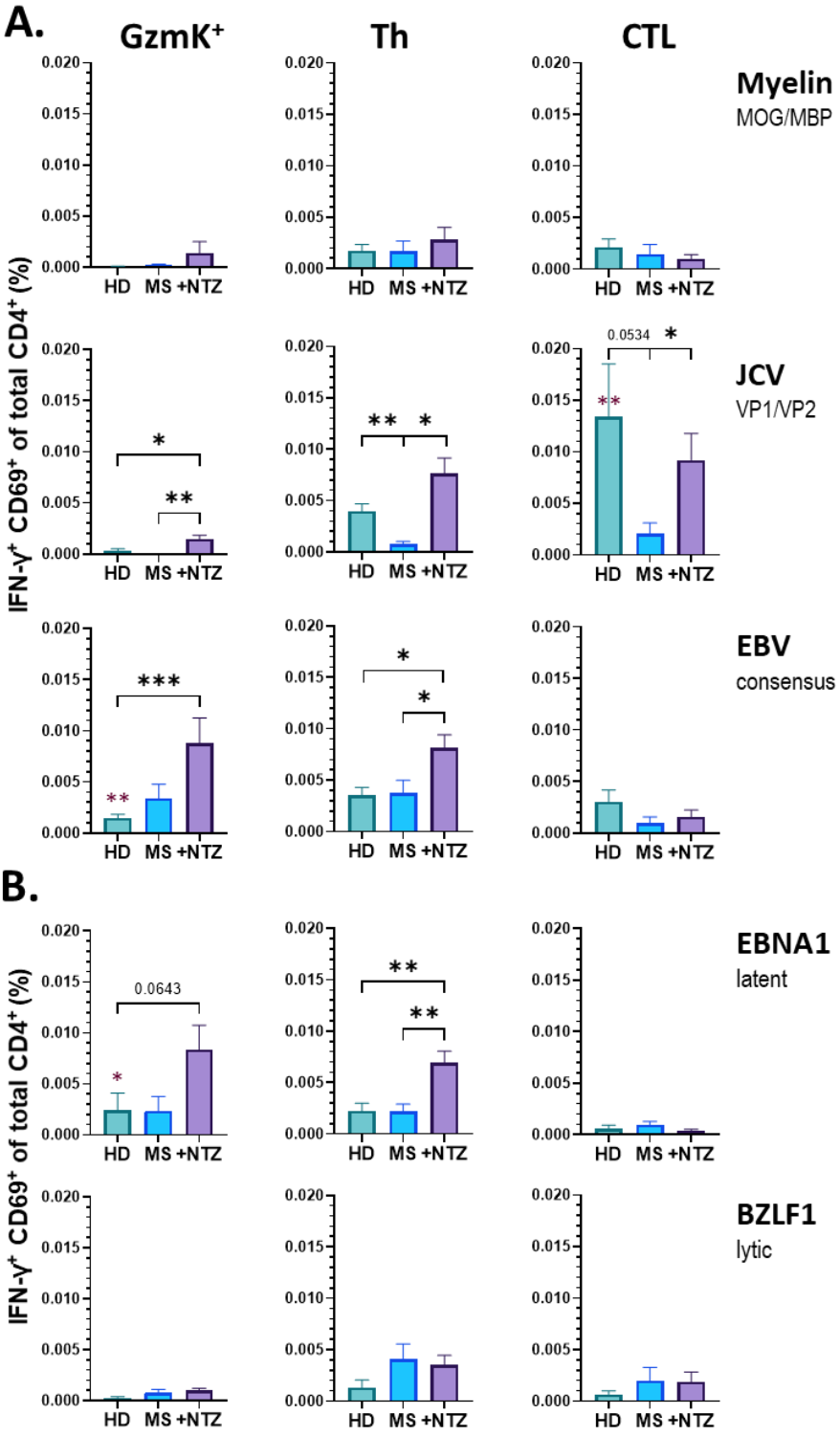
Figure 4.6 - Gating strategy used for antigen specificity assay.
Representative staining strategy to identify responses of several cell subsets among $CD4^+$lymphocytes by secretion of IFN-γ plus CD69 up-regulation. Treg ($FOXP3^+$), T helper (Th, $FOXP3^-GzmK^-GzmB^-$) and Cytotoxic T-Lymphocyte (CTL, $FOXP3^-GzmB^+$) cells, $GzmK^+$ cells are further subdivided in IL-$7R^-CCR6^-$Tr1 cells, IL-$7R^+CCR6^-$ "preTr1" cells and $IL7R^+CCR6^+$ cells. Dot plots shw that the expression of different lineage markers is not altered after 5h of incubation in presence (CTR+, SEB, Staphylococcal enterotoxin B) or absence (CTR-) of the stimulus.

$GzmK^+$ Tr1-enriched cells (Figure 4.7A, first column) in MS patients responded only to EBV, but not to myelin or JCV derived antigens. In contrast, EBV-specific $GzmK^+$ cells in healthy individuals were hardly detectable and had a lower response to EBV peptides. In addition, IFN-γ-producing T helper cells (Th1) are also specific for EBV but were present at similar frequencies in both RRMS patients and in healthy controls (Figure 4.7A, second column, third row). In contrast, $CD4^+$CTL responded poorly to EBV (Figure 4.7A, third column, third row). Notably, the response to JCV (Figure 4.7A, second row) had a completely different pattern characterized by a mixed CTL/Th1 response in healthy individuals and also in MS patients albeit to a lesser extent. Furthermore, even a low response to myelin-derived autoantigens could be detected in CTL and T helper cells (Figure 4.7A, first row) and in particular in $CCR6^+$ ($Eomes^-$) Th cells (Paroni et al., 2017). Finally, natalizumab-treated MS patients showed higher responses to EBV and also to JCV then untreated patients (Figure 4.7A,

first column), suggesting that, not myelin-, but viral-specific Tr1 cells are present and/or migrate efficiently to the CNS.

To understand if GzmK$^+$ T-cells were specific for EBV-derived proteins that are restricted to the lytic phase of the virus, or to proteins that are also expressed in the various latent stages, we next analysed the responses to EBNA1, the only protein expressed in all latent stages, and to proteins that are expressed exclusively in the lytic phase, such as BLZF1 and BMRF1 (Figure 4.7B and C). We found that GzmK$^+$ cells and also Th1 cells responded to EBNA1 in HD and MS patients, and that this latent-phase response is strongly higher in natalizumab-treated MS patients (Figure 4.7B, first row). However, the response to the lytic antigen BZLF1 is present in both Th1 cells and GzmB$^+$ CTL in MS patients (Figure 4.7B, second row).

When Tr1-containing GzmK$^+$cells were analysed more in detail according to CCR6 and IL-7R expression (Figure 4.7C), both IL-7R$^+$ pre-Tr1 cells and IL-7R$^-$ Tr1 cells were found to respond strongly to EBV, while CCR6$^+$ pro-inflammatory cell did not show a significant response (Figure 4.7C, first row, from left to right). Moreover, Tr1/pre-Tr1 cells responded strongly to EBNA1, but poorly to two lytic antigens BZLF1 and BMRF1 (Figure 4.7C, first and second column). Consistently, natalizumab-treated MS patients had higher responses. In addition, EBNA1-speficic CTL and BZLF1-specific pre-Tr1 cells were found only in MS patients treated with natalizumab. Lastly, we were also able to detect significant frequencies of IL-10$^+$ Tr1 cells among PBMC stimulated with the EBV pool or EBNA1, while also Tregs were modestly positive for IL-10 (Figure 4.8).


A.
GzmK+
Th
CTL
Myelin
MOG/MBP
JCV
VP1/VP2
EBV
consensus
IFN-γ+ CD69+ of total CD4+ (%)
HD MS +NTZ
0.020
0.015
0.010
0.005
0.000
*
**
***
0.0534
B.
EBNA1
latent
BZLF1
lytic
0.0643

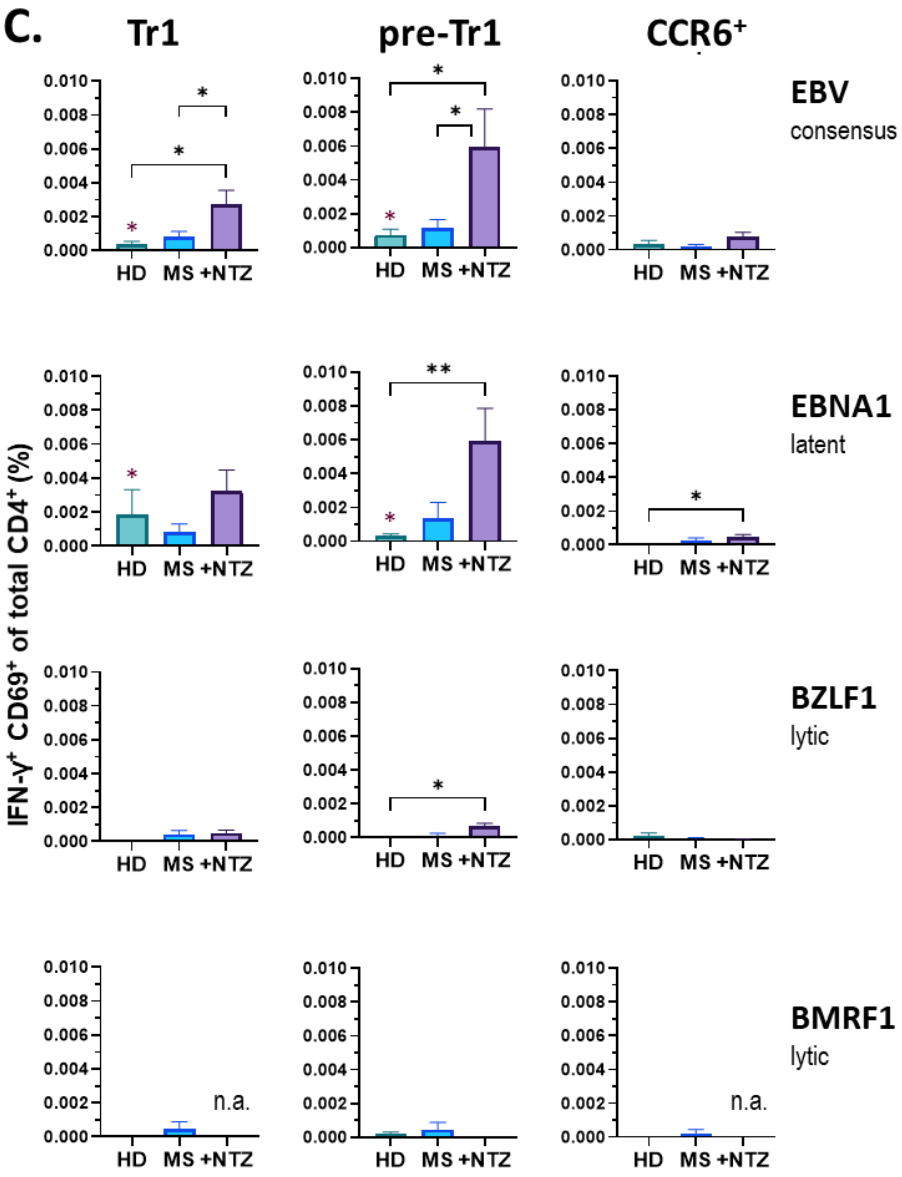

C.
Tr1
pre-Tr1
CCR6+
EBV
consensus
EBNA1
latent
BZLF1
lytic
BMRF1
lytic
IFN-γ+ CD69+ of total CD4+ (%)
HD
MS
+NTZ
n.a.

Figure 4.7 - Activation of $CD4^+$ T-cell subsets by MS-relevant antigens.
Percentages of total $CD4^+$ cells that were positive for CD69 and IFN-γ after brief stimulation with peptide pool, among gated $CD4^+GzmK^+$ cells, CTL ($GzmB^+$) and helper T-cells (Th, $GzmK^-/B^-$). PBMCs from peripheral blood of healthy donors (HD, *n=6 to 21*), relapsing-remitting (RR-) MS patients either untreated (MS, *n=13*) or natalizumab-treated (+NTZ, *n=13*). Specific responses are shown: **A.** to myelin-derived (MOG/MBP; first row), JCV-derived (VP1/VP2; second row) or EBV-derived (pool, third row) peptides and **B-C.** to EBV peptides derived from the latent EBNA1 antigen and the lytic antigens, BZLF1 and BMRF1. **C.** Distribution of EBV, EBNA1, BZLF1 and BMRF1 specific cells in $CD4^+GzmK^+$ T-cell subsets. Percentages of total $CD4^+$responding cells among Tr1 effector ($IL\text{-}7R^-$ $CCR6^-$), pre-Tr1 ($IL\text{-}7R^+$ $CCR6^-$) and $CCR6^+$ pro-inflammatory cells ($IL7R^+$). NTZ-treated MS patients are lacking for BMRF1 (not applicable, n.a.). Statistical significance was calculated by t-test (P-value: *≤0.0332, **≤0.0021, ***≤0.0002, ****≤0.0001). Significant differences of peptide response compared to unstimulated control in HD were indicated by a * on the top of a column, while significant differences in response to the same peptide in MS patients as compared to HD were indicated by * on top of a line. Graphs show means ±SEM.

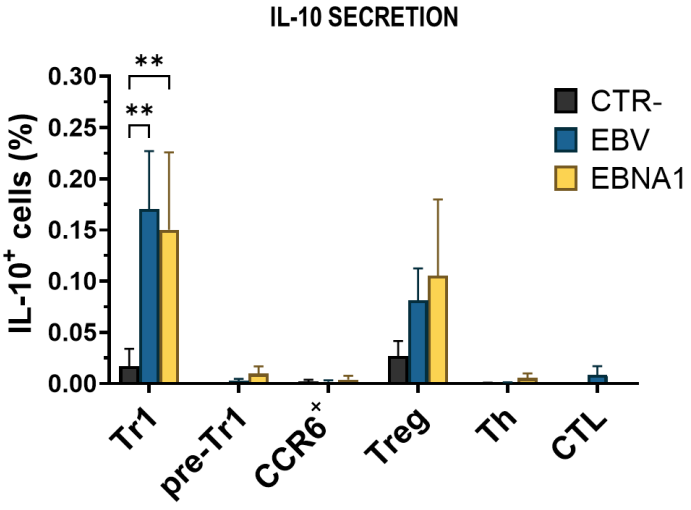


Figure 4.8 - Frequencies of IL-10$^+$ cells among EBVspecific T-cell subsets in MS patients.

Same experimental condition as in Figure 4.7, but IL-10 was detected using a different protocol, as for polyclonal stimulation of PBMCs. Graphs show the percentages of IL-10$^+$ cells among the indicated CD4$^+$T-cell subsets in MS patients treated with natalizumab (*n=10*). Statistical significance was calculated by t-test (P-value: *≤0.0332, **≤0.0021, ***≤0.0002, ****≤0.0001).

4.2.6. DISCUSSION

The purpose of this study was to characterize the phenotype and function of regulatory cell subsets with particular attention to Tr1 cells, in both untreated and natalizumab-treated MS patients in comparisons with healthy controls, with the aim of identifying a possible role of regulatory T-cell subset in MS and their use for cell-based therapies.

Phenotypic analysis of PBMCs obtained from healthy donors, according to Gruarin et al., confirmed that the expression of the cytotoxic molecule granzyme K, which is directly controlled by the transcription factor Eomes, specifically characterizes a Tr1-enriched subpopulation of $CD4^+FOXP3^-Eomes^+$ lymphocytes, and allows to distinguish it from $GzmB^+$CTL (Gruarin et al., 2019). Consistently, about 50% of the $GzmK^+$ Tr1-enriched population expresses the transcription factor Eomes, while is expressed in a smaller fraction of $GzmB^+$ CTL cells and is totally absent in T helper and $FOXP3^+$ lymphocytes. The $GzmK^+$ population can in turn be subdivided to identify a subset of $CCR6^+$ T-cells with pro-inflammatory properties (Mazzoni et al., 2019) and Tr1 cells ($CCR6^-$IL-$7R^-$) together with its putative "precursors" ($CCR6^-$IL-$7R^+$pre-Tr1) that show lower levels of Eomes expression. Furthermore, the characterization of the cytokine profile obtained by flow-cytometry analysis confirmed the anti-inflammatory properties of Tr1 cells that produce high level of the immunosuppressive cytokine IL-10 in the absence of CD40L upregulation (counterpart of the immune-stimulating molecule CD40 present on APCs) (Gruarin et al.,

2019). Moreover, also the CCR6- IL-7R$^+$ subset produce some IL-10, while the CCR6$^+$ IL-7R$^+$ subset was IL-10-negative and released some IL-17. These findings are consistent with the notions that the CCR6$^+$ subset has a pro-inflammatory cytokine profile (Mazzoni et al., 2019), while the CCR6- subset may represent precursors of Tr1 effector cells (pre-Tr1) (Gruarin et al., 2019).

We simultaneously monitored the frequencies and the *in vivo* turnover rate of Tr1 cells and FOXP3$^+$ Treg in healthy donors (HD) and in relapsing-remitting (RR) MS patients that were either untreated or treated with Natalizumab. We found that Tr1 cells, but not FOXP3$^+$ Treg cells or GzmB$^+$ CTL, were significantly reduced in peripheral blood of untreated RRMS patients compared to HD, and they also showed a higher *in vivo* proliferation rate. Strictly, the treatment with the anti-α4 antibody natalizumab restore Tr1 cell frequencies and proliferation rates, suggesting that circulating Tr1 cells in MS patients are recruited and activated in the CNS.

Conversely, RRMS patients had surprisingly higher frequencies of FOXP3$^+$ Tregs that showed a reduced *in vivo* cell turnover. These differences were however not dramatic and became statistically significant only in natalizumab-treated patients as compared to healthy individuals. This data suggested that an alteration of the homeostasis of Tregs may be present compared to healthy population, but is less relevant as compared to Tr1-cells. Notably, the reported frequencies of Tregs in RRMS patients in literature are controversial. Nevertheless, it is possible that FOXP3$^+$ Tregs may be dysfunctional in MS (Duffy, Keating, Perera, & Moalem-Taylor, 2018). Up to now, it is

not clear whether Tregs migrate into the CNS of MS patients, but results from EAE animal model show an accumulation of Tregs in CNS lesions (Duffy et al., 2018). Compartmentalization and trafficking appears to be tissue-specific as for Th effector cells, with distinct migration pattern contributing to selective retention and homing of Treg cells at sites where regulation is required (Duhen, Duhen, Lanzavecchia, Sallusto, & Campbell, 2012; Wei, Kryczek, & Zou, 2006), but until now no information has been available about the expression of adhesion molecules on Tregs in patients with MS. A report by Soilu-Hänninen *et al*. showed increased expression of the adhesion molecules CD44 and CD49d/α4-integrin in the total T-cell population during MS relapses (Soilu-Hanninen, Laaksonen, & Hanninen, 2005)**,** therefore, it would be interesting to investigate the expression not only of chemokine receptors, but also of adhesion molecules.

Moreover, our data showed that Tr1 cells were selectively and strongly enriched in CSF of active MS patients after an attack – while $FOXP3^+$ Tregs and $GzmB^+$ CTL were not – where they virtually all express high levels of CXCR3, but surprisingly only low levels of Ki67 proliferation marker (data not shown).

In 2019 Herich et al. showed similar enrichment of Tr1-like cells ($CD4^+CCR7^-CCR5^+GzmK^+$) in the blood of natalizumab-treated patients – but also in patients in remission – and proposed that these cells are responsible for immune-surveillance in brain parenchyma (CNS) during homeostasis, and could thus also play a role in MS. The authors showed that these cells use the adhesion molecules VLA-4/α4β1 for extravasation in permissive sites, and their stored GzmK to perform

trans-cellular diapedesis through the BBB (Herich et al., 2019). Since also our GzmK$^+$ T-cells express the CD49d/α4-integrin it is possible that these cells are directly recruited to the CNS from the circulation, or that they are generated in the CNS from circulating precursor cells, presumably from IL-7R$^+$ GzmK$^+$ pre-Tr1 cells, upon antigenic stimulation (Camell, 2021). In this regard, it is important to notice that in our data pre-Tr1 cell frequencies are not affected by natalizumab treatment. In addition, consistently with our published results and from other groups, the CNS-homing of GzmK$^+$ Tr1 cells could be controlled by CCR5 and CXCR3, both involved in MS and normally expressed on antiviral cells (Balashov, Rottman, Weiner, & Hancock, 1999; Paroni et al., 2017; Sorensen et al., 1999).

So far, our results obtained by cytokine profiling of polyclonal T-cell populations suggested that Tr1 cells play a key beneficial role in MS, since, in addition of their selective enrichment in CSF after an attack, they have increased anti-inflammatory properties in MS patients, different from what was observed in IBD patients (Alfen et al., 2018). In fact, Tr1-cells of MS patients produce low levels of the pathogenic cytokine GM-CSF and showed a higher production of the regulatory cytokine IL-10 as compared to healthy individuals. However, due to low number of CSF samples analysed, the results on IL-10 are still a bit preliminary. Thus, according to their beneficial anti-inflammatory role Geginat et al., 2016), IL-10 producing Tr1 cells could be suitable for cellular therapy in MS. Importantly, IL-10 is induced by antigenic stimulation, and the nature of antigens that can activate Tr1-cells are therefore critical.

Multiple sclerosis risk and pathogenesis has repeatedly been associated with viral infection by EBV (Ascherio & Munger, 2007), in addition to the presence of an autoreactive response against myelin (Geginat et al., 2017). Therefore, based on our previous studies on relevant antigen specificities of $CD4^+$T helper cells in MS patients (Paroni et al., 2017), we monitored responses to antigen derived from myelin, JCV and EBV. My novel assay to test the antigen specificity of Tr1 cells, based on multi-parametric flow cytometry, was successfully established, allowing me to identify the quality of very rare (<1:10.000) antigen-specific T-cells in only 10^6 PBMCs. Findings were very clear-cut and striking: MS is associated with an EBV/EBNA1-specific Tr1 response. Tr1- and pre-Tr1 cells in MS patients did not react with MOG/MBP or JCV, but responded strtongly to a mix of immundominant poeptides derived from various EBV proteins. They responded also strongly to EBNA1, a latent EBV antigen that is implicated in MS, but reacted poorly to two lytic EBV antigens. Strikingly, in healthy individuals EBV-specific pre-Tr1/Tr1 cells were in contrast rare. Interestingly, patients treated with natalizumab had significantly higher levels of EBV-specific Tr1 cells, suggesting that EBV-specific Tr1-/pre-Tr1 cells are recruited in the CNS and/or are generated there. Notably, we were also able to detect significant IL-10 production by Tr1 cells stimulated with the EBV pool or EBNA1. Conversely, we were unable to establish an antigen-specific suppression assay, presumably because the frequencies of antigen-specific responder and suppressor cells are too low. However, since Tr1 cells inhibit also $CD8^+$ T-cell responses (Groux, Bigler, de Vries, &

Roncarolo, 1998), the induction of EBNA1-specific Tr1 cells may represent an EBV immune evasion strategy, during the latent phase, that is apparently unique for MS. EBV infection is considered a major risk factor in MS, as is the presence of anti-EBNA1 antibodies. However, we did not observe an association between anti-EBNA1 antibodies and EBNA1-specific Tr1 or Th1 cells in untreated or natalizumab-treated patients (data not shown). Nevertheless, although EBNA1-specific Tr1 cells might play a beneficial role in attacks producing IL-10 after being activated by EBV, they could also promote EBV immune escape. The precise role of EBV in MS progression is debated, but in a scenario where EBV-infected B-cells in the CNS promote bystander recruitment of myelin-reactive Th1/17-cell (Geginat et al., 2017), or even cause immunopathology via the activation of $CD8^+$ CTL (Serafini, Rosicarelli, Veroni, Mazzola, & Aloisi, 2019), EBV-specific Tr1 cells might play also a detrimental role in the long run and promote relapses and/or disease progression. For this reason, expansion of Tr1 cells for cellular therapy of MS could be risky.

Instead, $CD4^+$CTL responded selectively to JCV both in MS patients and in healthy controls. So far, the levels of anti-JCV antibodies in the blood have been a useful marker to monitor patients following prolonged natalizumab treatment for the prevention of PML (Ho et al., 2017). The data obtained in this project add other evidences that a response to JCV such as of $CD4^+$CTL in healthy individuals contribute to immune-surveillance against this virus, but these cells accumulated in CNS of MS patients are potentially pathogenic in case of a reactivation of the virus in MS relapses and even PML (Stoner, 1991).

They could therefore be a useful biomarker for predicting the risk of PML in patients receiving natalizumab (Lanza Cariccio, Bramanti, & Mazzon, 2018). Notably, $CD4^+$CTL were proposed to be involved in progressive MS (Raveney et al., 2021). Moreover, IFN-γ producing T helper cells (Th1) respond both to EBV and JCV antigens in healthy individuals and accumulate in the CNS of MS patients, suggesting their involvement not only in antiviral immune-surveillance but also in MS respectively. In addition, myelin-specific auto-reactive T-cells were mainly $CCR6^+$ Th cells (data not shown), consistent with our previous published data (Geginat et al., 2017; Paroni et al., 2017). Finally, $FOXP3^+$ Tregs did not respond significantly to any of the tested antigens, but probably because IFN-γ and CD69 are not suitable activations markers to measure their response. However, we have detected some IL-10 production in response to EBV/EBNA1, suggesting a partially redundant role.

Overall, our results suggest a key role of Tr1-like cells in MS and are consistent with the notion that there is a dysregulated immune response against EBV in the CNS of active MS patients.

4.3. MATERIALS AND METHODS

4.3.1. PATIENTS AND SAMPLE COLLECTION

Patients samples were collected at the MS Center of the Fondazione Ca' Granda, IRCCS Ospedale Policlinico, upon written informed consent, after Institutional Ethical Committee approval. Patients were diagnosed according to the criteria of McDonald *et al*. and classified as clinically isolated syndrome (CIS), relapsing remitting MS (RRMS), secondary progressive MS (SPMS). In this study, we collected peripheral blood samples, by venous puncture, from 16 treatment-naïve RRMS patients affected by relapsing-remitting multiple sclerosis and 24 natalizumab-treated RRMS patients. However, untreated patients were in active phase, because those in remission were not available. Peripheral blood was collected before the therapy session. Three to seven milliliter of cerebrospinal fluid (CSF) samples were obtained by lumbar puncture from 6 to 8 untreated patients. All patients from whom CSF was analysed, had a RR disease and were naïve from any previous treatment. Demographic characteristics of MS patients and HD enrolled in the study are shown in Table 4.1. As controls, 35 age- and sex-matched healthy donors were enrolled/obtained at Policlinico (Milan, IT), upon informed consent.

	RR-MS	+NTZ	HD
count	16	24	35
male	7	10	15
female	9	14	20
age (median)	36.7	33.1	33.7
age (mean)	35.9	34.7	37.4
age (SD)	8.9	9.2	10.7

Table 4.1 - Demographic table

SD, standard deviation

4.3.2. SAMPLE PROCESSING

Ten to twelve milliliters of whole blood from each HD or patient were collected in BD Vacutainer Tubes cointaining Lithium Heparin as anticoagulant (cat, 454049, BD Biosciences, San Jose, CA). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood by Ficoll-Hypaque gradient according to manufacturer's protocol within 24 hours from blood withdrawal. While cells from CSF were separated by centrifugation.

Isolated PBMCs were freshly used for *ex vivo* phenotyping (Mix 1 table 4.3) or stimulated for functional assay, then stained for final flow cytometric analysis. Plasma was collected and decomplemented or stored at -80°C until usage.

4.3.3. POLYCLONAL STIMULATION FOR CYTOKINE DETECTION

Isolated cells were incubated for 4h at 37°C in a 5% CO2 humidified atmosphere at a density of 2,5 x 10^6 cell/mL in RPMI complete medium

(2 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 1% penicillin/streptomycin) supplemented with 10% FCS in presence or absence of PMA (50 ng/mL) and Ionomycin (500 ng/mL) (5×10^5 PBMC/well into a 96-well U bottom plate, Corning, cat. 3788). After 2h, Monensin (0.6 µl/ml, BD GolgiStop Protein Transport Inhibitor, cat. 554724) was added in order to block the secretion pathway and retain cytokines intracellularly. At the end, stimulated cells were stained for superficial and intracellular markers listed in table 4.5 (Mix 3). All details can be read in EJI, Flow cytometry guidelines, Tr1 chapter (Cossarizza et al., 2021).

4.3.4. ANTIGEN SPECIFICITY ASSAY

Isolated cells were stimulated with synthetic peptide pools of interest, i.e. derived from autologous- and/or viral- antigens (refer to Table 1.2), or with the super-antigen SEB as positive control. Incubation with a tumor-associated peptide or without peptides was also always analysed as negative control.

Brand	Origin	Protein	Protein sequence		cat. N.
Miltenyi	**Human**	Melan-A/MART-1	UniProt ID: Q16655	melanoma/vitiligo	130-094-597
PepTivator	**Human**	MOG	UniProt ID: Q16653	Myelin	130-096-770
	Human	MBP Isoform 1	UniProt ID: P02686-1	Myelin	130-096-763
	JCV	VP1	UniProt ID: P03089	late lytic, major capsid prot.	130-096-502
	JCV	VP2	UniProt ID: P03095-1	late lytic, minor capsid prot.	130-096-764
	EBV	EBV	consensus*		130-099-764
	EBV	BZLF1	UniProt ID: P03206	early lytic	130-093-611
	EBV	EBNA-1	UniProt ID: P03211	latent, nuclear protein	130-093-613
JPT					
PepMix	**EBV**	BMRF1	UniProt ID: P03191	early lytic	PM-EBV-BMRF1

Table 4.2 - List of peptides.

Pool of 15-mer peptides with 11-amino acid (aa) overlap, covering the complete sequence of the protein. 15 nmol (approx. 25 µg) per peptide. All peptide pools were dissolved according to manufacturer's protocol, aliquoted and kept frozen at -80 °C until further use. Final concentration in cell culture: 0.6 nmol (approximately 1 µg) of each peptide/mL. *consensus: is a pool of 43 lyophilized MHC class I and class II restricted peptides, derived from 13 different proteins; the peptides are restricted for the following HLA-molecules: HLA-A*02, -A*03, -A*11, -A*24, -A*26, -B*07, -B*08, -B*15, -B*18, -B*27, -B*35, -B*40, -B*4402, -DR.

Total PBMCs were incubated for 5h at 37°C in a 5% CO^2 humidified atmosphere at a density of $5x10^6$ cell/mL in RPMI complete medium (2 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 1% penicillin/streptomycin) supplemented with 5% autologous decomplemented plasma (1×10^6 PBMC/well into a 96-well V bottom plate, Nunc, cat. 249952) (Figure 4.9). Secretion was blocked with Monensin (0,6 µl/ml, BD GolgiStop Protein Transport Inhibitor, cat. 554724) after 90 min of incubation. Finally, the cells were stained for superficial and intracellular markers listed in Table 4.4 (Mix 2).

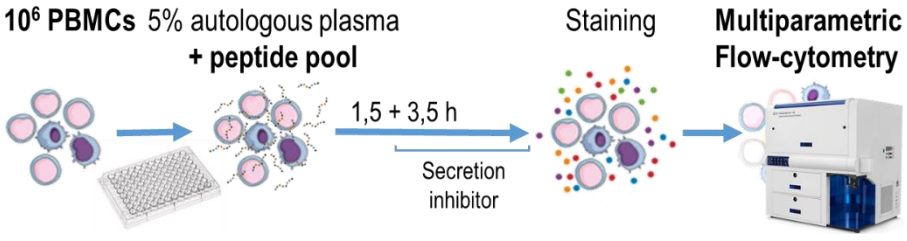


Figure 4.9 - Schematic representation of the antigen specificity assay.

4.3.5. FLOW CYTOMETRY

Lastly, cells are stained superficially and intracellularly with fluorochrome-conjugated monoclonal antibodies and live/dead exclusion dye. All details can be read in EJI, Flow cytometry guidelines, Tr1 chapter (Cossarizza et al., 2021). This optimized protocol for polychromatic flow cytometry allows the analysis of multiple cytokines, cell surface markers and functional markers such as Granzymes, Ki67 (a proliferation markers expressed in T-cells that have divided 2-5 days before *in vivo*) and chemokines receptors, up to 22 fluorochromes. All antibodies were previously titrated to determine optimal dilutions. The different panels of antibodies (Ab), used to stain specific markers in the present study are represented in following tables 3, 4 and 5 (surface markers highlighted in grey).

Samples were acquired using a BD FACSymphony flow cytometer with a 5 laser, 30 channels configuration (BD Biosciences, San Jose, CA, USA), using the BD FACSDiva Software.

4.3.5.1. SURFACE STAINING

For surface staining, total PBMCs were incubated for 20 min at 37°C protected from light, with the appropriate antibodies cocktail\mix including the live/dead (L/D) dye previously diluted in Brillant Staining Buffer (BSB, BD bioscience, cat. 563794). After incubation, unbound antibodies were removed by washing the cells with PBS supplemented with 1% FBS, and samples were fixed with the eBioscience Fixation/Permeabilization working solution (FOXP3/Transcription

Factor Concentrate and Diluent solutions, cat. 00-5521) or 2% PFA for later acquisition, in the case of *ex vivo*/antigen specificity or cytokine profiling respectively. After a 20 min incubation at RT, samples were further washed and centrifuged. Cells were resuspended in PBS + 1% FBS and stored at +4°C to continue successively with the intracellular staining protocol.

4.3.5.2. INTRACELLULAR STAININGS OF CYTOPLASMATIC MOLECULES AND TRANSCRIPTION FACTORS.

To stain for intracellular molecules, the PFA-fixed cells were permeabilized for 20 min at RT with the eBioscience Permeabilization Buffer 2x (cat. 00-8333) before adding the antibodies, thus allowing them to pass through the plasma membrane into the cell interior.

Fixed and permeabilized cells were washed and resuspended with the antibody cocktail previously diluted in permeabilization buffer: the eBioscience Permeabilization Buffer 2x was used for mix 3, while the BD TF Perm/Wash buffer 1x (cat. 51-9008102) was used for mixes 1 and 2. Then cells were incubated at 4°C, protected from light, for 120 or 50 minutes respectively. Finally, samples were washed and resuspended in PBS for data acquisition.

4.3.6. DATA AND STATISTICAL ANALYSIS

The data were analysed with FlowJo software (Tree Star Inc), while the statistical analyses were performed with Prism9 software (GraphPad). Student t-test and two-way ANOVA were performed throughout the study.

Table 4.3 - Mix 1 *ex vivo* phenotype

Marker	Fluorochrome	Clone	Brand	cat. N°	Dilution
IL-7R	BB700	HIL-Ti7R-M21	BD	566398	30
CXCR3	PE-Cy7	1C6/CXCR3	BD	560831	60
CCR6	BUV496	11A9	BD	612948	30
CD4	BUV563	SK3	BD	612912	180
CD8	BUV805	SK1	BD	564912	200
L/D dye	*FVS780*	/	*BD*	*565388*	*200*
CD45RA	BV605	HI100	BD	562886	140
CD69	BV786	FN50	BD	563834	100
/	/	/	/	/	/
CD3	BUV737	UCHT1	BD	612750	140
CCR7	PE-CF594	150503	BD	562381	60
CD161	PE-Cy5	DX12	BD	551138	40
CD25	APC-R700	2A3	BD	565106	40
CD27	BV480	L128	BD	566139	120
PD-1	BV650	EH12.1	BD	564104	30
CCR5	BUV395	2D7/CCR5	BD	565224	30
FOXP3	Alexa Fluor488	259D	BioLegend	320212	50
GzmK	PE	GM6C3	SantaCruz	SC56125	100
Eomes	eFluor 660	WD1928	Invitrogen	50-4877-42	25
GzmB	BV421	GB11	BD	563389	80
Ki-67	BV711	B56	BD	563755	80
IFN-γ	BV750	B27	BD	566357	140

Table 4.5 - Mix 2 antigen specificity

Marker	Fluorochrome	Clone	Brand	cat. N°	Dilution
IL-7R	BB700	HIL-Ti7R-M21	BD	566398	30
CXCR3	PE-Cy7	1C6/CXCR3	BD	560831	60
CCR6	BUV496	11A9	BD	564659	30
CD4	BUV563	SK3	BD	566000	180
CD8	BUV805	SK1	BD	564912	200
L/D dye	*FVS780*	/	*BD*	*565388*	*200*
CD107a	BV605	H4A3	BioLegend	328634	40
CD49d	BV786	9F10	BD	744588	120
CD29	BUV661	38047	BD	750084	60
CD69	BUV737	FN50	BD	612817	40
T-bet	PE-CF594	O4-46	BD	562467	40
CD40L	PE-Cy5	24-31	BioLegend	310808	50
IL-17A	APC-R700	N49-653	BD	565163	30
IL-2	BV510	5344.111	BD	563265	40
41-BB	BV650	H4A3	BioLegend	328637	40
TNFα	BUV395	MAb11	BD	563996	160
FOXP3	AlexaFluor488	259D	BD	320212	50
GzmK	PE	GM6C3	SantaCruz	SC56125	100
Eomes	eFluor 660	WD1928	Invitrogen	50-4877-42	25
GzmB	BV421	GB11	BD	563389	80
Ki-67	BV711	B56	BD	563755	80
IFN-γ	BV750	B27	BD	566357	140

Table 4.4 - Mix 3 polyclonal stimulation

Marker	Fluorochrome	Clone	Brand	cat. N°	Dilution
IL-7R	BB700	HIL-Ti7R-M21	BD	566398	30
CCR6	BUV496	11A9	BD	564659	30
L/D dye	*FVS780*	/	*BD*	*565388*	*200*
CD8	BUV805	SK1	BD	564912	200
CD4	BUV563	SK3	BD	566000	180
FOXP3	AF488	259D	BD	320212	50
GzmB	BV421	GB11	BD	563389	80
IFN-γ	BV750	B27	BD	566357	140
IL-17A	APC-R700	N49-653	BD	565163	30
Eomes	PE-Cy7	WD1928	Invitrogen	25-4877-42	30
GzmK	AlexaFluor647	G3H69	BD	566655	40
CD40L	BV605	24-31	BioLegend	310825	40
IL-10	PE	JES3.9D7	BioLegend	501404	30
GM-CSF	PECF594	BVD2-21C11	BioLegend	562857	30

Chapter 5. **Summary, Conclusion and Future Perspectives**

Our understanding of the role of the immune system in MS is evolving, unveiling the complex and increasingly realistic scenario involving novel pathogenic and regulatory processes, which includes Th1\Th17 pathogenic T-cells and regulatory subsets such as $FOXP3^+$ Treg and Tr1 cells (Dendrou, Fugger, & Friese, 2015; Filippi et al., 2018). Therefore, the identification of pathogenic mechanisms will certainly guide the development of more efficacious and specific therapies for MS. In fact, there is currently no cure for MS, but many drugs have been approved for the treatment of RRMS, which can greatly reduce disease progression and relapse rate (Hauser & Cree, 2020). However, although the root cause of MS is not yet defined, it has been proposed that the typical attacks of the relapsing-remitting course reflect the expansion of CNS antigen-specific effector T-cells, while remissions are the result of the control of the initial pathogenic response mediated by regulatory T-cells specific for the same antigen (Steinman, 2014). In fact, Treg cells expressing both IL-10 and FOXP3 are increased at the remission stage of MS (Frisullo et al., 2009; Lowther & Hafler, 2012).

Our results also confirm an increase in IL-10 producing Tr1 cells in RRMS patients, particularly in their CNS after an attack, and furthermore identify $Eomes^+$ Tr1-like cells as new key players in multiple sclerosis, which appear however to act as a double-edged sword. On the one hand they are efficiently recruited to the CNS and could produce very high levels of the anti-inflammatory cytokine IL-10,

suggesting a beneficial role in relapses. On the other hand, the selective response to EBV during its latency suggests that they are however activated by the “wrong” antigen and are likely to contribute substantially to the inefficient EBV control in MS, which in turn may promote disease progression.

It has recently been shown that Tr1 cells may also have another function besides the immune-regulation mediated by IL-10: Roessner et al. showed Eomes-mediated cytotoxic functions of IL-10 producing Tr1 cells in tumor control (Roessner et al., 2021). Instead, a study conducted in the mouse model of EAE showed that chronic neuro-inflammation depends on $CD4^+$ $Eomes^+$ cells, similarly to those that are increased in the peripheral blood and CSF of SPMS patients. These cells could include both Tr1 cells and CTLs. Therefore, the Tr1 cell functions and mechanisms involved in MS remain still to be unveiled, not only in MS patients with relapsing-remitting course but also in progressive forms.

To better investigate these mechanisms, I will analyze untreated progressive patients in the future. I already have analyzed an independent cohort of progressive MS patients treated with Ocrelizumab, a B-cell depleting antibody. Although these patients are of considerable interest for our study (EBV infects mainly B-cells), we were unfortunately unable to recruit a sufficient number of untreated, progressive control patients, because progressive patients are rare and the majority under therapy. Therefore, I cannot conclude if the obtained data from this cohort is characteristic for progressive patients, or due to B-cell depletion.

Furthermore, untreated RRMS patients are rather difficult to recruit, especially regarding CSF samples, but I plan to extend this analysis to a larger cohort of naïve RRMS patients. Furthermore, it is mandatory both to confirm the anti-inflammatory properties of Tr1 cells in CSF of active MS patients and to assess the suppressive capacities of regulatory T-cell populations towards MS-relevant viral or self-antigens T-cells response, in order to verify their potential pathogenic or beneficial function. Finally, we will analyse brain lesions of RR- and PR-MS patients for the presence of Tr1 cells and identify genes of CNS-infiltrating regulatory cells that could enhance their protective functions and that are specifically altered in MS.

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