Universitat Autònoma de Barcelona

ANTIGEN-SPECIFIC MDSCs INDUCE IMMUNOLOGICAL TOLERANCE IN AN EXPERIMENTAL MODEL OF MULTIPLE SCLEROSIS. GENERATION OF HUMAN MDSCs FROM HEMATOPOIETIC PROGENITORS AS A THERAPEUTIC TOOL

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That the experimental work and the writing of the memory of the doctoral thesis "Antigen-specific MDSCs induce immunological tolerance in an experimental model of multiple sclerosis. Generation of human MDSCs as a therapeutic tool" have been performed by Silvia Casacuberta Serra under their direction and consider that it is suitable to be presented for the degree of PhD in Immunology at the Universitat Autònoma de Barcelona.

Barcelona, 17th of December of 2015.

Director: Dr. Jordi Barquinero **Director: Dr. Carmen Espejo**

Tutor: Dr. Mª Dolores Jaraquemada **PhD student: Sílvia Casacuberta**

If you can dream it, you can do it.

Walt Disney

It's like Forrest Gump said, 'Life is like a box of chocolates.' Your career is like a box of chocolates - you never know what you're going to get. But everything you get is going to teach you something along the way and make you the person you are today. That's the exciting part - it's an adventure in itself.

Nick Carter

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You can't connect the dots looking forward; you can only connect them looking backwards. So you have to trust that the dots will somehow connect in your future. You have to trust in something - your gut, destiny, life, karma, whatever. Because believing that the dots will connect down the road will give you the confidence to follow you heart even when it leads you off the well worn path and that would make all the difference.

Steve Jobs

PRESENTATION

The work presented in this thesis has been carried out in the laboratory of Cell and Gene Therapy at the *Vall d'Hebron Institut de Recerca (VHIR)*. The laboratory is directed by Dr. Jordi Barquinero and this work is a result of a collaboration between his group and the group of Dr. Carmen Espejo and Dr. Xavier Montalban from *Servei de Neurologia-Neuroimmunologia, Centre d'Esclerosi Múltiple de Catalunya (Cemcat)* located in the *Hospital Universitari Vall d'Hebron (HUVH)*. For this reason both Dr. Jordi Barquinero and Dr. Carmen Espejo are co-directors of this thesis.

Dr. Barquinero's group has worked for several years in different aspects of preclinical gene therapy studies. In particular, the group has conducted an intense research in the area of hematopoietic gene therapy and the induction of immunological tolerance through the expression of self-antigens in hematopoietic cells using the murine model of multiple sclerosis (MS), the experimental autoimmune encephalomyelitis (EAE).

From this research line resulted a doctoral thesis and two publications (Eixarch, Espejo et al. 2009; Eixarch, Gomez et al. 2009). In the first publication it was demonstrated that the expression of a self-antigen in bone marrow (BM) cells induced immunological tolerance in the chronic progressive EAE model in both preventive and therapeutic approaches. Moreover, the absence of engraftment, the rapid recovery of the animals in the therapeutic arm and the fact that myeloablation was not required to induce immunological tolerance led to reconsider the initial hypothesis and it was postulated that the therapeutic effect observed was not mediated by cells with engrafting potential but rather by a more mature cell type that could express the self-antigen in a tolerogenic manner.

This new hypothesis and the subsequent research resulted in another doctoral thesis and one publication (Gomez, Espejo et al. 2014), in which the group found that in BM transduction cultures the most abundant cell types were of myeloid origin and that, indeed, these cells were myeloid-derived suppressor cells (MDSCs) with capacity to suppress T-cell responses *in vitro* in an antigen-specific manner.

Therefore, the first part of the work presented in this thesis was initiated with the purpose of better characterizing these MDSCs generated in BM retroviral transduction cultures and determining whether these cells were responsible for the induction of the

immunological tolerance observed in the EAE model as well as studying the potential mechanisms of action that may be involved.

The second part of this thesis was initiated after having promising results with the murine antigen-specific MDSCs generated *ex vivo* from BM cells. Therefore, we decided to move one step further and try to generate *in vitro* human MDSCs from hematopoietic progenitor cells. MDSCs constitute both a therapeutic target (e.g. in cancer) and a therapeutic tool (e.g. in autoimmunity). For this reason, this part of the thesis was aimed at developing efficient methods to generate human MDSCs from hematopoietic progenitor cells for its potential clinical application.

The results presented in this thesis have led to two scientific articles, one of them is currently under review for publication in an international journal and the other one is in preparation.

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I can't change the direction of the wind, but I can adjust my sails to always reach my destination.

Jimmy Dean

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Believe you can and you're halfway there.

Theodore Roosevelt

ABBREVIATIONS

5-FU: 5-Fluorouracil

ADA-SCID: adenosine deaminase severe combined immune deficiency

APC: allophycocyanin

APC-Cy7: allophycocyanin conjugated with cyanine 7

APCs: antigen presenting cells

B1: T-cell independent

B2: T-cell dependent

BBB: blood-brain barrier

BM: bone marrow

BCR: B-cell receptor

Breg: regulatory B cells

BSA: bovine serum albumin

BV421: brilliant violet 421

CB: cord blood

CBA: cytometric bead array

CFA: complete Freund's adjuvant

CGD: chronic granulomatous disease

CIA: collagen-induced arthritis

CIS: clinical isolated syndrome

CLIP: class II-associated invariant peptide

CLP: common lymphoid progenitor

CMP: common myeloid progenitor

CNS: central nervous system

CO_2: carbon dioxide

Cox-2 cyclooxygenase-2

Cy: cyanine

cpm: counts per minute

CTLA-4: cytotoxic T lymphocyte antigen 4

DCs: dendritic cells

DMEM: Dulbecco's modified Eagle's medium

DMTs: disease-modifying treatments

DNA: deoxyribonucleic acid

EAE: experimental autoimmune encephalomyelitis

EGFP: enhanced green fluorescent protein

ER: endoplasmic reticulum

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

FLT3-L: fms-like tyrosine kinase 3 ligand

FoxP3: forkhead box P3

FSC: forward scatter

G-CSF: granulocyte-colony stimulating factor

G-MDSCs: granulocytic MDSCs

GA: glatiramer acetate

GFAP: glial fibrillary acidic protein

GFP: green fluorescent protein

GM-CSF: granulocyte-macrophage colony-stimulating factor

GVHD: graft-versus-host disease

GVT: graft-versus-tumor

Gy: gray

h: hour

HE: hematoxylin and eosin

HIB: human isolation buffer

HLA: human leukocyte antigen

HSCs: hematopoietic stem cells

HSCT: hematopoietic stem cell transplantation

i.v.: intravenously

IFN-β: Interferon-β

IFN-γ: interferon-γ

IL: interleukin

IMCs: immature myeloid cells

IMDM: Iscove's Modified Dulbecco's Medium

iNOS: inducible nitric oxide synthase

iTreg: induced Treg

KB: Klüver-Barrera

LAG-3: lymphocyte-activation gene 3

LEA: lycopersicon esculentum agglutinin

Lin: lineage specific antigens

LMO2: LIM domain only 2

LPS: lipopolysaccharide

LSK: $Lin^{-}Sca^{-}1^{+}c\text{-}kit^{+}$ cells

LT-HSCs: long-term hematopoietic stem cells

M-CSF: macrophage colony-stimulating factor

M-MDSCs: monocytic MDSCs

MBP: myelin basic protein

MDSCs: myeloid-derived suppressor cells

MFI: mean fluorescence intensity

mHAgs: minor histocompatibility antigens

MHC: major histocompatibility complex

MOG: myelin oligodendrocyte glycoprotein

MoMuLV: Moloney murine leukemia virus

MPPs: multipotent progenitors

MS: multiple sclerosis

mTECs: thymic medullary epithelial cells

NI: non-immunized

NK: natural killer

NO: nitric oxide

NT: non-treated

nTreg: natural Treg

OD: optical density

p.i.: postimmunization

PB: peripheral blood

PBHSCs: peripheral blood hematopoietic stem cells

PBMCs: peripheral blood mononuclear cells

PBS: phosphate-buffered saline

PD-1: programmed death-1

PD-L1: programmed death-1 ligand-1

PD-L2: programmed death-1 ligand-2

PE: phycoerythrin

PE-Cy7: phycoerythrin conjugated with cyanine 7

PEG-ADA polyethylene glycol-ADA

PerCP: peridinin chlorophyll

PerCP-Cy5.5: peridinin chlorophyll conjugated with cyanine 5.5

PGE2: prostaglandin E2

PHA-L: phytohemagglutinin L

PICs: pre-integration complexes

PLP: proteolipid protein

PPMS: primary progressive multiple sclerosis

RA: rheumatoid arthritis

ROS: reactive oxygen species

RNA: ribonucleic acid

RRMS: relapsing-remitting multiple sclerosis

RT: room temperature

S1P: sphingosine-1-phosphate

Sca-1 stem cell associated antigen

SCID: severe combined immune deficiency

SCID-X1: X-linked severe combined immune deficiency

SCF: stem cell factor

SD: standard deviation

SEM: standard error of the mean

SIN: self-inactivating

SLE: systemic lupus erythematosus

SMI-32: non-phosporilated neurofilaments

SPMS: secondary progressive MS

SSC: side scatter

ST-HSCs: short-term hematopoietic stem cells

STAT: signal transducer and activator of transcription

T1D: type 1 diabetes

TAP: transporter associated with antigen processing

TCR: T-cell receptor

TGF- β transforming growth factor β

Th: T helper lymphocyte

TLR: Toll-like receptor

TNF-α: tumor necrosis factor α

TPO: thrombopoietin

Tr-1: T regulatory type 1 cell

Treg: regulatory T cell

VEGF: vascular endothelial growth factor

VHIR: Vall d'Hebron Institut de Recerca

WAS: Wiskott-Aldrich syndrome

γc: common gamma chain

Live as if you were to die tomorrow. Learn as if you were to live forever.

Mahatma Gandhi

INTRODUCTION

1. THE HEMATOPOIETIC SYSTEM

The hematopoietic system is responsible for the generation and renewal of all the cells that form the blood and the immune system through a process called hematopoiesis. During the life of an individual hematopoiesis occurs in many different organs but only one persists in the adult, the BM. In the adult BM we find the hematopoietic cells including hematopoietic stem cells (HSCs), myeloid and lymphoid progenitors, mature cells and other stromal cells (Figure 1).

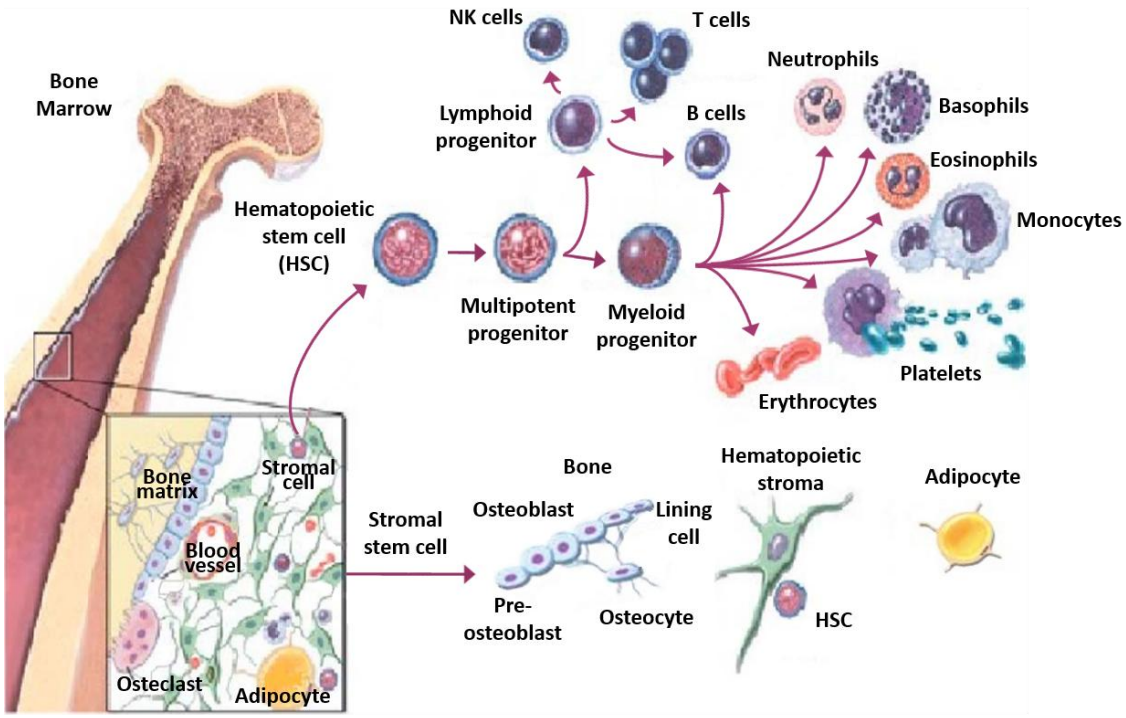


Figure 1. Structure of the hematopoietic system. The hematopoietic system is a hierarchical and heterogeneous tissue consisting of more or less immature cells of the various hematopoietic lineages (lymphoid and myeloid) that support hematopoiesis. The hematopoietic microenvironment, in addition to being the physical support for HSCs, regulates the maintenance of hematopoiesis by cell-cell contact or by secreting factors that regulate growth and differentiation of the cells that comprise. Figure adapted from Terese Winslow and Lydia Kibiuk, 2001©

The BM stroma consists of adipocytes, reticular cells, macrophages, vascular endothelial cells, terminations of the sympathetic nervous system, smooth muscle cells and mesenchymal stem cells and it is responsible for the production and deposition of the extracellular matrix and for the production of cytokines and growth factors. This

particular microenvironment, known as stem cell niche, interacts with HSCs via cell surface receptors, adhesive ligands, signalling pathways and secreted growth factors (Lo Celso and Scadden 2011; Morrison and Scadden 2014).

HSCs are adult stem cells that regulate the balanced turnover of erythrocytes, platelets and all immune cells by switching between quiescence, self-renewal and differentiation and, thereby, maintain homeostasis both in the steady state and in response to stress. Interactions of HSCs with their particular niche, as well as multiple stochastic fluctuations of HSCs autonomous processes, are critical for maintaining the stem cell properties. The best characterized hematopoietic model is the murine one, so most of what is explained below will refer to the murine system. The osteoblastic and vascular niches have long been identified as the two major components of the hematopoietic niche to support the maintenance, proliferation and differentiation of HSCs (Trumpp, Essers et al. 2010; Nakamura-Ishizu and Suda 2012). The osteoblastic niche contains quiescent (G_0) HSCs, also named long-term HSCs (LT-HSCs). By contrast, the vascular niche supports actively cycling HSCs, known as short-term HSCs (ST-HSCs), and their immediate progeny. ST-HSCs are the reservoir that gives rise to non-self-renewing multipotent progenitors (MPPs) and supports the daily production of billions of blood cells, whereas LT-HSCs function as a backup or reserved subpopulation (Suarez-Alvarez, Lopez-Vazquez et al. 2012).

As HSCs exit quiescence and re-enter the cell cycle, the choice between asymmetric and symmetric divisions constitutes one of the first important decision points governing their fate. Asymmetric division generates two cells with different fates and this allows maintenance of the HSC pool and the generation of differentiating progeny during homeostatic blood production. By contrast, symmetric division generates two cells with equivalent fates, and can either expand the HSC pool or increase the number of differentiating cells in conditions of emergency hematopoiesis (Congdon and Reya 2008; Kohli and Passegue 2014).

Even being the most studied stem cells, HSCs lack a clearly defined phenotype. The pool of murine HSCs is characterized as cells that do not express any lineage specific antigens (Lin^-); that are positive for the stem cell associated antigen (Sca-1) and

positive for the tyrosine kinase receptor c-kit ($Lin^-Sca\text{-}1^+c\text{-}kit^+$ cells). These cells are known as LSK cells and are able of long-term hematopoietic reconstitution when transplanted into lethally irradiated mice. The LSK cells can be divided in the different hierarchical classes of stem cells: LT-HSCs, ST-HSCs and MPPs (Figure 2)(Spangrude, Heimfeld et al. 1988; Morrison and Weissman 1994; Giebel and Punzel 2008; Seita and Weissman 2010). Some of these HSCs, termed side population, have the capacity to efflux dyes such as Hoechst 33342 or Rhodamine-123 and are characterized for having enriched capacity of long-term reconstitution (Bryder, Rossi et al. 2006).

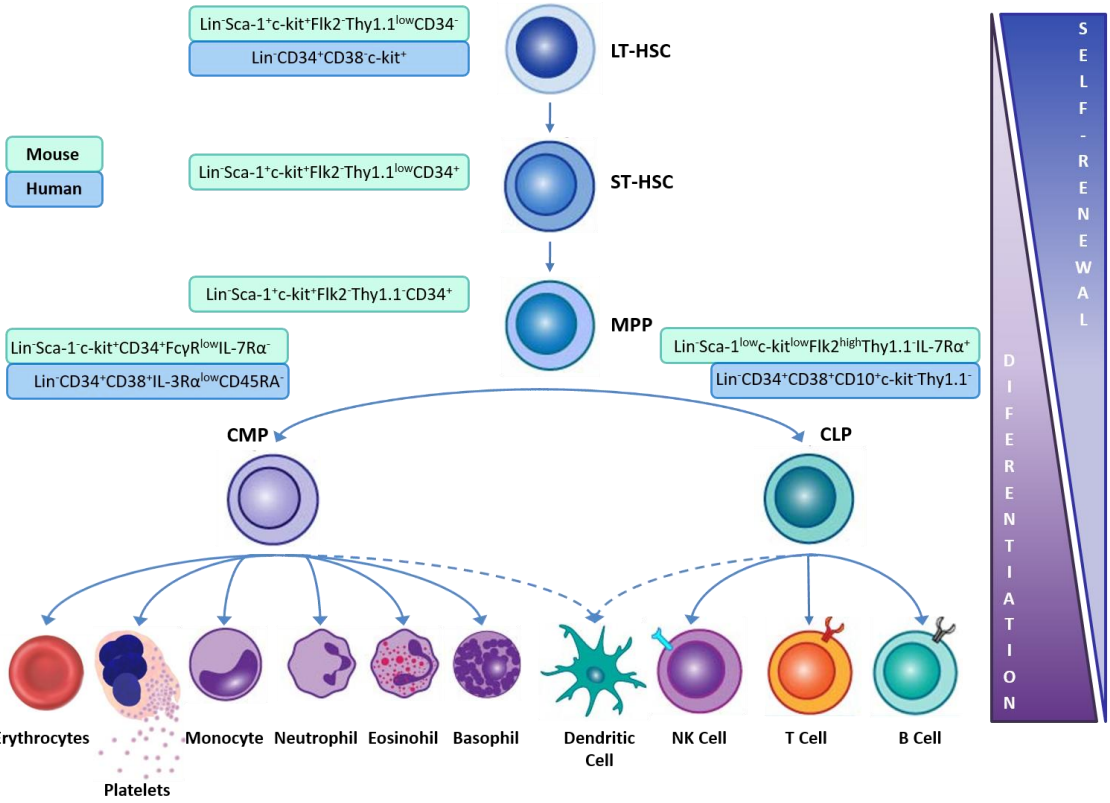


Figure 2. Model of the hematopoietic developmental hierarchy. Self-renewing HSCs reside at the top of the hierarchy, giving rise MPPs which in turn give rise to the common myeloid and common lymphoid oligopotent progenitors (CMP and CLP, respectively), which are responsible for the production of all the mature cells of their lineage. Both CMP and CLP have been proposed to give rise to dendritic cells. Development from the oligopotent progenitors to mature blood cells proceeds through a number of intermediate progenitors (not shown). The cell surface phenotype of many of these cells is shown for murine and human systems. It should be noted that although all the different HSCs subsets in mice have been characterized to a significant degree, this is still unresolved for the human system. Figure adapted from (Weissman and Shizuru 2008).

MPPs in turn give rise to oligopotent progenitors, which possess more restricted developmental potential with low self-renewal and high proliferative capacity. This represents a branching point in the hematopoietic hierarchy with the common lymphoid progenitor (CLP) giving rise to mature lymphoid cells including B, T and natural killer (NK) cells and the common myeloid progenitor (CMP) capable of giving rise to mature myeloerythroid cells including erythrocytes, platelets, basophils, eosinophils, neutrophils and macrophages. BM mature cells are differentiated cells that do not have self-renewal capacity and, depending on the cell type, nearly do not proliferate (Figure 2).

2. HEMATOPOIETIC STEM CELL TRANSPLANTATION

Hematopoietic stem cell transplantation (HSCT) is the infusion of HSCs to treat patients with defined congenital or acquired disorders of the hematopoietic system. There are two main objectives of HSCT, the first one is the replacement of the patient's defective hematopoietic system by a new healthy one which is restored by either its own cryopreserved HSCs or from HSCs of a healthy donor. Secondly, it allows the use of chemo and/or radiotherapy treatment at supralethal doses and re-establishing hematopoiesis through the administration of HSCs.

HSCs for transplantation are usually obtained from:

- **Bone marrow**

BM was the first source of HSCs used for transplantation. HSCs are removed from large bones, typically the iliac crests, while the donor is under general or local anesthesia. Initially it was thought that HSCs with capacity for regenerating hematopoiesis and the immune system of the recipient were only located in the BM. However, it was later confirmed that it was possible to mobilize these cells into the peripheral blood (PB).

- **Peripheral blood**

Peripheral blood hematopoietic stem cells (PBHSCs) are now the most common source of HSCs for transplantation and they are collected from the blood through a process known as apheresis. The PBHSC yield is boosted by granulocyte-colony stimulating

factor (G-CSF) that mobilizes stem cells from BM into the peripheral circulation. The number of HSCs obtained through apheresis after mobilization is higher than that obtained from BM extraction, and the cell content is also different since it contains more T cells. As compared with BM, PBHSCs produce more rapid hematopoietic reconstitution but they also entail higher risk of suffering from graft-versus-host disease (GVHD), since T cells from the donor are the main players in this complication.

- **Umbilical cord blood**

HSCs are obtained from cord blood (CB) immediately after birth. CB has a higher concentration of HSCs than that normally found in adult blood. However, the small quantity of blood obtained from an umbilical cord makes it more suitable for transplantation into small children than into adults. Newer techniques using *ex vivo* expansion of CB units or the use of two CB units from different donors allow CB transplants to be used in adults. The lower immunological reactivity of the lymphocytes allows some degree of incompatibility between the recipient and the donor, and correspondingly, the incidence and intensity of GVHD is usually lower.

HSCT is currently applied for the treatment of several types of diseases:

- **Primary immune deficiencies**

Children born with congenital immune deficiency syndromes can be treated with an allogeneic HSCT from a matched healthy donor. These syndromes include lymphocyte disorders, such as severe combined immune deficiency (SCID) and Wiskott-Aldrich syndrome (WAS), and several granulocyte disorders.

- **Hereditary hematological disorders**

Hereditary hematological disorders include BM failure syndromes and red cell disorders that can also be treated with allogeneic HSCT. These include severe aplastic anemia like Fanconi anemia and hemoglobinopathies such as sickle cell disease and thalassemia.

- **Autoimmune diseases**

Patients who develop severe autoimmune diseases that can be life-threatening or lead to significant morbidity can also be treated with HSCT. Patients with MS, rheumatoid

arthritis (RA) and systemic lupus erythematosus (SLE) have been reported to benefit from HSCT although it is still considered an experimental therapy (Hugle and Daikeler 2010).

- **Hematologic malignancies and solid tumors**

HSCT is commonly used to treat leukemia and lymphoproliferative disorders such as multiple myeloma, acute myeloid or lymphoblastic leukemia, Hodgkin's disease, among others. It is also used to treat solid tumors as neuroblastoma, germinal cancer, breast cancer, Ewing sarcoma and others. In these cases, autologous HSCT allows the use of high dose chemotherapy to ablate the tumor whereas allogeneic HSCT is used to induce graft-versus-tumor (GVT) effect, the beneficial aspect of the graft-versus-host phenomenon. GVT is due to therapeutic immune reaction of the grafted donor T lymphocytes that eliminate malignant host cells.

- **Tolerance induction for solid organ transplantation**

Researchers have observed that hematopoietic chimerism is associated with increased reciprocal tolerance between donor and host. This has allowed new means of inducing tolerance to recipients of allogeneic solid organ transplants, thus reducing the need for immunosuppressive medications and their long-term side effects.

2.1. Types of HSCT

The compatibility between donor and recipient is given by the genetic disparity in the major histocompatibility complex (MHC) or human leukocyte antigen (HLA) but also by the minor histocompatibility antigens (mHAgs). Although the donor and the recipient have the same HLA, the transplanted cells can be rejected by the incompatibility of the mHAgs. According to the degree of histocompatibility between the donor and recipient the following types of transplants are distinguished:

- **Xenogeneic transplant**

In xenotransplants, donor cells come from a different species. Xenotransplantation is not routinely used in HSCT due to the high risk of infectious and immunological complications. Although it is still in early stages, it is thought that in the future

xenotransplantation may become an alternative therapy for organ transplantation since the donation is generally scarce.

- **Allogeneic transplant**

In allogeneic transplantation, the donor and recipient are from the same species but genetically distinct. These genetic differences generally result in histocompatibility problems and rejection (host-versus-graft) and GVHD. However, as mentioned before, this type of immune reactivity can have a powerful anti-leukemia effect that autologous transplants do not have. Thus, in patients with hematologic malignancies, the ideal donor is the one that shares the two HLA haplotypes, usually a sibling, but not the minor antigens. The main drawback of allogeneic transplants, apart from rejection and toxicity associated with the pre-transplant conditioning regimen, is precisely the alloreactivity. This may result in the so-called GVHD caused by mature donor T cells present in the graft that react against the cells and tissues of the recipient.

- **Syngeneic transplant**

Syngeneic transplantation is rarely used in practice but highly safe and effective when dealing with solid organ transplants. The donor and recipient are identical twins so there are no genetic differences between them nullifying the possibility of rejection and GVHD. Therefore, in the case of HSCT this type of transplant would only be indicated for the treatment of some non-malignant conditions and malignant diseases where there is no other compatible donor. In hematological malignancies, the absence of alloreactivity constitutes a handicap for its lack of anti-leukemia effect but otherwise it ensures the engraftment of disease free cells.

- **Autologous transplant**

In this type of transplant the HSCs are obtained from the patient itself. It is used almost exclusively in cancer patients with solid tumors (to regenerate their hematopoietic system destroyed by high doses of chemotherapy and/or radiotherapy) or with hematological malignancies. The doses administered in this case are myeloablative, so the recovery of the hematopoietic function is not possible unless the progenitors, previously extracted from the patient and cryopreserved, are

re-administered to the patient after the conditioning treatment. This type of transplant involves fewer transplant related complications as it does not produce GVHD but, as it lacks alloreactivity, it is associated with an increased risk of tumor relapse.

In some hereditary diseases, an alternative to allogeneic transplant is gene therapy using autologous HSCs. Monogenic diseases affecting the hematopoietic system have been and are prime candidates to be treated with gene therapy. Gene correction at the level of autologous HSCs, which is performed *ex vivo*, greatly reduces the possibility of rejection. However, it does not rule out the possibility of an immune response against the product of therapeutic gene. This type of therapy is generally less toxic and better tolerated than an allogeneic transplant.

3. HEMATOPOIETIC GENE THERAPY

Gene therapy is a relatively recent and experimental approach to treat human diseases. It is defined as the intentional delivery of genetic material to a cell or tissue with the aim of correcting a specific genetic defect with therapeutic purposes. In other words, it is a therapeutic technique by which a functional gene, called transgene, is inserted into the cells of a patient to correct a genetic abnormality by providing a copy of a normal gene, directly repairing such gene or providing a gene that adds new functions or regulates the activity of other genes.

All cells in the human body contain genes, which make them potential targets for gene therapy. These cells can be divided into somatic cells (most cells of the body) or germinal cells (eggs or sperm). In theory it is possible to transform either somatic cells or germ cells.

- **Germ line gene therapy**

Gene therapy using germ cells results in permanent changes in all the cells of the body that are passed down to subsequent generations. The appeal of germ line gene therapy is its potential for offering a permanent therapeutic effect for the patient and

his descendants. However, this raises a whole set of concerns about technical aspects and bioethical and social implications.

- **Somatic gene therapy**

Somatic cells are non-reproductive. Somatic cell therapy is a safer approach because it only affects the targeted cells in the patient and is not passed on to future generations. However, this type of therapy presents specific problems as often the effects of somatic cell therapy are short-lived. Because the cells of most tissues ultimately die and are replaced by new cells, repeated treatments over the course of the individual's life span may be required to maintain the therapeutic effect. For this reason, stem cells are preferred target. Transporting the gene to the target cells or tissue is also problematic. However, all gene therapy to date on humans has been directed at somatic cells.

Gene therapy can be broadly classified into two categories:

- ***Ex vivo***

The patient cells are modified outside the body and then transplanted back again. This is the case of the hematopoietic gene therapy where HSCs from the patient are cultured in the laboratory and then are exposed to the virus carrying the therapeutic gene. The virus enters the cells and inserts the transgene into the cell's deoxyribonucleic acid (DNA). In some cases these cells can be expanded and then returned to the patient.

- ***In vivo***

In this type of gene therapy the gene is transferred directly into the patient body. It involves the administration of the vector containing the transgene directly to the target tissue (e.g. the liver in the case of hemophilia or the muscle in the case of muscular dystrophy) or directly into circulation. The latter implies a serious risk since you cannot control where the vector goes and there is the theoretically possibility to affect the germ line of the individual.

The concept of gene therapy first became feasible when in 1966 Edward Tatum published a paper evoking the effectiveness of viruses to be used in gene therapy

(Tatum 1966). Some years later, although unsuccessful, the first gene therapy trial in humans was performed (Rogers, Lowenthal et al. 1973; Terheggen, Lowenthal et al. 1975). During the 80s and 90s hematopoietic gene therapy experienced a dramatic growth both in basic research and in the numbers of clinical trials performed. In the 80s the first efficient packaging cell lines for recombinant viruses were developed and the first successful assays of gene transfer in animal models were performed (Williams, Lemischka et al. 1984; Kwok, Schuening et al. 1986; Yu, von Ruden et al. 1986; Kantoff, Gillio et al. 1987). During the 90's there was a boom of gene therapy clinical trials and since then this young discipline has not stopped evolving.

3.1. Gene transfer systems

To insert the therapeutic gene into the patient's targeted cells a carrier molecule, called a vector, must be used. For therapeutic purposes, the genetic material needs to be transferred into the appropriate cell and expressed at sufficient levels. In most cases, a relatively large piece of genetic material is required as it includes the promoter sequences that activate expression of the gene, the coding sequences that direct the production of the protein and signalling sequences that direct ribonucleic acid (RNA) processing such as polyadenylation. Gene therapy is based on a variety of gene transfer methods. The choice of one or the other depends on:

- The target cell or tissue and its characteristics.
- The desired stability of transgene expression in the target cell.
- The size of the genetic material to be transferred.

It is therefore not surprising that a wide variety of delivery systems have been developed. Generally, these can be divided into two groups: viral and non-viral.

- **Non-viral vectors**

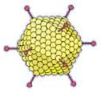
Non-viral or physico-chemical methods of gene transfer (transfection) were the first to be developed. These techniques introduce the DNA alone or conjugated to other molecules or polymers, such as liposomes. These delivery systems possess reduced biosafety risk compared to their viral analogous. However, they possess modest gene

delivery efficiency and short-term expression capacity. Therefore, in general, this group of techniques is used for applications that do not require a stable transgene expression in the target cells.

- **Viral vectors**

All viruses bind to their host cells and introduce their genetic material into the nucleus (transduction) as part of their replication cycle. Viral vectors are currently the most effective vectors and were the first to be employed in gene therapy clinical trials [reviewed in (Wirth, Parker et al. 2013)]. Important features that distinguish the different viral vectors most commonly used in gene therapy are summarized in Table 1. The main advantages of integrative viral vectors are their high gene transfer efficiency and the long-lasting expression of the transgene. The drawbacks of this type of systems rise from the random integration of viral sequences and their immunogenicity. Such is the case, for example, when an oncogene is activated due to non-specific integration of the vector. Obviously, application of viral vectors for clinical trials in humans requires serious consideration of safety aspects related to their use.

Table 1. Most commonly used viral vectors in gene therapy.

		Adenovirus	Adeno-associated virus	Alphavirus	Herpesvirus	Retrovirus/ Lentivirus	Vaccinia Virus
							
PARTICLE CHARACTERISTICS	**Genome**	dsDNA	ssDNA	ssRNA(+)	dsDNA	ssRNA (+)	dsRNA
	Coat	Naked	Naked	Enveloped	Enveloped	Enveloped	Enveloped
	Virion polymerase	Negative	Negative	Negative	Negative	Positive	Positive
	Genome size	39-38 kb	5 kb	12 kb	120-200 kb	8-12 kb	130-280 kb
GENE THERAPY PROPERTIES	**Target in cycle?**	No	No	No	No	Yes (except lentivirus)	No
	Packing capacity	High <36 kb (*gutless)	Low < 4 kb	Medium <7.5 kb	High > 30 kb	Medium <8 kb	High >25 kb
	Integration of transgene	No	No	No	No	Yes	No
	Transgene expression	Transient	Long lasting	Transient	Long lasting	Long lasting	Transient
	Immunogenicity	High/Low	Low	Low	Medium	Low	High

*Gutless: Third generation adenoviral vector with all the viral coding regions eliminated. No immune response generated but unlike its predecessors, it requires helper adenovirus to provide all viral proteins. ds: double strand; ss: single strand.

Generally, viral vectors can be classified into two main groups: non-integrative and integrative. Adenoviruses are the most used non-integrative vectors, especially for treating some cancers. Usually, in these cases, a sustained expression of the transgene over time is not desired but transient effects are sought, for example by the action of suicidal genes (oncolytic effect) or by the induction of an antitumoral immune response. Once the tumor has disappeared the effects of the transgene are no longer needed. On the other hand, the opposite is pursued for genetic diseases. In this case the expression of the therapeutic protein over the life span of the individual is sought, so the use of integrative viral vectors to achieve a long-term expression of the transgene is more suitable. The main integrative vectors are the ones based on the viruses from the family *Retroviridae*, two of which are widely used in gene therapy: the gammaretroviruses and lentiviruses. Although strictly speaking lentiviruses are also retroviruses, this term is generally applied to the vectors based on gammaretroviruses, so in this work will refer to gammaretrovirus based vectors as retroviral vectors.

The first integrative viral vectors that were developed were based on Moloney murine leukemia virus (MoMuLV) (Baum, Eckert et al. 1996; Dao and Nolta 1999) and until recenlty they have been the most widely used in gene therapy protocols for hematological inherited diseases.

3.2. Retroviral vectors

Retroviruses have a number of features that make them unique as gene delivery vehicles. Their life cycle includes an integrated state in the DNA of the host genome (provirus) where the retroviral promoter can direct high-level, efficient and stable expression of genes encoded within the confines of the viral genetic material.

3.2.1. Characteristics, structure and components of retroviruses

Retroviruses are infectious particles of about 80-120 nm of diameter consisting of two copies of positive-single strand RNA packed in a protein capsid and surrounded by a lipid envelope. They can be classified into two groups according to the complexity of its

genome: simple and complex. Simple retroviruses contain only the elementary genetic information common to all retroviruses whereas the complex ones also encode for additional regulatory proteins.

The two strands of RNA are physically linked as a dimer by hydrogen bonds and have a variable size of 8-12 Kb. They also have a 5' cap and a 3'poly-(A) equivalent to mRNA. The coding region common to all retroviruses contain three genes, ordered from 5' to 3' (Figure 3a):

- ***gag*** encodes a polyprotein (Gag) whose cleavage products are the major structural proteins of the matrix, the capsid and the nucleocapsid of the virus core.
- ***pol*** encodes a polyprotein whose cleavage products are the necessary enzymes for the viral replication: reverse transcriptase, integrase and protease.
- ***env*** encodes a polyprotein (Env) whose cleavage products are the structural proteins of the viral envelope. The surface envelope glycoprotein, the major antigen of the virus and responsible for receptor binding, and the trans-membrane glycoprotein which holds the surface protein in the lipid envelope and it is responsible for membrane fusion or receptor binding in the target cell.

In addition, the retroviral genome also contains a number of non-coding regions common to all retroviruses that include sequences necessary for the integration, transcription, translation and packaging of the viral RNA (Figure 3a).

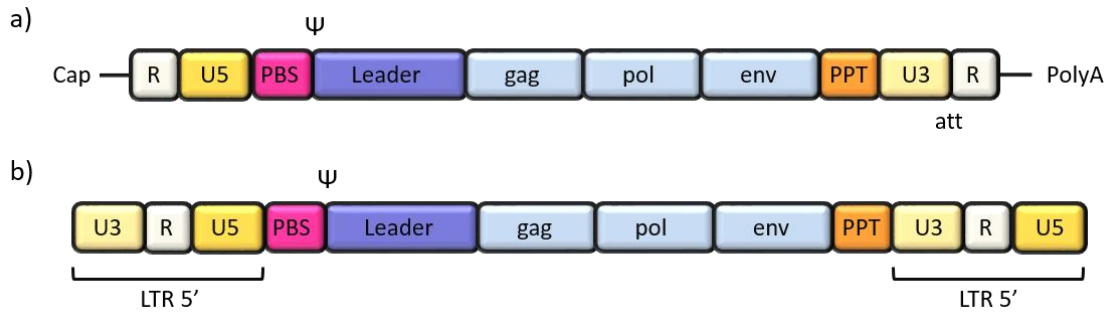


Figure 3. Simple retroviral genome diagram. a) Illustration of retroviral genomic RNA with its basics components. **b)** General diagram of the viral DNA once it is retrotranscribed in the host cell cytoplasm (provirus). PBS: primer binding site; Ψ: packaging signal; PPT: polypurine tract; LTR: long terminal repeat.

The retroviral envelope is a lipid bilayer derived from the host cell membrane during the budding process. As mentioned before, it contains the envelope glycoproteins that confer the virus its target specificity (tropism). The matrix proteins are linked to the envelope and surround the viral capsid which in turn contains the nucleocapsid proteins, tightly bound to the viral genomic RNA forming a ribonucleoprotein complex within the core, and all the proteins encoded by the *pol* gene necessary for the life cycle of the virus (reverse transcriptase, integrase and protease)(Figure 4).

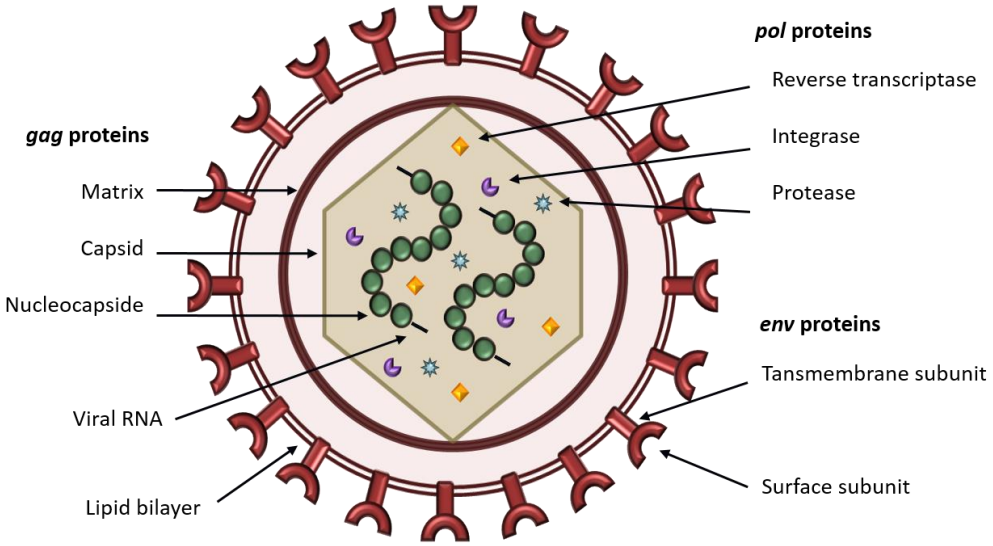


Figure 4. Schematic representation of a retrovirus and its components. Retroviruses are enveloped RNA viruses. The proteins encoded by the *gag* gene are part of the virus structure; the proteins encoded by the *pol* gene are the necessary enzymes for reverse transcription and integration of the viral genome into the host cell genome, and the membrane glycoproteins that confer virus tropism are encoded by the *env* gene.

The life cycle of retroviruses can be divided into two distinct phases: the early phase, which goes from the infection steps of recognition and cell binding to the integration of the viral complementary DNA (cDNA) into the host genome, and the late phase which begins with the expression of viral genes and continues to the release and maturation of progeny virions. The initial step of the early phase is the binding of viral particles to the surface of their target cells using cell surface proteins as specific receptors. Once the viral and cell membrane are fused, the virus is internalized and the capsid is disassembled releasing the viral proteins and genome into the cell cytoplasm

where the viral RNA is reverse transcribed by the reverse transcriptase generating a linear double-stranded DNA molecule, resulting in the so-called provirus (Figure 3b). The reverse transcription process generates the long terminal repeats (LTR), regions at both ends of the viral genome which contain essential sequences for the expression of the viral genes in the host cell as well as important sequences for the provirus integration. To achieve integration, the newly reversed transcribed DNA is associated with viral proteins to form the pre-integration complexes (PICs) and is transported to the nucleus. PICs from most of the retroviruses, as it is the case for gammaretrovirus, are relatively large and consequently are unable to enter intact nuclei and have to wait for the breakdown of the nuclear membrane, only occurring during cell mitosis, to reach the nucleus and be able to integrate into the host genome. Hence, these retroviruses are dependent on the cell cycle and cannot replicate into non-dividing cells. In contrast, lentiviruses are able to infect quiescent cells as their PICs, smaller and more nucleophilic, can actively cross the nuclear membrane.

Once the provirus is integrated into the cellular genome the late phase of the replicative cycle begins. The provirus uses the transcriptional machinery of the host cell to transcribe viral RNA molecules with two different objectives: some are translated to yield the proteins needed to form new virions (the structural and the enzymatic proteins) and others are packaged to generate the new virions. The newly synthesized envelope glycoproteins are expressed in the cell membrane, where the viral components are assembled and the newly synthesized RNA is packaged. Newly formed virions are extruded from the cell, through the so-called budding process, carrying with them part of the cell membrane which contains on its surface the envelope glycoproteins. In Figure 5 the retrovirus life cycle is outlined. The budding process does not lyse the host cell, so that all the progeny of the infected cell will carry the integrated provirus, which is going to be replicated with the genetic material of the host cells.

3.2.2. Production of recombinant retroviral vectors

Most retroviral vectors are based on the genome of MoMuLV gammaretrovirus. To use them for therapeutic purposes the viral genes (*gag*, *pol* and *env*) must be removed and

replaced with the gene of interest. The viral promoter region can also be replaced with one specific promoter. However, non-coding regions necessary for reverse transcription and integration need to be present in *cis* in the retroviral vector. Thus, the vectors integrate into the host cell genome but are unable to generate new viral particles since they lack the necessary genes for the production of the viral proteins.

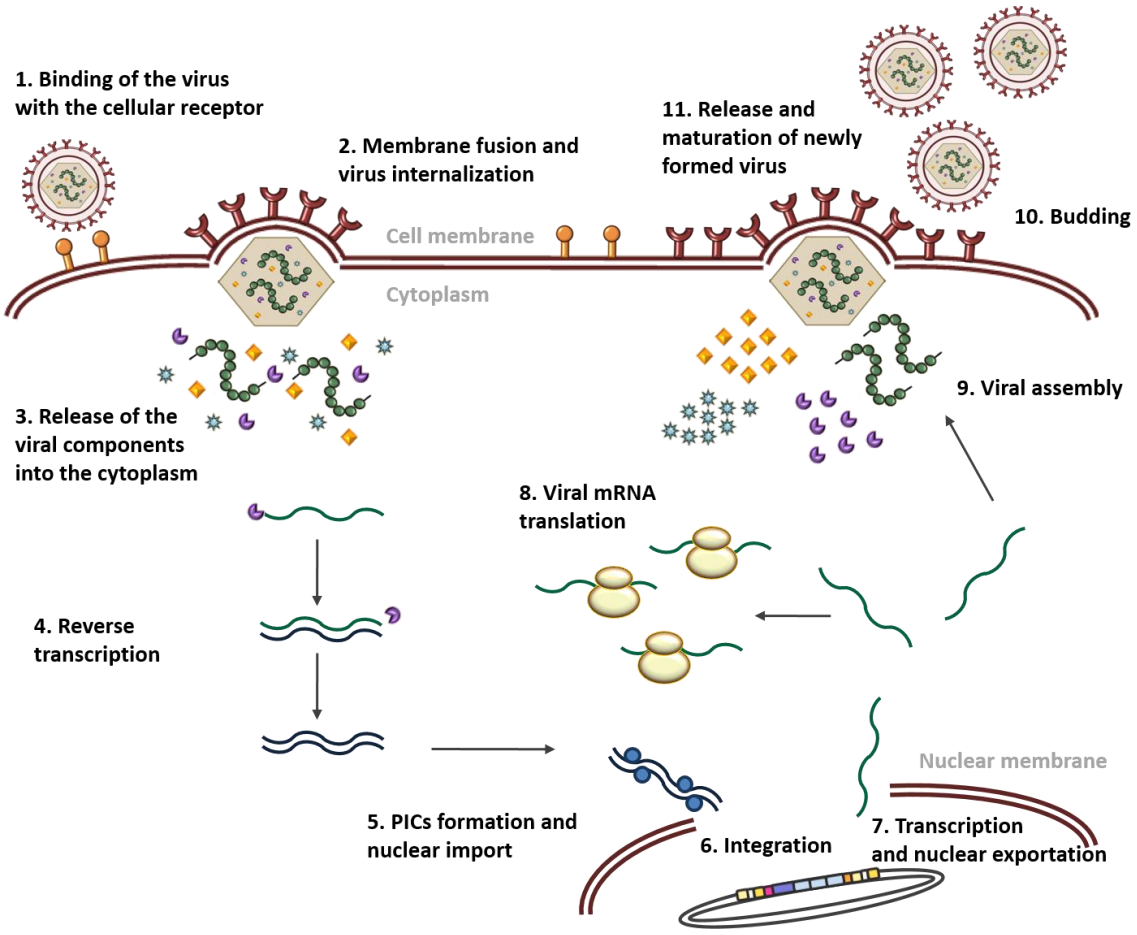


Figure 5. Life cycle of retroviruses. See text.

Therefore, the deletion of coding sequences from the retroviral vector makes it necessary to express the viral genes in *trans* in a packaging cell line which provides all the viral proteins required for capsid production and virion maturation. These packaging cell lines contain the viral genes separated in two plasmids, one contains the *gag* and *pol* genes and the second the *env* gene. With only these elements packaging

cells produce empty viral cores as they do not recognize any RNA containing the packaging signal (Ψ) (Figure 6).

When the retroviral vector with the transgene is expressed in the packaging cells, the viral structural proteins recognize the packaging sequence in the transcript derived from the retroviral vector and encapsidate it into the newly formed virions. Then the packaging cells become vector producing cells. Vector producing cells produce recombinant vectors capable of infection but not of replication. In this way, once the recombinant vector infects the target cell, the transgene is integrated into the cell's genome and the therapeutic protein is expressed (Figure 6).

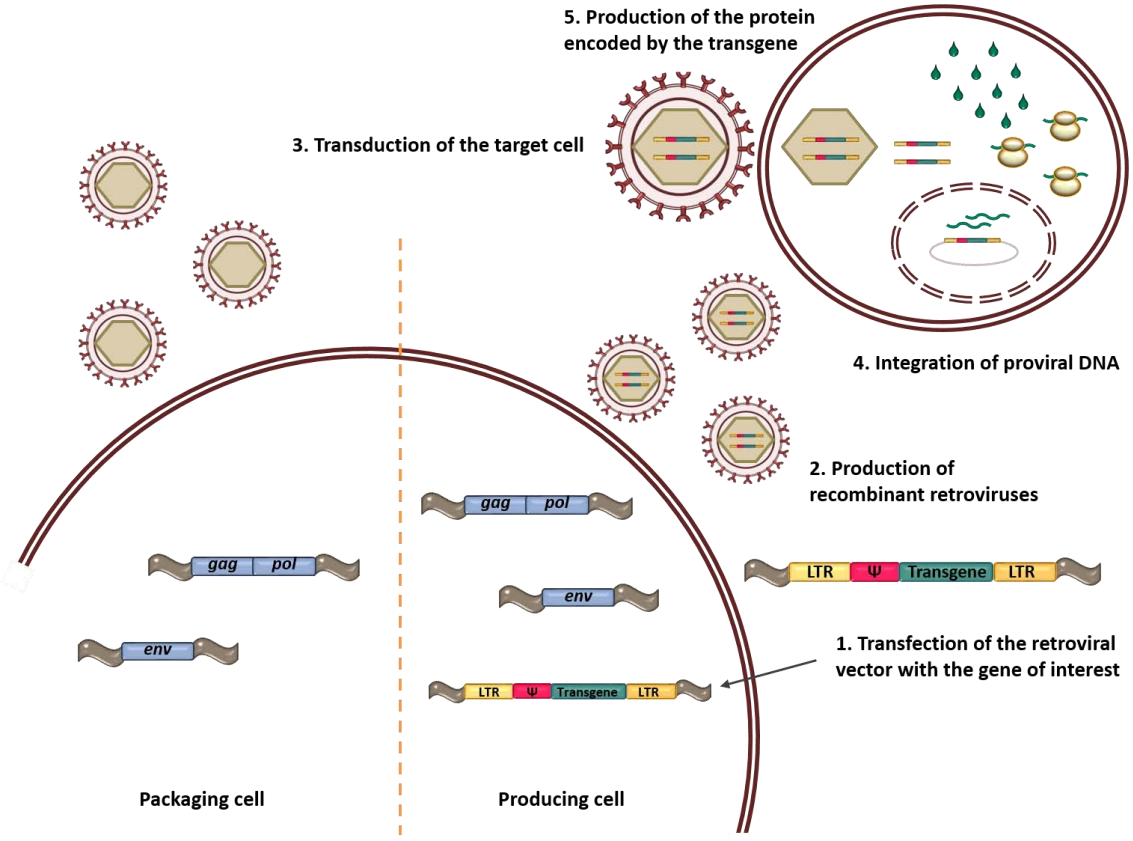


Figure 6. Packaging and producing cells. Packaging cells (left panel), producing cells (right panel) and gene transfer to target cell.

3.2.3. Pseudotyping of retroviral vectors

A major breakthrough for gene therapy using retroviral vectors has been pseudotyping. Viral vector pseudotyping is a strategy used to create vectors with new tropism and trafficking properties. Attachment to and entry into potential target cells requires a favorable interaction between the envelope glycoprotein of the virus and a protein on the surface of the cell. For the purposes of gene therapy, one might either want to limit or expand the range of cells susceptible to infection by the therapeutic vector. To this end, many vectors have been developed in which the endogenous viral envelope proteins have been replaced by envelope proteins from other viruses. Viral vectors in which the envelope proteins have been replaced are referred to as pseudotyped vectors.

Wild type MoMuLV can have an ecotropic envelope, which only allows infection of murine cells, or the amphotropic one, which allows infection of cells from various species, including humans (Palu, Parolin et al. 2000). The first retroviral vectors developed for transducing human HSCs were derived from MoMuLV and presented the amphotropic envelope, which was later replaced by the envelope of the gibbon ape leukemia virus (GALV) (Kiem, Heyward et al. 1997), which proved to be much more efficient to transduce human HSCs (van Hennik, Verstegen et al. 1998; Barquinero, Segovia et al. 2000). However, the envelope most commonly used to efficiently transduce murine HSCs is the ecotropic one.

3.3. EGFP as a marker gene

Recombinant vectors, apart from coding for the therapeutic gene, usually also carry a marker gene for quantifying or selecting the cells expressing the vector. Marker genes have also been used for optimizing transduction protocols, studying long- and short-term repopulating ability after BM transplantation, *in vivo* cell tracking after transplant (Persons, Allay et al. 1998) or for studies of transgene immunogenicity (Eixarch, Gomez et al. 2009).

There are different types of marker genes. The first ones were genes that confer resistance to antibiotics or chemotherapeutics (e.g. neomycin) and those encoding enzymes such as β-galactosidase, alkaline phosphatase and luciferase. But gene marking experienced a breakthrough with the appearance of the green fluorescent protein (GFP) from the jellyfish *Aquorea Victoria*. The GFP gene was cloned in 1992 (Prasher, Eckenrode et al. 1992) and some years later a variant was developed, the enhanced GFP (EGFP), with two mutations in the chromophore zone which emits a stronger fluorescence compared to the wild type protein when excited at 488 nm (Cormack, Valdivia et al. 1996). The EGFP and the other fluorescent proteins have advantages over other gene markers, since they can be directly visualized in the fluorescence microscope and detected by flow cytometry without prior manipulation of the cells. This implies that the cells expressing EGFP can be easily separated from those which do not. For these reasons, since its appearance, EGFP has been widely used in gene transfer studies displacing other gene marking systems (Limon, Briones et al. 1997).

3.4. HSC gene therapy trials using retroviral vectors

HSCs, together with epithelial stem cells, are the stem cells that best meet the requirements to be used as target cells in gene therapy due to their capacity of self-renewal, their easy obtention, and the fact that they can survive, and even expand, in *ex vivo* cultures and can be handled and transplanted relatively easily. For that reasons, HSCs are particularly suited for the correction of genetic defects that affect the hematopoietic system. Evolution of human HSC gene therapy is shown in Figure 7.

The first genetic disease treated with gene therapy was a type of SCID due to adenosine deaminase deficiency (ADA-SCID) which is characterized by systemic toxicity due to the accumulation of purine metabolites and defects in survival and function of T, B and NK cells. The first clinical trials in the early 90s were based on the infusion of genetically corrected lymphocytes or HSCs (Blaese, Culver et al. 1993; Blaese, Culver et al. 1995; Bordignon, Notarangelo et al. 1995; Kohn, Weinberg et al. 1995; Kohn, Hershfield et al. 1998). However, it was not until 2000 when the first great success of

gene therapy was reported by the French group of Marina Cavazzana-Calvo and Alain Fischer for X-linked SCID (SCID-X1) disease (Cavazzana-Calvo, Hacein-Bey et al. 2000). SCID-X1 is characterized by mutations in the common gamma chain (γc) of different cytokine receptors that block the differentiation and function of T and NK cells. Using a retroviral vector, functional versions of the γc gene were introduced into $CD34^+$ cells from patients, which were then reinfused without prior conditioning. Nowadays more than 20 patients have been successfully treated in France and the United Kingdom (Hacein-Bey-Abina, Le Deist et al. 2002; Gaspar, Parsley et al. 2004; Hacein-Bey-Abina, Hauer et al. 2010; Gaspar, Cooray et al. 2011).

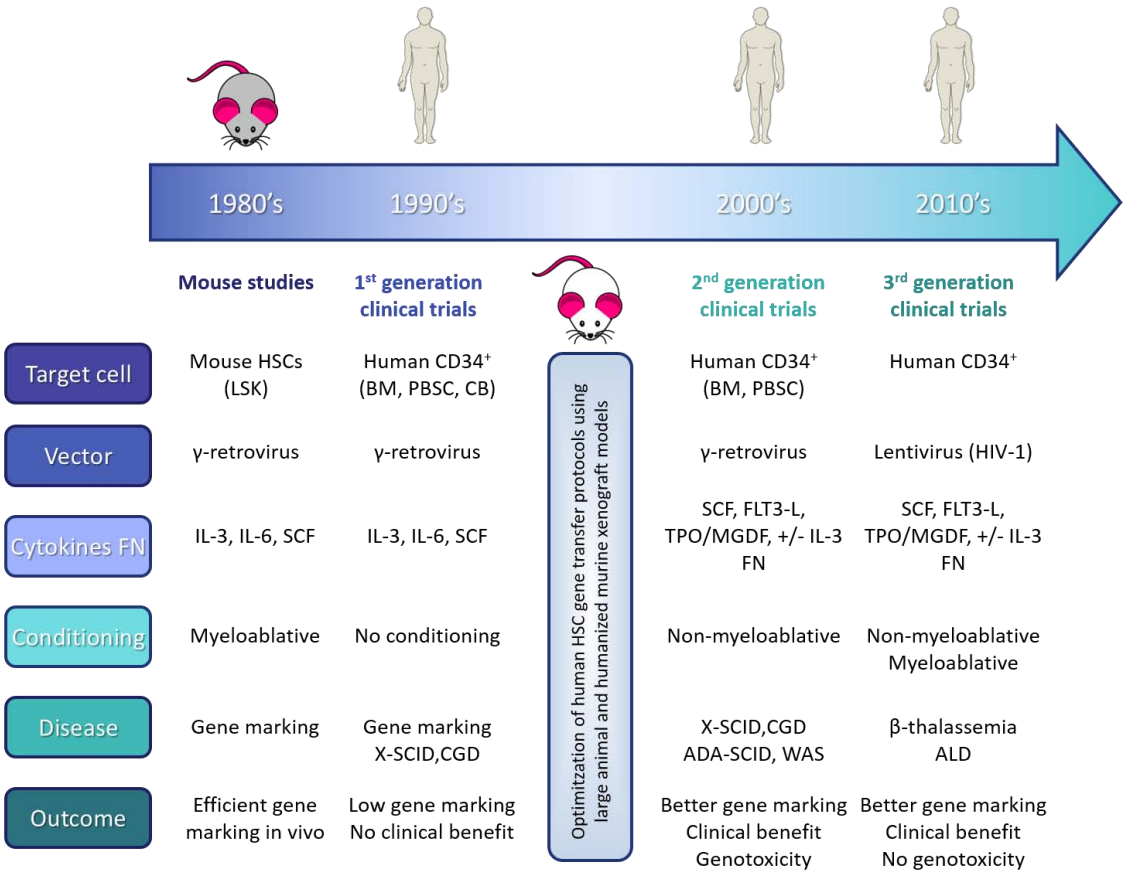


Figure 7. Timeline of the development of human HSC gene therapy. The main phases in the evolution of HSC gene therapy clinical trials are ordered chronologically. The six principal features defining each phase are outlined. ALD: adrenoleukodystrophy; CGD: chronic granulomatous disease; FLT3-L: fms-related tyrosine kinase 3 ligand; FN: fibronectin; HIV: human immunodeficiency virus; IL-3: interleukin 3; MGDF: megakaryocyte growth and development factor; SCF: stem cell factor; TPO: thrombopoietin. Figure adapted from (Larochelle and Dunbar 2013).

The second great success of gene therapy for genetic diseases was published in 2002 by an Italian group (Aiuti, Slavin et al. 2002) again in children with ADA-SCID. Unlike in the previous clinical trial, patients received low intensity conditioning prior to transplantation with genetically modified autologous HSCs and polyethylene glycol (PEG)-ADA was withdrawn. The authors postulated that the continuous administration of PEG-ADA prevented the potential selective advantage of the corrected cells. Since then, more than 40 patients have been successfully treated in Italy, the United Kingdom, Japan and the United States of America (Aiuti, Cattaneo et al. 2009; Gaspar, Cooray et al. 2011; Candotti, Shaw et al. 2012; Montiel-Equihua, Thrasher et al. 2012). Moreover, GlaxoSmithKline has recently announced the commercialization of a gene therapy for ADA-SCID. Besides SCID-X1 and ADA-SCID, other hematopoietic diseases have been successfully treated with gene therapy as X-linked chronic granulomatous disease (Kang, Choi et al. 2010), WAS (Boztug, Schmidt et al. 2010; Aiuti, Biasco et al. 2013; Hacein-Bey Abina, Gaspar et al. 2015) and β-thalassemia (Cavazzana-Calvo, Payen et al. 2010).

3.5. Gene therapy genotoxicity

One of the major risks of gene therapy based on integrative vectors is insertional mutagenesis. Retroviral vectors have the potential risk of oncogenesis by insertion, either by activation or overexpression of oncogenes or by inactivation of tumor suppressor genes, which is intrinsic to its semi-random integration preferences.

Despite the absence of any reported toxic effect in all the previous studies in animal models (mice, dogs and monkeys) using retroviral vectors, insertional mutagenesis occurred in the first SCID-X1 clinical trial, leading to T-cell leukemia. Genetic analysis of malignant cells showed integration of the retroviral vector near the regulatory region of the proto-oncogene LIM domain only 2 (LMO2) (Hacein-Bey-Abina, Von Kalle et al. 2003). So far, six of the 20 patients have developed T-cell leukemia (Hacein-Bey-Abina, Garrigue et al. 2008; Howe, Mansour et al. 2008) and had one or two provirus integrations within LMO2 locus. LMO2-associated leukemic event was also observed in four of nine WAS treated patients (Boztug, Schmidt et al. 2010). It is noteworthy that

despite the use of a similar gene transfer technology in the ADA-SCID trials, none of the successfully treated patients developed leukemia (Aiuti, Cattaneo et al. 2009; Ferrua, Brigida et al. 2010; Gaspar, Cooray et al. 2011; Candotti, Shaw et al. 2012).

Many efforts have been made to improve the safety of integrating retroviral vectors. A key advance has been the creation of self-inactivating (SIN) vectors in which the promoter and enhancer elements in their LTR are eliminated. For this reason these vectors must contain an additional internal promoter to drive transgene expression. This type of vector has been shown to be less genotoxic in *in vitro* assays (Yu, von Ruden et al. 1986; Cattoglio, Pellin et al. 2010; Kustikova, Brugman et al. 2010). The use of HIV-derived lentiviral vectors might constitute an additional safeguard, since this type of vector does not have a tendency to integrate close to the transcription start site. Clinical trials using SIN vectors have recently started for primary immunodeficiencies with promising results reporting stable and high-level hematopoietic reconstitution and with no genotoxicity effects [reviewed in (Naldini 2015)]. However, there are other aspects that have to be taken into consideration as the type of transgene, the transduction protocol (cytokines, medium and ratio of viruses per cell), cell dose administered to the patient, patient age and the tendency of the patient itself to develop leukemia that may have favored the development of malignancies.

3.6. Gene therapy and immune response

Besides the risks of gene therapy discussed in the previous section there is another major problem in gene therapy, the potential immunogenicity of the vector or the transgene product. In HSC gene therapy transgene products constitute a source of potential antigens as the proteins encoded by the therapeutic gene may contain epitopes that the patient immune system has never “seen” before and, therefore, do not recognize them as self. In fact, immune responses to transgene products or to cells expressing transgenes have been reported in several preclinical settings (Yuasa, Sakamoto et al. 2002; Gao, Lebherz et al. 2004) as well as in some gene therapy clinical trials (Traversari, Marktel et al. 2007; Zaldumbide and Hoeben 2008). Immunogenicity

of transduced cells is determined or influenced by many factors, including the route of entry, the molecular structure of the transgene product and antigen dose (Eixarch, Gomez et al. 2009). Factors related to the patient itself as the level of immunocompetence or immune suppression, the genetic background and the histocompatibility molecules may also influence the immune response against the transgene.

On the other hand, it is well accepted that under certain conditions transferring gene modified HSCs can induce tolerance to the specific transgene, thus saving the problem of transgene immunogenicity. This last aspect is unique to the hematopoietic system as a therapeutic target, since no other tissue, except perhaps the liver, has the capacity to create tolerance. Therefore, it is plausible to use gene therapies based on HSCs to induce tolerance in situations such as autoimmunity (Eixarch, Espejo et al. 2009) and transplantation (Uchida, Weitzel et al. 2014).

4. IMMUNE TOLERANCE

Immune tolerance is defined as specific unresponsiveness to an antigen, either self or foreign. When specific lymphocytes encounter antigens, they may become activated leading to an immune response, or may be inactivated or eliminated leading to tolerance. Tolerance to foreign antigens is important to avoid immune reactions to the food we eat or to the particles we inhale. When this tolerance is disrupted for certain antigens, one suffers allergic processes when comes into contact with them. On the other hand, tolerance to self-antigens prevents the immune system to react against these antigens, to trigger an immune response against them and, consequently, to produce an autoimmune disease.

Tolerance to self-antigens, also called self-tolerance, is a fundamental property of the normal immune system which is acquired very early, during the development of the immune system. This concept was evidenced by Peter Medawar in the 1950s when he elegantly demonstrated that tolerance to donor tissues was associated to allogeneic chimerism when induced neonatally (Billingham, Brent et al. 1953)(Figure 8).

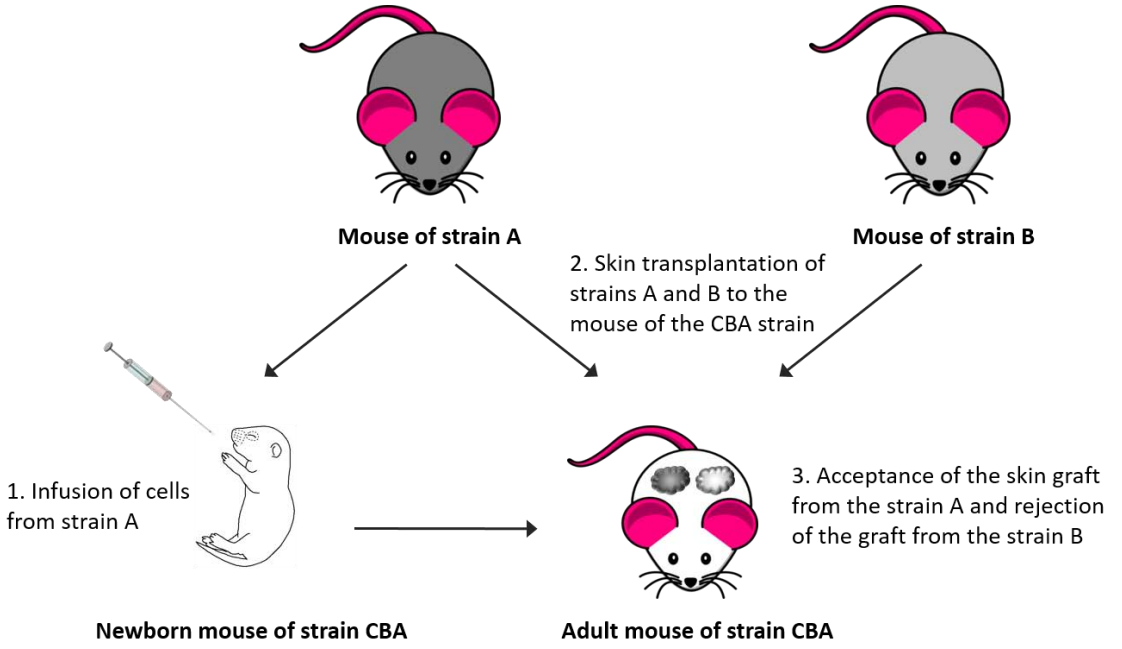


Figure 8. Diagram of the experiment carried out by Medawar and colleagues. Cells from the strain A were injected to newborn mice from the strain CBA. When they reached adulthood these received skin grafts from the allogeneic strains A and B. The skin graft from the B strain was rejected while the one from the A strain was tolerated by the recipients.

4.1. Antigen presentation

The immune system can be classified into two general systems, the innate or natural immune system and the adaptive or acquired immune system. Defence against microbes is mediated by the early and rapid reactions of innate immunity and the later but potent responses of the adaptive immunity. The main components of adaptive immunity are lymphocytes and their secreted products, such as antibodies. Substances that induce specific immune responses are called antigens. To induce an adaptive immune response, cells present antigens on their surface through the MHC molecules so that these are specifically recognized by the T-cell receptor (TCR).

Antigens can be lipopolysaccharides (LPS), lipoproteins, glycolipids or, usually, proteins. T lymphocytes specific for one antigen do not recognize it in its soluble form, they only recognize antigenic peptides when they are bound to the MHC molecules and presented by cells. The MHC is a region of highly polymorphic genes whose products are expressed on the surface of different cell types. MHC was discovered as a

system that determined the outcome of the transplanted tissues but now it is known that the physiological function of MHC is the presentation of peptides to T lymphocytes. There are two main types of MHC molecules, the MHC class I and class II. Class I molecules are expressed on the surface of all nucleated cells and present intracellular peptides to $CD8^+$ cytotoxic T lymphocytes. In contrast, class II molecules are expressed only in specialized cells called antigen presenting cells (APCs), such as dendritic cells (DCs), macrophages and B lymphocytes, and present extracellular antigens, that have been previously endocyted, to $CD4^+$ T helper (Th) lymphocytes.

4.1.1. Endogenous (intracellular) antigens: class I pathway

Class I molecules consist of two noncovalently linked polypeptide chains, a heavy chain encoded by the MHC genes called α chain and a light chain encoded outside the MHC loci called β_2-microglobulin. The fully assembled class I molecule is a heterotrimer consisting of an α chain, a β_2-microglobulin and a bound antigenic peptide. Stable expression of MHC-I molecules on cell surface requires all three components. The antigenic peptides that are presented by class I pathway derive from cytosolic proteins synthesized inside the cells. These strange peptides are usually products of intracellular pathogens such as viruses or, in the case of tumoral cells, aberrant products of oncogenes.

In the cytoplasm, intracellular proteins are degraded by the proteasome into small antigenic peptides of 8 to 11 amino acids. These proteins are targeted for proteosomal degradation by ubiquitination. These small peptides are then transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) to bind to the molecules of the MHC (synthesized in the ER). The TAP protein is located in the ER membrane where it mediates the active transport of peptides from the cytosol into the ER lumen. Inside the ER, the newly formed class I molecules remain attached to the TAP complex. When the peptide enters the ER through the TAP, it binds to the class I molecule. Then the MHC-I loaded with the peptide is released and transported to the cell membrane to present the antigen on the surface of the cell (Figure 9a). Through its receptor (TCR), the $CD8^+$ T cells recognize specifically

the MHC-I/antigen complex and, with the adequate co-stimulatory signals, they are activated triggering the so called cytotoxic T-cell response that results in the lysis of the cell which is presenting the recognized antigen.

4.1.2. Exogenous (extracellular) antigens: class II pathway

Class II molecules are composed of two noncovalently associated polypeptide chains, an α chain and a β chain. Unlike class I molecules, the genes encoding both chains of class II molecules are polymorphic and present in the MHC locus. Like class I molecules, MHC-II is a heterotrimer composed of an α chain, a β chain and a bound antigenic peptide. Its stable expression on the cell surface needs the presence of all three components. The antigenic peptides that are presented by class II pathway derive from extracellular or exogenous antigens that have been endocyted by APCs in peripheral tissues. These antigens usually come from pathogens (bacteria or parasites) and toxins, among others. In contrast to class I molecules that are expressed in virtually all the cells of the organism, class II molecules are only expressed in APCs either constitutively, as in DCs or B lymphocytes, or upon induction by interferon-γ (IFN-γ) as in macrophages.

MHC class II molecules are synthesized in the ER and are linked to a polypeptide called the invariant chain (Ii or CD74), which stabilizes the complex and prevents the premature and inappropriate binding of antigens. The MHC-II/Ii complex is transported from the Golgi to an antigen processing compartment that has characteristics of both endosomes and lysosomes. Acidic endosomes with exogenous peptides, where they have been partly degraded by proteases and chaperone proteins, fuse with the compartment (Cresswell 1994). Then the Ii chain is gradually cleaved by cathepsins into small fragments until there is only a small piece attached to the MHC-II groove. This small fragment, called CLIP (class II-associated invariant peptide), is finally released by the interaction of the HLA-DM molecule (in humans) or by the H2-M (in mice) leaving the groove of the MHC-II empty so that the antigenic peptides can bound to it and be presented on the surface of the cell (Neefjes, Stollorz et al. 1990; Roche and Cresswell 1991) (Figure 9b). These special features of the Ii have been used to

direct sequences of interest (e.g. those encoding for antigenic peptides) towards the class II compartment, thereby ensuring an efficient presentation by this route of antigen presentation (Carstens, Newman et al. 2000; Bischof, Wienhold et al. 2001). In fact, our laboratory used this technique to successfully induce immunological tolerance in a mouse model of MS (Eixarch, Espejo et al. 2009). Through its TCR, $CD4^+$ T cells specifically recognize the MHC-II/antigen complex, but they are only activated if they receive the appropriated co-stimulatory signals.

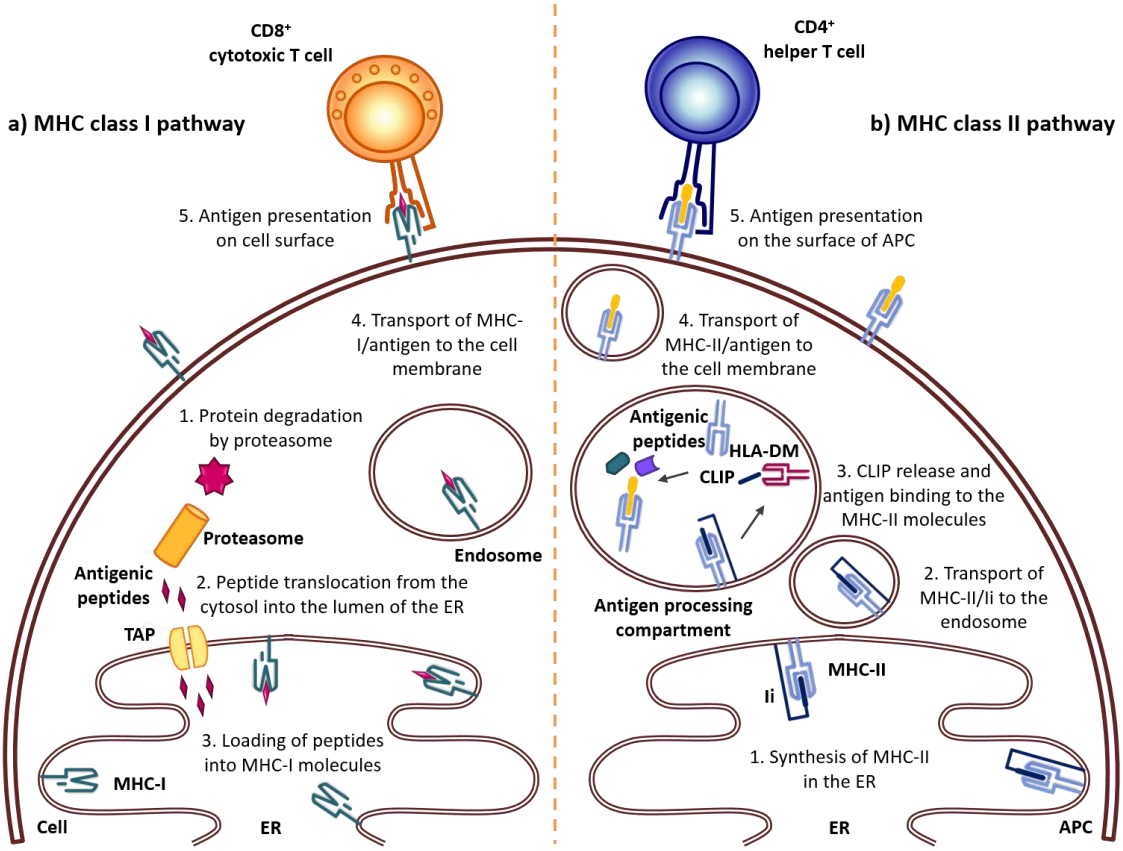


Figure 9. Class I and class II antigen presentation pathways. See text.

4.2. Tolerance mechanisms

There are several mechanisms of tolerance depending on the place of occurrence. A primary mechanism called central tolerance operates in the generative organs, the thymus and BM before the maturation and circulation of T and B cells respectively. However, not all antigens that lymphocytes need to be tolerant to are expressed in

these organs, and thus central tolerance mechanisms alone are not sufficient. Fortunately, there is an additional tolerance mechanism called peripheral tolerance that act on mature circulating T and B cells, which eliminates remaining autoreactive lymphocytes.

4.2.1. Central tolerance in T lymphocytes

Immature T cells generated in the BM migrate to the thymus for their maturation. These immature thymocytes are called double-negative thymocytes as do not express either CD4 or CD8 molecules. Thymocytes at this stage are considered to be at the proT-cell stage of maturation. First, the rearrangement of the TCR genes takes place to generate the repertoire of T-cell clones of each individual. At the next stage of maturation, proT lymphocytes express both CD4 and CD8 co-receptors forming a double-positive population. TCR expression in the double-positive stage leads to the formation of the complete αβ TCR, which is expressed on the cell surface in association with CD3 and ζ proteins. In the maturation process, about 90-95% of the immature lymphocytes die due to the aberrant TCR gene rearrangement or to the processes of positive and negative selection to which they are exposed during maturation in the thymus.

- **Positive selection**

In the thymic cortex, these immature cells encounter epithelial cells that are displaying a variety of self-peptides bound to self-MHC molecules. Positive selection is the process by which lymphocytes able to recognize these self-peptide/self-MHC complexes with low affinity are selected to survive, while lymphocytes incapable of recognizing self-MHC molecules die by apoptosis due to the lack of appropriate survival signals; this phenomenon is called death by neglect. In this recognition process T cells become committed to the $CD4^+$ (helper lymphocytes) or $CD8^+$ (cytotoxic lymphocytes) lineage based on whether its TCR respectively recognizes MHC class II or MHC class I molecules. Thus, positive selection ensures that T cells are self-MHC restricted but this process does not discriminate autoreactive lymphocytes.

- **Negative selection**

Negative selection is the process by which thymocytes whose TCRs recognize peptide/MHC complexes with high affinity are eliminated by apoptosis (Figure 10A). The deletion of immature self-reactive T cells may occur both at the double-positive stage in the cortex and in committed T cells in the medulla, although it is this last one which seems to be specialized in this process. The thymic medullary cells that mediate negative selection include hematopoietic (DCs, macrophages and B cells) and epithelial cells (Kyewski and Klein 2006). Thymic medullary epithelial cells (mTECs) express a transcription factor called AIRE (autoimmune regulator) that induces the expression of a number of tissue-specific genes in the thymus that otherwise are only expressed in specific peripheral organs, making many tissue-specific peptides available for presentation to developing T cells. A mutation in the gene that encodes AIRE leads to tissue-specific autoimmune diseases (Anderson, Venanzi et al. 2002). The mechanism of negative selection in the thymus is the induction of apoptosis. Unlike the phenomenon of death by neglect, which occurs in the absence of positive selection, in this process active death-promoting signals are generated when the TCR of immature thymocytes binds with high affinity to self-peptide/self-MHC complexes (Allen 1994; Alam, Travers et al. 1996) (Figure 10A). However, immature lymphocytes with low affinity for these complexes can escape the negative selection, so there must be other mechanisms of tolerance in the periphery to control the potentially autoreactive clones which escape from the thymic negative selection. In fact, a population of AIRE-expressing cells able to present autoantigens that are not presented in the thymus and capable of eliminating autoreactive naïve T cells has been identified in the peripheral lymphoid organs (Gardner, Devoss et al. 2008; Poliani, Kisand et al. 2010).

Although clonal deletion is the main mechanism of central tolerance, antigen presentation by mTECs also promotes the development of specific regulatory T (Treg) cells by recognition of self-antigens at intermediate affinity. Once in the periphery, these cells modulate other immune cells by inhibiting the proliferation and effector function of autoreactive T cells (Itoh, Takahashi et al. 1999; Baldwin, Hogquist et al. 2004; Aschenbrenner, D'Cruz et al. 2007) (Figure 10A).

4.2.2. Peripheral tolerance in T lymphocytes

Peripheral tolerance is the mechanism by which mature T lymphocytes that recognize self-antigens and have escaped central tolerance are eliminated or become unresponsive to these antigens. Peripheral tolerance can be due to anergy, suppression of self-reactive lymphocytes and deletion (Figure 10B). The same mechanisms may induce unresponsiveness to tolerogenic forms of foreign antigens.

- **Anergy (functional unresponsiveness)**

Full activation of T cells requires at least two signals: the recognition of the antigen by the TCR (signal 1) and recognition of co-stimulatory molecules, mainly the B7-1 (CD80) and B7-2 (CD86), present in the surface of APCs by the CD28 receptor (signal 2). Anergy is the process that occurs when the lymphocyte is activated in an incomplete form. In other words, the lymphocyte recognizes the antigen/MHC complex but it does not receive the second co-stimulatory signal necessary for activation (Appleman and Boussiotis 2003). T cells can also become anergic if they recognize antigens with little affinity (Alam, Travers et al. 1996). Apart from not receiving the second stimulatory signal, T cells may become anergic by engaging inhibitory receptors of the CD28 family, whose function is to inhibit T-cell responses. Among the many inhibitory receptors that have been described, the best established ones are cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1). The binding of the CD80/CD86 molecules on the APCs to CTLA-4 instead of CD28 and the binding of PD-1 to its ligands, the programmed death-1 ligand-1 (PD-L1) and the programmed death-1 ligand-2 (PD-L2), leads to T-cell anergy [reviewed in (de la Fuente, Cibrian et al. 2012; McGrath and Najafian 2012; Dai, Jia et al. 2014)].

- **Suppression of self-reactive lymphocytes by Treg cells**

Suppression of self-reactive lymphocytes is a mechanism conducted by Treg cells and usually requires cell-cell contact. Treg cells differ from the rest of T-cell subtypes by the simultaneous expression of CD4, CD25 and the transcription factor forkhead box P3 (FoxP3), which plays a crucial role in the generation of these cells. It was previously thought that Treg cells could only arise from the thymus, however, it is now widely

accepted that they can also develop in the periphery from naïve $CD4^+$ T cells (O'Garra and Vieira 2004; Bilate and Lafaille 2012). Thus, thymus-derived Treg cells are known as natural Treg (nTreg) cells, and those that are extrathymically derived are known as adaptative or induced Treg (iTreg) cells. The most common type of iTreg is the T regulatory type 1 (Tr-1) population. Tr-1 cells are distinguished by their unique cytokine profile. They secrete high amounts of interleukin 10 (IL)-10, a cytokine with anti-inflammatory and immunosuppressive activity, and transforming growth factor β (TGF-β), low levels of IL-2, IL-5 and IFN-γ and no IL-4 (Roncarolo, Gregori et al. 2006; Fujio, Okamura et al. 2010). There is no specific marker for Tr-1 cells although CD49b and lymphocyte-activation gene 3 (LAG-3) has recently been proposed as specific markers of the Tr-1 cell population (Gagliani, Magnani et al. 2013). FoxP3 is not a marker of Tr-1 cells as its expression is low and transient after activation (Roncarolo, Gregori et al. 2011). Another T-cell population that has been shown to have regulatory function includes inducible $CD8^+$ Treg cells, they are well characterized in rodents but not in humans (Ke and Kapp 1996; Kapp and Bucy 2008).

- **Deletion by apoptosis**

In addition to deletion of self-reactive lymphocytes by negative selection at thymic level, T lymphocytes can also be eliminated in the periphery by the expression of molecules such as CTLA-4 or Fas and its ligand (FasL) that promote T-cell apoptosis. T lymphocytes that recognize self-antigens without co-stimulation or that are repeatedly stimulated by antigens undergo apoptosis. Exposure to high concentrations of antigen was also described as a mechanism of deletion in the periphery (Ferber, Schonrich et al. 1994).

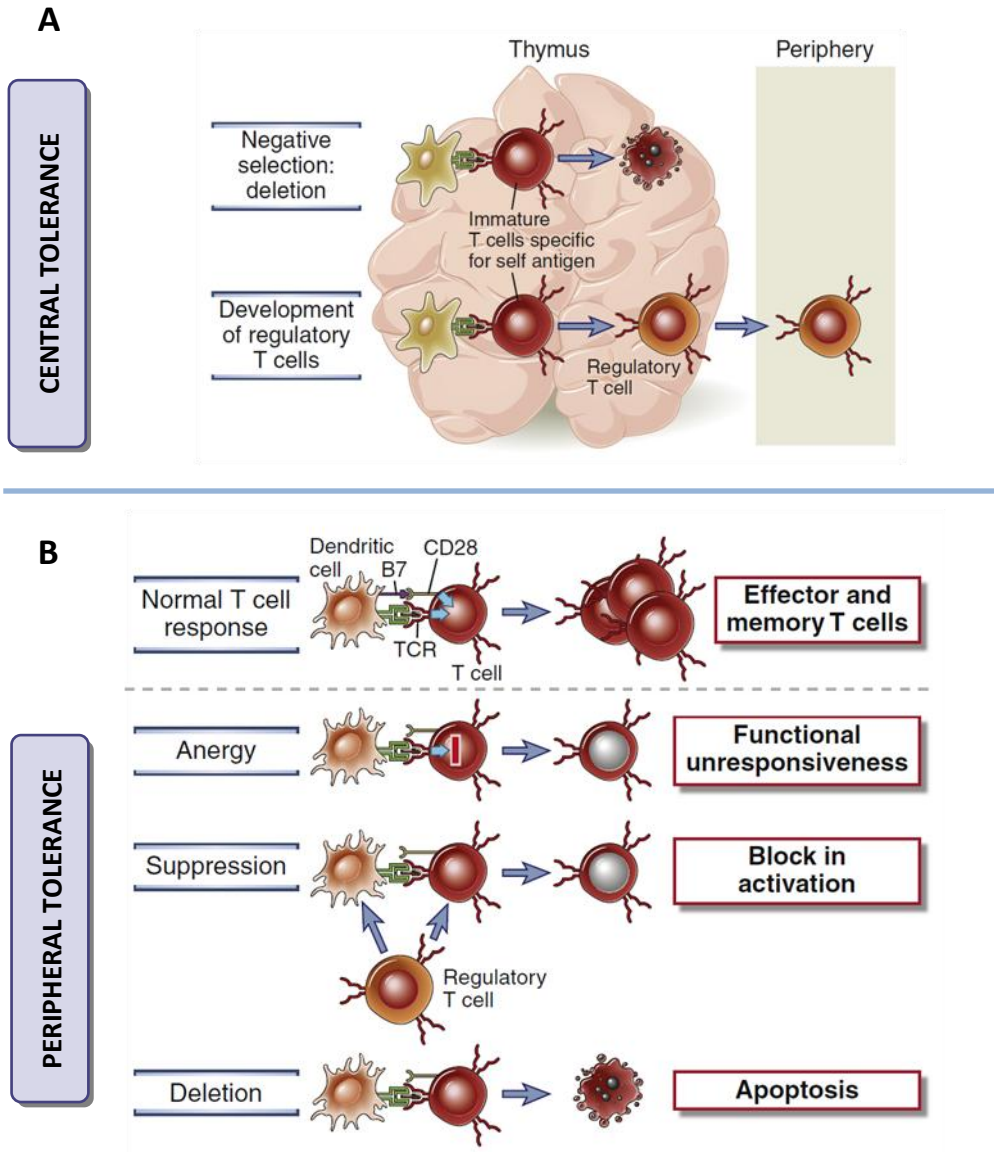


Figure 10. Mechanisms of central and peripheral T-cell tolerance. A) Central T-cell tolerance. Recognition of self-antigens with high affinity by immature T cells in the thymus leads to the death of the cells by apoptosis (negative selection). Some $CD4^+$ T cells that recognize self-antigens in the thymus are not eliminated and instead differentiate into Treg cells that enter peripheral tissues. **B)** Peripheral T-cell tolerance. The signals involved in a normal immune response and the three major mechanisms of peripheral T-cell tolerance (anergy, suppression by Treg cells and deletion) are shown. Figure adapted from Abbas, Lichtmanand and Pillai. Cellular and Molecular Immunology. Publisher: Saunders. Eighth edition (2014).

4.2.3. B cell tolerance

B cells are generated and developed in the BM. Tolerance in B lymphocytes is necessary for maintaining unresponsiveness to thymus-independent self-antigens, such as polysaccharides and lipids.

- **Central tolerance**

In the BM, once immature B lymphocytes express antigen receptors, these cells are subjected to both positive and negative selection processes. In positive selection, lymphocyte precursors with antigen receptors that bind to self-antigens with low affinity are selected to survive and continue to mature in peripheral lymphoid tissues. In negative selection, lymphocytes that bind to self-antigens with high affinity receive signals that lead to cell death by apoptosis or induce rearrangement of antigen receptor genes, a process known as receptor editing. If the editing fails, B lymphocytes are also deleted (Figure 11A).

- **Peripheral tolerance**

The process of central tolerance for B cells is less strict than the one for T cells; therefore, many B cells escape central tolerance and migrate to the periphery, where the most important mechanism of tolerance is anergy (Goodnow 1992). Mature B lymphocytes that recognize self-antigens in peripheral tissues in the absence of specific Th lymphocytes, which involves absence of co-stimulatory signals, may enter anergy or die by apoptosis. B cells that recognize self-antigens with low affinity may also be prevented from responding by the interaction with inhibitory receptors (Figure 11B).

Similar to Treg cells, there is a population of B cells with regulatory profile. These regulatory B (Breg) cells, also called B10 cells, suppress pathogenic and self-reactive T and B cells through the production of IL-10 and TGF-β and the expression of inhibitory molecules (Mizoguchi and Bhan 2006; Lundy 2009). The exact origin and molecular identity of Breg cells remain unclear; however, recent evidence suggests that they can be derived from all B cells under the correct stimulatory context. Antigen and B-cell receptor (BCR) signalling are critical in their early development, although additional

stimuli such as CD40 ligation, IL-21 and Toll-like receptor (TLR) ligands appear to be involved in their developmental process (Gray and Gray 2010; Kalampokis, Yoshizaki et al. 2013; Tedder and Leonard 2014). In mice, Breg cells have been identified as $CD1d^{high}CD5^{+}$ IL-10-producing population (Yanaba, Bouaziz et al. 2008; Tedder 2015) but in humans it still remains elusive. However, human IL-10-producing B cells have been found within the transitional $CD38^{+}CD24^{+}$ B-cell population and in the $CD27^{+}$ memory cell pool (Blair, Norena et al. 2010).

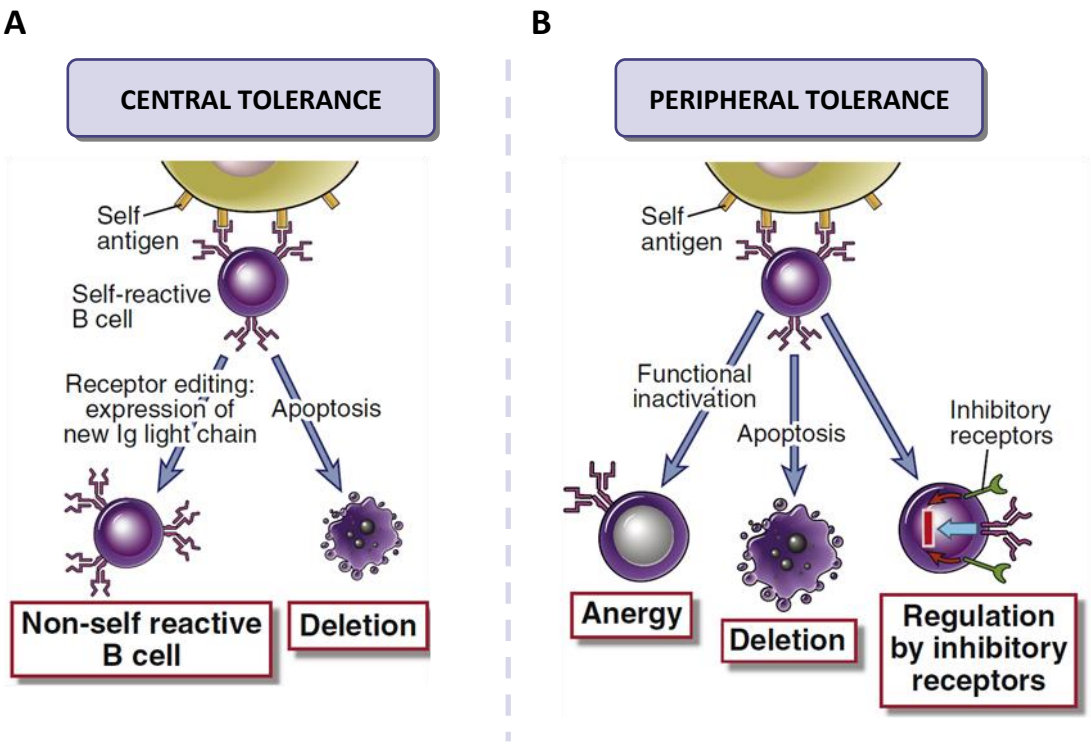


Figure 11. Mechanisms of central and peripheral B-cell tolerance. A) Central B-cell tolerance. Recognition of self-antigens with high affinity by immature B cells in the BM die by apoptosis (negative selection) or undergo receptor editing. **B)** Peripheral B-cell tolerance. B cells that recognize self-antigens in peripheral tissues become anergic, die by apoptosis or remain inactive through the signals of inhibitory receptors. Figure adapted from Abbas, Lichtmanand and Pillai. Cellular and Molecular Immunology. Publisher: Saunders. Eighth edition (2014).

4.3. Breakdown of immunological tolerance: autoimmunity

Despite the existence of central and peripheral tolerance mechanisms that keep the immune system under control so that it does not react to self-antigens, these

mechanisms can fail leading to autoimmune diseases such as SLE, RA, MS and type 1 diabetes (T1D) among others.

Interaction of genetic determinants and environmental influences can induce tolerance breakdown and development of pathogenic autoreactive T or B cells in susceptible individuals. It has been shown that the level of expression of autoantigens in the thymus is an important factor in developing tolerance to them (Liston, Lesage et al. 2005; Taubert, Schwendemann et al. 2007). Moreover, not all antigens are presented in the thymus; some late-developing antigens or antigens confined to specialized organs (e.g. testis or brain) may not be exposed to developing lymphocytes. Under certain circumstances, a release of antigens from these organs by tissue damage can lead to an immune response and autoimmunity. Antigens from certain pathogens can cross react with self-antigens due to its structural similarity, called molecular mimicry, and therefore trigger an autoimmune response. For example, coxsackie virus has been implicated in T1D (Kaufman, Erlander et al. 1992) and several putative mimicry peptides have also been proposed that may trigger specific central nervous system (CNS) autoimmune responses such as Epstein Barr Virus (EBV) (Fourneau, Bach et al. 2004; Berer and Krishnamoorthy 2014)

5. MYELOID-DERIVED SUPPRESSOR CELLS

In addition to regulatory T and B cells, there are other regulatory cells of myeloid origin which include MDSCs, tolerogenic DCs, type 2 or M2 tumor-associated macrophages and type 2 or N2 tumor-associated neutrophils (Keskinov and Shurin 2015). These regulatory myeloid cells are both a therapeutic target, for example in cancer where an enhancement in the immune response against the tumor is needed, and a therapeutic tool used to promote immune tolerance in transplantation and autoimmune diseases (Nagaraj, Collazo et al. 2009).

MDSCs comprise a heterogeneous population of immature cells of myeloid origin with potent immunosuppressive activity. MDSCs were first described more than 20 years ago in patients with cancer and since then MDSCs have been broadly studied in many

pathological conditions. In healthy individuals, immature myeloid cells (IMCs) that are generated in the BM migrate to the peripheral organs where they differentiate into mature macrophages, DCs or granulocytes. However, factors that are produced during pathological conditions such as acute or chronic infections, trauma and in the tumor microenvironment promote the accumulation of IMCs at these sites, block their differentiation and induce their expansion and activation (Gabrilovich and Nagaraj 2009)(Figure 12). In mice these cells are broadly defined by the co-expression of CD11b and Gr-1 markers (Bronte, Wang et al. 1998; Kusmartsev and Gabrilovich 2002). However, these cells are not a homogeneous population. More recently two main subpopulations were described based on their differential expression of the two epitopes of the Gr-1: Ly6G and Ly6C (encoded by different genes). Thus, we can distinguish between granulocytic MDSCs (G-MDSCs) with the phenotype $CD11b^{+}Ly6G^{+}Ly6C^{low}$ or $CD11b^{+}Gr\text{-}1^{high}$ and monocytic MDSCs (M-MDSCs) with the phenotype $CD11b^{+}Ly6G^{-}Ly6C^{high}$ or $CD11b^{+}Gr\text{-}1^{low}$ (Youn, Nagaraj et al. 2008; Ostrand-Rosenberg and Sinha 2009).

In contrast to murine MDSCs, the phenotype to clearly identify human MDSCs still remains elusive due to the absence of a homolog Gr-1 gene in humans. Different phenotypes have been reported for MDSCs depending on the disease, the type of cancer and the anatomic site. Nevertheless, it seems that a consensus is emerging to define human MDSCs as $CD33^{+}CD11b^{+}HLA\text{-}DR^{low/-}$, with M-MDSCs being $CD14^{+}CD15^{low/-}$ and G-MDSCs $CD14^{-}CD15^{+}CD66b^{+}$, which looks consistent with their morphology (Serafini 2013; Gantt, Gervassi et al. 2014).

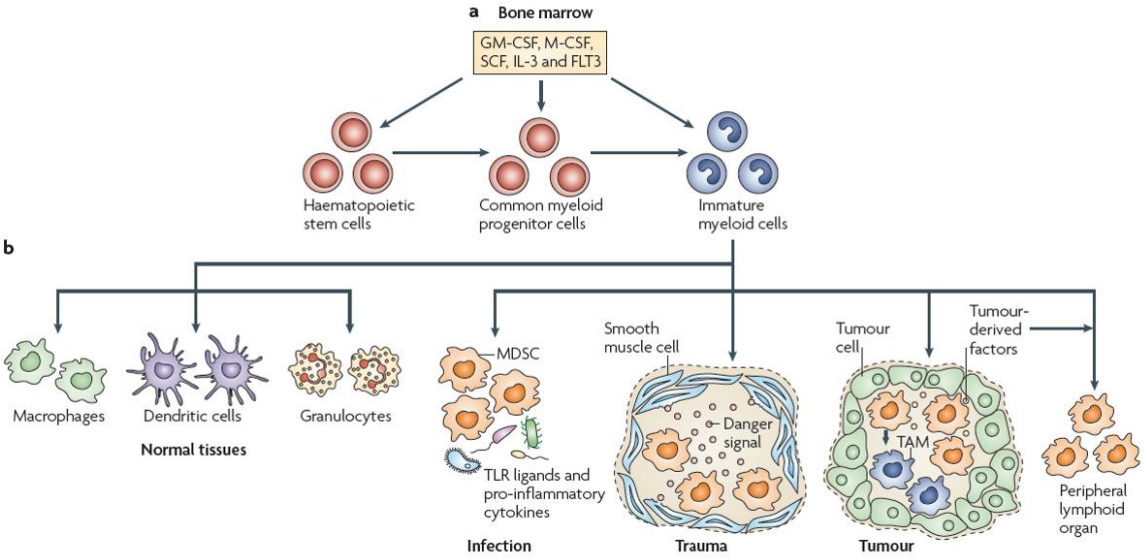


Figure 12. Origin of MDSCs. See text. Figure from (Gabrilovich and Nagaraj 2009).

5.1. Mechanisms of MDSCs immune suppression

MDSCs are able to suppress T-cell function both by antigen-specific and non-specific mechanisms. Many studies have shown that the immunosuppressive activities of MDSCs require direct cell-cell contact, which suggest that they exert their function either through cell surface receptors such as PD-L1 (Ioannou, Alissafi et al. 2011; Noman, Desantis et al. 2014) and CD80/CD86 as well as through the release of short-lived immune suppressive soluble factors as arginase-1, inducible nitric oxide synthase (iNOS), nitric oxide (NO) and reactive oxygen species (ROS) (Gabrilovich and Nagaraj 2009).

Both arginase-1 and iNOS are enzymes that metabolize arginine and convert it to urea and L-ornithine. The high production of arginase-1 by MDSCs upon activation leads to the depletion of this non-essential amino acid from the microenvironment. Arginine deprivation inhibits T-cell proliferation through decreasing the expression of the ζ chain of the CD3 which is necessary for the correct assembly and signal transduction of the TCR (Rodriguez, Zea et al. 2002). In addition to ζ chain down-regulation, the absence of arginine arrests T cells in G_0-G_1 phase of the cell cycle by preventing the up-regulation of cyclin D3 and cdk4 and increasing cdk6 expression (Rodriguez, Quiceno et

al. 2007). iNOS produced by MDSCs converts L-arginine to citrulline and finally to NO, which impairs T-cell activation by inhibiting the Janus kinase/signal transducer and activator of transcription (Jak/STAT) signalling cascade downstream the IL-2 receptor, reducing MHC-II expression and inducing T-cell apoptosis. Because T cells lack the enzymes and transporters necessary for the cysteine metabolism, T cells depend on APCs as an essential source of this amino acid. MDSCs can also reduce the available extracellular cysteine and thus prevent T-cell activation. In addition to amino acid starvation, another important mechanism in suppressing T-cell responses is the production of ROS. ROS and peroxynitrite produced by MDSCs cause the loss of the ζ chain of the CD3 and induce the nitration of the TCR resulting in conformational changes and in an impaired TCR/MHC-peptide recognition, leaving T cells unresponsive to antigen-specific stimulation (Gabrilovich and Nagaraj 2009; Serafini 2013; Gantt, Gervassi et al. 2014) (Figure 13).

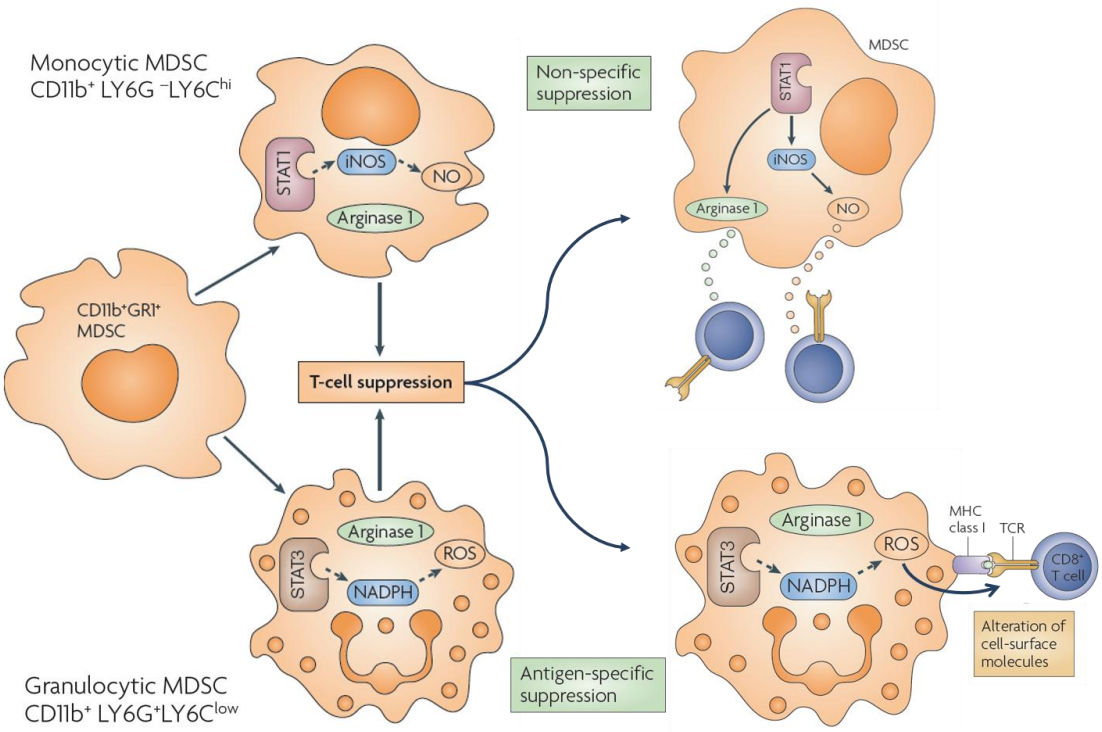


Figure 13. Suppressive mechanisms mediated by M- and G-MDSCs. G-MDSCs produce high levels of ROS and low levels of NO due to the increased activity of STAT3 and NADPH. ROS and peroxinitrite induce conformational changes of TCRs leading to antigen-specific T-cell unresponsiveness. M-MDSCs have increased expression of STAT1 and iNOS which suppress T-cell function through the inhibition of Jak3, STAT5 and MHC-II leading to T-cell apoptosis. Both MDSC subsets suppress T-cell proliferation through L-arginine starvation through increased levels of arginase-1. Figure adapted from (Gabrilovich and Nagaraj 2009).

Moreover, in addition to the direct suppressive mechanisms of MDSCs, these cells may also have an indirect action on the inhibition of T-cell responses by promoting the development of iTreg cells. Recent findings suggest that MDSCs are able to induce the generation of iTreg cells *in vitro* and *in vivo* (Huang, Pan et al. 2006). However, studies are not consistent with the factors involved in iTreg differentiation. It seems that both the production of cytokines such as IFN-γ, IL-10 and TGF-β and direct cell-cell interactions through CD40 and CTLA-4 are required, although the induction of iTreg cells by MDSCs remains elusive (Gabrilovich and Nagaraj 2009; Nagaraj, Youn et al. 2013).

The nature of the immune suppression mediated by MDSCs is regulated by several factors: the subset of MDSC involved, the local microenvironment, the state of T-cell activation and the retrograde signalling provided to MDSCs from T cells. Recent reports have demonstrated that M- and G-MDSCs use different mechanisms of immune suppression. The immunosuppressive activity of M-MDSCs depends on the production of high levels of NO and immunosuppressive cytokines and low levels of ROS, resulting in an antigen-independent suppression. In contrast, G-MDSCs produce high levels of ROS and low levels of NO which require closer and prolonged cell-cell contact resulting in an antigen-specific suppression of both $CD8^+$ and $CD4^+$ T cells leading to tolerance (Nagaraj, Youn et al. 2013). However, some studies have also reported an antigen-specific suppression by M-MDSCs (Movahedi, Guilliams et al. 2008). The local microenvironment and the interaction of MDSCs with the different subsets of T lymphocytes can also define the type of immune suppression mediated by MDSCs. It has been reported that in peripheral lymphoid organs MDSCs only suppress antigen-specific T-cell responses whereas at the tumor site they can inhibit T cells by both antigen-specific and non-specific suppression (Corzo, Condamine et al. 2010) (Figure 14).

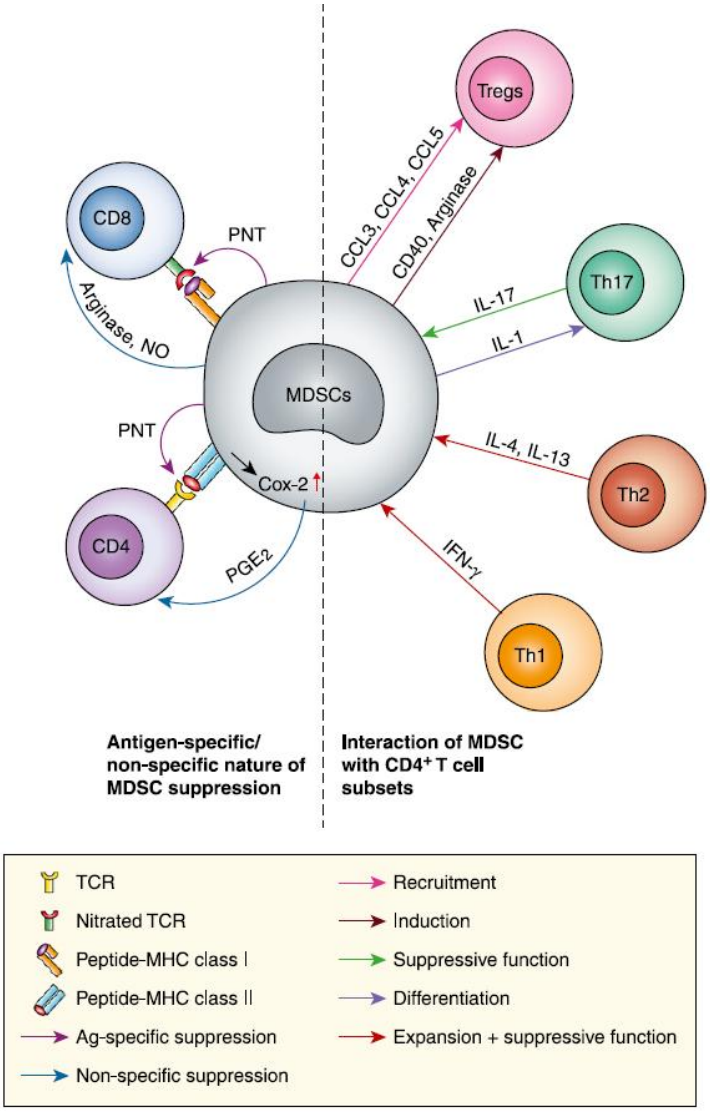


Figure 14. Complex interaction between MDSCs and the different subsets of T lymphocytes. The interaction between MDSCs and T cells define the nature of MDSC-mediated immune suppression and promote their expansion and activation. Ag: antigen; PNT: peroxynitrite; Cox-2: cyclooxygenase-2; PGE_2: prostaglandin E2. Figure from (Nagaraj, Youn et al. 2013).

5.2. Mechanisms of MDSCs generation, expansion and activation

In pathological conditions MDSCs are generated, expanded and activated in response to different factors that have partially overlapping activities. Two models have been proposed: the one-signal model and the two-signal model. In the one-signal model a single factor, usually granulocyte-macrophage colony-stimulating factor (GM-CSF), induces the differentiation, expansion and activation of MDSCs. In contrast, the two-signal model suggests that the generation of MDSCs can be divided into two processes,

one responsible for the expansion and the other for the activation of MDSCs. The process of expansion is driven by various cytokines and growth factors such as GM-CSF, macrophage colony-stimulating factor (M-CSF), G-CSF, IL-6 and vascular endothelial growth factor (VEGF) produced by tumors or BM stroma in response to chronic stimulation that activates the signalling pathways of STAT3 and STAT5. This type of signalling promotes de proliferation of IMCs while blocks their differentiation into mature cells. However, this model suggests that MDSCs need a second activating signal that induces the up-regulation of arginase-1, NO and the production of immunomodulatory cytokines. This activating signal is provided by proinflammatory factors such as IFN-γ, IL-1β, IL-4, IL-13, prostaglandin E2 (PGE2) and TLR ligands produced by activated T cells or tumor stromal cells that trigger the STAT1 and NF-ƙB signalling pathways as well as cyclooxygenase-2 (Cox-2) up-regulation (Condamine and Gabrilovich 2011).

MDSCs can be targeted for different clinical applications. As MDSCs are one of the main immunosuppressive factors that inhibit anti-tumor immune responses, several therapeutic strategies to reduce or eliminate these cells or their suppressive activity are currently being investigated. Agents such as sunitinib and gemcitabine have been reported to decrease the numbers of MDSCs whereas Cox-2 inhibitors and sildenafil seem to inhibit MDSCs function. However, one of the most promising approaches is to promote the differentiation of MDSCs into mature stimulatory macrophages and DCs. This can be achieved by administration of clinically approved agents such as vitamin D3, vitamin A and all-trans retinoic acid (ATRA) (Gabrilovich and Nagaraj 2009; Gantt, Gervassi et al. 2014). On the other hand, the powerful immunosuppressive features of MDSCs make these cells good candidates to inhibit antigen-specific immune responses in autoimmune diseases (Li, Tu et al. 2014), GVHD (Highfill, Rodriguez et al. 2010; Lv, Zhao et al. 2014; Messmann, Reisser et al. 2015) or organ transplantation (Hongo, Tang et al. 2014). Therefore, the *ex vivo* generation of immune-suppressive MDSCs could be used for therapeutic purposes. Murine MDSCs can be differentiated from HSCs by culturing them with IL-3, IL-6, stem cell factor (SCF), thrombopoietin (TPO), fms-like tyrosine kinase 3 ligand (FLT3-L), VEGF and M-CSF (Zhou, French et al. 2010). Also, incubation of human monocytes from PB mononuclear cells (PBMCs) in the

presence of the combination of various cytokines such as GM-CSF, IL-6, IL-4, G-CSF or PGE2 resulted in functional $CD33^{+}HLA\text{-}DR^{low/-}$ MDSC-like cells (Marigo, Bosio et al. 2010; Lechner, Megiel et al. 2011; Obermajer and Kalinski 2012).

6. MULTIPLE SCLEROSIS

MS is a chronic, inflammatory and demyelinating disease of the CNS that mainly affects adults aged 20 to 40, being more common in women than in men (ratio 2-3:1). It is the main cause of disability in young adults, after traumatisms.

MS is caused by the destruction of the myelin sheaths surrounding neuronal axons, resulting in improper nerve conduction and giving rise to a wide spectrum of clinical signs depending on the affected area of the CNS. There are three defined clinical forms of MS (Figure 15). The most common one is called relapsing-remitting MS (RRMS), occurring in approximately 80-90% of patients. It is characterized by the appearance of episodes of disease worsening (exacerbations or clinical outbreaks) followed by complete or partial recovery periods (remissions). Approximately 50% of patients with RRMS, after a variable period of time, evolve into a progressive clinical form of MS called secondary progressive (SPMS). In 10-20% of patients the disease evolves progressively from the beginning, called primary progressive MS (PPMS). The new clinical classification considers that patients having a single outbreak of the disease but not yet diagnosed of MS are referred to as clinically isolated syndromes (CIS), which is considered the first manifestation of the disease (Lublin, Reingold et al. 2014).

The etiology of MS remains elusive, although numerous studies indicate that the combination of genetic components that confer susceptibility along with certain environmental factors may be involved in the breakdown of tolerance and the beginning of an autoimmune response (Comabella and Khoury 2012). Genes associated with MS have been identified; this is the case for the alleles of the HLA of class II, in particular the HLA-DR and HLA-DQ (Compston and Coles 2008; Hoppenbrouwers and Hintzen 2011). Microbial agents, in particular viruses as Epstein-

Barr (EBV) and human herpes virus-6 (HHV-6), have also been proposed as MS risk factors (Cermelli, Berti et al. 2003; Haahr and Hollsberg 2006).

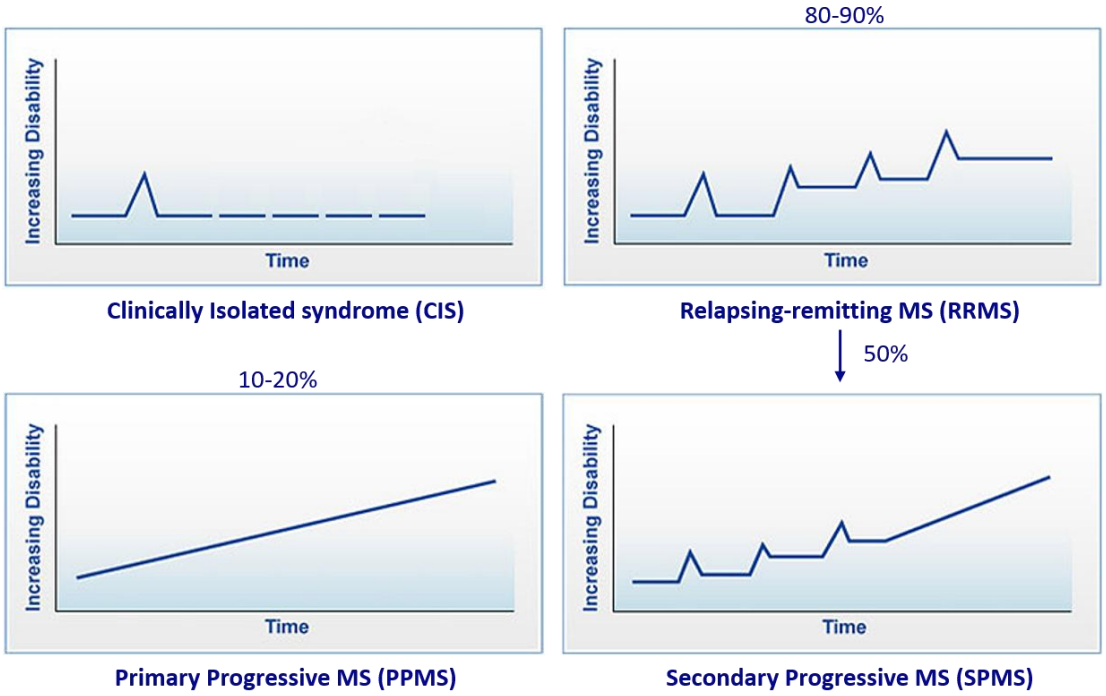


Figure 15. Clinical forms of MS. The new clinical classification of Lublin et al. 2014 defines the types of MS as RRMS, SPMS, PPMS and includes the CIS as the first manifestation of the disease. Adapted from Types of MS. http://www.msactivesource.com/types-of-ms.xml

MS is considered a disease mediated by $CD4^+$ T cells (mainly Th1 and Th17 cells) reactive to antigens of the myelin sheath, although many other immune cells are involved in the pathogenesis of the disease such as B lymphocytes and $CD8^+$ T cells. Autoreactive cells enter the CNS and release proinflammatory cytokines and other soluble factors that induce the destruction of the myelin sheath by activating microglia and astrocytes (Comabella and Khoury 2012). However, the myelin antigens responsible for the initial autoimmune response remain to be defined. The myelin sheath of the CNS is a complex structure generated by oligodendrocytes that covers the neural axons allowing rapid transmission of nerve impulses. It consists of a lipid fraction (70-80%) and a protein fraction (20-30%), where the main candidate antigens are found. Among them are the myelin basic protein (MBP), proteolipid protein (PLP)

and myelin oligodendrocyte glycoprotein (MOG). The main candidate antigens have been extensively used to induce the animal model of MS, the EAE (Schmidt 1999).

6.5. Current and emerging therapies for MS

Currently, there is no cure for MS. Existing treatments are intended to reduce the frequency and intensity of clinical outbreaks and to delay the progression of the disease. Since MS is considered to be an immune-mediated disease, therapeutic strategies have been focused on modulating or suppressing the immune response. A first type of treatments are those aimed to treat acute disease exacerbations with high doses of corticosteroids such as prednisolone or metilprednisolone (Miller, Weinstock-Guttman et al. 2000). The other group is the so-called disease-modifying treatments (DMTs). Within this group we find the beta-interferons (IFN-β), IFN-β-1b and IFN-β-1a, glatiramer acetate (GA), teriflunomide and dimethyl fumarate. This treatments constitute the first line of action since they have been shown to regulate the immune response, reduce the rate of outbreaks, the accumulation of lesions and, to a lesser extent, the progression of disability (Paty and Li 1993; Johnson, Brooks et al. 1995). It has been demonstrated that IFN-β decreases blood-brain barrier (BBB) disruption and antigen presentation and that have many immunomodulatory effects on T and B cells. GA is a synthetic copolymer that is believed to reduce MBP autoreactive T cells, to induce Treg cells and to activate Th2 cells in the periphery. Although the exact mechanism of action of these two DMTs still remains unknown, it seems that they shift the proinflammatory environment to an anti-inflammatory one by inhibiting Th1 cells and down-regulating the inflammatory immune response (Minagar 2013). The Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have recently approved two other oral treatments for MS, teriflunomide and dimethyl fumarate. Teriflunomide inhibits autoreactive T and B cell proliferation by blocking pyrimidine synthesis in rapidly proliferating cells and dimethyl fumarate reduces the levels of proinflammatory cytokines [reviewed in (Minagar 2013; Cross and Naismith 2014)]. The monoclonal antibody alemtuzumab has also been recently approved for the treatment of active RRMS. It targets the antigen CD52, which is expressed on

leukocytes, and depletes circulating T and B cells by antibody-dependent cytolysis (Havrdova, Horakova et al. 2015).

Second-line DMTs are more effective but have important associated side effects. For this reason, these treatments are used in patients who do not respond to first-line treatments or with a rapid progression of the disease. This group of therapies includes mitoxantrone, natalizumab and fingolimod. Mitoxantrone is an antineoplastic agent with immunosuppressive and immunomodulatory features. It blocks DNA and RNA synthesis of proliferating T and B cells, decreases secretion of IFN-γ, tumor necrosis factor (TNF-) α and IL-2 and inhibits T-cell activation. Natalizumab is a humanized monoclonal antibody that blocks the alpha-4 subunit of the very late activating antigen-4 (VLA-4) expressed on activated T cells and inhibits the migration of inflammatory cells to the CNS. Fingolimod, the first oral DMT approved for MS, is an antagonist of the sphingosine-1-phosphate (S1P) receptors expressed on resting and naïve T and B cells. By binding to S1P receptors, fingolimod retains autoreactive lymphocytes in the lymph nodes preventing them to migrate to the CNS. Other new therapeutic strategies mostly based on the use of monoclonal antibodies as daclizumab (anti-CD25), ocrelizumab and ofatumumab (both anti-CD20) and the oral drug laquinimod are currently being tested in ongoing clinical trials (Cross and Naismith 2014).

However, despite using these medications some patients continue to have exacerbations and accumulate disability. New molecules are continually being developed and tested in EAE to effectively block the autoimmune response and improve the course of the disease. The armamentarium of approved therapies for MS has grown substantially in the past two decades; however, although these therapies are more effective, all inhibit immune responses in an unspecific manner, require long-term application and are associated with sometimes serious- to life-threatening risks. Thus, more effective and specific strategies to suppress the autoimmune response are needed. To this end, antigen-specific approaches represent an emerging strategy to induce tolerance and improve the disease. Compared to unspecific immunomodulatory or immunosuppressive interventions, antigen-specific therapies

aim to target only the immune cells that react to a specific antigen while preserving the integrity of physiological immune responses. Targeting the pathogenic autoreactive T-cell responses by direct intervention offers the opportunity to treat the disease with few side effects and has the potential of long-lasting clinical benefit, since it aims to correct the causes of autoimmune diseases at their roots (Garber 2014; Lutterotti and Martin 2014).

Early-stage trials using antigen-specific strategies to induce tolerance show encouraging results in people with MS and T1D. In the case of MS, tested treatments are well tolerated and a reduction in the frequency of myelin-reactive T cells has been reported as well as a reduction in disease activity in several patients. This is the case of the ETIMS trial conducted by Martin's team. Nine MS patients (seven RRMS and two SPMS) were given a single injection of autologous lymphocytes chemically coupled to a cocktail of seven myelin peptide antigens (derived from MBP, MOG and PLP proteins) in escalating doses. Results showed that the four patients receiving the highest doses presented a reduction in the proliferation response to some or all of the antigens tested (Lutterotti, Yousef et al. 2013). Another strategy to induce tolerance that has come to the clinic is the one that employs DNA vaccines. Steinman's group designed a plasmid which encoded the full-length MBP that was given intramuscularly to RRMS patients. The tolerizing vaccine was well tolerated and provided favorable results regarding magnetic resonance imaging data in MS patients with active disease. In addition, this effect was accompanied by a decrease in Th1 $CD4^+$ T cells reacting against peptides from three myelin proteins MBP, MOG and PLP and reduced titers of specific autoantibodies (Bar-Or, Vollmer et al. 2007). Selmaj and colleagues have some of the most promising results so far with the application of antigen-specific tolerance for treatment of RRMS. Selmaj's group designed a strategy in which three myelin peptides were delivered through transdermal skin patches to 30 RRMS patients. Compared with a placebo, this treatment achieved a statistically significant drop in MS disease activity and patients had fewer relapses. Moreover, only a minority of patients had detectable immune responses to the antigens of interest and antigen-specific production of IFN-γ also decreased (Walczak, Siger et al. 2013). Apart from these antigen-specific strategies, several novel and promising approaches are already in

preclinical development such as the use of tolerogenic dendritic cells (Mansilla, Selles-Moreno et al. 2015).

7. EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

EAE is an experimental autoimmune and demyelinating disease of the CNS that shares many clinical, pathogenic and histopathological features with MS such as chronic neuroinflammation, demyelination and neuronal damage and is mediated by autoimmune attack to the CNS. It is considered the animal model that better mimics the human disease (Lassmann and Wisniewski 1979; Wekerle, Kojima et al. 1994) and represents a powerful tool for studying disease pathogenesis as well as potential therapeutic interventions.

7.1. EAE induction

EAE is based on the autoimmune reaction against antigens of the myelin sheath and is induced to susceptible animals by immunization with myelin antigens or by adoptive transfer of myelin-specific T cells. The first EAE experiments were conducted by Rivers *et al.* who showed that EAE could be induced in primates by immunization with CNS homogenates (Rivers, Sprunt et al. 1933). Since then, different species and strains of animals, including rodents and primates, have been used to generate different models of EAE. Similar to MS, susceptibility to EAE is influenced by genetic and environmental factors as well as the protocol of disease induction (Simmons, Pierson et al. 2013). Active immunization consists of subcutaneous administration of myelin antigens emulsified with complete Freund's adjuvant (CFA) containing *Mycobacterium tuberculosis* to enhance the immune response, followed by administration of pertussis toxin (Stromnes and Goverman 2006), which although its specific function is unknown, it seems that it is involved in the permeabilization of the BBB facilitating the entry of inflammatory cells to the CNS (Bruckener, el Baya et al. 2003). On the other hand, EAE can be passively induced by the adoptive transfer of activated myelin-specific $CD4^+$ T

lymphocytes into naïve mice, which shows the involvement of the immune system in the pathogenesis of the disease (Stromnes and Goverman 2006).

The antigens used to induce EAE can be CNS homogenates, myelin proteins such as MBP, MOG and PLP or peptides derived from these (Miller and Karpus 2007). Other encephalitogenic proteins include the lipidic form of MBP and non-myelin antigens as the calcium-binding protein derived from astrocytes S100β (Massacesi, Vergelli et al. 1993; Kojima, Berger et al. 1994) among others.

7.2. Clinical course

After immunizing the animals, either actively or passively, a specific immune response against the encephalitogenic antigen is triggered giving rise to the first clinical signs. Signs are presented as an ascending paralysis, first affecting only the tail causing its partial or total paresis (loss of tail tone) followed by paraparesis or paraplegia of hind limbs and progressing to the forelimbs reaching tetraparesia or quadriplegia, which can sometimes lead to the animal's death (Figure 16). The beginning and progression of clinical signs is accompanied by a progressive loss in body weight that is maintained until stabilization or recovery from disease (Moreno, Espejo et al. 2012).

Different clinical courses of EAE can be experimentally induced depending on the combination of species, strain and encephalitogenic antigen. For example, by immunizing Lewis or Dark Agouti rats with MBP or peptides derived from it a type of acute and monophasic EAE can be developed, which is presented with a single clinical episode of disease (Stepaniak, Wolf et al. 1997; Lenz, Wolf et al. 1999). In SJL mice the disease is characterized by a relapsing-remitting course of paralysis if are immunized with PLP or a peptide derived from it (Polman, Dijkstra et al. 1986). Finally, the most commonly used model is the chronic progressive EAE in which the animals develop a progressive clinical course without outbreaks (Figure 16). The animals that develop this clinical course are rats and mice with genetic endowment H-2^b, usually C57BL/6J, immunized with the MOG protein or with peptides derived from it (Mendel, Kerlero de Rosbo et al. 1995; Weissert, Wallstrom et al. 1998) and mice with genetic endowment

H-2^{u} immunized with MBP (Acha-Orbea, Mitchell et al. 1988; Wraith, Smilek et al. 1989).

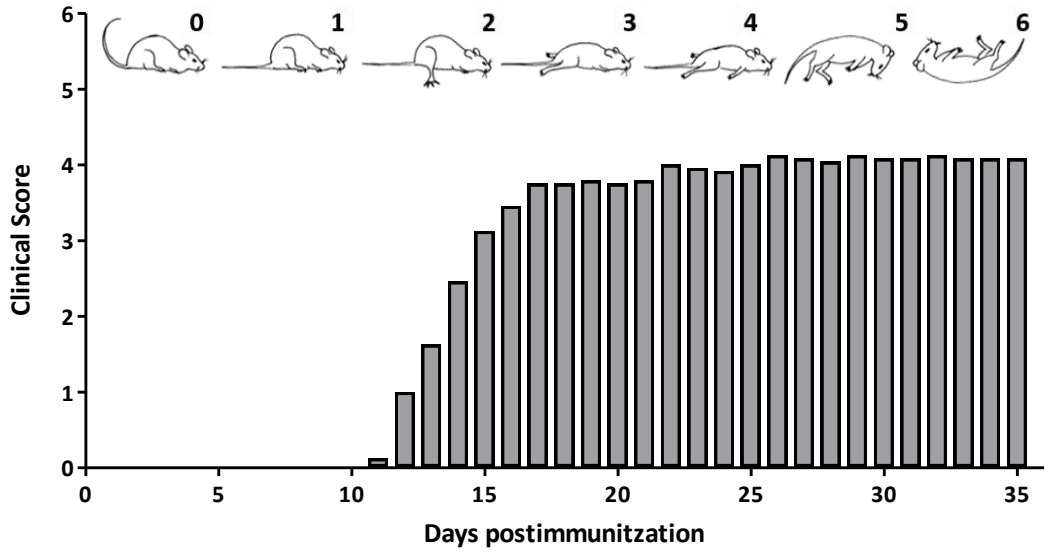


Figure 16. Evolution of neurological signs in C57BL/6J mice immunized with MOG$_{40-55}$ peptide. The graph shows the clinical score given to mice according to the different degrees of paralysis on the days after immunization. The clinical score ranges from the absence of clinical signs (score = 0) to paraplegia of the hind limbs (score = 3) and up to the death of the mouse due to the disease (score = 6).

7.3. Histopathology

In contrast to MS, EAE classic models are characterized by the appearance of an ascending paralysis, since infiltration of inflammatory cells in the CNS takes place predominantly in the spinal cord while it is weak in the brain. In EAE, the characteristic CNS inflammatory lesion is the perivascular cuff, which consists of inflammatory infiltrates mainly composed of T and B lymphocytes and macrophages that have crossed the BBB, being predominantly located in the peripheral white matter of the spinal cord. In the CNS lesions of most of the EAE models, CD4^{+} T cells predominate in association with overall inflammation and relatively little demyelination, compared to CD8^{+} T cell mediated demyelination that occurs in MS (Babbe, Roers et al. 2000). However, over the years, several EAE models have been developed in which the CNS lesions more closely resemble those of MS, such as the presence of focal inflammatory and demyelinating lesions with variable degree of remyelination in brain and spinal

cord (Storch, Stefferl et al. 1998), axonal damage (Kornek, Storch et al. 2000), as well as cortical demyelination including extensive subpial demyelination (Pomeroy, Matthews et al. 2005; Storch, Bauer et al. 2006).

7.4. Pathogenesis

Due to its homology with MS, the different EAE models represent a useful tool to study the autoimmune and inflammatory responses involved in the pathogenesis of MS. In addition, the wide availability of transgenic and knockout mice has enabled the study of the function of multiple molecules like cytokines, chemokines, receptors and the role of different cell types in the development and pathogenesis of the disease.

EAE, like MS, has been traditionally considered as a $CD4^+$ T cell mediated autoimmune disease. After active immunization, myelin-specific $CD4^+$ T cells are activated in the periphery, egress the secondary lymphoid organs and cross the BBB. Once inside the CNS, these effector T cells are reactivated by resident APCs presenting myelin antigens through MHC class II molecules, triggering the inflammatory and demyelinating process. However, it has become increasingly clear that the pathogenesis of MS and EAE expands far beyond the idea of only being mediated by $CD4^+$ T cells; it involves various types of immune cells of both arms of the innate and adaptive immune system, as well as immune-like glial cells of the CNS (Duffy, Lees et al. 2014)(Figure 17).

According to the classic paradigm, MHC class II-restricted $CD4^+$ T cells are phenotypically classified as Th1 or Th2 cells based on cytokine production and transcription factor expression (Mosmann, Cherwinski et al. 1986). MS and EAE have long been considered to be mediated by Th1 cells, which characteristically secrete proinflammatory cytokines such as IFN-γ, TNF-α and IL-2. The first two activate macrophages, which mediate the destruction of the myelin sheaths and in turn secrete other cytokines such as IL-12, IL-23 and TNF-α. By contrast, Th2 cells seem to protect animals from the disease by secreting anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13 (Hemmer, Archelos et al. 2002; Duffy, Lees et al. 2014). A dysregulation in the balance between Th1 and Th2 cytokines has long been implicated in MS

immunopathogenesis, although this theory has had to be reconsidered due to the discovery of IL-23. It was found that mice deficient in IL-12 (IL-$12^{-/-}$) (Becher, Durell et al. 2002; Gran, Zhang et al. 2002), necessary for the generation of Th1 cells, and mice deficient in IFN-γ (IFN- $\gamma^{-/-}$) (Ferber, Brocke et al. 1996) were susceptible to the development of EAE while mice deficient in IL-23 (IL-$23^{-/-}$), critical for Th17 cell differentiation and proliferation, were found to be resistant (Cua, Sherlock et al. 2003). Th17 cells have been defined as a distinct subset of $CD4^+$ T cells which secrete a unique proinflammatory cytokine profile, including IL-17A, IL-17F, IL-22, IL-21, IL-9 and TNF-α (Langrish, Chen et al. 2005). Th17 cells promote inflammation and are pathogenic in many autoimmune and allergic disorders (MS, RA, SLE and bronchial asthma, among others) (Fletcher, Lalor et al. 2010). IL-17 stimulates the production of other proinflammatory cytokines, chemokines and adhesion molecules, and is also involved in the recruitment of neutrophils to the focus of inflammation. However, the role of Th17 cells is still unclear since the overexpression of IL-17 in T cells does not exacerbate EAE and the neutralization of IL-17A and IL-17F does not reduce the incidence or the severity of the disease (Haak, Croxford et al. 2009).

In EAE, as in MS, the participation of other cell types such as B cells has also been considered. Antibodies against myelin antigens have been found in acute and chronic lesions of MS patients but this seems independent of the type of disease, duration or staging (Sadaba, Tzartos et al. 2012). B cells have a paradoxical role in both EAE and MS. B cells primed by Th1 cells secrete proinflammatory cytokines including IFN-γ, IL-12 and TNF-α that contribute to the pathogenesis of EAE and MS while B cells primed by Th2 cells secrete cytokines of a more anti-inflammatory profile such as IL-4 and IL-13 (Duffy, Lees et al. 2014). In EAE, the production of anti-myelin antibodies contributes to demyelination and to the pathogenesis of the disease. The depletion of B cells by using an anti-CD20 antibody protected the animals from developing the disease (Monson, Cravens et al. 2011) and attenuated the established EAE by reducing inflammation and MOG-specific Th1 and Th17 cells (Weber, Prod'homme et al. 2010). Actually, B-cell depleting therapies using genetically engineered anti-CD20 monoclonal antibodies such as rituximab, ocrelizumab and ofatumumab are currently being tested in clinical trials with promising results (von Budingen, Palanichamy et al. 2015).

However, it has also been reported that B cells may have a protective role through reducing inflammation and the opsonization of myelin debris which facilitates clearance by phagocytic cells (Van der Goes, Kortekaas et al. 1999; Mann, Ray et al. 2012).

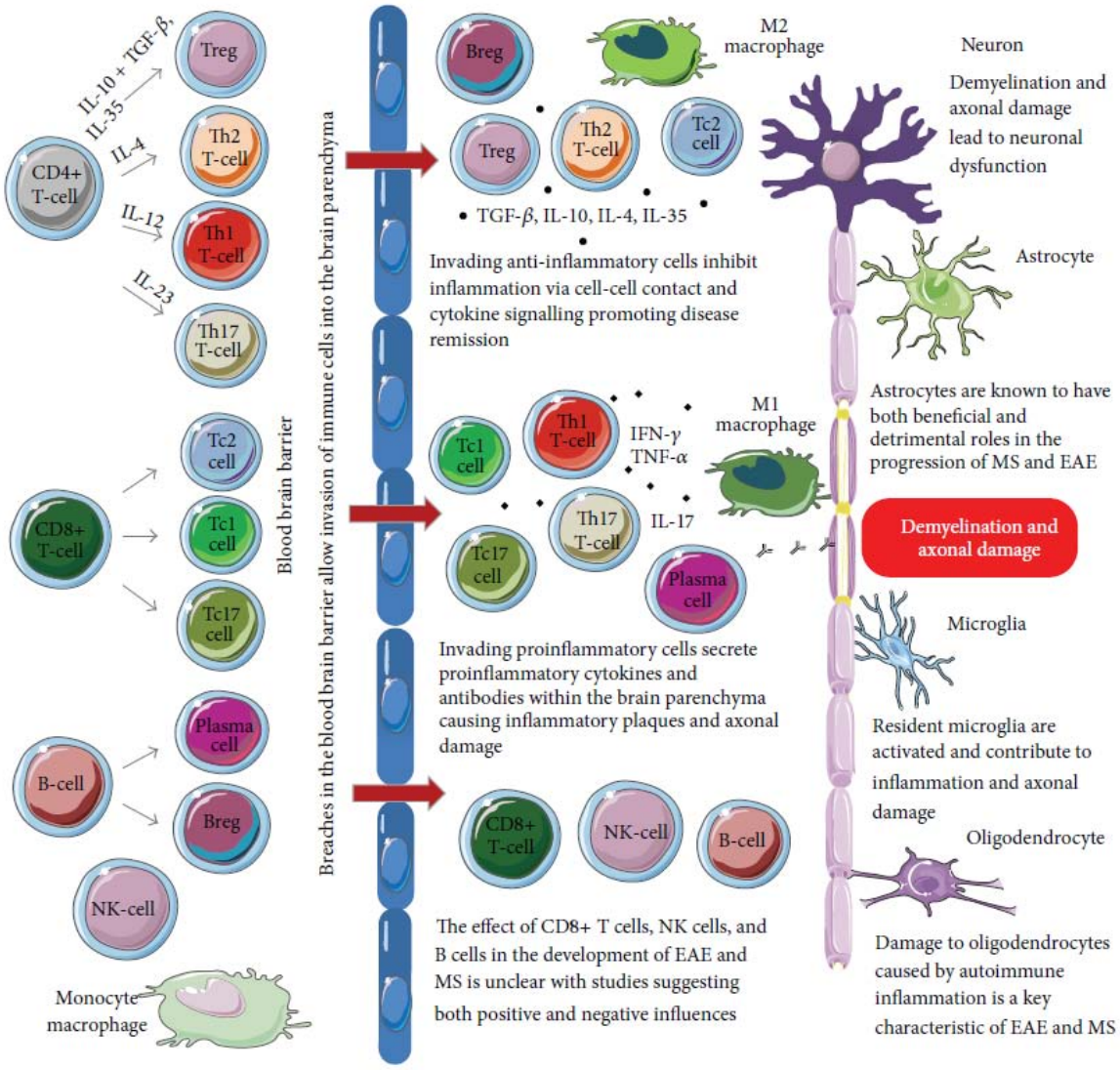


Figure 17. Contribution of immune and glial cell subtypes to the pathogenesis of EAE and MS. During the development and progression of MS and EAE immune cells from both the innate and adaptive immune system are activated in the periphery and cross the BBB invading the CNS. Infiltrating proinflammatory cells promote demyelination and axonal damage and in turn activate resident glial cells that also contribute to the pathogenesis of EAE and MS. On the other hand, cells with anti-inflammatory and/or regulatory properties inhibit disease progression and promote tissue repair. Figure from (Duffy, Lees et al. 2014). Tc: cytotoxic T cell.

The mechanisms involved in recovery from EAE and remission in MS are still unclear, but a shift from a predominantly proinflammatory cell infiltrate to a more anti-inflammatory microenvironment seems to play an important role (Duffy, Lees et al. 2014). It has been reported that recovery from EAE is associated with increased numbers of antigen-specific Treg cells into the CNS which are able to suppress the production of IFN-γ by MOG-stimulated T cells *in vitro* (O'Connor, Malpass et al. 2007). Moreover, the reduction in $CD4^+$ T cells in the CNS may be due to the immunosuppressive action of IL-10 and TGF-β secreted by Treg cells (Groux, Bigler et al. 1996; Lin, Martin et al. 2005).

Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning.

Albert Einstein

HYPOTHESIS

In previous studies conducted at our laboratory we demonstrated that infusion of BM cells transduced with a self-antigen induced immunological tolerance in an experimental model of MS in both preventive and therapeutic approaches (Eixarch, Espejo et al. 2009). The absence of molecular chimerism and the rapid recovery of the animals led us to reconsider that the therapeutic effect observed was not mediated by cells with engrafting potential and their progeny, but rather by a more mature cell type that was necessarily present in BM transduction cultures and that could present antigens to T cells in a tolerogenic manner. More recently, we found that the most abundant cell types in BM transduction cultures were of myeloid lineage and that these cells were indeed MDSCs (Gomez, Espejo et al. 2014). These observations led us to hypothesize that these antigen-specific MDSCs generated in BM retroviral transduction cultures were responsible for the induction of the immunological tolerance previously observed in the EAE model.

Furthermore, since MDSCs could be a potential therapeutic tool and after having promising results with the murine antigen-specific MDSCs generated *ex vivo* from BM cells, we considered the hypothesis that human MDSCs could be generated *in vitro* from hematopoietic progenitor cells for their potential clinical application.

Aim for the moon. If you miss, you may hit a star.

W. Clement Stone

OBJECTIVES

The main objective of the first part of this doctoral thesis was to determine whether the antigen-specific MDSCs generated in BM retroviral transduction cultures were responsible for the induction of the immunological tolerance previously observed in the EAE model.

To this end, the following specific objectives were proposed:

1. Characterize phenotypically and functionally the MDSCs generated in BM retroviral transduction cultures.
2. Determine whether these MDSCs were able to induce immunological tolerance *in vivo* and ameliorate the clinical and histopathological signs of EAE in both preventive and therapeutic approaches.
3. Study the immune response in animals preventively and therapeutically treated with antigen-specific MDSCs.

The main objective of the second part of this doctoral thesis was to develop an efficient method to generate human MDSCs from hematopoietic progenitor cells for its potential clinical application.

To this end, the following specific objectives were proposed:

1. Determine the optimal culture conditions, the cytokine combination and the culture length, to efficiently generate human MDSCs from hematopoietic progenitor cells.
2. Phenotypically characterize the *in vitro* generated MDSCs.
3. Determine whether the generated MDSCs were able to suppress T-cell responses.

Tell me and I forget. Teach me and I remember. Involve me and I learn.

Benjamin Franklin

MATERIALS AND METHODS

PART 1: ANTIGEN-SPECIFIC MDSCs INDUCE IMMUNOLOGICAL TOLERANCE IN THE EAE IN BOTH PREVENTIVE AND THERAPEUTIC APPROACHES

1. RETROVIRAL VECTORS

The retroviral vectors used in this work had previously been generated in our laboratory (Eixarch, Espejo et al. 2009) from the retroviral vector SF91-IRES-EGFP (provided by Christopher Baum, Hannover, Germany) (Hildinger, Schilz et al. 1999) based on the MoMuLV. This family of retroviral vectors is optimized for a high expression of transgenes in myeloid hematopoietic progenitors. Two retroviral vectors have been used containing either the coding sequence of the wild-type murine Ii (SF91-Ii-IRES-EGFP, control vector) or the Ii with the coding sequence for the CLIP region replaced by the one encoding the MOG_{40-55} peptide (SF91-IiMOG-IRES-EGFP, therapeutic vector) (Figure 18).

Figure 18. Diagram of the retroviral vectors used. The top one corresponds to the SF91-Ii-IRES-EGFP vector expressing the murine Ii as well as the EGFP region. This vector was used as a control in different experiments. The bottom one corresponds to the therapeutic vector SF91-IiMOG-IRES-EGFP in which the CLIP region has been replaced by the sequence encoding the MOG_{40-55} autoantigen.

2. RETROVIRAL VECTOR PRODUCER CELL LINES

In this project two producer lines called NX-e/Ii7 and NX-e/IiMOG23 were used. Both cell lines were derived from the ecotropic packaging cell line NX-e (a gift from G. Nolan, Standford University, CA, USA), in turn derived from the human cell line HEK-293 of embryonic kidney. Both cell lines stably express three elements. The first one, *env* gene, encodes for the ecotropic glycoprotein of the envelope of the retrovirus with tropism for murine cells. The second one, *gag* and *pol* genes, encodes both for

structural proteins to form new virions and for the necessary enzymes for the life cycle of the virus. The third element is the retroviral vector containing the transgene and the sequences that regulate its expression; the NX-e/Ii7 cell line contains the SF91-Ii-IRES-EGFP retroviral vector and the NX-e/IiMOG23 contains the SF91-IiMOG-IRES-EGFP. Both producer cell lines were generated in our laboratory and have a viral titer of $0.8x10^6$ and $2x10^6$ infectious particles / mL respectively (Eixarch, Espejo et al. 2009).

Producer cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with:

- 10% of fetal bovine serum (FBS) Premium
- 2 mM L-glutamine
- 50 IU/ml penicillin
- 50 µg/ml streptomycin (all from Biowest, Nuaillé, France)
- 50 µM of β-mercaptoetanol (Sigma-Aldrich, St Louis, MO, USA)

Cells were cultured at 37 °C, 5% carbon dioxide (CO_2) and 100% of relative humidity.

3. BONE MARROW TRANSDUCTION CULTURE

3.1. Mice

Six- to ten-week old female B6/SJL-*Ptprc*^{a}*Pepc*^{b}/BoyCrl mice (referred as B6/SJL) purchased from Charles River (Wilmington, MA) were used as BM donors. The animals were maintained in the animal facilities of the Vall d'Hebron Research Institute (VHIR) in rooms with controlled temperature (22ºC) and with 12-h light/12-h dark cycle. Water and food were provided *ad libitum*. All experimental procedures were approved by our institutional Ethics Committee on Animal Experimentation and were performed in strict accordance with European Union (Directive 2010/63/UE) and Spanish regulations (Real Decreto 53/2013; Generalitat de Catalunya Decret 214/97).

3.2. Isolation of BM cells

Donor mice were given an intraperitoneal (i.p.) dose of 50 mg/Kg of 5-Fluorouracil (5-FU) in phosphate-buffered saline (PBS) (Ferrer Farma, Barcelona, Spain) five days before BM extraction. This treatment eliminates dividing cells in the BM, enriches it in more immature hematopoietic progenitors (more quiescent) and in turn drives them into cycle, an essential condition to be transduced with retroviral vectors.

Five days after 5-FU administration, mice were euthanized by CO_2 asphyxiation. Then the ventral area of the animals was sterilized with 70% ethanol and dissected to extract tibias, femora and iliac crests, which were devoid of their adjacent soft tissues. The clean bones were placed in a 50 mL tube (BD Falcon™, Bedford, MA, USA) containing ice-cold Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Gran Island, NY, USA) supplemented with:

- 20% of FBS Premium
- 50 IU/ml penicillin
- 50 µg/ml streptomycin

Working in a laminar flow cabinet, the bones were placed in a sterile mortar containing 6 mL of supplemented IMDM and were crushed with a pestle to obtain the cells. Cells were collected into a 50 mL tube and a single-cell suspension was obtained by passing them through a 70 µm cell strainer (BD Biosciences, San Diego, USA) to remove debris and remaining cellular aggregates. After several washes with IMDM to collect all the cells released from the bones, cells were centrifuged 5 minutes at 453 g. Cells were resuspended in supplemented IMDM and counted in the Neubauer chamber using Türk solution as diluent. This solution contains Giemsa stain and 1% of acetic acid that allows the lysis of erythrocytes and facilitates the counting of nucleated cells.

3.3. BM transduction

3.3.1. Coating of culture plates with RetroNectin®

Prior seeding the cells and in order to improve transduction efficiency, 6-well culture plates (Nunc, Roskilde, Denmark) were incubated with 1 mL of RetroNectin® (CH-296 recombinant fibronectin fragment, Takara Bio Inc., Otsu, Japan) diluted in PBS at a concentration of 48 μg/ml for 2 hours (h) at room temperature (RT). Once removed the solution containing fibronectin, plates were incubated for 30 minutes at RT with 1 mL of PBS with 2% bovine serum albumin (BSA; Sigma-Aldrich). Finally, plates were washed with 1 mL of Hank's buffered salt solution (HBSS; Biowest) containing 25 mM Hepes (Biowest).

3.3.2. Prestimulation of BM cells with cytokines

Cells were seeded at a density of 0.5-1x10^6 cells/ml in a final volume of 2 mL on pre-coated 6-well plates as described above. The medium used for this culture was IMDM with GlutaMAX™ (Gibco) supplemented with:

- 20% of FBS Premium
- 50 IU/ml penicillin
- 50 μg/ml streptomycin
- 10% of conditioned medium from the WEHI-3B$^+$ cell line, source of murine IL-3 (Lee, Hapel et al. 1982)
- 8% conditioned medium from the BHK/MKL cell line, source of murine SCF and provided by S. Tsai (*Fred Hutchinson Cancer Research Center,* Seattle, WA, USA)

Prestimulation with cytokines was carried out for 48 h prior to transduction in an incubator at 37°C, 5% CO_2 and with 100% of relative humidity in order to induce stem cells to divide and prevent their apoptosis.

3.3.2. Obtaining the supernatant rich in retroviral vectors

NX-e/Ii7 and NX-e/IiMOG23 recombinant retroviral producer cell lines were thawed following standard techniques. They were cultured in supplemented DMEM medium as specified in part 2 of this section. When they reached confluence, cells were passaged to a new culture flask of adequate volume in order to obtain the necessary supernatant for transduction and to maintain the optimal cell confluency. The day prior to the collection of the supernatant rich in retroviral vectors, the usual growth medium of NX-e producer cell lines was changed to the suitable medium for the culture of the target cells to be transduced (see part 3.3.2, but without adding the conditioned media from the WEHI-3B$^+$ and BHK/MKL cell lines and with 10% FBS Premium). At 16-18 h after changing the culture medium, the supernatant was collected and filtered through a 0.45 µm filter to prevent the possible transfer of vector producing cells. The supernatants were used immediately or were aliquoted and criopreserved at -80°C until needed.

3.3.3. Retroviral transduction of BM cells

Two cycles of transduction were performed on two consecutive days (two and three of culture) using a multiplicity of infection (MOI) of approximately 1 in each cycle (Eixarch, Espejo et al. 2009) (Figure 19). For transduction, 90% of the culture medium of each well was collected in a 50 mL tube and centrifuged for 5 minutes at 453 g. The pellet was resuspended in the following mixture:

- 10% of FBS Premium
- 10% of WEHI-3B$^+$ conditioned medium
- 8% of BHK/MKL conditioned medium
- 4 µg/ml of protamine sulfate (Rovi S.A., Madrid, Spain)
- 72% of supernatant from the retrovirus producer cell line

The volume of each well was restored with medium rich in recombinant vectors in which the collected cells had been resuspended. Plates were then centrifuged for 1 h at 652 g. This same procedure was repeated the next day.

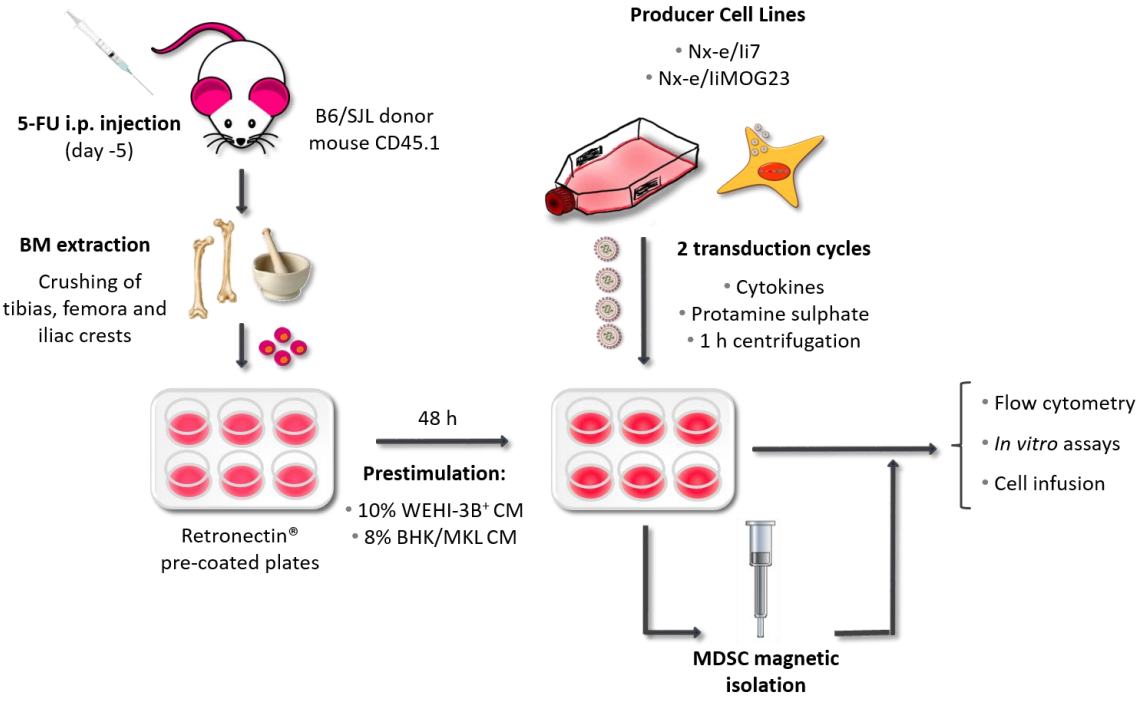


Figure 19. Diagram of extraction and transduction of murine BM. See text. CM: conditioned medium.

3.4. Harvesting transduced BM cells

Transduced BM cells were harvested 24 h after the second cycle of transduction. To this end, BM cells were detached from the culture plates using Cell Dissociation Buffer (Gibco) and were collected in a 50 mL tube. After washing them with PBS, cells were centrifuged for 5 minutes at 453 g, resuspended in PBS and counted diluted in Türk solution using a Neubauer chamber. They were then incubated with 10 IU of DNAase (Sigma-Aldrich) per million of cells for 15 minutes at 37°C. Once collected, BM cells were used for *in vitro* assays, to isolate the MDSCs or to be infused into mice.

3.5. Transduction efficiency

Before using BM cells in the different experiments, 10^5 cells were separated from the culture 24 h after the second cycle of transduction to evaluate the transduction efficiency, which was assessed by analyzing the expression of EGFP by flow cytometry using a FACSCanto (BD Biosciences) flow cytometer. Untransduced cells were used as

negative controls. Data were analyzed using the FCS Express v4 software (De Novo Software, Los Angeles, CA, USA).

4. CHARACTERIZATION OF TRANSDUCED BM CELLS AND MDSCs

4.1. Characterization of transduced BM cells

To study what kind of cells were generated during BM retroviral transduction cultures, BM cells were characterized by flow cytometry prior to culture (day 0) and 24 h after the second cycle of transduction (day 4). Antibodies used for phenotypic characterization of BM cells are listed in Table 2.

Cells were stained for flow cytometry following the protocol of direct staining of cell surface antigens described below:

1. $5x10^5$ cells were added to each tube and washed with 1 mL of PBS + 1% of BSA + 0.1% sodium azide (PBA-azide).
2. Cells were centrifuged at 300 g for 5 minutes and the supernatant was discarded leaving 50 µL of liquid.
3. Pellet was resuspended with a vortex pulse and Fc receptors were blocked by incubating the cells with 1 µL of anti-mouse CD16/32 antibody (clone 93, BioLegend, San Diego, CA, USA) for 10 minutes at RT.
4. Cells were then incubated for 20 minutes at 4ºC with specific antibodies and isotype controls protected from light.
5. Cells were washed with 1 mL of PBA-azide and centrifuged at 300 g for 5 minutes.
6. Cells were resuspended in 200-300 µL of PBA-azide and acquired using a FACSCanto flow cytometer.

Due to the presence of erythrocytes on day 0 in BM cells preparations, an additional step was required when staining these samples. After labelling the cells, erythrocytes were lysed by incubating the cells with 1 mL of BD Pharm Lyse (BD Biosciences) solution for 10 minutes at 37ºC.

Table 2. Antibodies used for BM culture characterization.

Antibody	Isotype	Clone	µg for 10^5 cells	Manufacturer
PE anti-mouse CD3e	Hamster IgG1,k	145-2C11	0.2	BD Biosciences
PerCP-Cy5.5 anti-mouse CD3e	Hamster IgG1,k	145-2C11	0.125	eBioscience
PerCP anti-mouse CD4	Rat IgG2b,k	GK1.5	0.2	BioLegend
APC anti-mouse CD8a	Rat IgG2a,k	53-6.7	0.1	BioLegend
PE-Cy7 anti-mouse CD45R (B220)	Rat IgG2a,k	RA3-6B2	0.2	eBioscience
PE anti-mouse NK1.1	Mouse IgG2a,k	PK135	0.3	BD Biosciences
PerCP anti-mouse CD11c	Hamster IgG	N418	0.2	BioLegend
APC anti-mouse CD11b	Rat IgG2b,k	M1/70	0.1	BioLegend
PE-Cy7 anti-mouse Gr-1 (Ly-6G/Ly-6C)	Rat IgG2b,k	RB6-8C5	0.1	BioLegend
APC mouse lineage antibody cocktail	Isotype cocktail	-	5 µL	BD Biosciences
PE anti-mouse CD45	Mouse IgG2a,k	A20	0.1	BioLegend

PE: Phycoerythrin; PerCP: Peridinin chlorophyll; APC: allophycocyanin; Cy: Cyanine.

Data were analyzed using the FCS Express v4 software. First and in all cases, the forward (FSC) and the side scatter (SSC) parameters were used to identify and select the population to study. Then, different parameters were combined to study the different cell populations present in BM transduction cultures. Results are presented as percentages of positive cells. Combinations of these antibodies were used to study the following cell populations:

- T lymphocytes: $CD3e^+$
- $CD4^+$ T lymphocytes: $CD3e^+CD4^+$
- $CD8^+$ T lymphocytes: $CD3e^+CD8a^+$
- B lymphocytes: $B220^+$

- NK cells: $CD3e^{-}NK1.1^{+}$
- DCs: $CD11b^{+}CD11c^{+}$
- M-MDSCs: $CD11b^{+}Gr\text{-}1^{low}$
- G-MDSCs: $CD11b^{+}Gr\text{-}1^{high}$
- HSCs: $CD45^{+}Lineage^{-}$
- Mesenchymal stem cells: $CD45^{-}Lineage^{-}$

4.2. Phenotypic characterization of MDSCs

In order to phenotypically characterize the generated MDSCs, the expression of the PD-L1, PD-L2, $I\text{-}A^{b}$ (MHC-II), CD80 and CD86 molecules was analyzed by flow cytometry in both M-MDSCs ($CD11b^{+}Gr\text{-}1^{low}$) and G-MDSCs ($CD11b^{+}Gr\text{-}1^{high}$) at day 4 of BM transduction culture. The expression of these molecules was studied both in a steady state and after activation. To activate MDSCs, cells were cultured for 18 h in the presence of inflammatory stimuli. As inflammatory stimuli 2 ng/ml of IFN-γ and/or 100 ng/ml of LPS were used. MDSCs were stained for flow cytometry using the antibodies listed in Table 3 and following the protocol described in the above section. Samples were acquired using a FACSCanto flow cytometer and data were analyzed using the FCS Express v4 software. Results are presented as percentages of positive cells and as the mean fluorescence intensity (MFI) of the relevant cell populations.

Table 3. Antibodies used for MDSCs phenotypic characterization.

Antibody	Isotype	Clone	µg for 10^5 cells	Supplier
APC anti-mouse CD11b	Rat IgG2b,k	M1/70	0.1	BioLegend
PE-Cy7 anti-mouse Gr-1 (Ly-6G/Ly-6C)	Rat IgG2b,k	RB6-8C5	0.1	BioLegend
PerCP anti-mouse Gr-1 (Ly-6G/Ly-6C)	Rat IgG2b,k	RB6-8C5	0.1	BioLegend
PE anti-mouse PD-L1	Rat IgG2b,k	10F.9G2	0.2	BioLegend
Biotin anti-mouse PD-L2	Rat IgG2a,k	TY25	0.2	BioLegend
PE anti-mouse I-A^b	Mouse IgG2a,k	AF6-120.1	0.2	BioLegend
PE anti-mouse CD80	Hamster IgG1	16-12A1	0.2	BD Biosciences
PE-Cy7 anti-mouse CD86	Rat IgG2a,k	GL1	0.2	eBioscience
PerCP streptavidin	-	-	0.1	BD Biosciences

4.3. ROS production by MDSCs

ROS production by both populations of MDSCs was measured at day 4 of BM transduction culture using the oxidation-sensitive probe CellROX™ Deep Red Reagent (Invitrogen, Carlsbad, CA, USA). This probe is a cell-permeable dye that is non-fluorescent in a reduced state but that becomes fluorescent upon oxidation by ROS. The fluorophor emission (665 nm) can be measured by flow cytometry.

At day four of culture and 24 h after the second cycle of transduction, cells were incubated in the presence of 5 µM of CellROX™ for 30 min at 37ºC, according to the manufacturers' instructions. Cells were then washed with cold PBS and labelled with conjugated anti-CD11b and anti-Gr-1 antibodies (Table 4) as described in part 4.1 of this section. The murine fibroblastic NIH 3T3 and the WEHI-3B cell lines were also incubated with the probe and were used as negative and positive controls,

respectively. ROS production was detected using a FACSCanto flow cytometer and data was analyzed using the FCS Express v4 software. The analysis was done by discarding dead cells by simultaneous staining with the viable cell dye 7-aminoactinomicin D (7-AAD). If the plasma membrane is disrupted this dye enters into cells and intercalates into DNA, so that only apoptotic or dead cells are stained. In this way, dead cells can be excluded from analysis to avoid the interference of its autofluorescence. Results are represented as the MFI of the relevant cell populations.

Table 4. Antibodies used to stain MDSCs in ROS production assays

Antibody	Isotype	Clone	µg for 10^5 cells	Supplier
PE anti-mouse CD11b	Rat IgG2b,k	M1/70	0.1	eBioscience
PE-Cy7 anti-mouse Gr-1 (Ly-6G/Ly-6C)	Rat IgG2b,k	RB6-8C5	0.1	BioLegend

5. ISOLATION OF MDSCs

Once transduced BM cells were obtained, MDSCs were magnetically isolated from the other cell populations by positive selection using CD11b MicroBeads selection kit (Miltenyi Biotec, Teterow, Germany) according to manufacturer's instructions (Figure 20):

1. BM cells were centrifuged at 300 g for 10 minutes and the pellet was resuspended in 90 µL of human isolation buffer (HIB) per 10^7 total cells. HIB consisted of ice-cold sterile PBS pH 7.2 containing 0.5% human serum albumin (Grifols, Parets del Vallès, Spain) and 2mM EDTA (ethylenediaminetetraacetic acid; SERVA, Heidelberg, Germany). When working with higher cell numbers, all reagents and total volumes were scaled up accordingly (e.g. for $2x10^7$ total cells, twice the volume of all indicated reagent and total volumes were used).
2. 10 µL of CD11b MicroBeads per 10^7 total cells were added. The cell suspension was vortexed to mix well and incubated for 30 minutes at 4ºC.

3. After incubating the cells with the antibody, 1 mL of buffer per 10^7 total cells was added in order to wash the cells. Then they were centrifuged at 300 g for 10 minutes and resuspended in 500 µL of buffer per 10^8 cells.
4. To perform the magnetic separation, two LD columns (Miltenyi Biotec), one for the cells transduced with the control vector and one for the cells transduced with the therapeutic one, were placed in the magnetic field of a quadroMACS Separator (Miltenyi Biotec). A collection tube was placed under each LD column to collect the negative fraction eluted from the column.
5. The columns were prepared by rinsing with 2 mL of buffer. Once all the buffer had run through, cell suspension was added and unlabelled cells were collected. The columns were washed twice with 1 mL of buffer, adding the buffer each time once the column reservoir was empty.
6. To collect the MDSCs, the columns were removed from the magnetic separator and placed onto a 15 mL collection tube (BD Falcon™). 3 mL of buffer was applied and the magnetically labelled cells were flushed out with a plunger supplied with the column. A second elution with 3 mL of buffer was performed to increase recovery.
7. Both negative (unlabelled cells) and positive (labelled cells) fractions were centrifuged at 300 g for 10 minutes, resuspended in 1 mL of PBS and counted in the Neubauer chamber using trypan blue as diluent solution (0.4% of trypan blue in PBS). Viability was assessed by counting the number of living (exclude the dye) and dead cells (incorporate the dye).

Isolated MDSCs were used for *in vitro* assays or were infused to the animals for *in vivo* studies. The purity of the separated MDSCs and the cell populations present in the isolated fraction were assessed by flow cytometry using the antibodies listed in Table 2.

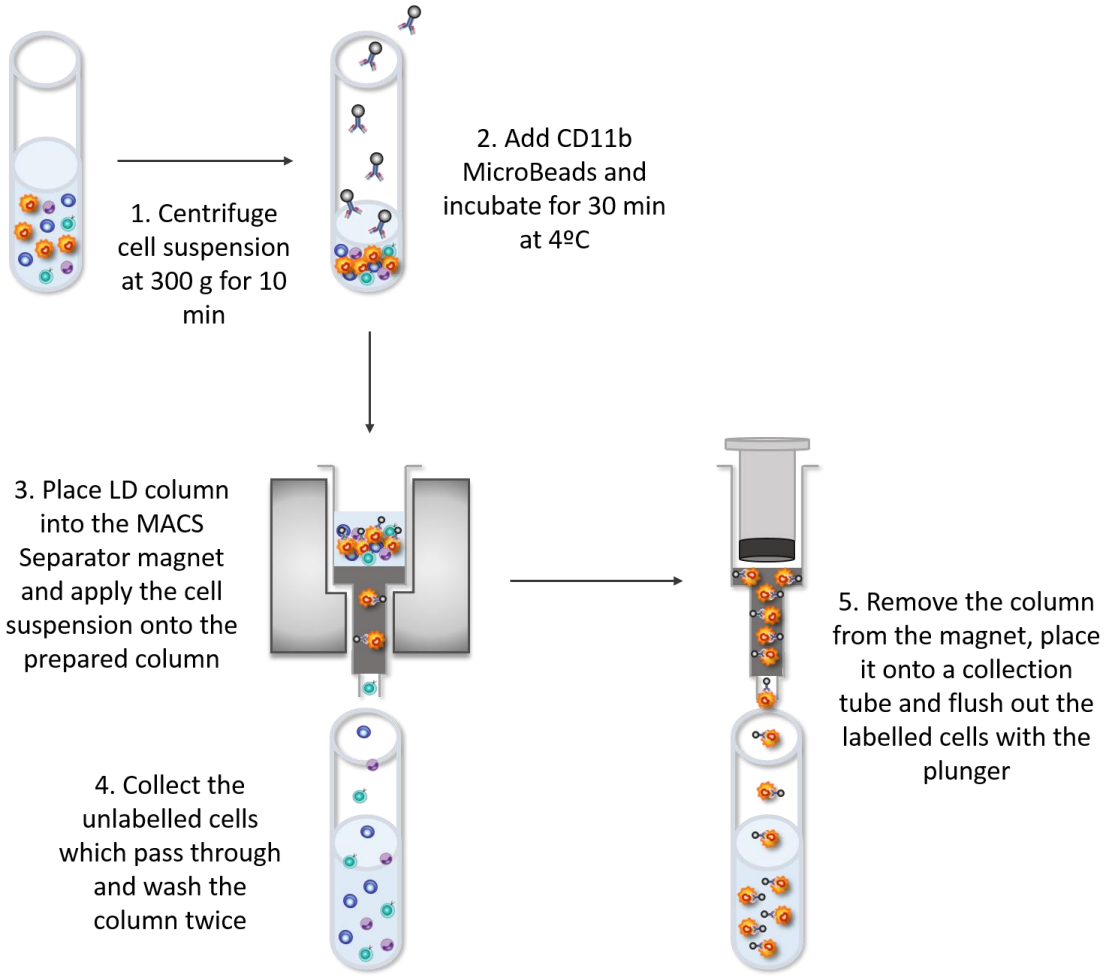


Figure 20. Diagram of MDSC magnetic isolation. See text. Min: minutes.

6. TRANSDUCED BM CELLS AND ISOLATED MDSCs INFUSION

6.1. Recipient mice

As recipient mice, females aged between six and ten weeks of the strain C57BL/6J purchased from Harlan Laboratories (Udine, Italy) were used. Upon arrival, they were distributed in groups of five mice per cage and remained in the room assigned for the EAE model during all the experiment.

6.2. Cell infusion

Both unfractionated BM cells and isolated MDSCs were infused to mice 7 days before (preventive arm) or 13-14 days after (therapeutic arm) EAE induction. Cells were diluted in PBS and injected intravenously (i.v.) in the lateral tail vein at a dose of $1x10^6$

cells for BM cells and 0.5-1x10^6 for isolated MDSCs per mouse in a volume of 200 µL (Figure 21).

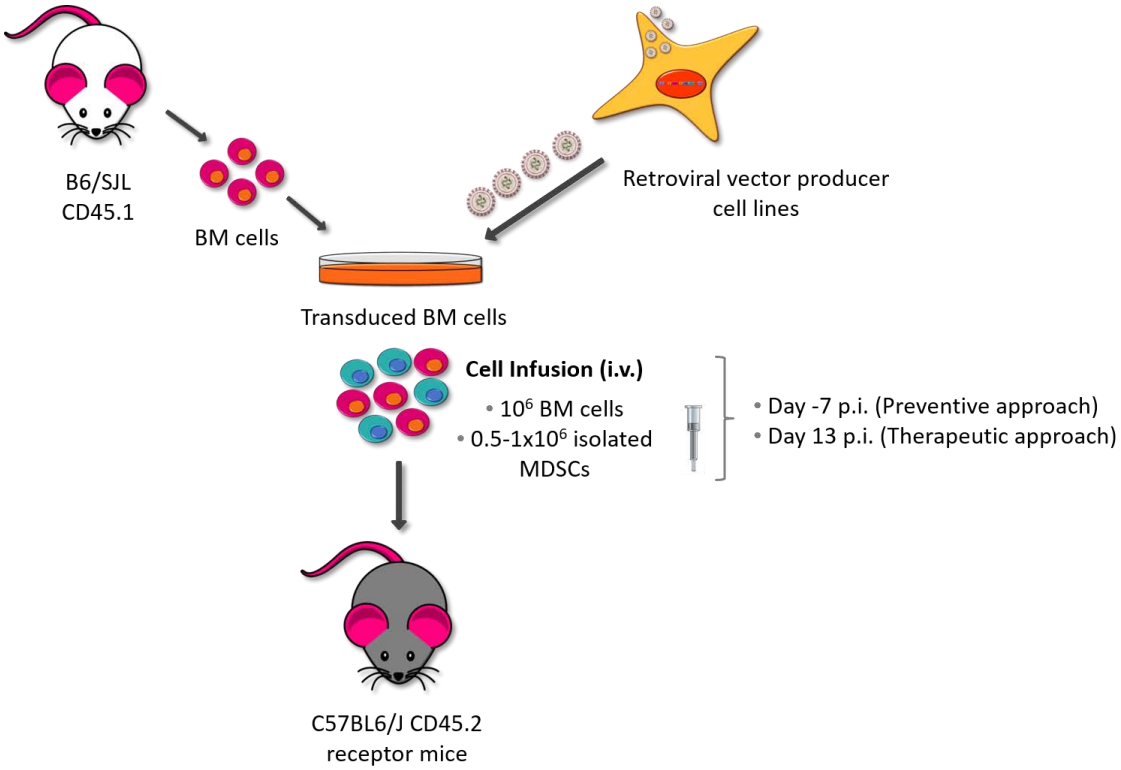


Figure 21. Diagram of the infusion of transduced unfractionated BM cells and isolated MDSCs. BM cells or isolated MDSCs transduced with retroviral vectors were infused to mice 7 days before or 13-14 days after EAE induction. p.i.: postimmunization.

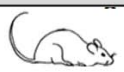
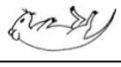
7. EAE INDUCTION AND CLINICAL FOLLOW-UP

Female mice of the strain C57BL/6J aged six to ten weeks were immunized to induce a chronic progressive EAE. Prior to immunization, animals were anesthetized with 50 µL of a solution containing 37 mg/kg ketamine (Ketolar, Parke-Davis, Pfizer, New York, NY, USA) and 5.5 mg/kg xylazine (Xilagesic, Laboratorios Calier, Barcelona, Spain) by i.p. injection. Once anesthetized, animals were weighed and an identification mark was made in the ears of each animal to enable an individualized clinical follow-up. Then the animals were immunized by subcutaneous injection with a solution containing 100 µg of the MOG_{40-55} peptide (synthesized by the *Secció de Proteòmica, Serveis Cientificotecnics de la Universitat Pompeu Fabra*, Barcelona, Spain) diluted in saline and emulsified with CFA (Sigma-Aldrich) containing 4 mg/ml of *Mycobacterium*

tuberculosis H37RA (Difco Laboratories, Detroit, MI, USA). Each mouse received 50 µL of the emulsion at four different points near the inguinal and axillary lymph nodes. At the same day of immunization and 2 days later mice received 250 ng of pertussis toxin (Sigma-Aldrich) diluted in saline i.v. Mice immunized in the same way but without the peptide were included as negative controls of the immunization process. Mice were weighed and examined daily to assess the presence and severity of neurological signs using the criteria specified in Table 5 [modified from (Espejo, Carrasco et al. 2001)]. A cumulative clinical score was calculated by summing up each individual score from each mouse from day zero until the end of the experiment. Animals who scored 6 kept this score until the end of the experiment.

Weight loss was calculated as the percentage of the variation in daily weight compared to the weight of each animal on the day of immunization. All experiments were performed in a blinded manner throughout the entire experimental process in such a way that the investigator examining and evaluating the animals was kept unaware of the treatment administrated to each subject. All data presented here are in accordance with the guidelines suggested for EAE publications (Baker and Amor 2012).

Table 5. Evaluation of EAE neurological signs

	Clinical score	Neurological signs
	**0**	No clinical sings
	**0.5**	Partial tail paresis
	**1**	Paralysis of whole tail
	**2**	Mild paraparesis of one or both hind limbs
	**2.5**	Severe paraparesia or paraplegia of hind limbs
	**3**	Mild tetraparesis
	**4**	Tetraparesis (severe in hind limbs)
	**4.5**	Severe tetraparesis
	**5**	Tetraplegia (end point criteria)
	**6**	Death

7.1. Motor performance test

Motor performance was tested at the end of each experiment (only in the therapeutic approaches) by using a rotarod apparatus (Ugo Basile, Comerio, Italy). Rotarod analysis is one of the most widely used tests to assess motor coordination in rodents (Moreno, Espejo et al. 2012). The rotarod was set to accelerate from a speed of 4 to 40 rotations per minute in a 300 second time trial. Each mouse was then placed on the rotating cylinder and the amount of time the mouse remained walking on the cylinder without falling (rotarod latency) was recorded. Each mouse was given two trials on the rotarod. The two trials were averaged to report a single value for an individual mouse and mean values were then calculated for all animals within a given treatment group.

8. HISTOPATHOLOGY AND IMMUNOSTAINING OF THE CNS

8.1. Obtainment and fixation of the CNS

Mice were euthanized at the end of the experiment (approximately 30 days after immunization) by CO_2 asphyxiation. Brain and spinal cord were removed from the animals and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 24 h at 4ºC. Then, brain and spinal cord were cut into transversal sections and sent to the Department of Pathology of our hospital (Hospital Universitari Vall d'Hebron, Barcelona, Spain) to be embedded in paraffin wax.

8.2. Histopathology of the CNS

Tissues embedded in paraffin were cut into 4 µm serial sections and stained with hematoxylin and eosin (HE) and Klüver-Barrera (KB) to assess the degree of inflammation and demyelination, respectively.

The presence of cell infiltration was evaluated using HE staining according to the following criteria (Figure 22):

0. No lesion
1. Cellular infiltration only in the meninges

2. Very discrete and superficial infiltrates in parenchyma
3. Moderate infiltrate (less than 25%) in the white matter
4. Severe infiltrates (less than 50%) in the white matter
5. More severe infiltrates (more than 50%) in the white matter

The presence of demyelination areas was evaluated using KB staining according to the following criteria (Figure 22):

0. No demyelination areas
1. Little demyelination, only around infiltrates and involving less than 25% of the white matter
2. Demyelination involving less than 50% of the white matter
3. Diffuse and widespread demyelination involving more than 50% of the white matter

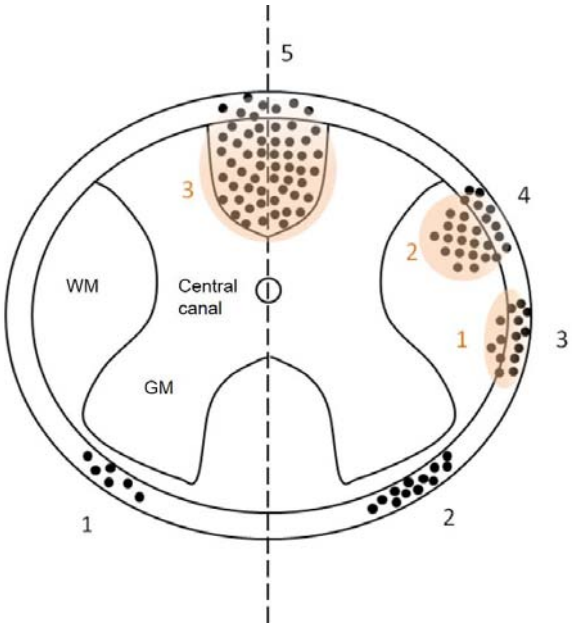


Figure 22. Evaluation criteria used to determine the degree of cell infiltration and demyelination of the spinal cord of mice with EAE for histopathological studies. Numbers in black (cell infiltration): 1, infiltration only in meninges; 2, superficial infiltration in parenchyma; 3, moderate infiltration of white matter (WM); 4, severe infiltration, less than 50% of WM; 5 very severe infiltration exceeding 50% in WM and possible affectation of grey matter (GM). Numbers in orange (demyelination): 1, little demyelination, less than 25% and only around infiltrates; 2, demyelination, less than 50% of the WM; 3, diffuse and widespread demyelination exceeding 50% of WM.

8.3. Immunostaining of the CNS

Spinal cords from three representative animals of the different experimental groups were also immunostained to evaluate additional histopathological parameters (Table 6). Again, 4 µm serial sections of the CNS were performed and were attached to glass slides pretreated with poly-L-lysine (Leica Microsystems Plus Slides, Barcelona, Spain). Tissues were then deparaffinized in xylene and rehydrated through a descending alcohol battery ending in PBS. Afterwards, antigen retrieval was performed in citrate 10 mM (pH=6) for non-phosporilated neurofilaments (SMI-32) or in protease type XIV (Sigma-Aldrich) for CD3. Non-specific protein binding was blocked by incubating sections with blocking solution (0.2% BSA in PBS) for 1 h at RT. Tissues were then incubated overnight at 4ºC with primary antibodies diluted in blocking solution (see Table 6). After several washes with PBS, sections were incubated at RT with their respective secondary antibodies or streptavidin diluted in PBS (Table 7). Three randomly chosen areas (1 mm^2) along the spinal cord were analyzed in a blinded manner. CD3-positive cells were counted in infiltrates manually and results are expressed as the number of CD3 positive cells / mm^2. Lycopersicon esculentum agglutinin (LEA), glial fibrillary acidic protein (GFAP), SMI-32 and MBP quantification was performed with the ImageJ analysis software (http://imagej.nih.gov/ij/) and results are expressed as percentage of stained pixels / area.

Table 6. Primary antibodies used for immunostaining of the CNS of mice with EAE.

Target	Antibody	Dilution	Manufacturer
T lymphocytes	Rabbit anti-CD3	1/100	DakoCytomation, Glostrup, Denmark
Macrophages / Microglia	LEA	1/100	Sigma-Aldrich
Astrocytes	Rabbit anti-GFAP conjugated with cyanine 3 (Cy3)	1/500	DakoCytomation
Axonal damage	Mouse anti-200kD neurofilament heavy (SMI-32)	1/100	Abcam, Cambridge, UK
Myelin	Rabbit anti-MBP	1/400	Chemicon®, Millipore, Billerica, MA, USA

Table 7. Secondary antibodies and streptavidin used for immunostaining of the CNS of mice with EAE.

Antibody	Dilution	Manufacturer
Alexa 488 goat anti-rabbit	1/200	Life Technologies, Carlsbad, CA, USA
FITC streptavidin	1/100	eBioscience
Alexa 488 goat anti-mouse	1/200	Life Technologies

FITC: Fluorescein isothiocyanate.

9. IMMUNOLOGICAL ASSAYS

9.1. Splenocyte isolation

For the immunological assays, mice were euthanized at the end of the experiment, their abdominal surfaces sterilized with 70% ethanol and their spleens were removed and kept in supplemented IMDM at 4ºC. The splenocytes were obtained by pressing the spleens with a sterile syringe plunger on a 70 µm filter. Cells were washed with supplemented IMDM and centrifuged at 300 g for 5 minutes. Next, erythrocytes were lysed by diluting the cell pellet with 1 mL of sterile water. Immediately after, the lysis was blocked by the addition of 20 mL of PBS supplemented with 20% of FBS and the cell suspension was filtered again through a 70 µm filter to remove cellular aggregates. Cells were centrifuged at 300 g for 5 minutes and resuspended in proliferation medium consisting of IMDM supplemented with:

- 10% of HyClone® FetalClone (Thermo Fisher Scientific, Waltham, MA, USA)
- 2 mM L-glutamine
- 50 IU/ml penicillin
- 50 µg/ml streptomycin
- 2 µM of β-mercaptoetanol

Cell concentration was determined using a hematological counter (Coulter® Act Diff™, Beckman Coulter, Inc. L'Hospitalet de Llobregat, Spain).

9.2. Study of the different lymphoid populations in the spleens of mice with EAE treated with BM cells or MDSCs

To study the different cell populations, $5x10^5$ splenocytes were separated from the cell suspension and stained for flow cytometry as specified in part 4.1 of this section using the antibodies listed in Table 8. Combinations of these antibodies were used to study the following cell populations:

- T lymphocytes: $CD3^+$
- $CD4^+$ T lymphocytes: $CD3^+CD4^+$
- $CD8^+$ T lymphocytes: $CD3^+CD4^-$
- Treg cells: $CD3^+CD4^+CD25^+FoxP3^+$
- Activated $CD4^+$ T lymphocytes: $CD3^+CD4^+CD25^+FoxP3^-$
- B lymphocytes: $CD45^+B220^+$
- B1 lymphocytes (T-cell independent): $CD45^+B220^+CD5^+$
- B2 lymphocytes (T-cell dependent): $CD45^+B220^+CD5^-$
- Breg lymphocytes: $CD45^+B220^+CD1d^{high}CD5^+$

To study Treg cells, the staining protocol was slightly modified since the marker characteristic of this population requires an intracellular staining. FoxP3 was stained using anti-mouse/rat FoxP3 staining kit (eBiosicence) according to manufacturers' instructions:

1. $5x10^5$ cells were added to each tube and were washed with PBA-azide.
2. Cells were centrifuged at 300 g for 5 minutes and the supernatant was discarded leaving 50 µL of liquid.
3. Pellet was resuspended with a vortex pulse and Fc receptors were blocked by incubating the cells with 1 µL of anti-mouse CD16/32 antibody for 10 minutes at RT.
4. Cells were then incubated for 20 minutes at 4ºC with specific antibodies for cell surface molecules and isotype controls protected from light.

5. Cells were washed with 1 mL of PBA-azide and centrifuged at 300 g for 5 minutes and the supernatant was discarded.
6. Cell pellet was resuspended with a vortex pulse and incubated for 30 minutes at 4ºC protected from light with 1 mL of fixation/permeabilization solution.
7. Tubes were centrifuged at 300 g for 5 minutes and the supernatant was discarded.
8. 1 mL of permeabilization buffer was added and tubes were centrifuged at 300 g for 5 minutes and the supernatant was discarded.
9. Anti-FoxP3 antibody or isotype control was added and cells were incubated for 30 minutes at 4ºC protected from light.
10. Cells were washed with 1 mL of permeabilization buffer and centrifuged at 300 g for 5 minutes and the supernatant was discarded.
11. Cells were resuspended in 200-300 µL of PBA-azide and acquired using a FACSCanto flow cytometer.

Data were analyzed using the FCS Express v4 software.

Table 8. Antibodies used to study the different cell populations

Antibody	Isotype	Clone	µg for 10^5 cells	Supplier
FITC anti-mouse CD3e	Hamster IgG1	145-2C11	0.5	BioLegend
PerCP anti-mouse CD4	Rat IgG2b,k	GK1.5	0.2	BioLegend
PE anti-mouse CD25	Rat IgG1,λ	PC61.5	0.3	eBioscience
APC anti-mouse FoxP3	Rat IgG2a,k	FJK-16s	0.3	eBioscience
APC-Cy7 anti-mouse CD45	Mouse IgG2a,k	104	0.2	BioLegend
PE-Cy7 anti-mouse CD45R (B220)	Rat IgG2a,k	RA3-6B2	0.1	eBioscience
APC anti-mouse CD5	Rat IgG2a,k	53-7.3	0.2	eBioscience
Alexa 488 anti-mouse CD1d	Rat IgG2b,k	1B1	0.5	eBioscience

9.3. Study of the proliferation of splenocytes of mice with EAE treated with unfractionated BM cells or MDSCs

To evaluate the proliferative capacity of splenocytes, mice were euthanized at the end of the experiment and splenocytes were obtained as explained in part 9.1 of this section. After calculating cell concentration, cells were seeded at $2x10^5$ cells/well in 96-well plates (Nunc) in 200 µL of proliferation medium. Cells were stimulated with 5 µg/ml of MOG_{40-55} or 5 µg/ml of phytohemagglutinin L (PHA-L) (Sigma-Aldrich) and cells without any stimulus were used as baseline controls.

After 48 h of incubation, 50µl of the supernatant of each well were collected and stored at -80°C for further quantification of cytokines levels. Then 1µCi/well of [^{3}H]-thymidine (PerkinElmer, Waltham, MA, USA) was added and cells were incubated for 18 h. After this time, cells were transferred to a filter using a harvester (Harvester 96, TomTec®, Unterschleissheim, Germany) following the manufacturer's instructions. The levels of incorporated radioactivity (proliferating cells) were determined using a beta scintillation counter (Wallac, Turku, Finland). Five replicas for each mouse and condition were performed and results were expressed as the mean of counts per minute (cpm).

9.4. Suppression of splenocyte proliferation

To measure the ability to suppress splenocyte proliferation by unfractionated BM cells and isolated MDSCs, mice with EAE were sacrificed by CO_2 asphyxiation at day 11-13 postimmunization (p.i.) (inflammatory phase of EAE) and splenocytes were obtained as specified in part 8.1 of this section. Then, 10^5 splenocytes/well were seeded in 96-well plates with proliferation medium. Total BM cells or the isolated MDSCs were irradiated at 25 grays (Gy) and were added to the wells at different proportions as indicated in Table 9. The proportions used for MDSCs were equivalent to those of BM cells taking into account the proportions of MDSCs within BM cells.

Cells were then cultured with 5 µg/ml of MOG_{40-55} for 48h. Splenocytes were cultured with and without the peptide as proliferation controls. Splenocyte proliferation was measured as specified in part 9.3 of this section. Five replicas for condition were

performed and the percentage of suppression of splenocyte proliferation was calculated using the following formula:

$$\text{Percentage of suppression} = \left[1 - \left(\frac{\text{cpm in the presence of BM or MDSCs}}{\text{cpm in the absence of BM or MDSCs}}\right)\right] \times 100$$

Table 9. BM cell and MDSC proportions used in the suppression assays

Ratio (BM:SPL)	BM	Splenocytes (SPL)
8:1	$8x10^5$	10^5
4:1	$4x10^5$	10^5
2:1	$2x10^5$	10^5
1:1	10^5	10^5
1:2	$0.5x10^5$	10^5
1:4	$0.25x10^5$	10^5
Ratio (MDSCs:SPL)	**MDSCs**	**Splenocytes (SPL)**
4:1	$4x10^5$	10^5
2:1	$2x10^5$	10^5
1:1	10^5	10^5
1:2	$0.5x10^5$	10^5
1:4	$0.25x10^5$	10^5
1:8	$0.125x10^5$	10^5

9.5. Quantification of secreted cytokines

The analysis of the secreted cytokine profile was analyzed in culture supernatants from splenocytes stimulated with MOG_{40-55} obtained from proliferation assays (see part 8.1 of this section) with the Mouse FlowCytomix Th1/Th2 10 plex kit (Bender MedSystems Inc., Burlingame, CA, USA). This kit is a bead based detection system which allows the quantification of the mouse cytokines GM-CSF, IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17 and TNF-α by flow cytometry. Beads are coated with antibodies specifically

reacting with each cytokine to be detected in the multiplex system. There are two sets of beads of different sizes (4 µm and 5 µm) so they can be differentiated on the cytometer combining the FSC and SSC parameters. Moreover, for each sphere size five distinct populations can be distinguished as they are internally dyed with different intensities of a fluorescent dye emitting in red (660 nm) (Figure 23).

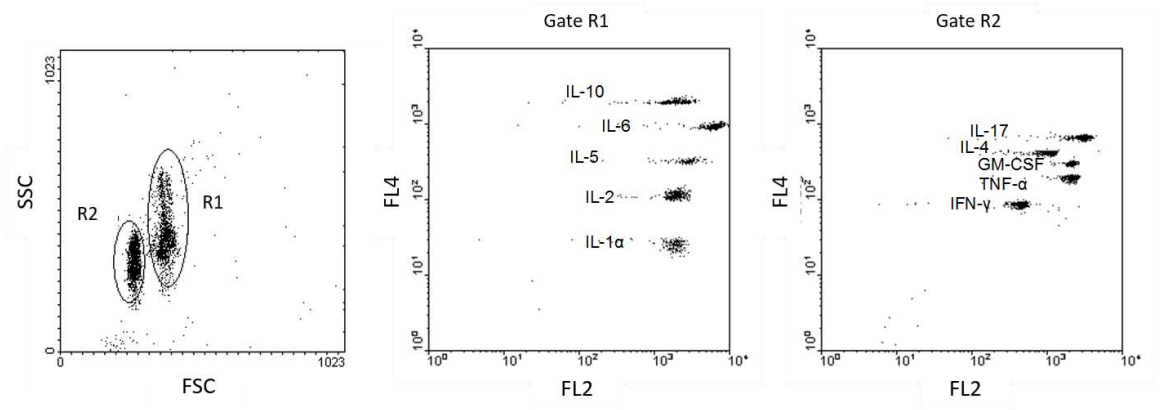
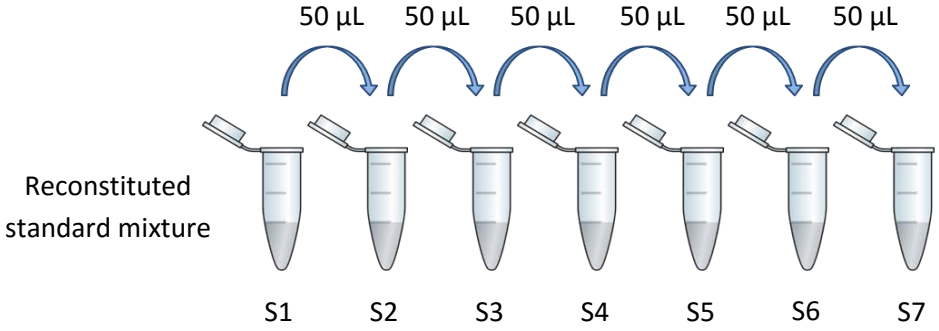


Figure 23. Cytometric bead array detection system. Two bead populations can be distinguished by FSC and SSC (R1 and R2). In each bead population five cytokines can be distinguished according to the different fluorescence intensity detected in the FL4 channel (660nm).

The detection of cytokines from each sample was determined following the manufacturer's instructions:

1. Total number of samples including standards for the standard curve and blanks were calculated.
2. Lyophilized standard was reconstituted by adding 210 µL of assay buffer provided with the kit. Standard curves were created by making serial dilutions of the standard mixture:



3. Afterwards, 25 µL of sample or standard was added to each corresponding tube.
4. Then 25 µL of bead mixture was added to all tubes including blanks.
5. Next, 50 µL of biotinylated antibody mixture was added, tubes were mixed well and incubated for 2 h at RT protected from light with aluminum foil.
6. Tubes were washed with 1 mL of assay buffer and centrifuged at 200g for 5 minutes. Supernatant was discarded leaving 100 µL of liquid in each tube.
7. Next, 50 µL of streptavidin-PE solution was added and tubes were mixed and incubated for 1 h at RT protected from light with aluminum foil.
8. Tubes were washed with 1 mL of assay buffer and centrifuged at 200g for 5 minutes. Supernatant was discarded leaving 100 µL of liquid in each tube.
9. Finally, 500 µL of assay buffer was added to all tubes and samples were acquired using a FACSCanto flow cytometer.

Cytokine concentration from the different samples was extrapolated from the standard curve for each cytokine using the FlowCytomix™ Pro 3.0 software (eBioscience).

9.6. Detection of anti-MOG_{40-55} antibodies in serum by ELISA

In order to detect anti-MOG_{40-55} antibodies in serum of mice with EAE, mice were euthanized by CO_2 asphyxiation at the end of each experiment and PB was obtained by cardiac puncture, collected into a 1.5 mL eppendorf tube and kept in ice. Samples were then centrifuged at 3000g for 30 minutes and the serum was collected and stored at -80ºC until analyzed.

96-well flat bottom plates (Costar, Sigma-Aldrich) were coated overnight with 0.1 µg/well (1 µg/ml) of $MOG_{40–55}$ peptide in sodium carbonate solution at pH 9.6 at 4ºC. The next day plates were washed twice with PBS containing 0.1% of Tween-20 (washing solution) and subsequently blocked with blocking solution (PBS with 5%

skimmed milk powder) for 1 h at 37ºC. After two washes, serum samples previously diluted (1:25) in blocking solution were incubated in duplicate for 1 h at 37ºC. Afterwards, the plate was washed and the secondary antibody goat anti-mouse IgG conjugated with horseradish peroxidase (BD Biosciences) diluted 1:3000 in washing solution was added and incubated for 2 h at RT. After three washes, substrate solution TMB Substrate Reagent Set (BD Biosciences) containing hydrogen peroxide and a chromogen was added. After 20 minutes the reaction was stopped by the addition of 1M sulfuric acid [modified from (Eixarch, Espejo et al. 2009)]. The optical density (OD) was read on a spectrophotometer (Anthos Labtec, Lagerhausstr, Austria) for plates using a 450 nm filter. Serum samples from saline-immunized mice were included as negative controls. Results were presented as the mean OD of each sample and positivity was defined as an OD greater than the mean OD + 2 standard deviations (SD) of sera from non-immunized (NI) control mice.

10. STATISTICAL ANALISIS

The statistical analysis of the data was performed using the SAS® 9.3 program (SAS Institute Inc., Cary, NC, USA), the SPSS v21.0 program (SPSS Inc., Chicago, USA) or the GraphPad Prism program version 5.1 (GraphPad Inc., San Diego, CA, USA), which was also used to do the graphs. Depending on the distribution of the variables different statistic analyses were performed. In all cases it was considered that the differences were statistically significant when the *p*-value was ≤ 0.05.

Parametric statistical tests were applied when the data of the groups to compare were normally distributed. The tests used included Student's *t* test when comparing the means of two independent data sets and paired Student's *t* test when comparing the means of two paired data sets. Generalized estimating equation method was used when comparing the means of three or more independent groups with the repeated measures design and a Bonferroni correction was applied to compare data sets in pairs. When data were not normally distributed, non-parametric statistical tests were applied. The Mann-Whitney U test was used to compare the means of two independent data sets and the Wilcoxon test and Wilcoxon rank-sum tests reporting

exact *p*-values were used to compare the means of two paired data sets. In cases where the hypothesis was uni-directional and was supported by strong previous evidence, the statistical tests mentioned above were used but applying only one tail. Otherwise, two-tail analyses were applied.

PART 2: GENERATION OF HUMAN MDSCs FROM HEMATOPOIETIC PROGENITOR CELLS

1. HUMAN HEMATOPOIETIC PROGENITOR CELLS

Human hematopoietic progenitors ($CD34^+$) were obtained from apheresis products from healthy donors treated with G-CSF. Apheresis products were kindly gifted by Dr. Gregorio Martín-Henao from *Banc de Sang i Teixits de Catalunya* (BST; Barcelona, Spain) and by Dr. Pedro Marín from *Servei d'Hemoteràpia de l'Hospital Clínic de Barcelona* (Barcelona, Spain). The hematopoietic progenitors were stored in liquid nitrogen until needed.

2. DETERMINATION OF CULTURE CONDITIONS FOR GENERATING MDSCs FROM HEMATOPOIETIC PROGENITORS

$CD34^+$ cells from apheresis products were thawed following standard techniques. Cells were counted in the Neubauer chamber and viability was assed using trypan blue. Cells were seeded at 50.000 cells/well in round bottom 96-well plates with StemSpan™ Serum-Free Expansion Medium II (SFEM II; StemCell Technologies, Grenoble, France) supplemented with 50 IU/ml penicillin and 50 µg/ml streptomycin. In order to generate MDSCs, different combinations of the cytokines listed in Table 10 were added to culture medium. Every three days, 90% of the culture medium of each well was collected in a 15 mL tube and centrifuged for 5 minutes at 300 g. Cell pellet was resuspended in fresh medium and recombinant cytokines were added.

To determine the optimal conditions to generate MDSCs from hematopoietic progenitors, cells were cultured at 37ºC, 5% CO_2 and with 100% of relative humidity for 9, 14 or 20 days and the following cytokine cocktails were tested. At least three replicates per condition were performed.

For cultures of 9 days the following cytokine combinations were tested:

- SCF + TPO + FLT3-L + GM-CSF + IL-6
- SCF + TPO + FLT3-L; adding GM-CSF and IL-6 at day 4 of culture
- SCF + TPO + FLT3-L + GM-CSF + IL-6 + IL-3
- SCF + TPO + FLT3-L + IL-3; adding GM-CSF and IL-6 at day 4 of culture

For cultures of 14 and 20 days the following cytokine combinations were tested:

- SCF + TPO + FLT3-L + GM-CSF + IL-6
- SCF + TPO + FLT3-L + GM-CSF + IL-6 + IL-3

To maintain the adequate cell density, cells were transferred to 24-well plates at day 8 for cultures of 14 and 20 days and to 12-well plates at day 15 for cultures of 20 days. At the end of the culture, cells were harvested by collecting the content of each well and washing them with PBS. Cells were centrifuged for 5 minutes at 300 g and were resuspended in culture medium or PBS, depending on the experiment. Cells were then counted in the Neubauer chamber using trypan blue as diluent.

Table 10. Cytokines used to generate MDSCs from HSC

Cytokine	Activity	Culture concentration	Supplier
SCF	Increases the survival of HSCs *in vitro* and stimulates their proliferation in culture	50 ng/ml	CellGenix (Freiburg, Germany)
TPO	Stimulates the development of megakaryocyte precursors	10 ng/ml	Peprotech (Rocky Hill, NJ, USA)
FLT3-L	Regulates proliferation of early hematopoietic cells	50 ng/ml	Humanzyme (Chicago, IL, USA)
IL-3	Promotes differentiation of committed progenitor cells into myeloid progenitors	20 ng/ml	Peprotech
IL-6	Proinflammatory cytokine known to induce MDSCs differentiation	40 ng/ml	CellGenix
GM-CSF	Stimulates the development of neutrophils and macrophages from myeloid progenitors	40 ng/ml	Genzyme (Cambridge, MA, USA)

3. CHARACTERIZATION OF HEMATOPOIETIC PROGENITORS AND GENERATED MDSCs

$CD34^+$ hematopoietic progenitors were phenotypically characterized by flow cytometry prior to culture (day 0) and the generated MDSCs at days 9, 14 and 20 of culture. Cells were stained with the antibodies listed in Table 11 following the protocol of direct staining of cell surface antigens described below:

1. $5x10^5$ cells were added to each tube and were washed with 1 mL of PBA-azide.
2. Cells were centrifuged at 300 g for 5 minutes and the supernatant was discarded leaving 50 µL of liquid.
3. Pellet was resuspended with a vortex pulse and Fc receptors were blocked by incubating the cells with 5 µg of purified human IgG (Flebogamma®, Grifols) for 15 minutes at RT.
4. Cells were then incubated for 20 minutes at 4ºC with specific antibodies and isotype controls protected from light.
5. Cells were washed with 1 mL of PBA-azide and centrifuged at 300 g for 5 minutes.
6. Cells were resuspended in 200-300 µL of PBA-azide and acquired using a LSR Forstessa (BD Biosciences) flow cytometer.

Table 11. Antibodies used to characterize hematopoietic progenitors and MDSCs

Antibody	Isotype	Clone	µg for 10^5 cells	Supplier
PerCP anti-human CD34	Mouse IgG1,k	8G12	0.05	BD Biosciences
APC anti-human CD33	Mouse IgG1,k	WM53	0.09	BioLegend
APC-H7 anti-human HLA-DR	Mouse IgG2a,k	G46-6	0.075	BD Biosciences
BV421 anti-human CD14	Mouse IgG2a,k	M5E2	0.1	BioLegend
PE anti-human CD15	Mouse IgG1,k	W6D3	0.16	BioLegend

BV421: Brilliant violet 421.

Moreover, in MDSCs generated in cultures of 20 days using the SCF + TPO + FLT3-L + GM-CSF + IL-6 combination, the expression of PD-L1, CD80 and CD86 markers was also determined by flow cytometry. The expression of these molecules was studied both in a steady state and in an activated state. To activate MDSCs, cells were cultured 18 h in the presence of inflammatory stimuli [10 ng/ml of IFN-γ (Peprotech) and 100 ng/ml of LPS]. MDSCs were stained for flow cytometry analysis using the antibodies listed in Table 12 and following the protocol described above.

Table 12. Antibodies used to characterize hematopoietic progenitors and MDSCs

Antibody	Isotype	Clone	µg for 10^5 cells	Supplier
APC anti-human CD33	Mouse IgG1,k	WM53	0.09	BioLegend
APC-H7 anti-human HLA-DR	Mouse IgG2a,k	G46-6	0.075	BD Biosciences
BV421 anti-human CD14	Mouse IgG2a,k	M5E2	0.1	BioLegend
PE anti-human CD15	Mouse IgG1,k	W6D3	0.16	BioLegend
PE-Cy7 anti-human PD-L1	Mouse IgG2b,k	29E.2A3	0.5	BioLegend
FITC anti-human CD80	Mouse IgG1,k	L307.4	0.19	BD Biosciences
PerCp-Cy5.5 anti-human CD86	Mouse IgG1,k	2331	0.125	BD Biosciences

3. SUPPRESSION OF ALLOGENEIC PBMCs PROLIFERATION

3.1. MDSC cell sorting

MDSCs generated in cultures of 14 and 20 days, using the SCF + TPO + FLT3-L + GM-CSF + IL-6 combination of cytokines, were purified by cell sorting. Cells were stained in a laminar flow cabinet with anti-CD33 and anti-HLA-DR antibodies listed in Table 12 following the protocol of direct staining of cell surface antigens described below:

1. Cell suspension was centrifuged at 300 g for 5 minutes and pellet was resuspended in 1-1.5 mL of HIB solution.

2. Fc receptors were blocked by incubating the cells with 5 µg of purified human IgG per $5x10^5$ cells for 15 minutes at RT (human IgG volume was scaled up accordingly).
3. Cells were then incubated for 20 minutes at 4ºC with specific antibodies and isotype controls protected from light (antibodies volume was scaled up accordingly).
4. Cells were washed with 3 mL of HIB and centrifuged at 300 g for 5 minutes.
5. Cells were resuspended at a concentration of 10^7 cells/ml in HIB solution and sorted using a FACSAria II (BD Biosciences) cell sorter.

MDSCs were sorted following the gating strategy shown in Figure 23 and collected into 15 mL tubes containing 6 mL of proliferation medium (see part 9.1 of this section). $CD33^+HLA\text{-}DR^+$ cells were also sorted to be used as positive controls. Then cells were centrifuged at 300 g for 5 minutes and resuspended in 1 mL of proliferation medium. Cells were counted in the Neubauer chamber using trypan blue to assess cell viability.

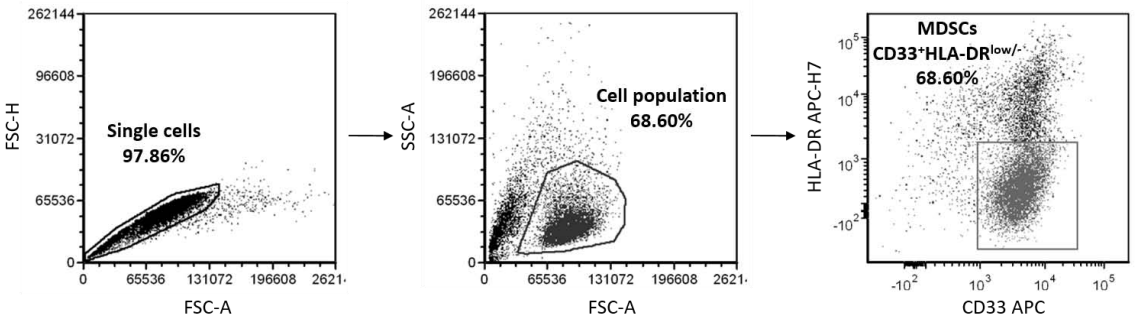


Figure 23. MDSC gating strategy. This image shows the gating strategy used to isolate $CD33^+HLA\text{-}DR^{low/-}$ MDSCs. First of all single cells were selected using FSC-A and FSC-H parameters as singlet events are presented in a more diagonal display than doublets. Second, the population of live cells were selected using FSC-A and SSC-A. Finally, MDSCs were identified selecting the cells with $CD33^+HLA\text{-}DR^{low/-}$ phenotype.

3.2. Isolation of PBMCs from whole blood

PBMCs were obtained from PB samples of healthy donors collected in 10 mL tubes containing EDTA acquired from *Banc de Sang i Teixits de Catalunya*. PBMCs were

isolated by a density gradient using Lympholyte®-H (CEDARLANE®, Burlington, Canada) which is a separation medium specifically designed for the isolation of viable lymphocytes and monocytes from human PB (Figure 24).

PB was transferred to a 50 mL tube and diluted to a final volume of 30 mL in PBS supplemented with 10% FBS and 50 IU/ml penicillin and 50 µg/ml streptomycin. Diluted blood was carefully layered over 10 mL of Lympholyte®-H (3:1 proportion) and centrifuged at 800 g for 25 minutes at RT without acceleration or brake. Afterwards, the well-defined mono-lymphocyte layer at the interface was carefully removed using a plastic Pasteur pipette and transferred into a new 50 mL tube. Cells were washed twice by adding 25 mL of supplemented PBS and centrifuging them at 300 g for 5 minutes. Finally, cells were resuspended in 1 mL of proliferation medium and counted with the Neubauer chamber using Türk solution as diluent.

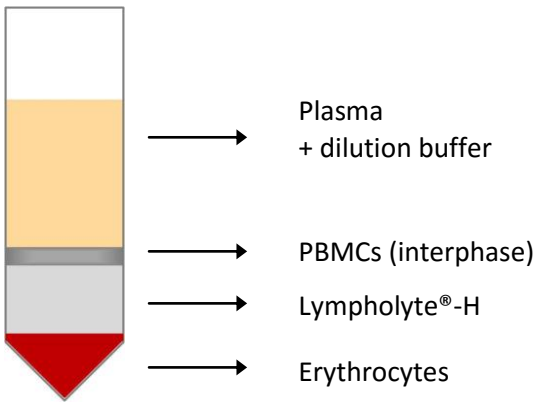


Figure 24. Representative figure of a PBMCs density gradient. Diagram showing the different phases that are formed after a density gradient from PB.

3.3. Suppression assays of PBMC proliferation

Due to the inability to use MDSCs and PBMCs from the same donor, we co-cultured the generated MDSCs with allogeneic PBMCs. To measure the ability of the MDSCs generated in 14 and 20 days cultures to suppress PBMC proliferation induced by an allogeneic stimulus, 10^5 PBMCs/well were seeded in round bottom 96-well plates with proliferation medium. Sorted $CD33^+HLA\text{-}DR^{low/-}$ MDSCs or $CD33^+HLA\text{-}DR^+$ cells were irradiated at 25 Gy and added in the same proportion to the allogeneic PBMCs.

$CD33^{+}HLA\text{-}DR^{+}$ cells were used as a positive control of an allogeneic-induced proliferative response. Results are expressed as cpm.

In order to assess the capacity of MDSCs to suppress PHA-L-induced T-cell proliferation, 10^5 PBMCs/well were cultured with 2 µg/mL of PHA-L. Sorted MDSCs were irradiated at 25 Gy and added to PBMCs at different proportions:

Ratio (MDSCs:PBMCs)	MDSCs	PBMCs
2:1	$2x10^5$	10^5
1:1	10^5	10^5
1:5	$0.5x10^5$	10^5
1:10	$0.25x10^5$	10^5

Cells were then cultured for 96 h at 37ºC, 5% CO_2 and with 100% of relative humidity. PBMCs were cultured alone with and without PHA-L as proliferation controls. Then supernatants (50 µL/well) were collected and stored at -80ºC for further quantification of cytokines levels. Then 1 µCi/well of [^{3}H]-thymidine was added and cells were incubated for 18 h. Cells were then transferred to a filter using a harvester following the manufacturer's instructions. The levels of incorporated radioactivity (proliferating cells) were determined using a beta scintillation counter. Five replicas for condition were performed and the percentage of suppression of PBMC proliferation was calculated using the formula described in part 9.4 of this section.

3.4. Quantification of the cytokines secreted in the culture supernatants

The profile of secreted cytokines was analyzed in culture supernatants from suppression assays of PHA-L-induced T-cell proliferation (see part 3.3 of this section). The levels of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A were measured with the BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences)

and the levels of IL-1β, IL-5, IL-12, IL-13, IL-21 and GM-CSF were measured with the BD™ CBA Human Soluble Protein Flex Set Capture Beads in conjunction with BD™ CBA Human Soluble Protein Master Buffer Kit (all from BD Biosciences). As in the case of mouse secreted cytokines quantification (mentioned in part 9.5 of the above section), this is a bead based detection system which allows the simultaneous quantification of human multiple soluble proteins by flow cytometry. Each single bead population is coated with a capture antibody which specifically reacts with each cytokine to be detected. PE-conjugated detection antibodies form sandwich complexes with the cytokines and the capture beads. The intensity of PE fluorescence of each sandwich complex reveals the concentration of each particular cytokine. The seven beads of the Human Th1/Th2/Th17 Cytokine Kit are resolved in the red channel (660 nm) and the different Flex Set Capture Beads can be distinguished in the red and far red fluorescence channels (660 and 780 nm) of a flow cytometer.

The detection of cytokines using the Human Th1/Th2/Th17 Cytokine Kit was performed following the manufacturer's instructions:

1. Total number of samples including standards for the standard curve and blanks were determined.
2. The lyophilized standard spheres were transferred to a 15 mL conical polypropylene tube and were reconstituted with 2 mL of assay diluent.
3. Standard curves were created by making serial dilutions of the standard mixture with a 1:2 dilution factor (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256). One additional tube was added with only assay diluent to serve as a negative control.
4. A mixture containing all the capture beads was prepared by adding 10 µL of each capture bead for each sample to be analyzed.
5. Afterwards, 50 µL of the mixed capture beads was added to each tube.
6. Then 50 µL of standard dilution or sample was added to each corresponding tube.

7. Next, 50 µL of the Human Th1/Th2/Th17 PE detection reagent was added, tubes were mixed well and incubated for 3 h at RT protected from light with aluminum foil.
8. Tubes were washed with 1 mL of wash buffer and centrifuged at 200g for 5 minutes. Supernatant was discarded leaving 100 µL of liquid in each tube.
9. Finally, 300 µL of assay buffer was added to all tubes and samples were acquired using a LSR Forstessa flow cytometer.

The detection of cytokines using the CBA Human Soluble Protein Flex Set Capture Beads in conjunction with the CBA Human Soluble Protein Master Buffer Kit was performed following the manufacturer's instructions:

1. Total number of samples including standards for the standard curve and blanks were determined.
2. The lyophilized standard spheres from each CBA Human Soluble Protein Flex Set were pooled to a 15 mL conical polypropylene tube and were reconstituted with 4 mL of assay diluent.
3. Standard curves were created by making serial dilutions of the standard mixture with a 1:2 dilution factor (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256). One additional tube was added with only assay diluent to serve as a negative control.
4. A mixture containing all the capture beads was prepared by adding 1 µL of each capture bead for each sample to be analyzed. Mixed capture beads were diluted with capture bead diluent. The diluent volume was calculated subtracting the volume for each bead tested from the total volume of diluted beads needed to perform the assay.
5. A mixture of all the different PE detection reagents was prepared by adding 1 µL of each PE detection reagent for each sample to be analyzed. Mixed PE detection reagents were diluted with detection reagent diluent. The diluent volume was calculated subtracting the volume for each PE detection reagent

tested from the total volume of diluted detection reagent needed to perform the assay.

6. Then 50 µL of standard dilution or sample was added to each corresponding tube.

10. Afterwards, 50 µL of the mixed capture beads was added, tubes were mixed well and incubated for 1 h at RT protected from light with aluminum foil.

11. Next, 50 µL of the mixed PE detection reagent was added, tubes were mixed well and incubated for 2 h at RT protected from light with aluminum foil.

7. Tubes were washed with 1 mL of wash buffer and centrifuged at 200g for 5 minutes. Supernatant was discarded leaving 100 µL of liquid in each tube.

8. Finally, 300 µL of assay buffer was added to all tubes and samples were acquired using a LSR Forstessa flow cytometer.

Cytokine concentration from the different samples was extrapolated from the standard curve for each cytokine using the FCAP Array 3.0 software (BD Biosciences).

4. STATISTICAL ANALISIS

The statistical analysis of the data was performed using the SPSS v21.0 program (SPSS, Inc.) or with the GraphPad Prism program version 5.1, which was also used to do the graphs. Depending on the distribution of the variables different statistic tests were performed. In all cases it was considered that the difference was statistically significant when the *p*-value was ≤ 0.05.

Parametric statistical tests were applied when the data of the groups to compare were normally distributed. The tests used included the Student's *t* test when comparing the means of two independent data sets and paired Student's *t* test when comparing the means of two paired data sets. One-way ANOVA test was used when comparing the means of three or more independent groups and Dunnett's test was used when comparing the means of multiple groups with a single control, in both cases the Bonferroni correction was applied to compare data sets in pairs.

Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.

Thomas A. Edison

RESULTS

PART 1: ANTIGEN-SPECIFIC MDSCs INDUCE IMMUNOLOGICAL TOLERANCE IN THE EAE IN BOTH PREVENTIVE AND THERAPEUTIC APPROACHES

1. CHARACTERIZATION OF BM CULTURE

In order to identify and characterize the population of cells responsible for the induction of the immunological tolerance that our group previously observed in the EAE model (Eixarch, Espejo et al. 2009), immunophenotypic analyses of BM cells were performed before and after retroviral transduction. For this purpose the phenotype of BM cells was analyzed by flow cytometry in fresh BM obtained from 5-FU treated mice (50 mg/kg at day -5) and 24 h after the second cycle of transduction.

Myeloid, T and B lineage markers were analyzed both at the beginning and at the end of the culture (day 0 and 4). On day 4, the proportions of T $CD4^+$, T $CD8^+$ and B ($B220^+$) lymphocytes were greatly reduced whereas those of immature hematopoietic progenitors ($CD45^+Lin^-$) and cells co-expressing the CD11b and Gr-1 myeloid markers were significantly increased compared with fresh BM cells (day 0). Indeed, in agreement with our previous results (Gomez, Espejo et al. 2014), the vast majority of cells that were generated in BM retroviral transduction cultures consisted of two main myeloid cell populations $CD11b^+Gr\text{-}1^{low}$ (16.9 ± 4.6%) and $CD11b^+Gr\text{-}1^{high}$ (52.2 ± 6.6%), which correspond, respectively, to the phenotypes of M- and G-MDSCs. The percentages of the two subtypes of MDSCs increased by twenty- and tenfold, respectively (Figure 25). On day 4, the absolute numbers of M-MDSCs increased nearly thirty-fold and those of G-MDSCs increased more than ten-fold as shown in Figure 26, indicating that these cells were expanded during culture or were generated de novo.

The effectively transduced cells quantified by the expression of the EGFP marker mainly consisted of M- and G-MDSCs, which represented 19.6 ± 0.9% and 35.9 ± 1.5% of the total transduced cells, respectively, accounting for more than the half of the transduced cells (Figure 27). Since the therapeutic effect observed in EAE occurred only when the transplanted cells were transduced with the autoantigen and not with

the control vector (Eixarch, Espejo et al. 2009), we can infer that the cells responsible for the beneficial effect must be transduced. For this reason, both populations of MDSCs represent the best candidates for mediating the induction of immunological tolerance in our EAE model.

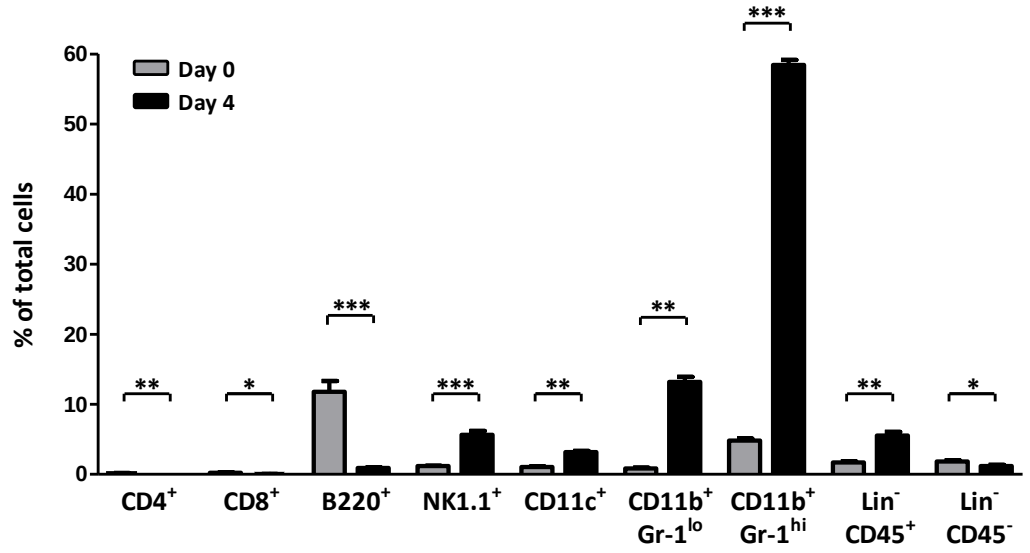


Figure 25. Cell populations in fresh BM cells and after 4 days of transduction culture . Bars indicate the percentage of the different cell populations within the BM cells at day 0 (grey bars) and at day 4 24 h after the second cycle of transduction (black bars). Data are represented as the mean ± standard error of the mean (SEM). * p<0.05, ** p<0.01, *** p<0.001; (n=3).

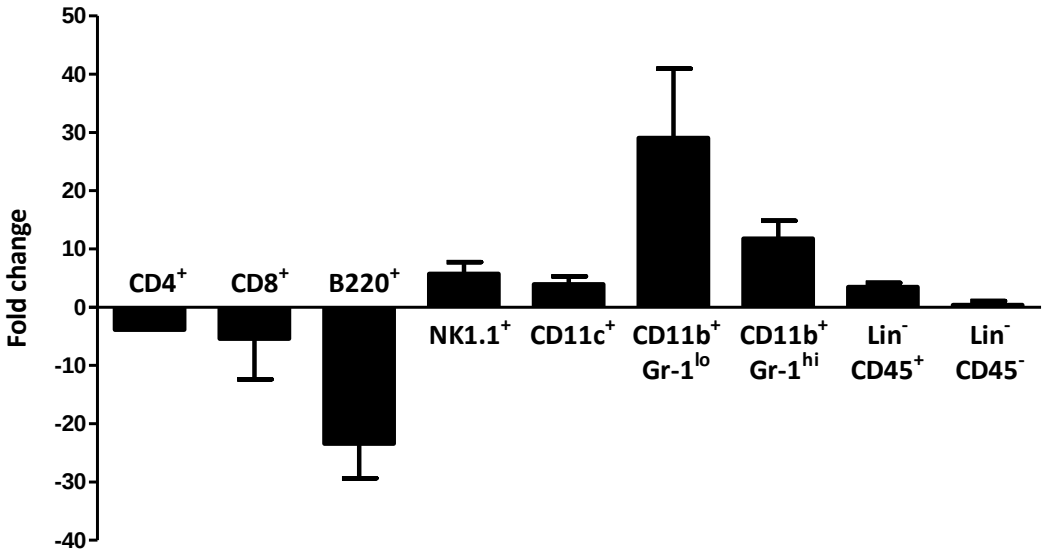


Figure 26. Fold change in cell number of BM cells. Bars indicate the fold change in cell numbers after the second cycle of transduction. Data are represented as the mean ± SEM. (n=3).

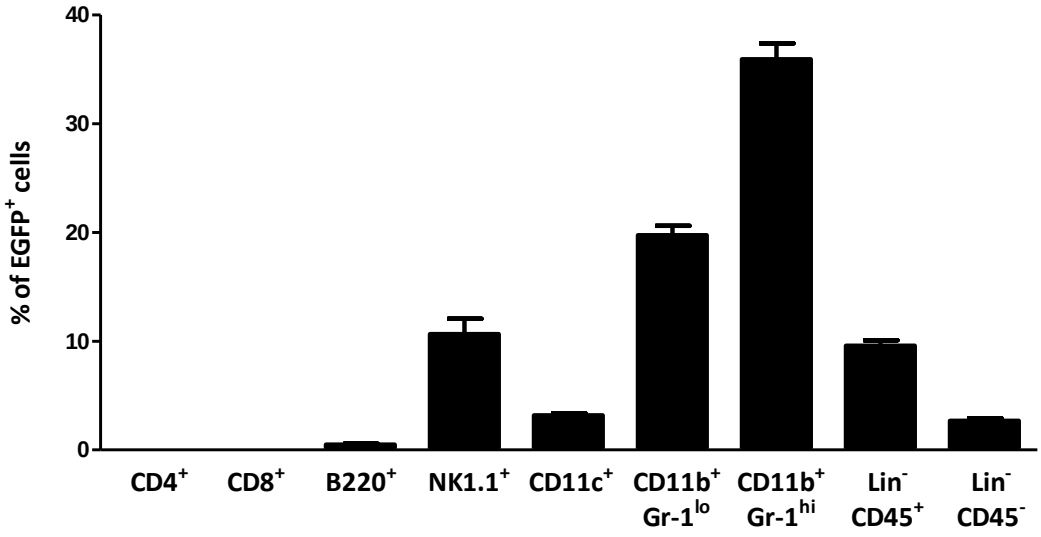


Figure 27. Transduced cell populations present in BM transduction cultures. The percentage of each cell subpopulation within the transduced BM cells was analyzed by gating on $EGFP^+$ cells. Data are represented as the mean ± SEM. (n=3).

2. PHENOTYPIC CHARACTERIZATION OF MDSCs

To better define the MDSCs generated during BM retroviral transduction cultures, further phenotypic characterization of both subsets of MDSCs was performed. We decided to study the expression of several molecules associated with the ability to suppress T-cell responses in both M- and G-MDSCs. The expression of PD-L1 and PD-L2, which are the ligands of the inhibitory receptor PD-1, and CD80 and CD86 which are the ligands of CD28 and CTLA-4, as well as the expression of MHC-II molecules ($I\text{-}A^b$) was analyzed by flow cytometry at day 4 of BM transduction culture. As it is known that MDSCs are activated under inflammatory conditions, the expression of these molecules was studied both at baseline and in the presence of inflammatory stimuli.

Both M- and G-MDSCs expressed PD-L1 and its expression was significantly increased upon stimulation with IFN-γ, LPS and with IFN-γ plus LPS (Figure 28A and 28C). None of the two populations showed expression of PD-L2 in any of the cases (data not shown). Consequently, we focused on the study of the MFI of PD-L1. As expected, both subsets of MDSCs showed significantly up-regulation of PD-L1 expression upon stimulation, which was significantly higher when stimulated with IFN-γ plus LPS (Figure 28B and

28D). In this condition, PD-L1 expression was increased by more than threefold in both M- and G-MDSC subsets.

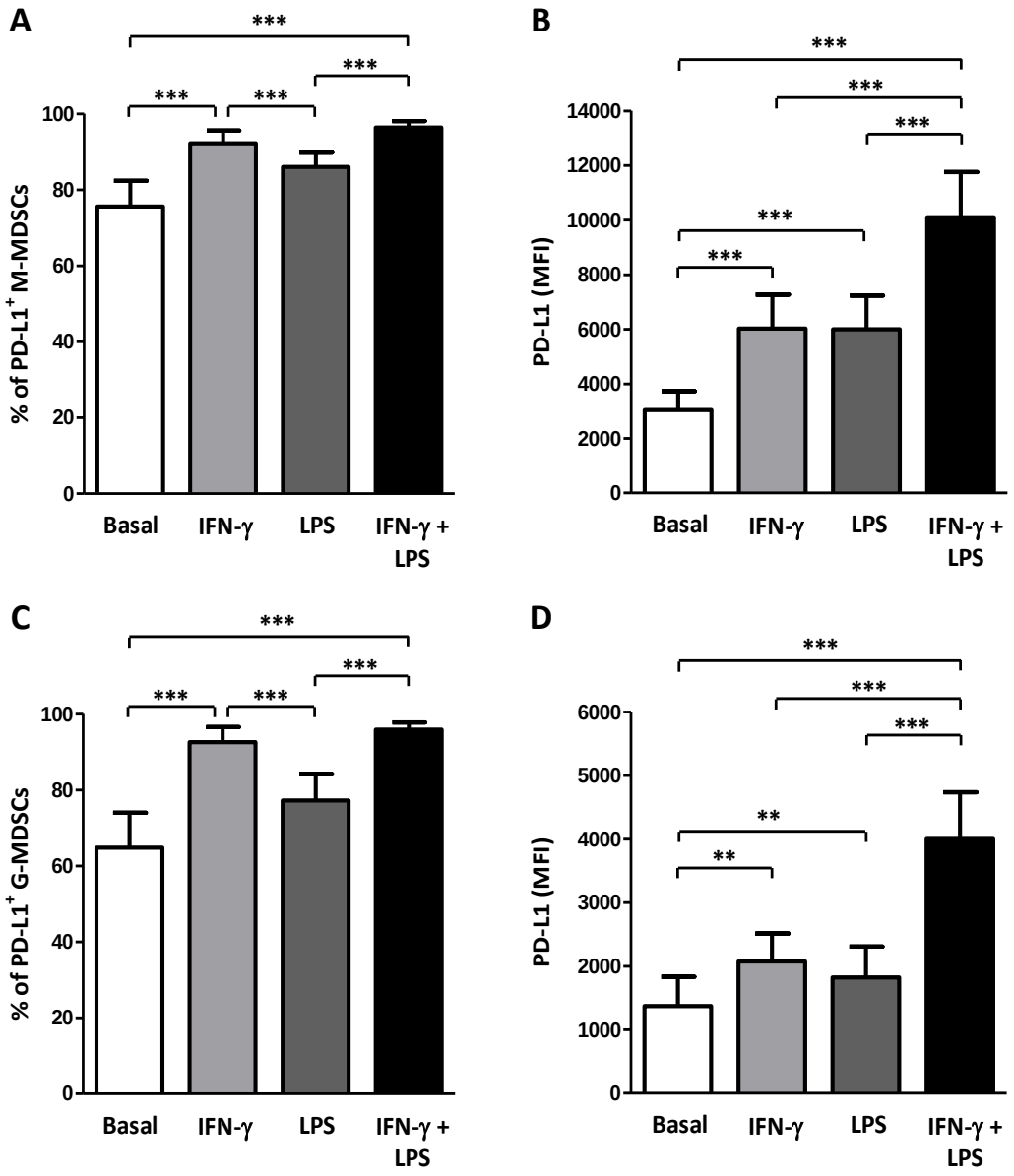


Figure 28. PD-L1 expression in M- and G-MDSC subsets. Expression of PD-L1 was assessed both at baseline and after 18 h of IFN-γ and LPS stimulation. (A) Percentage of M-MDSCs expressing PD-L1, (B) MFI of PD-L1 expression of M-MDSCs, (C) percentage of G-MDSCs expressing PD-L1 and (D) MFI of PD-L1 expression of G-MDSCs. Data are represented as the mean ± SEM. ** p<0.01, *** p<0.001; (n=4).

As it became apparent that using IFN-γ and LPS in combination was the condition that had a more potent stimulatory effect on the cells, we decided to study the expression of the rest of the markers using only this condition. Both subsets of MDSCs expressed CD80 and CD86 and its expression was significantly increased upon stimulation, although M-MDSCs showed a higher level of expression of both markers (Figure 29A and 29C). Regarding the study of the MFI, both M- and G-MDSC populations showed a significant up-regulation of CD80 and CD86 expression when stimulated (Figure 29B and 29D). Interestingly, 45.2 ± 1.6% of M-MDSCs expressed MHC-II while only 2.0 ± 0.2% of G-MDSCs did. However, MHC-II expression was significantly increased in both subpopulations upon stimulation (Figure 29E and 29F).

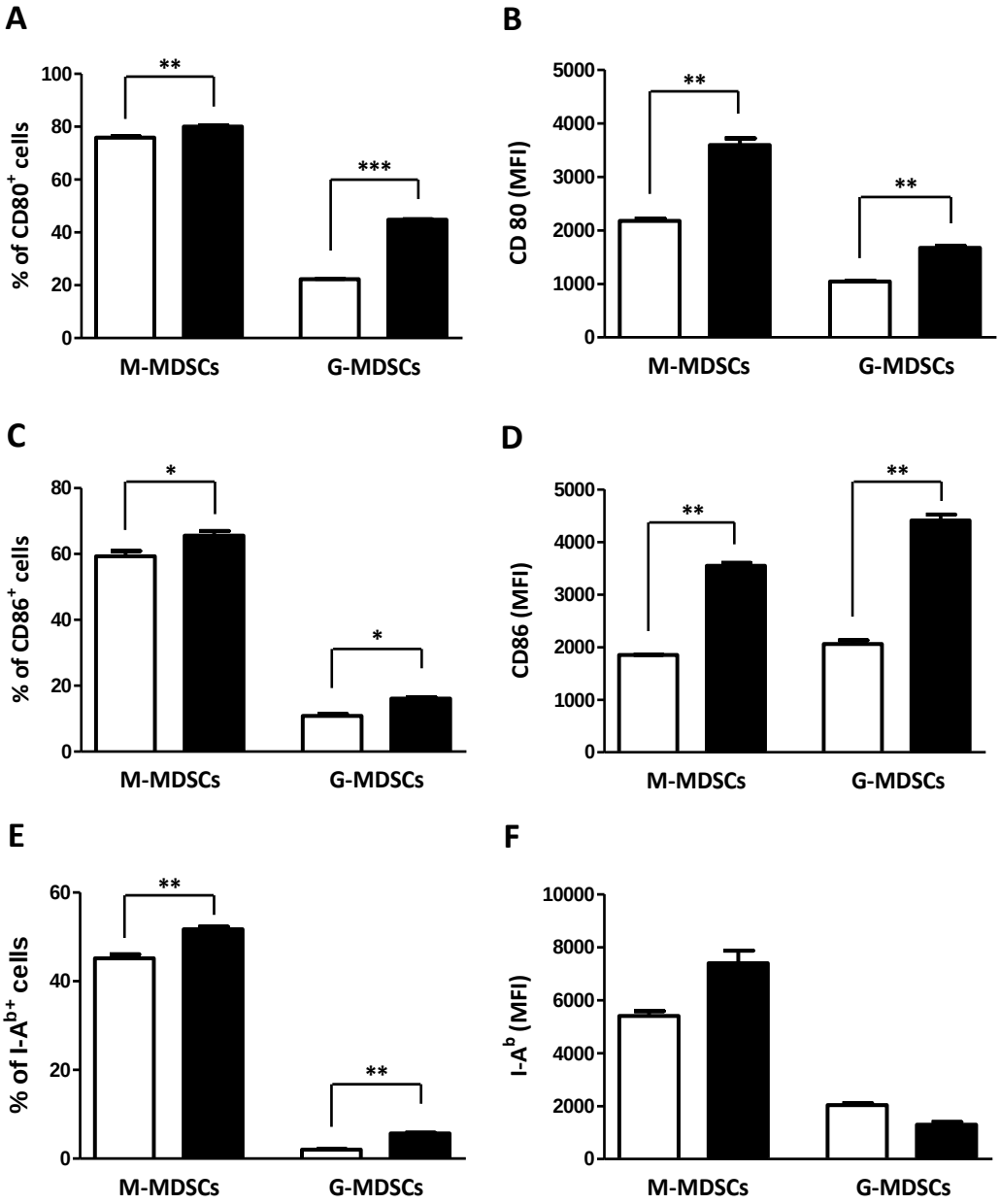


Figure 29. Phenotypic characterization of M- and G-MDSCs generated in BM transduction cultures. Expression of CD80, CD86 and MHC-II (I-A^b) molecules was assessed both at baseline (white bars) and after 18 h of IFN-γ and LPS stimulation (black bars). (A) Percentage of cells expressing CD80, (B) MFI of CD80, (C) percentage of cells expressing CD86, (D) MFI of CD86, (E) percentage of cells expressing I-A^b, (F) MFI of I-A^b. Data are represented as the mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; (n=3).

3. ROS PRODUCTION BY MDSCs

As in previous studies from our group it was already demonstrated that MDSCs generated in BM transduction cultures had arginase-1 and iNOS activity (Gomez, Espejo et al. 2014), we aimed to further characterize these cells by studying the

production of ROS. We found that both unfractionated BM cells and the two types of MDSCs produced high levels of ROS. M-MDSCs presented greater levels of ROS production in comparison with G-MDSCs and unfractionated BM cells, although this difference did not reach statistical significance (Figure 30). These results indicate that M-MDSCs are the main population responsible for ROS production within unfractionated BM.

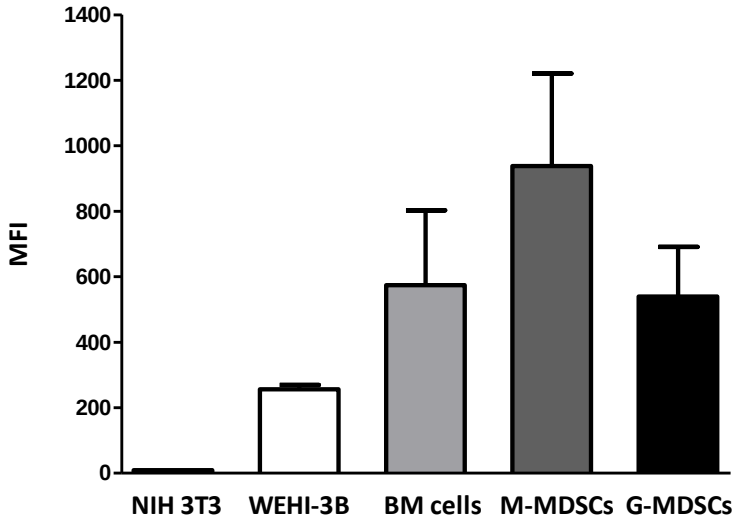


Figure 30. ROS production by BM cells and MDSCs. This graph shows the production of ROS by unfractionated BM cells and by the two subpopulations of MDSCs. NIH 3T3 and WEHI-3B cells were used as negative and positive controls, respectively. Error bars correspond to SEM. (n=3).

4. TRANSDUCTION EFFICIENCY OF BM CELLS AND MDSCs

In each experiment, just before performing the functional assays or the cell infusion, the transduction efficiency of both BM cells and isolated MDSCs was evaluated 24 h after the last cycle of transduction. The average transduction efficiency for all the experiments was 29.1 ± 6.0% for the vector SF91-IiMOG-IRES-EGFP and 13.0 ± 7.6% for SF91-Ii-IRES-EGFP.

5. CHARACTERIZATION OF ISOLATED MDSCs

5.1. MDSC isolation efficiency

In order to test if the generated MDSCs were responsible for the therapeutic effect observed in the EAE model (Eixarch, Espejo et al. 2009), these cells were isolated from total BM cells. As in the case of the transduction efficiency, the purity of the isolated MDSCs was assessed by flow cytometry prior to each experiment by staining the isolated cells with CD11b and Gr-1 antibodies (Figure 31). The average purity of the isolated MDSCs in the set of experiments was of 97.9 ± 1.4% for therapeutic vector SF91-IiMOG-IRES-EGFP (IiMOG) transduced cells, of which 22.0 ± 11.0% corresponded to M-MDSCs and 66.2 ± 8.8% to G-MDSCs. The purity of the isolated MDSCs transduced with the control vector SF91-Ii-IRES-EGFP (Ii) was of 98.0 ± 1.1%, of which 22.6 ± 10.1% corresponded to M-MDSCs and 66.6 ± 8.9% to G-MDSCs. No significant differences in the proportions of M- and G-MDSCs between the cells transduced with the therapeutic or control vector were observed.

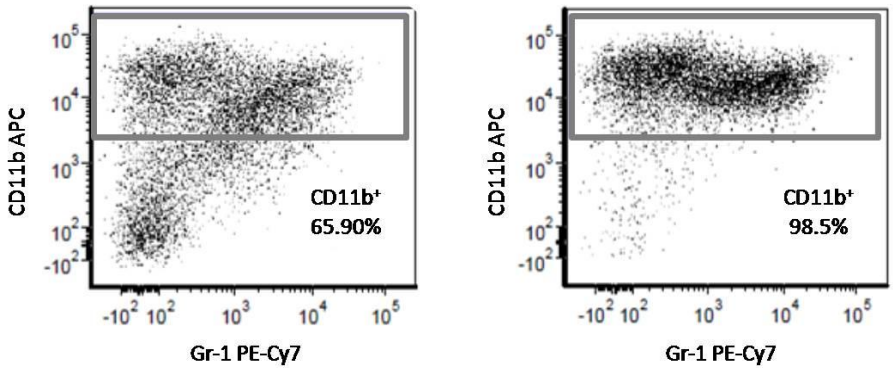


Figure 31. Purity of isolated MDSCs. Dot plots representative of the purity of MDSCs pre (left) and post (right) magnetic isolation.

5.2. Characterization of isolated MDSCs

With the purpose of identifying what type of cell populations were present in the isolated cells after magnetic separation through positive selection by CD11b, cells were stained with myeloid, T and B lineage markers and analyzed by flow cytometry. As shown in Figure 32A, the vast majority of cells corresponded to MDSCs, of which 19.7 ±

3.2% were M-MDSCs and 68.7 ± 4.8% were G-MDSCs. As expected, the fraction of isolated cells did not contain immature hematopoietic progenitors or any kind of lymphocytes but there was a small percentage of positive cells for the CD11c marker (5.1 ± 2.0%), which were Gr-1$^{-/low}$. The effectively transduced cells mainly consisted of M- and G-MDSCs, which represented 40.5 ± 10.0% and 38.9 ± 8.6% of the total transduced cells, respectively (Figure 32B).

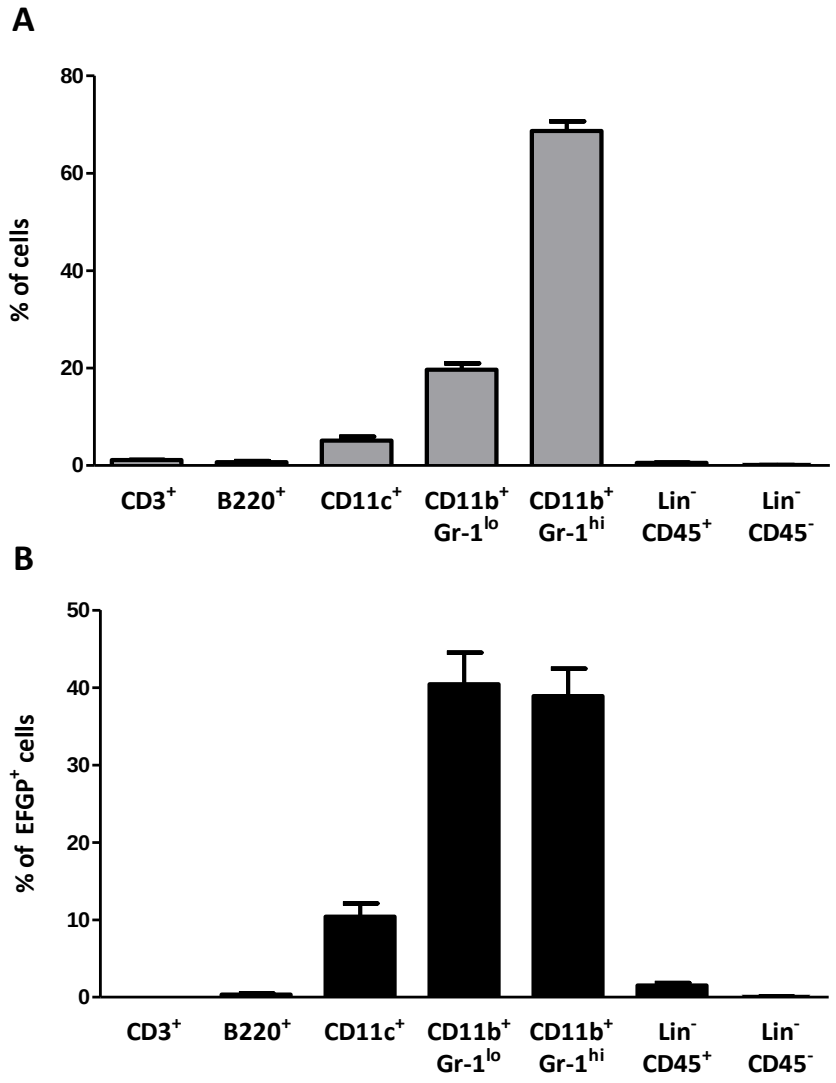


Figure 32. Cell populations in isolated MDSCs. (A) Bars indicate the percentage of the different cell populations within the isolated cells after magnetic separation. (B) Percentage of each cell subpopulation within the transduced cells. It was analyzed by gating on the EGFP$^+$ cell population. Data are represented as the mean ± SEM. (n=3).

6. TRANSDUCED BM CELLS AND MDSCs SUPPRESS MOG_{40-55}-INDUCED SPLENOCYTE PROLIFERATION

In our laboratory we previously demonstrated that BM cells, M- and G-MDSCs were able to suppress T-cell proliferation (Gomez, Espejo et al. 2014). Due to some changes in BM culture conditions, we decided to reevaluate if the MDSCs generated in BM transduction cultures were still able to suppress T-cell proliferation. Thus, once both populations of MDSCs were phenotypically characterized and after analyzing that they were able to produce ROS, we proceeded to verify the ability of these cells to suppress the proliferative response of splenocytes against antigenic stimulus. To this end, splenocytes from mice immunized with MOG_{40-55} peptide were co-cultured with different proportions of either unfractionated BM cells or isolated MDSCs transduced with the therapeutic vector SF91-IiMOG-IRES-EGFP (IiMOG) or with the control vector SF91-Ii-IRES-EGFP (Ii). Cell proliferation was quantified after 72 h by measuring the incorporation of [^{3}H]-thymidine.

As expected, the results obtained in the suppression assays show that BM cells were able to actively suppress MOG_{40-55}-induced splenocyte proliferation in a dose-dependent manner (Figure 33A). Isolated MDSCs exhibited a suppressive activity that was equivalent to that of BM cells, suggesting that these cells may be responsible for the suppressive effect observed with unfractionated BM cells (Figure 33B). In both cases no significant differences were observed between Ii- and IiMOG-transduced cells, suggesting that *in vitro* these cells only have the ability to suppress splenocyte proliferation in an unspecific manner or that antigen-specific suppression by IiMOG-transduced cells is masked.

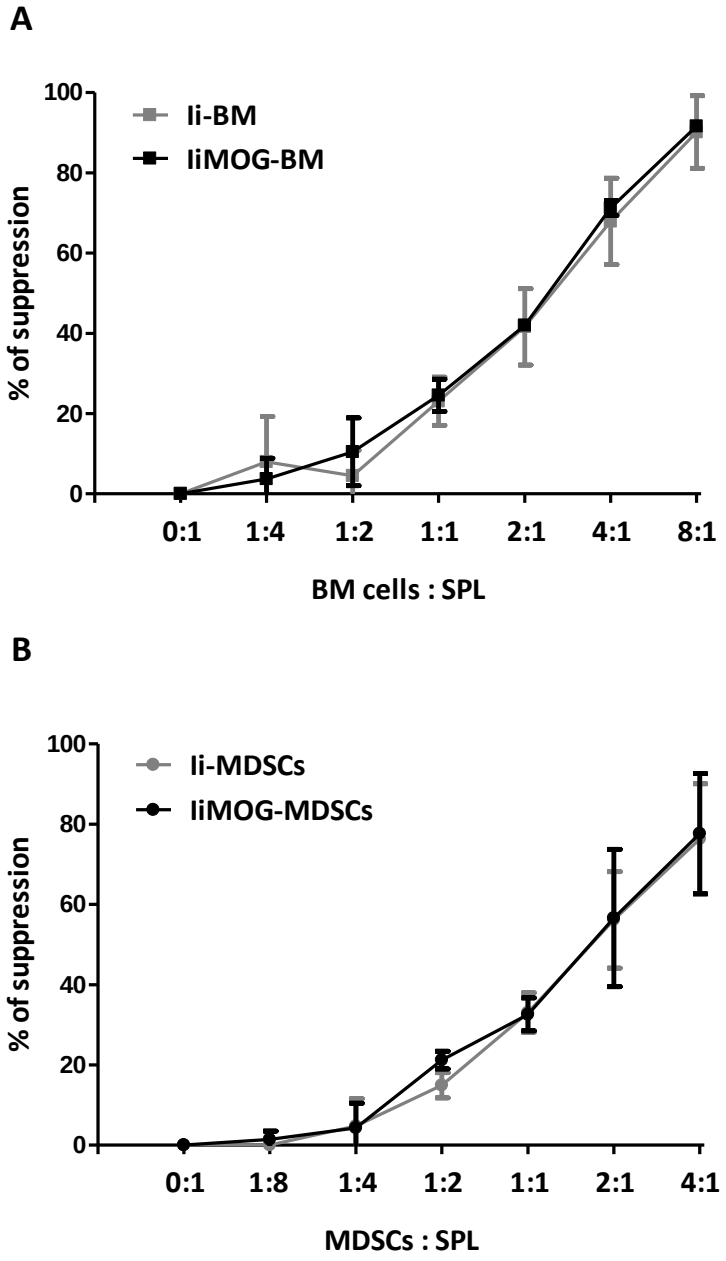


Figure 33. Transduced BM cells and MDSCs suppress MOG_{40-55}-induced splenocyte proliferation. Charts show the percentage of suppression of splenocyte proliferation by irradiated total BM cells (A) and by isolated MDSCs (B) transduced with either the vector encoding the MOG antigen (black) or the control vector (grey). SPL: splenocytes. Data represent the mean ± SEM of two independent experiments.

7. INFUSION OF MOG-SPECIFIC BM CELLS AND MDSCs AMELIORATES THE CLINICAL COURSE OF EAE

7.1. Infusion of MOG-specific BM cells and MDSCs protects against EAE

To evaluate whether BM cells or isolated MDSCs were capable of protecting mice from EAE, non-myeloablated C57BL/6J mice were infused with $1x10^6$ unfractionated BM cells or $0.5x10^6$ total MDSCs either transduced with the control vector (Ii-treated mice) or with the therapeutic vector (IiMOG-treated mice) seven days before EAE induction (preventive approach). Additionally, a group of non-treated mice (NT) was included as disease control.

Both BM cells and MDSCs IiMOG-treated groups were significantly protected against the disease, effect that was not observed in their respective control groups. Both IiMOG-treated groups had significantly lower maximum and cumulative clinical score compared to their counterparts (Table 13). A single infusion of MOG-specific MDSCs ameliorated the clinical course of the disease to a similar extend as unfractionated MOG-specific BM cells (Figure 34A and 34B), suggesting that MDSCs contribute to the therapeutic effect. In addition, this improvement in clinical parameters was paralleled by a lower weight loss in IiMOG-treated animals compared to their respective controls (BM cells: p=0.010; MDSCs: p=0.054) (Figure 34C and 34D).

Table 13. Infusion of IiMOG-BM cells and IiMOG-MDSCs protects against EAE

	Incidence (%)	Maximum clinical score	Cumulative clinical score
Ii-BM cells	7/8 (87.5)	4.2 ± 1.9	58.3 ± 28.4
IiMOG-BM cells	4/7 (57.1)	2.1 ± 2.0	22.9 ± 24.0
p value	ns	0.012	0.011
Ii-MDSCs	6/6 (100.0)	4.3 ± 0.3	61.7 ± 16.7
IiMOG-MDSCs	6/8 (75.0)	2.8 ± 2.1	35.3 ± 31.8
p value	ns	0.020	0.044

The incidence is expressed as frequency and percentage and clinical scores are expressed as mean ± SD.
ns: non-significant

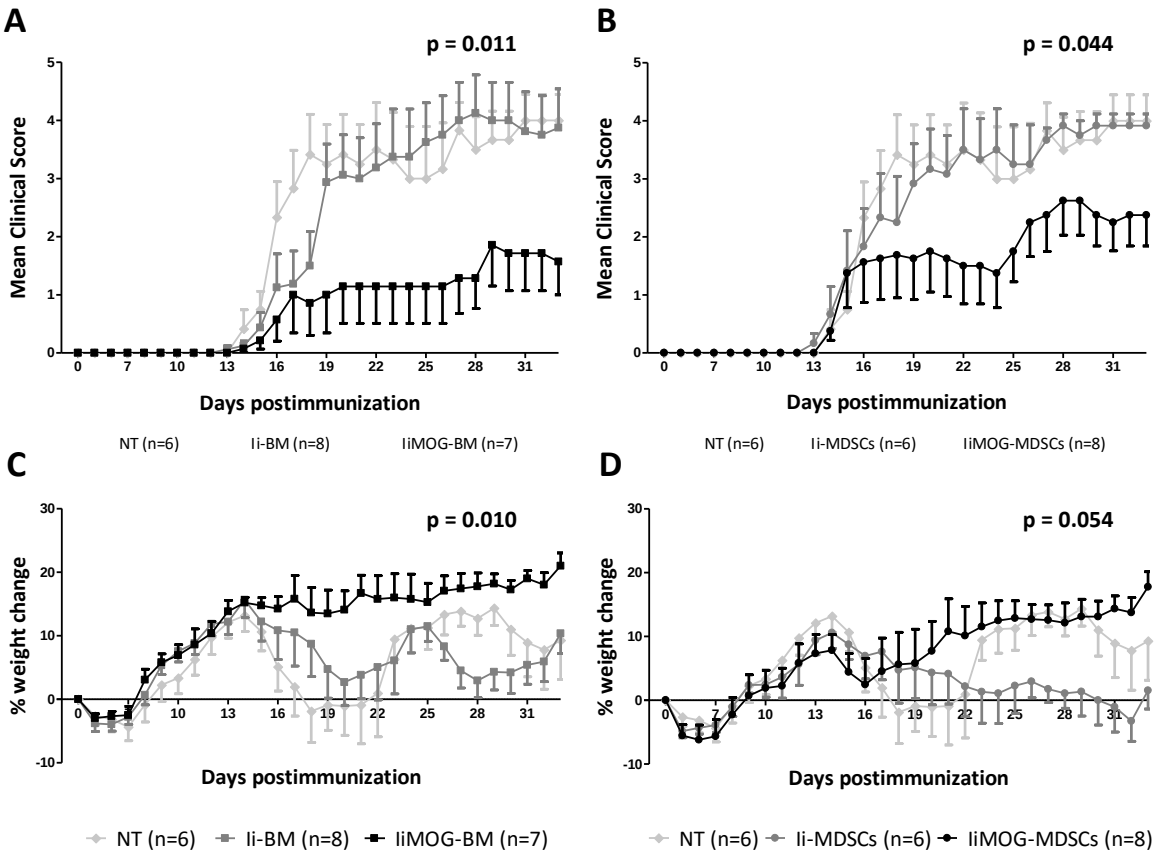


Figure 34. Preventive approach. Mice treated with BM cells (A) and MDSCs (B) expressing MOG_{40-55} peptide were protected against EAE or developed a milder disease compared to their respective control groups. Graphs represent the mean of the daily clinical score for every experimental group ± SEM. Mice treated with IiMOG-BM cells (C) and IiMOG-MDSCs (D) presented lower weight loss compared with the Ii-treated animals. Graphs represent the mean of the percentage of daily weight change ± SEM.

Moreover, incident mice from IiMOG-treated groups did not develop the typical clinical course of the chronic EAE model that is observed in C57BL/6J mice immunized with MOG_{40-55} peptide, as most of them presented milder clinical signs and in some cases the disease was similar to the relapsing-remitting model (Figure 35C and 35D). For this reason, when the clinical parameters of the incident mice (those that developed clinical signs) from IiMOG groups were analyzed in comparison with the ones of incident animals from the Ii groups, we also found that maximum and cumulative scores were lower in the IiMOG-treated groups, although the cumulative clinical scores only reached statistical significance in mice treated with BM cells (Table 14)(Figure 35A). However, in the case of mice treated with MDSCs, there are periods of time in which the clinical scores of mice treated with MOG-specific MDSCs were significantly lower than those of their counterparts (Figure 35B).

Table 14. Incident mice from IiMOG-treated groups developed milder EAE.

	Maximum clinical score	Cumulative clinical score
Ii-BM cells	4.8 ± 0.9	66.6 ± 17.1
IiMOG-BM cells	3.8 ± 0.5	40.0 ± 15.3
p value	0.018	0.021
Ii-MDSCs	4.3 ± 0.3	61.7 ± 16.7
IiMOG-MDSCs	3.9 ± 0.6	52.9 ± 21.11
p value	0.045	ns

Clinical scores are expressed as mean ± SD. ns: non-significant.

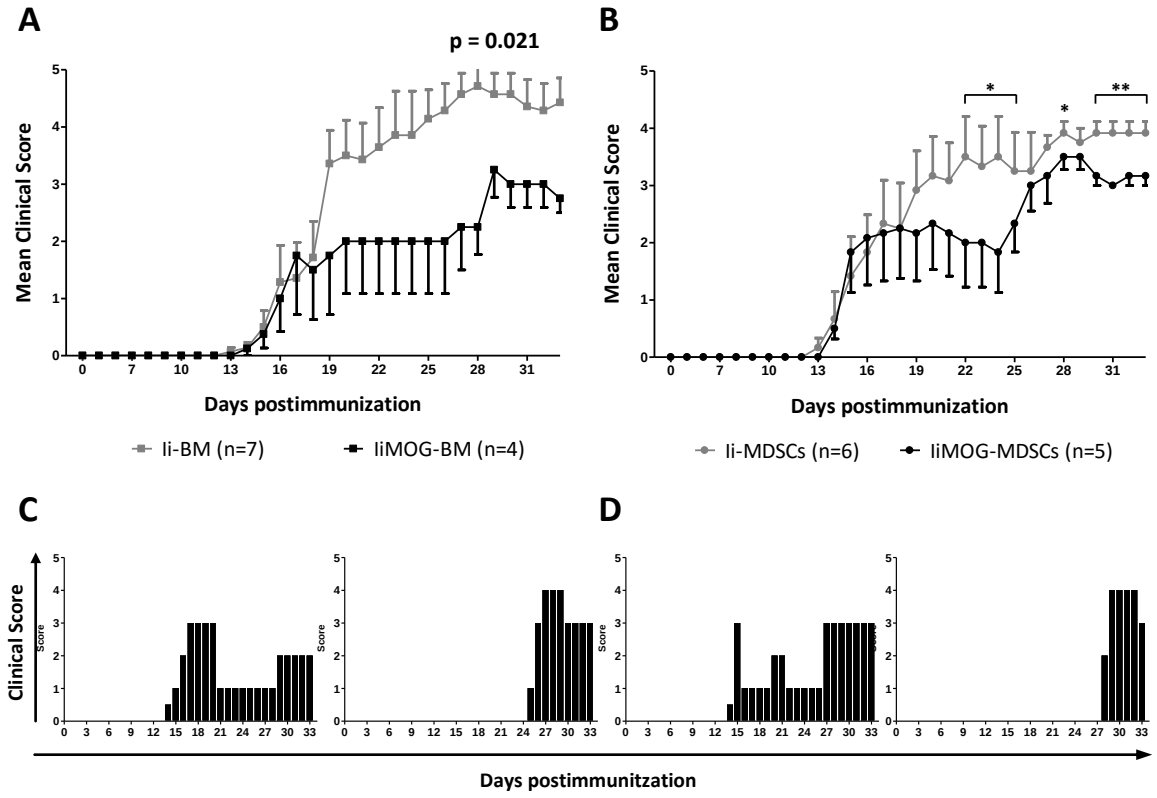


Figure 35. Milder EAE in incident IiMOG treated mice. Both groups treated with BM cells (A) and MDSCs (B) expressing MOG_{40-55} developed a milder disease compared to control groups. Graphs represent the mean of the daily clinical score for every experimental group ± SEM. Mice treated with IiMOG-BM cells (C) and IiMOG-MDSCs (D) did not develop a clinical course typical of the chronic EAE model. Bars represent the daily clinical scores of two representative mice from each IiMOG-treated group. * p<0.05, ** p<0.01.

7.2. Infusion of MOG-specific BM cells and MDSCs improves established EAE

After the onset of the first clinical signs in the majority (77.0%) of the animals (day 13-14 p.i.), mice were randomized into the different experimental groups (NT, Ii-BM cells, IiMOG-BM cells, Ii-MDSCs and IiMOG-MDSCs) in such a way that the clinical parameters would be comparable between them. Mice were infused with BM cells or with MDSCs. Thereafter, the clinical follow-up was carried out by a single researcher in a blinded manner to avoid bias and in all the experiments the animals were followed at least for 30 days.

Three experiments were performed. In the first one, $1x10^6$ BM cells and $0.5x10^6$ MDSCs were infused. Because the effect of this dose of MDSCs was weak, we decided to double this dose, so that in the second and third experiments we injected $1x10^6$ MDSCs. Pooled results of the clinical data of these last two experiments are shown.

Mice treated with $1x10^6$ IiMOG-BM cells and with $1x10^6$ IiMOG-MDSCs presented significantly less cumulative clinical score compared to their counterparts (Table 15, Figure 36A and 36E), a difference that was not observed when mice were treated with $0.5x10^6$ IiMOG-MDSCs (Figure 36D). In addition, mice treated with MOG-specific cells had lower maximum clinical scores compared with their controls, although these differences only reached statistical significance in the case of mice treated with IiMOG-BM cells (Table 15).

Table 15. EAE clinical scores of mice treated with $1x10^6$ transduced BM cells and MDSCs

	Maximum clinical score	Cumulative clinical score
Ii-BM cells	4.31 ± 0.26	94.44 ± 8.40
IiMOG-BM cells	3.14 ± 1.57	51.57 ± 34.80
p value	0.005	0.002
Ii-MDSCs	3.86 ± 1.28	54.57 ± 20.28
IiMOG-MDSCs	3.32 ± 1.3	36.39 ± 20.99
p value	0.075	0.006

Clinical scores are expressed as the mean ± SD.

It is well recognized that the onset of the clinical signs of EAE is accompanied by a loss of weight in the animals that is maintained over time until recovery or stabilization of the disease. In accordance with the clinical outcome, animals treated with both MOG-specific BM cells and MDSCs presented a significantly lower weight loss than control mice (BM cells: $p=3.1x10^{-4}$; MDSCs: $p=0.019$) (Figure 36B and 36H, respectively).

To determine if improvement in the clinical score was accompanied by an improvement in the motor performance, mice were tested for motor coordination and balance using a rotarod apparatus at the end of each experiment. Normal, healthy mice remain on the cylinder for long periods of time while clinically severe EAE mice fall off the apparatus much earlier, indicating functional motor disability. In accordance with the clinical score, mice treated with IiMOG cells remained on the rotating cylinder for longer periods of time than mice treated with control cells, although the differences were only statistically significant between mice treated with BM cells.

Moreover, after cell infusion, most of the animals treated with IiMOG-BM cells and with IiMOG-MDSCs did not develop the common clinical course of this chronic non-remitting model, in contrast to the animals from control groups that continued to develop a chronic EAE (Figure 37). In addition, 37.5% of mice from the IiMOG-BM cells group and 28.6% from the IiMOG-MDSCs group were disease remissions, which were not observed in their respective controls. In this study remission was defined as an improvement in the clinical score for three consecutive days with respect to the score of the day of cell infusion.

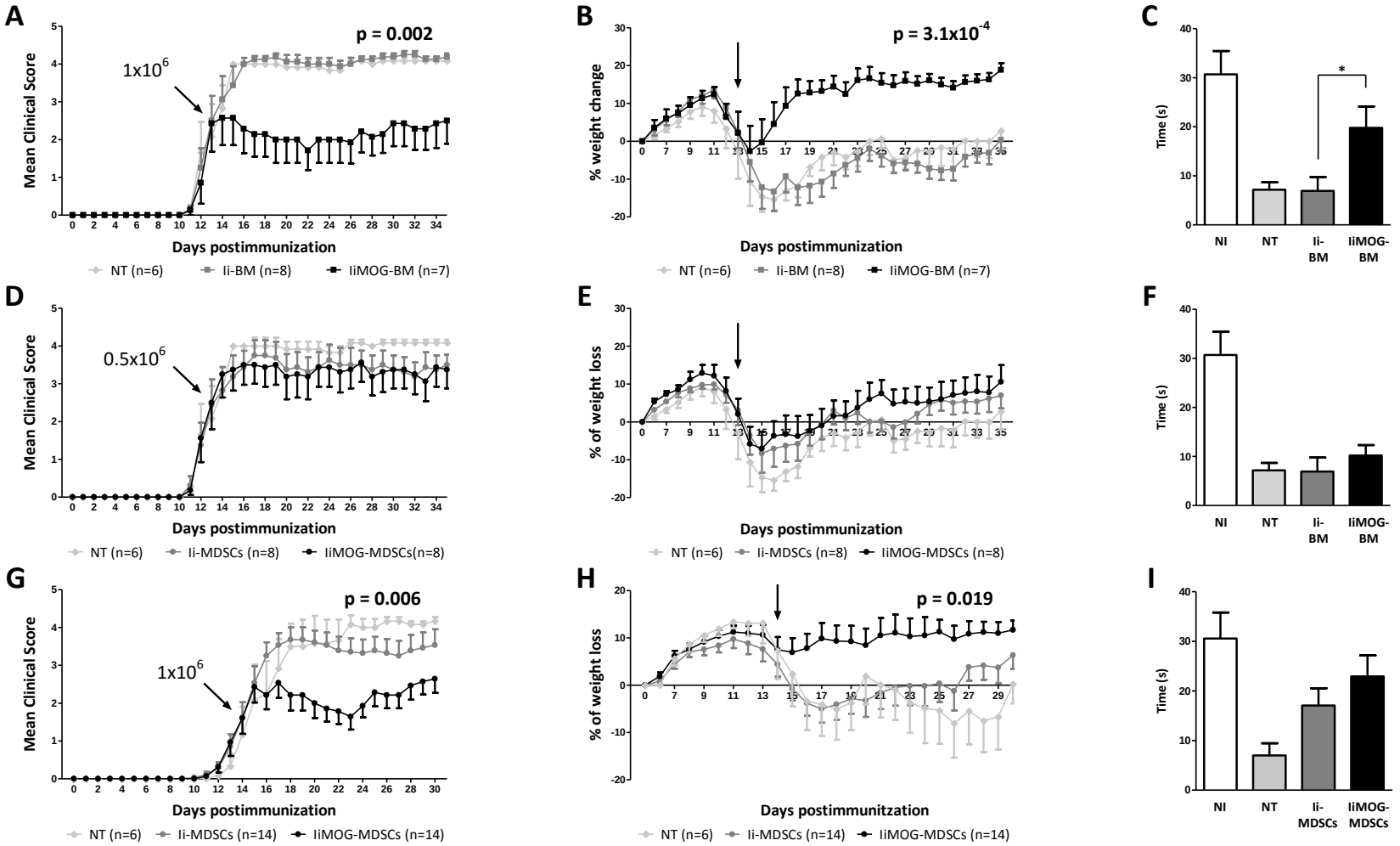


Figure 36. Improvement of EAE clinical course after MOG-specific BM cells and MDSCs infusion. After the appearance of the first neurological signs (day 13-14 p.i.) mice were infused either with $1x10^6$ BM cells (A), with $0.5x10^6$ MDSCs (D) or with $1x10^6$ MDSCs (E). After the injection of $1x10^6$ of IiMOG-BM cells or $1x10^6$ of IiMOG-MDSCs mice presented a milder EAE, while this was not observed in their Ii-treated controls. Moreover, this amelioration of the clinical signs was paralleled by a reduction in the weight loss of IiMOG-treated animals when they received $1x10^6$ of IiMOG-BM cells (B) or $1x10^6$ of IiMOG-MDSCs (H) but not with $0.5x10^6$ MDSCs (E). Charts C, F and I show rotarod performance of mice treated with $1x10^6$ BM cells, with $0.5x10^6$ MDSCs or with $1x10^6$ MDSCs, respectively. Arrows indicate the day of cell infusion. Data represent the mean ± SEM. * $p<0.05$.

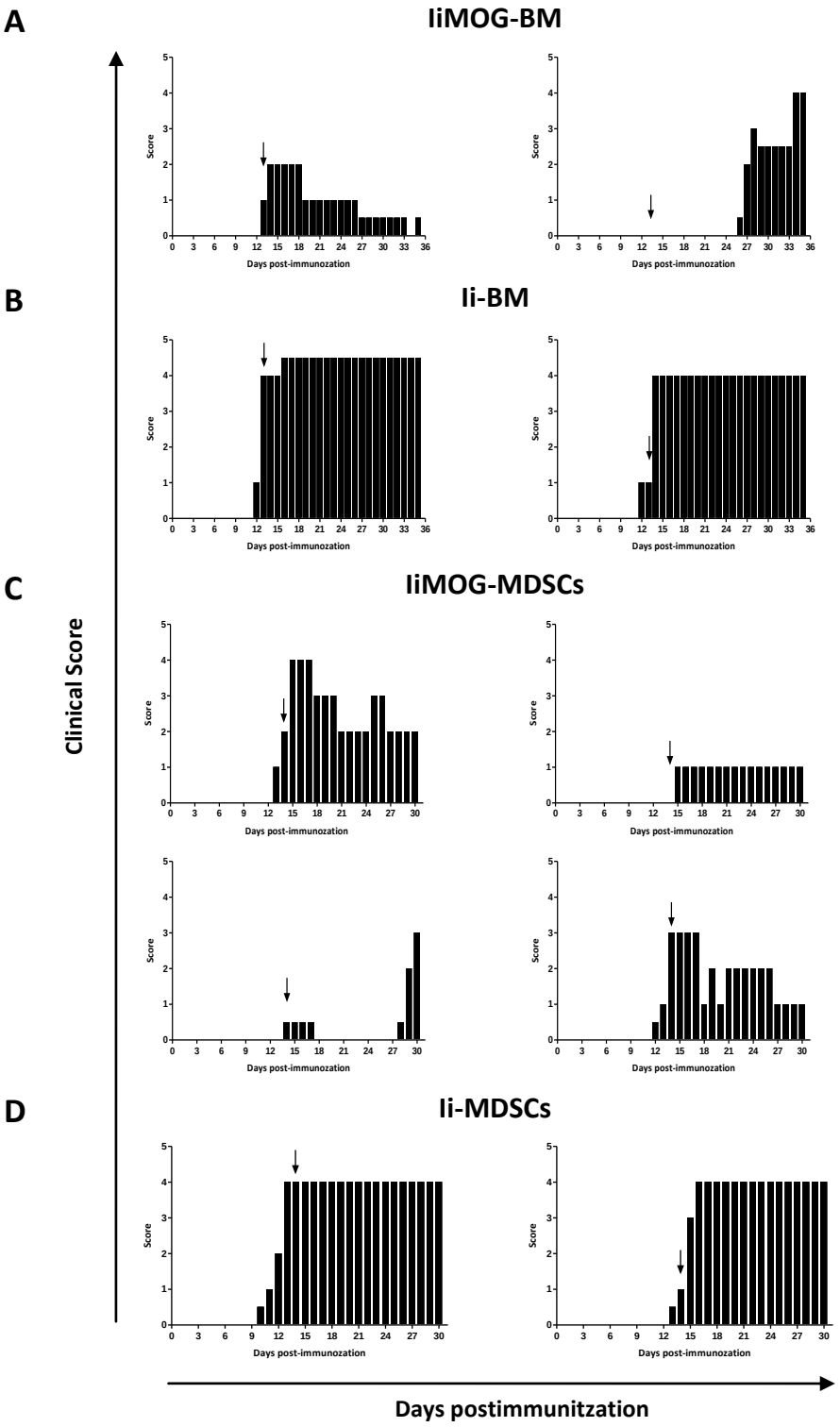


Figure 37. Animals treated with IiMOG-BM cells (A) and IiMOG-MDSCs (C) did not develop a chronic progressive clinical course whereas mice treated with Ii-BM cells (B) and Ii-MDSCs (D) developed the common chronic EAE course of this model. Graphs represent the daily clinical score of individual mice representative of each experimental group.

7.3. CNS pathology is improved in IiMOG-treated animals

To assess whether EAE improvement was accompanied by a decreased neuropathology, histopathological studies were performed in the brain and spinal cord of all the animals at the end of the experiment (day 33-35 p.i.). The presence of inflammation was assessed through HE staining and demyelination by KB staining. All histopathological analyses were carried out in a blinded manner to avoid bias.

In both the preventive and therapeutic approaches, in accordance with the clinical score, all of the IiMOG-treated animals presented milder CNS pathological findings than their respective controls, which showed extensive inflammatory infiltrates in the spinal cord white matter (mainly consisting of lymphocytes and macrophages) and demyelination in areas with moderate to severe inflammatory infiltration (Figure 38). However, with this semi-quantitative evaluation, inflammation and demyelination was only significantly reduced in animals preventively treated with MDSCs expressing the self-antigen (HE: 2.4 ± 1.7 vs 4.3 ± 0.5, p=0.008; KB: 1.1 ± 0.8 vs 2 ± 0.6, p=0.035) (Figure 38C and 38D).

Moreover, spinal cords of three representative mice from each group were also immunostained to evaluate T-cell infiltration (CD3), microglia activation (LEA), reactive astrogliosis (GFAP), axonal damage through specific detection of non-phosphorylated neurofilaments (SMI-32) and myelin (MBP)(Figure 39). Results show that mice treated either with BM cells or MDSCs expressing the MOG_{40-55} peptide presented significantly less T-cell infiltration, microglia activation, reactive astrogliosis, axonal damage and demyelination in the CNS (Figure 40, Figure 41 and Table 16).

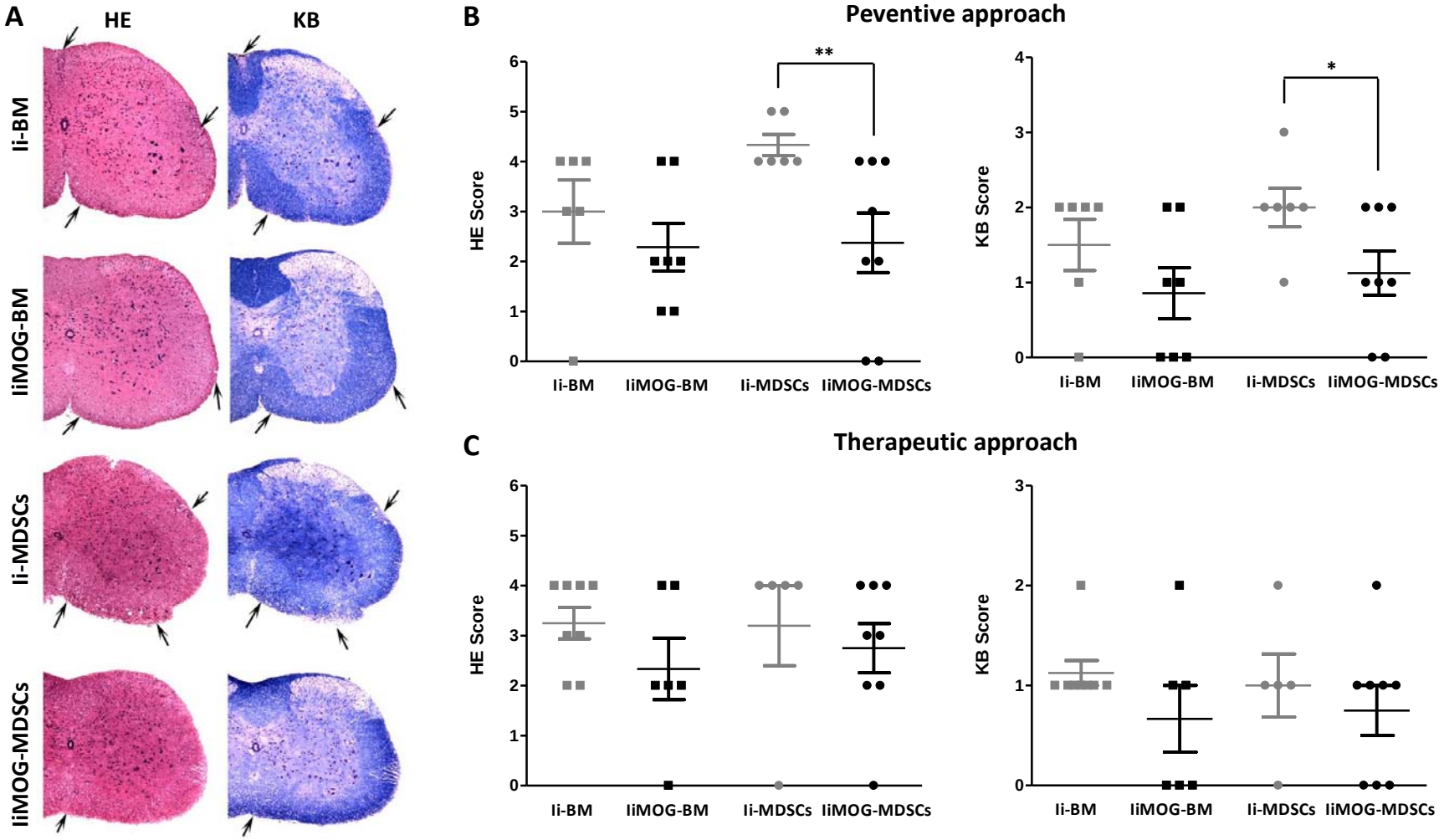


Figure 38. Mice treated with MOG-specific cells presented reduced inflammation and demyelination in the CNS. (A) Representative histopathologies of the spinal cord of mice treated with BM cells or MDSCs. Spinal cords of mice preventively (B) and therapeutically (C) treated with $1x10^6$ cells were stained to evaluate inflammation (HE) and the degree of demyelination (KB) semi-quantitatively. Data are represented as the mean ± SEM. * $p<0.05$, ** $p<0.01$.

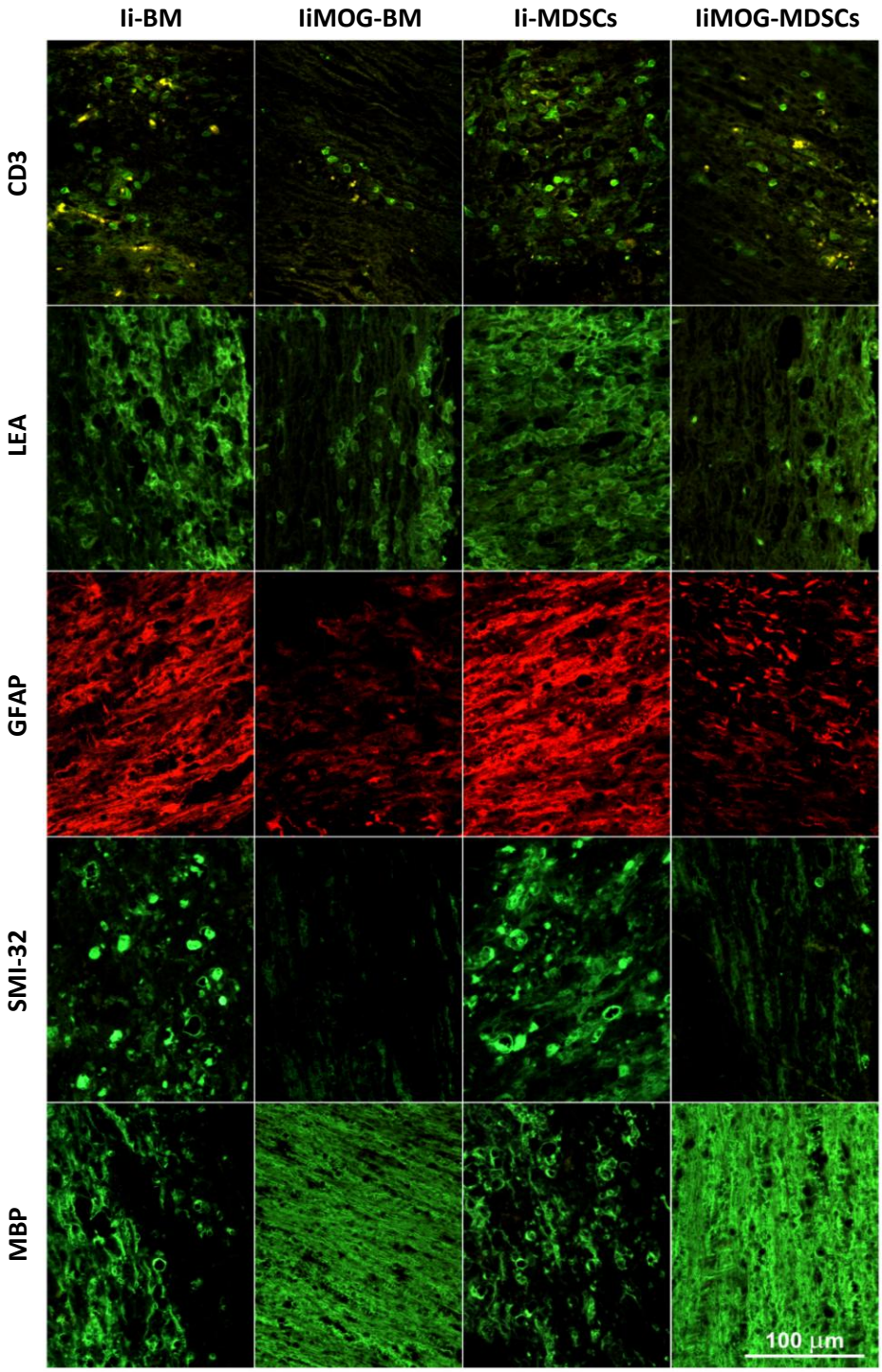


Figure 39. Representative microphotographs of CNS immunostainings to evaluate T-cell infiltration (CD3), microglia activation (LEA), reactive astrogliosis (GFAP), axonal damage (SMI-32) and myelin (MBP) . Each column corresponds to a sample of an animal representative of each experimental group.

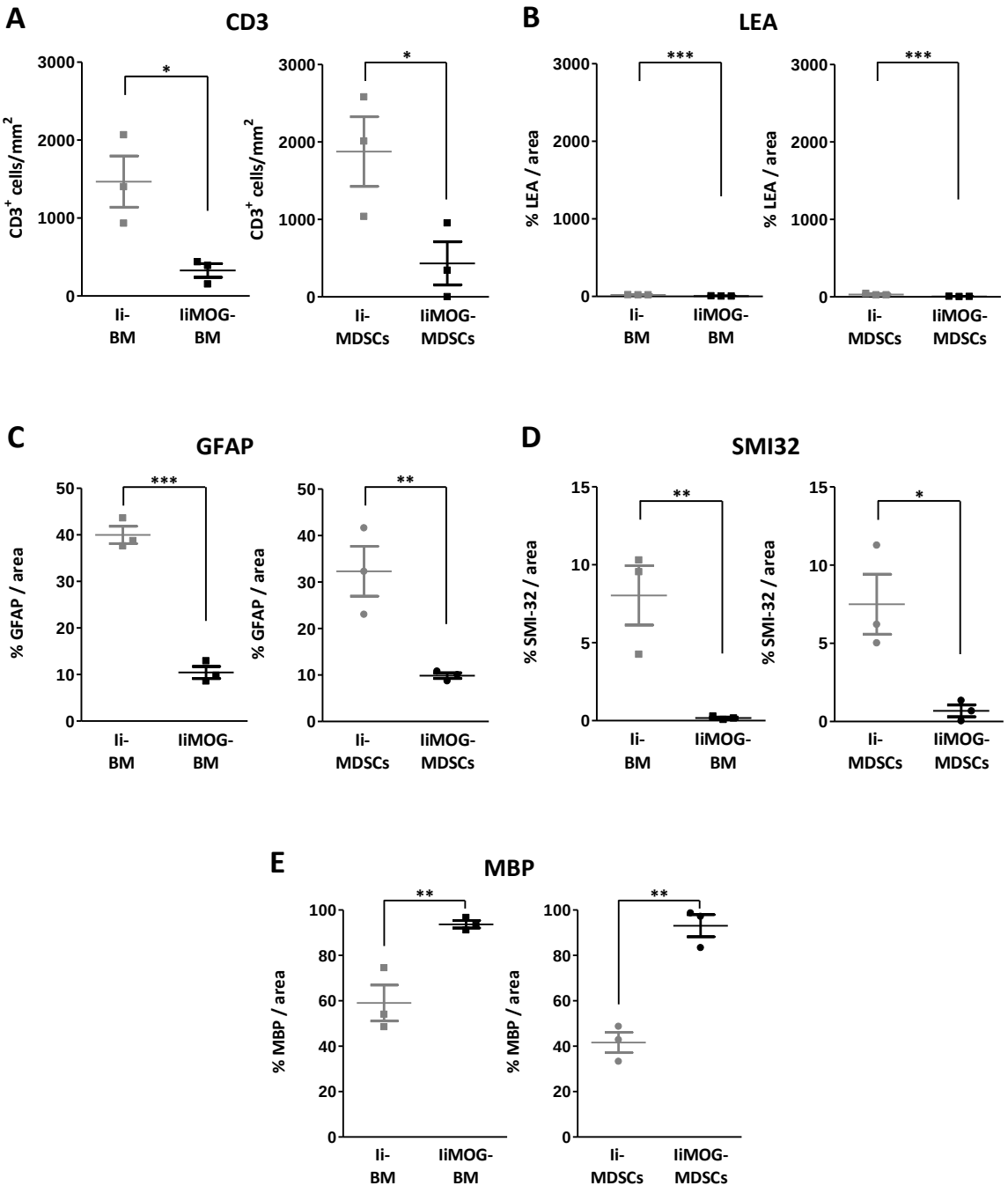


Figure 40. Significant reduction of the inflammatory component, demyelination and axonal damage in mice preventively treated with MOG-expressing BM cells and MDSCs. T-cell infiltration (CD3, A) was reduced in IiMOG-treated groups. Moreover, the animals of these groups presented less microglia activation (LEA, B), astrogliosis (GFAP, C) and axonal damage (SMI-32, D). In addition, these animals showed a lower degree of demyelination (E), which was evaluated with the amount of myelin (MBP) present in the white matter of the spinal cord. Data are represented as the mean of the percentage of stained pixels/area ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

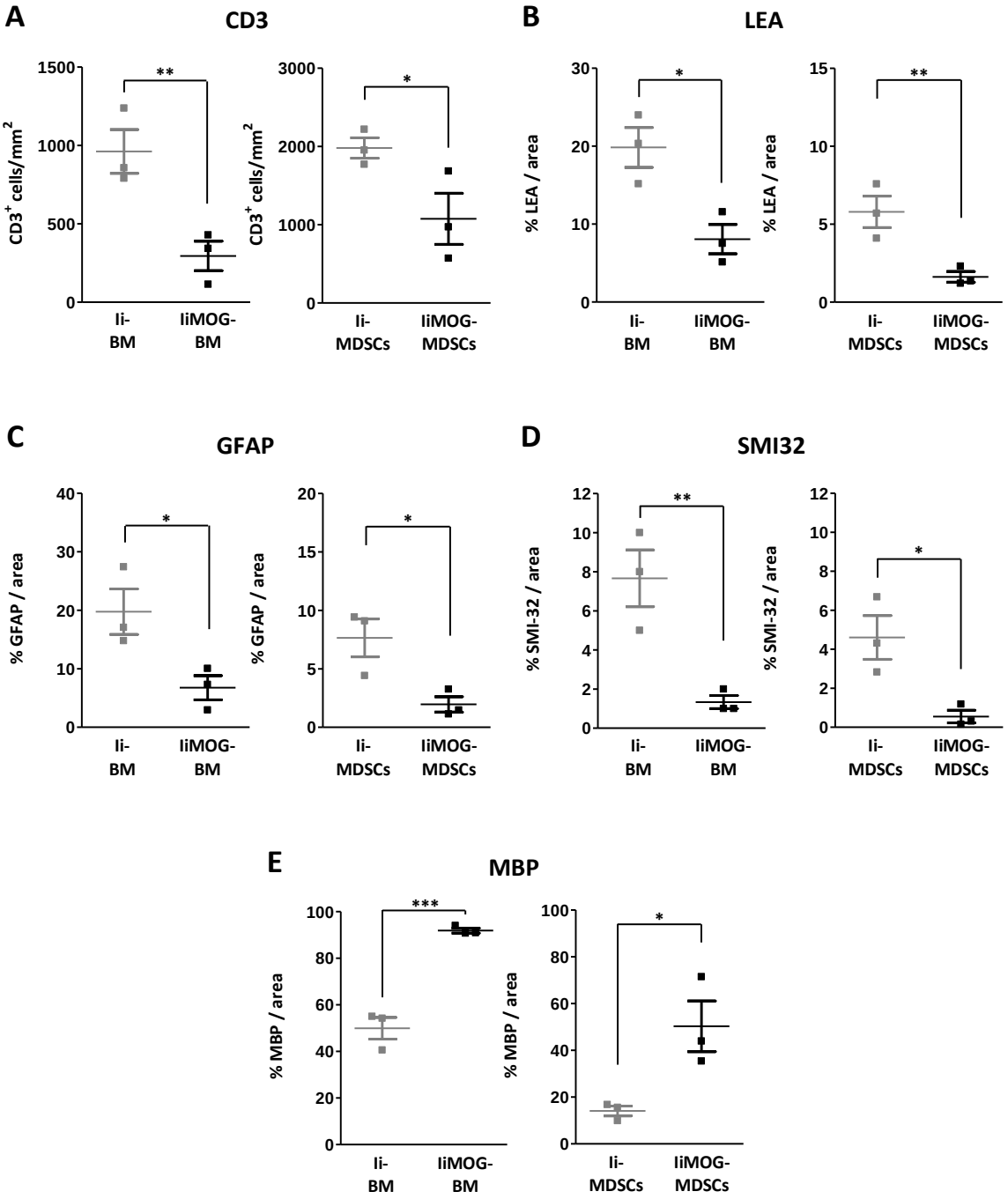


Figure 41. Significant reduction of the inflammatory component, demyelination and axonal damage in mice therapeutically treated with 1x10^6 MOG-expressing BM cells and 1x10^6 MDSCs. T-cell infiltration (CD3, A) was reduced in IiMOG-treated groups. Moreover, the animals of these groups presented less microglia activation (LEA, B), astrogliosis (GFAP, C) and axonal damage (SMI-32, D). In addition, these animals showed a lower degree of demyelination (E), which was evaluated with the amount of myelin (MBP) present in the white matter of the spinal cord. Data are represented as the mean of the percentage of stained pixels/area ± SEM. * p<0.05, ** p<0.01, *** p<0.001.

Table 16. Reduced neuropathology in mice treated with MOG-specific cells

Preventive approach					
	CD3	**LEA**	**GFAP**	**SMI-32**	**MBP**
Ii-BM	1466.7 ± 569.6	20.0 ± 1.1	40.0 ± 3.2	8.0 ± 3.3	59.0 ± 13.7
IiMOG-BM	327.0 ± 153.1	4.0 ± 2.5	10.0 ± 2.2	0.0 ± 0.1	94.0 ± 2.8
***p* value**	p=0.014	p=0.0003	p<0.0001	p=0.007	p=0.006
Ii-MDSCs	1876.2 ± 780	31.0 ± 11.1	32.0 ± 9.3	8.0 ± 3.3	42.0 ± 7.8
IiMOG-MDSCs	431.7 ± 482.4	4.0 ± 1.5	1.0 ± 1	1.0 ± 0.6	93.0 ± 8.4
***p* value**	p=0.026	p=0.008	p=0.007	p=0.013	p=0.007
Therapeutic approach					
	CD3	**LEA**	**GFAP**	**SMI-32**	**MBP**
Ii-BM	961.9 ± 241.5	19.8 ± 4.4	19.8 ± 6.7	7.7 ± 2.5	49.9 ± 8.1
IiMOG-BM	295.2 ± 162.5	8.1 ± 3.3	6.8 ± 3.6	1.3 ± 0.6	91.9 ± 1.8
***p* value**	p=0.008	p=0.01	p=0.021	p=0.007	p=0.0005
Ii-MDSCs	1981.0 ± 225.2	5.8 ± 1.7	7.6 ± 2.8	4.6 ± 1.9	14.1 ± 3.7
IiMOG-MDSCs	1076.0 ± 564.5	1.6 ± 0.6	2.0 ± 1.1	0.5 ± 0.6	50.3 ± 18.8
***p* value**	p=0.031	p=0.009	p=0.016	p=0.013	p=0.015

Results are expressed as the mean ± SD of $CD3^+$ cells/mm^2 and as the percentage of stained pixels/area.

7.4. Mice treated with MOG-specific cells present less activated T cells and more B cells with a regulatory phenotype

Based on the findings of a reduction in both EAE disease severity and CNS pathology in IiMOG-treated mice, we decided to study different lymphoid populations that are relevant in the pathogenesis of EAE and MS in order to better characterize the mechanisms by which MOG-expressing BM cells and MDSCs produce their beneficial effect in EAE. To do this, we studied the populations of T and B lymphocytes present in the spleen of treated mice at end of each experiment (day 33-35 p.i.) by flow cytometry in both preventive and therapeutic approaches.

In the preventive approach, regarding the populations of T lymphocytes, we did not observe any differences in the percentages of $CD4^+$ ($CD3^+CD4^+$), $CD8^+$ ($CD3^+CD4^-$) and Treg cells ($CD3^+CD4^+CD25^+FoxP3^+$) between animals treated with MOG-specific BM cells and MDSCs and their respective controls. Nonetheless, mice treated with IiMOG-MDSCs presented a reduced percentage of activated T cells ($CD3^+CD4^+CD25^+FoxP3^-$) than their counterparts (IiMOG-MDSCs: 2.0 ± 0.9% vs Ii-MDSCs: 3.2 ± 0.9%, p=0.043). In relation to B cells, we also observed no differences in the percentages of total B cells ($CD45^+B220^+$), B1 ($CD45^+B220^+CD5^+$) and B2 ($CD45^+B220^+CD5^-$) cells between the animals from the different experimental groups. However, mice treated with either BM cells or MDSCs expressing the MOG_{40-55} peptide presented an increased percentage of B cells with a regulatory phenotype ($CD45^+B220^+CD1d^{high}CD5^+$) compared to their respective controls, although it only reached statistical significance in the case of IiMOG-MDSCs treated animals (Ii-BM: 2.2 ± 0.6% vs IiMOG-BM: 2.9 ± 0.8%, p=0.060; Ii-MDSCs: 2.0 ± 0.5% vs IiMOG-MDSCs: 2.7 ± 0.6%, p=0.030) (Figure 42).

In the therapeutic approach, we did not observe any differences in the percentages of $CD4^+$ ($CD3^+CD4^+$), $CD8^+$ ($CD3^+CD4^-$) and Treg cells ($CD3^+CD4^+CD25^+FoxP3^+$) between animals treated with MOG-specific BM cells and MDSCs and their respective controls either. However, mice treated with IiMOG-BM cells presented a reduced percentage of activated T cells ($CD3^+CD4^+CD25^+FoxP3^-$) compared to their counterparts (Ii-BM: 2.8 ± 0.7% vs IiMOG-BM: 1.9 ± 0.4%, p=0.021), fact that in this case was not observed in mice treated with IiMOG-MDSCs. Regarding the populations of B cells, no differences were observed in the percentages of total B cells ($CD45^+B220^+$), B1 ($CD45^+B220^+CD5^+$) and B2 ($CD45^+B220^+CD5^-$) cells between the animals from the different experimental groups. However, like in the preventive approach, mice treated with MDSCs expressing the MOG_{40-55} peptide presented an increased percentage of B cells with a regulatory phenotype ($CD45^+B220^+CD1d^{high}CD5^+$) compared to their respective controls, although in this case it did not reach statistical significance (Ii-MDSCs: 3.5 ± 1.6% vs IiMOG-MDSCs: 5.0 ± 1.6%, p=0.110) (Figure 43).

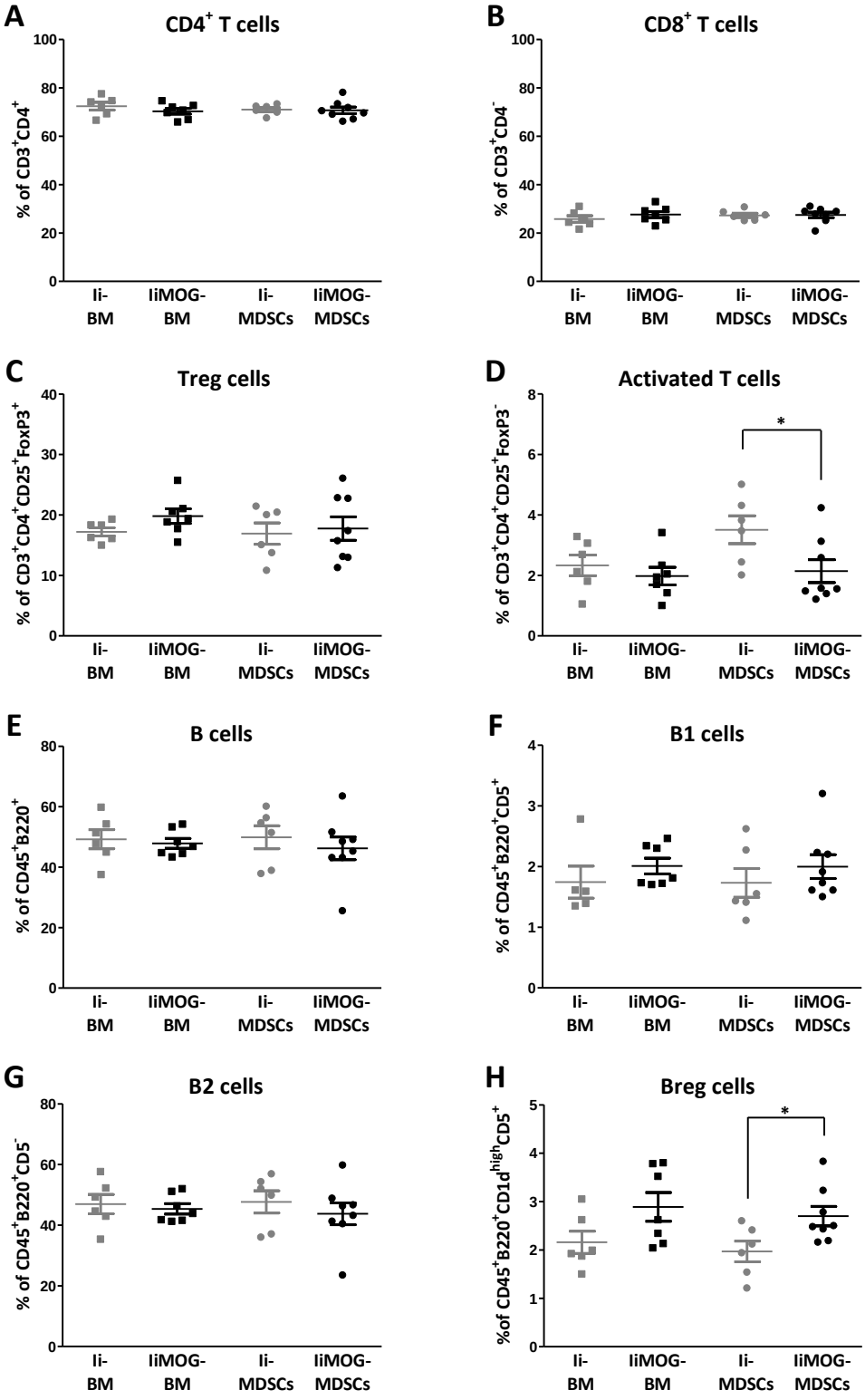


Figure 42. Frequency of splenic T and B cell populations in mice preventively treated with MOG-specific BM cells and MDSCs. Percentages of $CD4^+$ T cells (A) and $CD8^+$ T cells ($CD4^-$) (B) are calculated from $CD3^+$ T-cell population. Percentages of Treg cells ($CD25^+FoxP3^+$) (C) and activated T cells ($CD25^+FoxP3^-$) (D) are calculated from $CD3^+CD4^+$ T-cell population. Percentages of total B cells ($B220^+$) (E), B1 T-independent cells ($B220^+CD5^+$) (F) and B2 T-dependent cells ($B220^+CD5^-$) (G) are calculated from $CD45^+$ cell population. Percentages of B cells with a regulatory phenotype ($CD1d^{high}CD5^+$) (H) are calculated from $CD45^+B220^+$ B cell population. Data are represented as the mean ± SEM. * $p<0.05$.

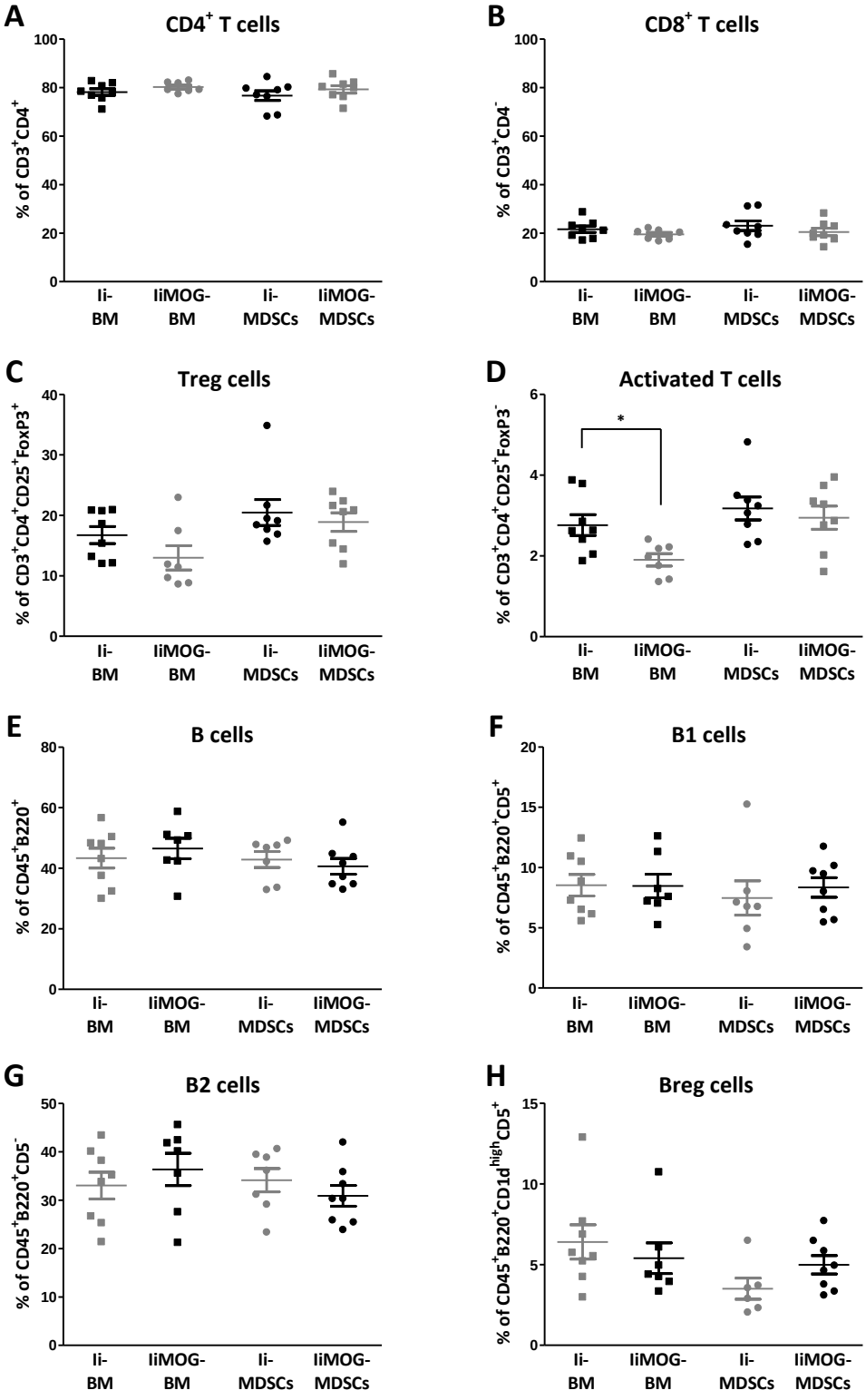


Figure 43. Frequency of splenic T and B cell populations in mice therapeutically treated with MOG-specific BM cells and MDSCs. Percentages of $CD4^+$ T cells (A) and $CD8^+$ T cells ($CD4^-$) (B) are calculated from $CD3^+$ T-cell population. Percentages of Treg cells ($CD25^+FoxP3^+$) (C) and activated T cells ($CD25^+FoxP3^-$) (D) are calculated from $CD3^+CD4^+$ T-cell population. Percentages of total B cells ($B220^+$) (E), B1 T-independent cells ($B220^+CD5^+$) (F) and B2 T-dependent cells ($B220^+CD5^-$) (G) are calculated from $CD45^+$ cell population. Percentages of B cells with a regulatory phenotype ($CD1d^{high}CD5^+$) (H) are calculated from $CD45^+B220^+$ B cell population. Data are represented as the mean ± SEM. * $p<0.05$.

7.5. Splenocytes of mice treated with BM cells and MDSCs have an altered proliferative response

To study what possible mechanisms were playing a role in the beneficial effect of the antigen-specific cells, the proliferation capacity of splenocytes from mice of the different experimental groups was evaluated against different stimuli. To this end, splenocytes were cultured in the presence of the encephalitogenic peptide (MOG_{40-55}), in the presence of a polyclonal stimulus (PHA-L) or in culture medium used as a basal proliferation control.

Contrarily to what we expected, we found no differences in the proliferation capacity among the splenocytes of mice treated with MOG-specific cells and their respective controls, neither when they were stimulated with PHA-L nor with MOG_{40-55}, in both preventive and therapeutic approaches. This could be due to the fact that the injection of cells *per se* caused an increased basal response in the splenocytes from Ii-treated animals compared to that of splenocytes from NT mice. In the preventive approach, this effect was observed in mice treated with both types of cells [BM cells: (NT: 3257.0 ± 863.6 cpm vs Ii-BM: 27624.0 ± 9840.0 cpm, p=0.004) and MDSCs: (NT: 3257.0 ± 863.6 cpm vs Ii-MDSCs: 28250.0 ± 7872.0 cpm, p=0.004)] (Figure 44A and 44B) whereas in the therapeutic approach it only reached statistical significance in the case of animals treated with MDSCs (NT: 1312.0 ± 838.1 cpm vs Ii-MDSCs: 22647.0 ± 6821 cpm, p=0.001) (Figure 44D). This fact could mask the response against antigen-specific and polyclonal stimuli and may explain why there were no differences between the proliferative capacity of splenocytes from IiMOG-treated mice and their controls, although splenocyte proliferation of IiMOG-MDSCs treated mice in the presence of PHA-L was significantly decreased compared to their counterparts (IiMOG-MDSCs: 23799.0 ± 12967.0 cpm vs Ii-MDSCs: 36271.0 ± 9779.0 cpm, p=0.021) (Figure 44D).

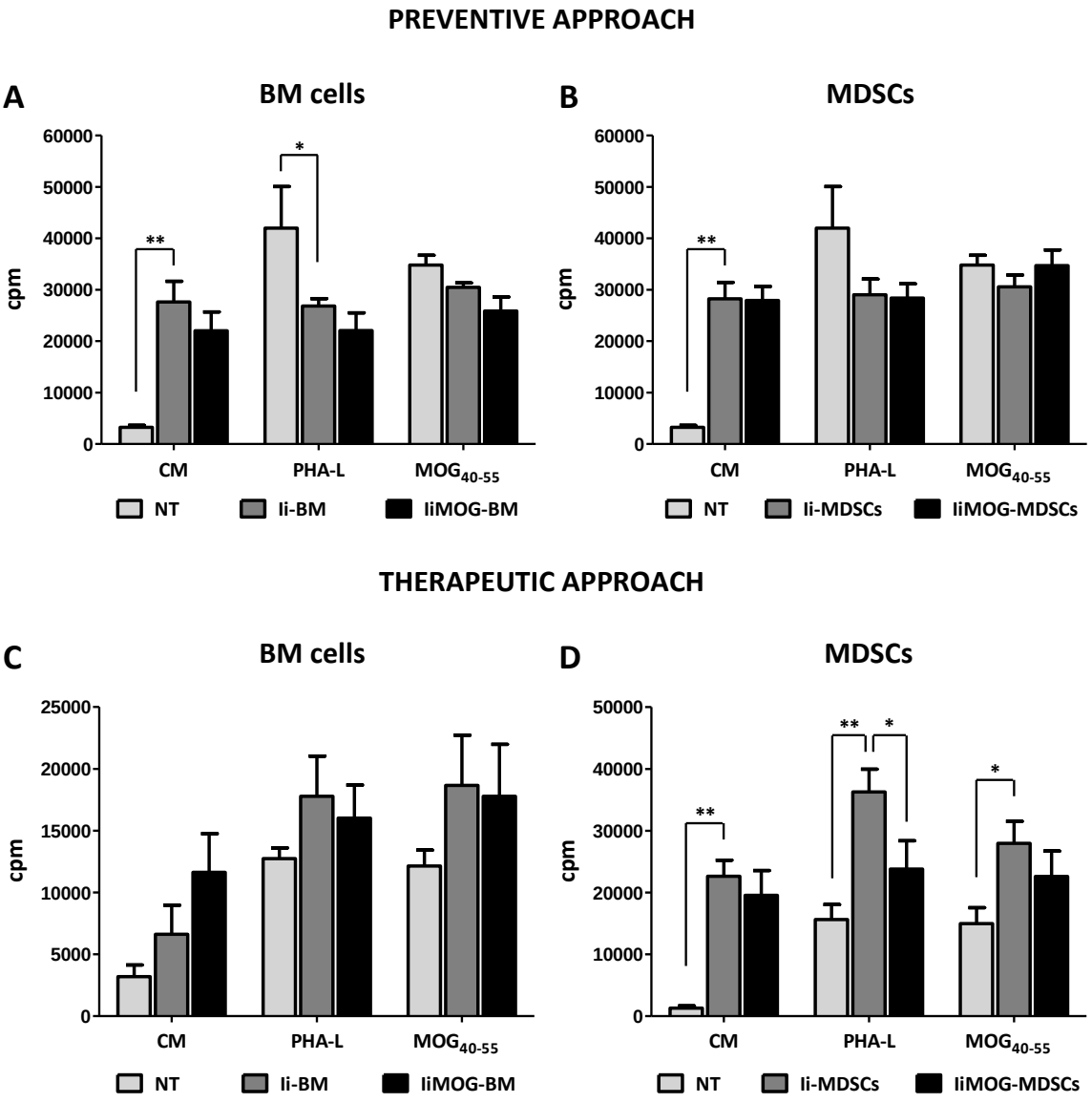


Figure 44. Proliferative response of the splenocytes from BM cell- and MDSC-treated animals in the different culture conditions. Charts A and B show the proliferative response of splenocytes from preventively treated animals and charts C and D from the therapeutically treated ones. CM: culture medium. Data are represented as the mean ± SEM. * $p<0.05$, ** $p<0.01$.

7.6. Treatment with BM cells and MDSCs modifies the cytokine secretion profile

To study whether the treatment with MOG-specific cells modifies the cytokine secretion profile (Th1, Th2 or Th17), secreted cytokines were quantified in the supernatants of splenocytes stimulated with the encephalitogenic peptide. In accordance with the results obtained in the proliferation assays, no differences were found between the levels of cytokines secreted by the splenocytes from mice treated with MOG-specific BM cells and MDSCs and those from their controls.

The splenocytes of treated animals secreted increased levels of cytokines compared to those of NT mice. In the preventive approach, splenocytes from Ii-BM cell treated mice secreted significantly more IL-2, IL-4, IL-5, IL-6, TNF-α, GM-CSF and IFN-γ than those from NT animals (Figure 45A). Regarding the cytokines secreted by splenocytes from Ii-MDSCs treated animals, the amounts of IL-2, IL-5, IL-6, TNF-α, GM-CSF and IFN-γ were also found significantly higher than those secreted by the splenocytes from NT mice (Figure 45B). In the therapeutic approach, splenocytes from Ii-BM cells treated mice secreted significantly more IL-2, IL-4, IL-5 and IL-10 than those from NT animals (Figure 45C). Regarding the cytokines secreted by splenocytes from animals treated with Ii-MDSCs, the amounts of IL-2, IL-4 and IL-10 were also found significantly higher than those secreted by the splenocytes from NT mice (Figure 45D). These results in conjunction with the ones from the proliferation assays could indicate that the injection of cells *per se* causes an alteration in the cytokine secretion profile of the splenocytes from treated mice and masks an eventual antigen-specific effect of IiMOG-cells, which may explain why there were no differences between the cytokines secreted by the splenocytes from IiMOG-treated mice and their controls.

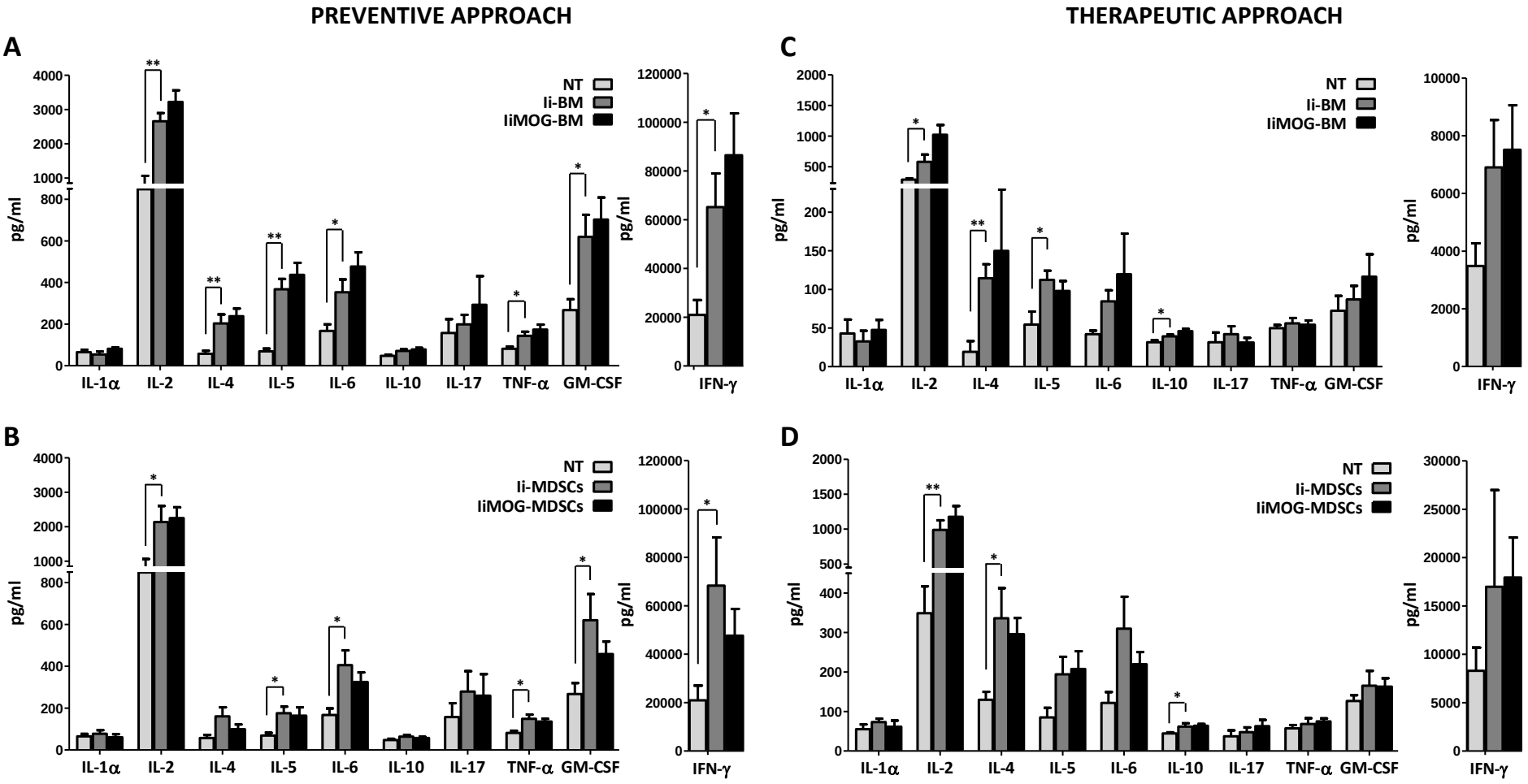


Figure 45. Determination of the Th1/Th2/Th17 cytokine secretion profile of the splenocytes from preventively and therapeutically treated mice. Levels of cytokines secreted by MOG_{40-55}-stimulated splenocytes from mice preventively treated with BM cells (A) and MDSCs (B) and therapeutically treated with BM cells (C) and MDSCs (D). Data are represented as the mean ± SEM. * $p<0.05$, ** $p<0.01$.

7.7. Presence of specific antibodies against MOG_{40-55} does not affect EAE outcome

Serum samples were collected at the end of each experiment (day 33-35 p.i.) and the presence of specific IgG antibodies against the encephalitogenic peptide was assessed using an ELISA technique.

No differences were found between the levels of specific antibodies between the IiMOG-treated groups and the Ii-treated groups in both preventive and therapeutic approaches (Figure 46). In the preventive approach, specific antibodies were found in 85.71% of the animals treated with IiMOG-BM cells, a percentage that was similar in the Ii-BM cell group (83.3%) and in NT animals (80%). In the case of mice treated with MDSCs, specific antibodies were found in 100% of the animals of both groups. Moreover, no differences were observed between protected and non-protected animals from IiMOG-treated groups (data not shown). In the therapeutic approach, positivity for specific antibodies was detected in 85.71% of the animals treated with IiMOG-BM cells, in 62.5% in the Ii-BM cell group and 58.33% in NT animals. In the case of mice treated with MDSCs, similar percentages of specific antibodies were found in animals treated with IiMOG-MDSCs (75%) and Ii-MDSCs (71.43%). In addition, no differences were observed between recovered and unrecovered mice from IiMOG-treated groups (data not shown), which strongly suggests a lack of impact of these antibodies on this model of EAE.

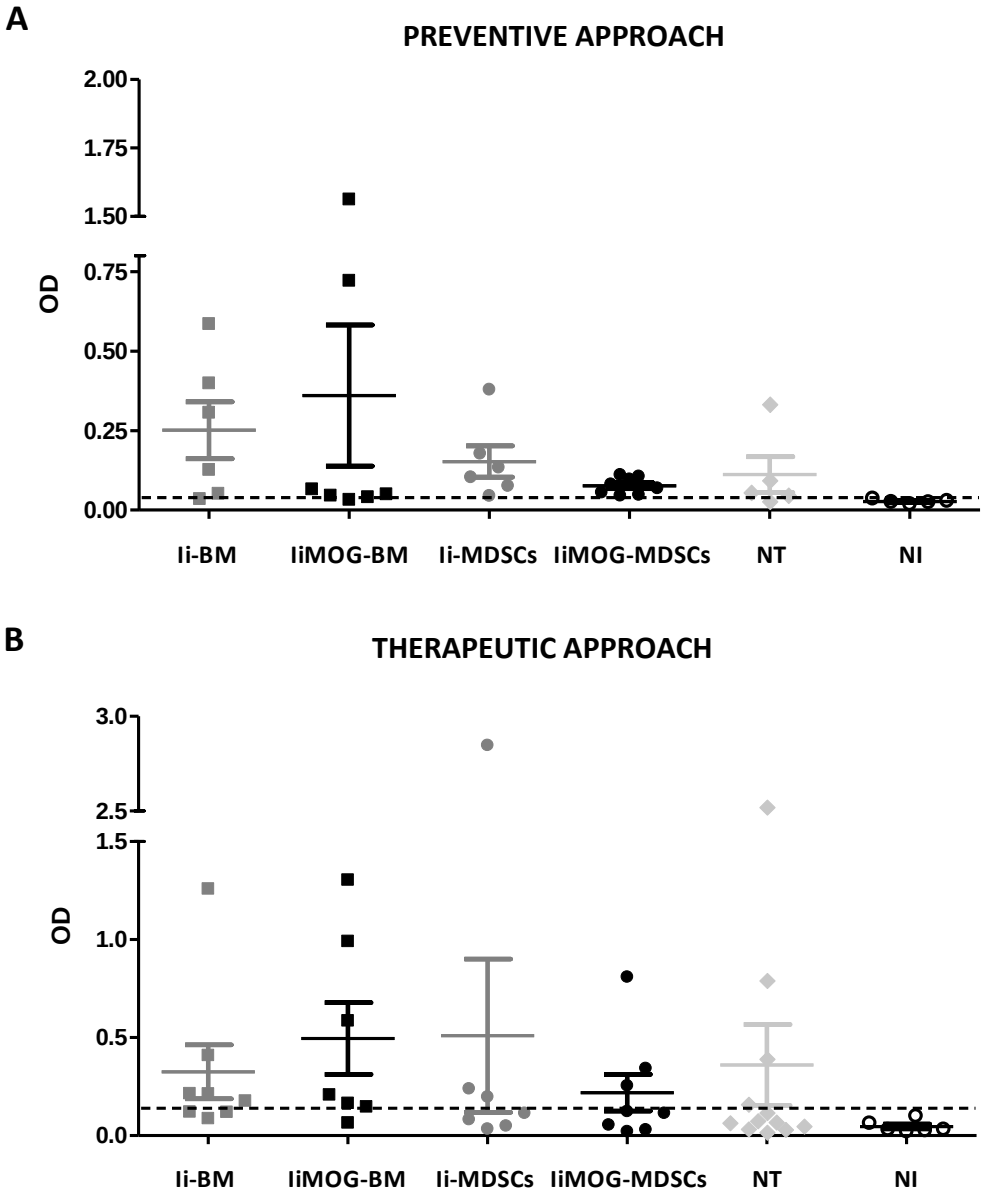


Figure 46. Detection of anti-MOG_{40-55} antibodies in mice treated with transduced BM cells and MDSCs. Charts show anti-$MOG_{40–55}$ IgG levels in mice sera. Both the prevalence and the mean levels of anti-$MOG_{40–55}$ antibodies were similar in all experimental groups from both preventive (A) and therapeutic (B) approaches. Dotted lines represent the mean OD of the NI mice sera plus 2 SD, which corresponds to 0.04 in the preventive approach and to 0.11 in the therapeutic approach.

PART 2: GENERATION OF HUMAN MDSCs FROM HEMATOPOIETIC PROGENITOR CELLS

1. DETERMINATION OF CULTURE CONDITIONS FOR GENERATING MDSCs FROM HSCs

As mentioned in the previous part of this thesis, in our laboratory we have successfully generated functional murine MDSCs from BM cells able to suppress T-cell responses. As a continuation of the murine studies, we hypothesized that human MDSCs could also be generated *in vitro* and eventually be used for the induction of immune tolerance in clinical settings. To generate human MDSCs, purified $CD34^+$ hematopoietic progenitors from apheresis products were cultured for nine days with different combinations of cytokines and the cultured cells were phenotypically characterized by flow cytometry.

After nine days of culture no significant differences were observed between the different combinations of cytokines since in all the cases about 20% of the cells presented a MDSC phenotype ($CD33^+HLA\text{-}DR^{low/-}$) (Figure 47A). Regarding the two subtypes of MDSCs, a reduction in the percentage of M-MDSCs ($CD33^+HLA\text{-}DR^{low/-}CD14^+CD15^-$) was observed when cells were cultured without IL-3 and when GM-CSF and IL-6 were added at day 4 of culture, although these differences did not reach statistical significance (Figure 47B). However, the use of this combination of cytokines resulted in the highest percentage of G-MDSCs ($CD33^+HLA\text{-}DR^{low/-}CD14^-CD15^+$), although again the differences were not statistically significant (Figure 47C). Interestingly, after nine days of culture the majority of the cells still expressed the immature hematopoietic progenitor marker CD34 (Figure 47D), indicating that they still had potential to further differentiate.

For this reason, we decided to extend the length of the culture and the $CD34^+$ cells were cultured for 14 and 20 days to determine whether greater amounts of MDSCs could be generated. To perform these cultures the combinations of cytokines in which GM-CSF and IL-6 were added at day 4 were discarded since, as mentioned above, in the presence of IL-3 no differences were observed and without IL-3 the percentage of M-MDSCs was reduced.

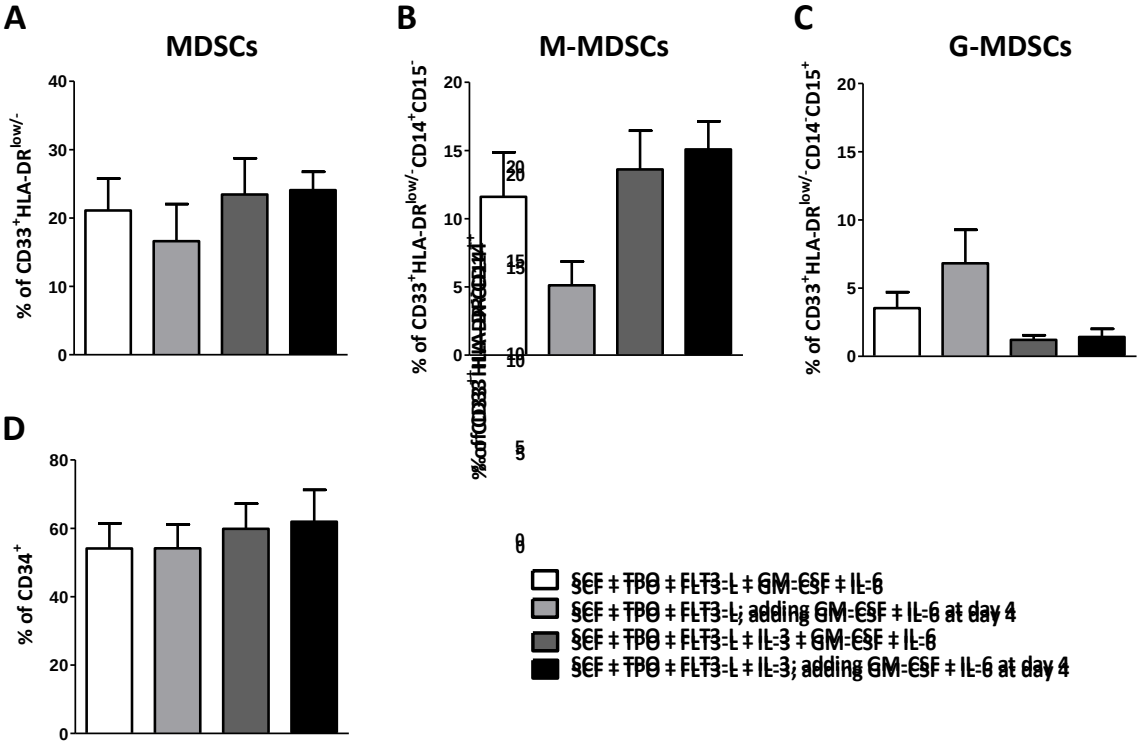


Figure 47. Percentages of the different types of MDSCs obtained after nine days of culture in the presence of different combinations of cytokines. (A) Percentage of total MDSCs. (B) Percentage of M-MDSCs. (C) Percentage of G-MDSCs. (D) Percentage of $CD34^+$ cells. Data are represented as the mean ± SEM; (n=3).

As shown in Figure 48A, the percentage of cells expressing the CD34 marker significantly decreased over time, in such a way that after 14 days of culture only 19.4 ± 11.6% and 10.3 ± 10.4% expressed the immature hematopoietic progenitor marker and after 20 days almost there were no $CD34^+$ cells (4 ± 4.0% and 1.3 ± 2.2%), when cultured with the cocktails of cytokines without IL-3 and with IL-3 respectively.

Concerning the percentages of total MDSCs, these were significantly higher after 20 days of culture compared with only nine days in the presence of both cocktails of cytokines (without IL-3: 73.7 ± 9.2% vs 31.2 ± 14.3%, p = 0.003; with IL-3: 69.1 ± 17.4% vs 30.3 ± 12.4%, p = 0.005). These percentages were also higher compared with those obtained after 14 days of culture, although it only reached statistical significance in the absence of IL-3 (without IL-3: 73.7 ± 9.2% vs 43.1 ± 9.9%, p = 0.043; with IL-3: 69.1 ± 17.4% vs 44.6 ± 5.5%, p = 0.123) (Figure 48B).

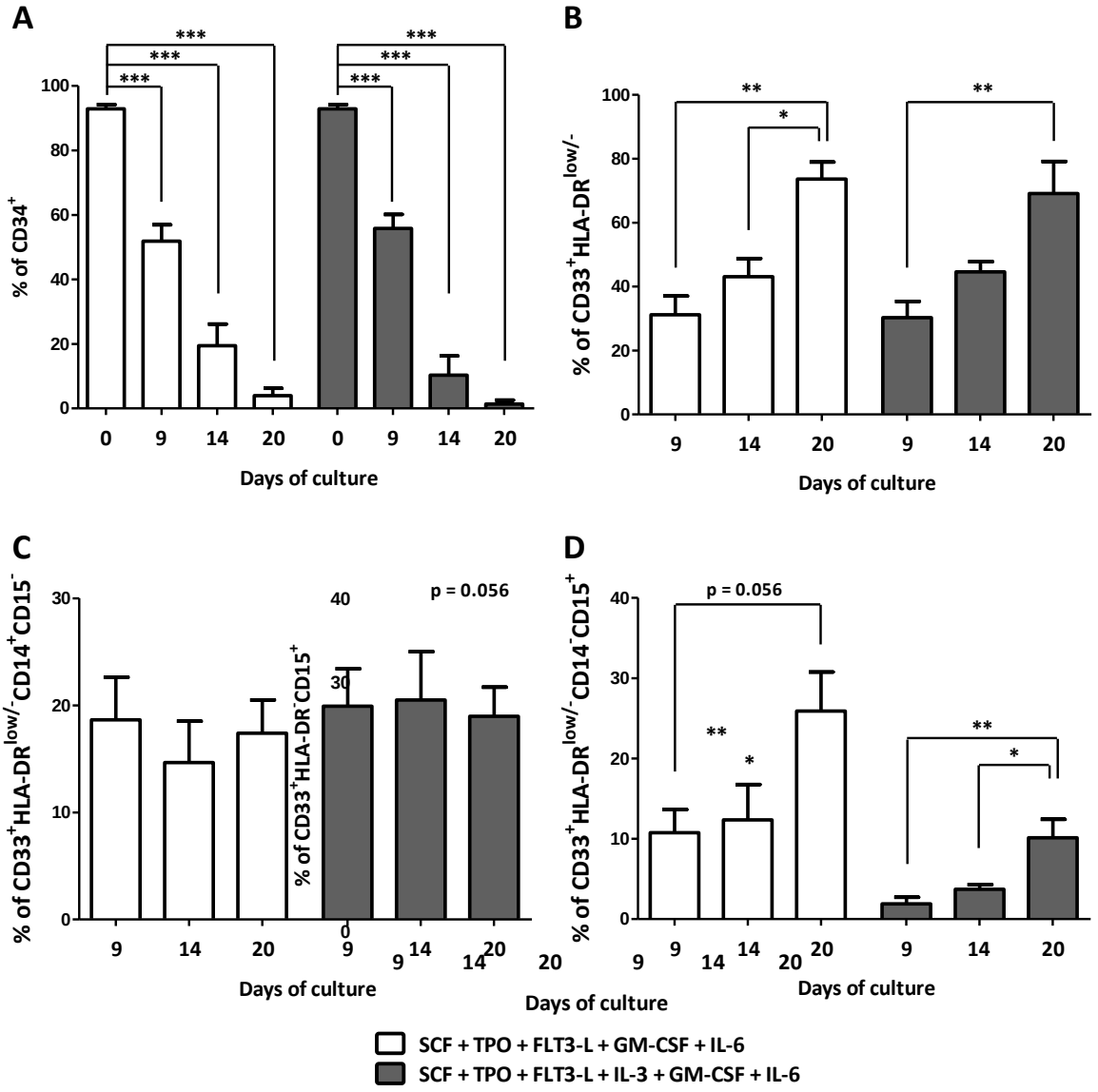


Figure 48. Evolution of the percentages of MDSCs over time in the presence of two different cytokine combinations. (A) Loss of the CD34 marker over time. (B) Percentages of total MDSCs ($CD33^{+}HLA\text{-}DR^{low/-}$) in cultures of 9, 14 and 20 days respectively. (C) Percentages of M-MDSCs ($CD33^{+}HLA\text{-}DR^{low/-}CD14^{+}CD15^{-}$) in cultures of 9, 14 and 20 days respectively. (D) Percentages of G-MDSCs ($CD33^{+}HLA\text{-}DR^{low/-}CD14^{-}CD15^{+}$) in cultures of 9, 14 and 20 days respectively. Data are represented as the mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; (n=3-6).

Regarding the proportions of the two subtypes of MDSCs, no significant differences were observed in the percentages of M-MDSCs over time (Figure 48C). On the contrary, the percentages of G-MDSCs were increased after 20 days of culture compared to the cultures of nine days (without IL-3: 25.9 ± 8.4% vs 10.8 ± 7.0%, p = 0.056; with IL-3: 10.1 ± 4.0% vs 1.9 ± 2.0%, p = 0.003) and 14 days (without IL-3: 25.9 ± 8.4% vs 12.4 ± 7.6%, p = 0.161; with IL-3: 10.1 ± 4.0% vs 3.7 ± 1.0%, p = 0.033),

although in both cases the differences only reached statistical significance in the presence of IL-3 (Figure 48D).

When analyzing which combination of cytokines was better to generate MDSCs, no significant differences were observed in the percentages of total MDSCs and M-MDSCs in the presence or absence of IL-3 in cultures of 14 days, but a reduction in the percentages of G-MDSCs was observed in the presence of IL-3, although this difference did not reach statistical significance (without IL-3: 12.4 ± 7.6% vs with IL-3: 3.7 ± 1.0%, $p = 0.151$) (Figure 49A).

In cultures of 20 days, no significant differences were found in the percentages of total MDSCs and M-MDSCs either. However, as already noted above in the results after 14 days of culture, a statistically significant reduction in the percentages of G-MDSCs was observed when cells were cultured in the presence of IL-3 (without IL-3: 25.9 ± 8.4% vs with IL-3: 10.1 ± 4.0%, $p = 0.025$) (Figure 49B).

For these reasons, we decided to perform all the subsequent experiments using only the cytokine cocktail without IL-3: SCF + TPO + FLT3-L + GM-CSF + IL-6.

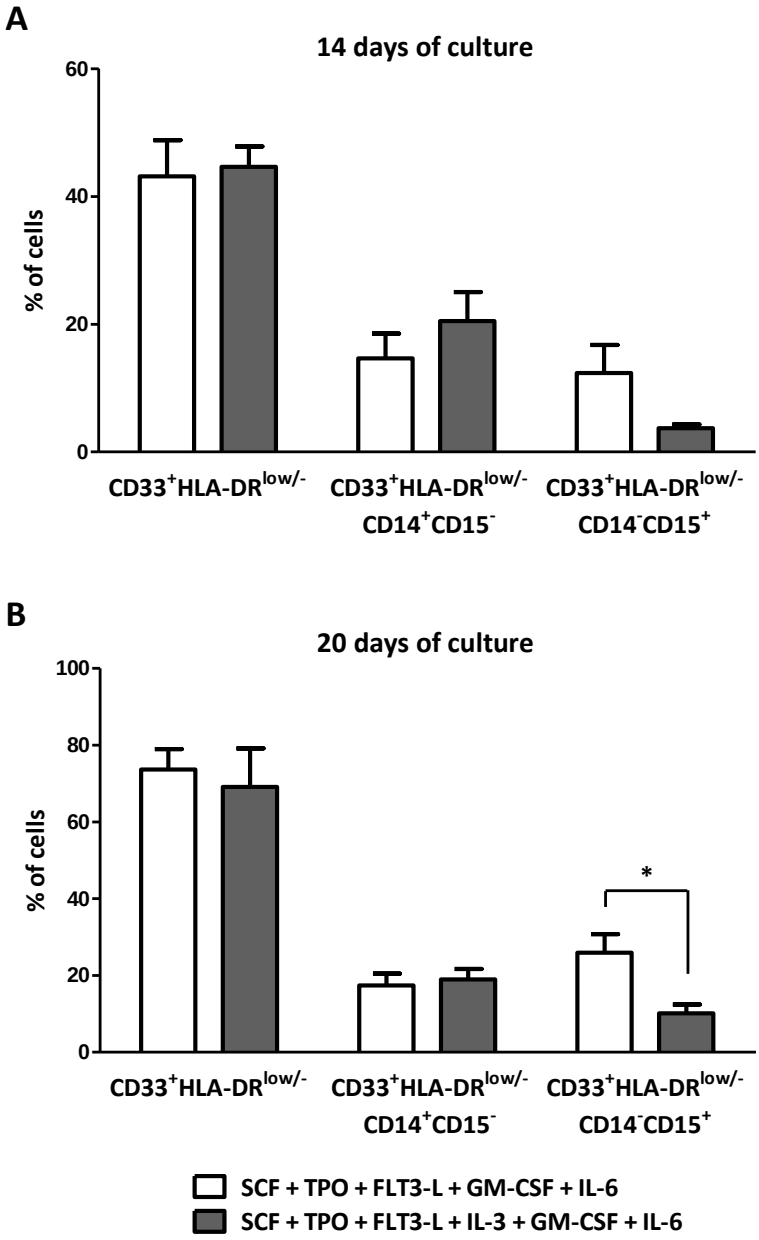


Figure 49. Proportions of the different types of MDSCs cultured for 14 and 20 days with the two combinations of cytokines. (A) Percentages of total MDSCs ($CD33^{+}HLA\text{-}DR^{low/-}$), M-MDSCs ($CD33^{+}HLA\text{-}DR^{low/-}CD14^{+}$) and G-MDSCs ($CD33^{+}HLA\text{-}DR^{low/-}CD15^{+}$) generated in 14 day cultures. (B) Percentages of total MDSCs ($CD33^{+}HLA\text{-}DR^{low/-}$), M-MDSCs ($CD33^{+}HLA\text{-}DR^{low/-}CD14^{+}$) and G-MDSCs ($CD33^{+}HLA\text{-}DR^{low/-}CD15^{+}$) generated in 20 days cultures. Data are represented as the mean ± SEM. * $p<0.05$; (n=3).

2. CHARACTERIZATION OF THE *IN VITRO* GENERATED MDSCs

As in the case of murine MDSCs generated in BM transduction cultures, we decided to further characterize the human MDSCs generated in cultures of 20 days using the selected cocktail of cytokines. We decided to study the same markers we analyzed in the murine MDSCs (PD-L1, CD80 and CD86) by flow cytometry. As already mentioned, MDSCs are known to be activated under inflammatory conditions, so the expression of these molecules was studied both at baseline and in the presence of inflammatory stimuli (IFN-γ and LPS).

In a basal state 14.4 ± 3.8% of the generated MDSCs expressed PD-L1 and this percentage was significantly increased up to 86.8 ± 7.0% upon stimulation with IFN-γ plus LPS (p=0.003) (Figure 50A). Regarding the two subpopulations of MDSCs, M-MDSCs presented a higher percentage of cells positive for PD-L1 than G-MDSCs but although this percentage increased upon stimulation the differences did not reach statistical significance (37.4 ± 16.5% vs 67.5 ± 25.9%, p=0.25) (Figure 50B). Conversely, the percentage of G-MDSCs positive for PD-L1 was significantly increased under inflammatory conditions since it raised from 9.9 ± 3.1% in the basal state to 97.6 ± 1.9% in the activated state (p=0.0009) (Figure 50C), indicating that the increase of PD-L1$^+$ cells in total MDSCs was mainly due to the increment of the expression of this molecule in G-MDSCs. We also looked at the MFI of this molecule in all types of MDSCs and, although it was increased in all the populations upon activation, the differences did not reach statistical significance (Figures 50B, 50D and 50F).

Regarding the expression of CD80 and CD86 molecules, only M-MDSCs showed a certain degree of expression. No differences in the percentages of CD80$^+$ cells were observed between the basal and the activated states. On the other hand, the percentage of positive cells for CD86 appeared to decrease under inflammatory conditions (Figure 50C). No differences were observed in the MFI of these molecules upon stimulation (Figure 50B, 50D, and 50F).

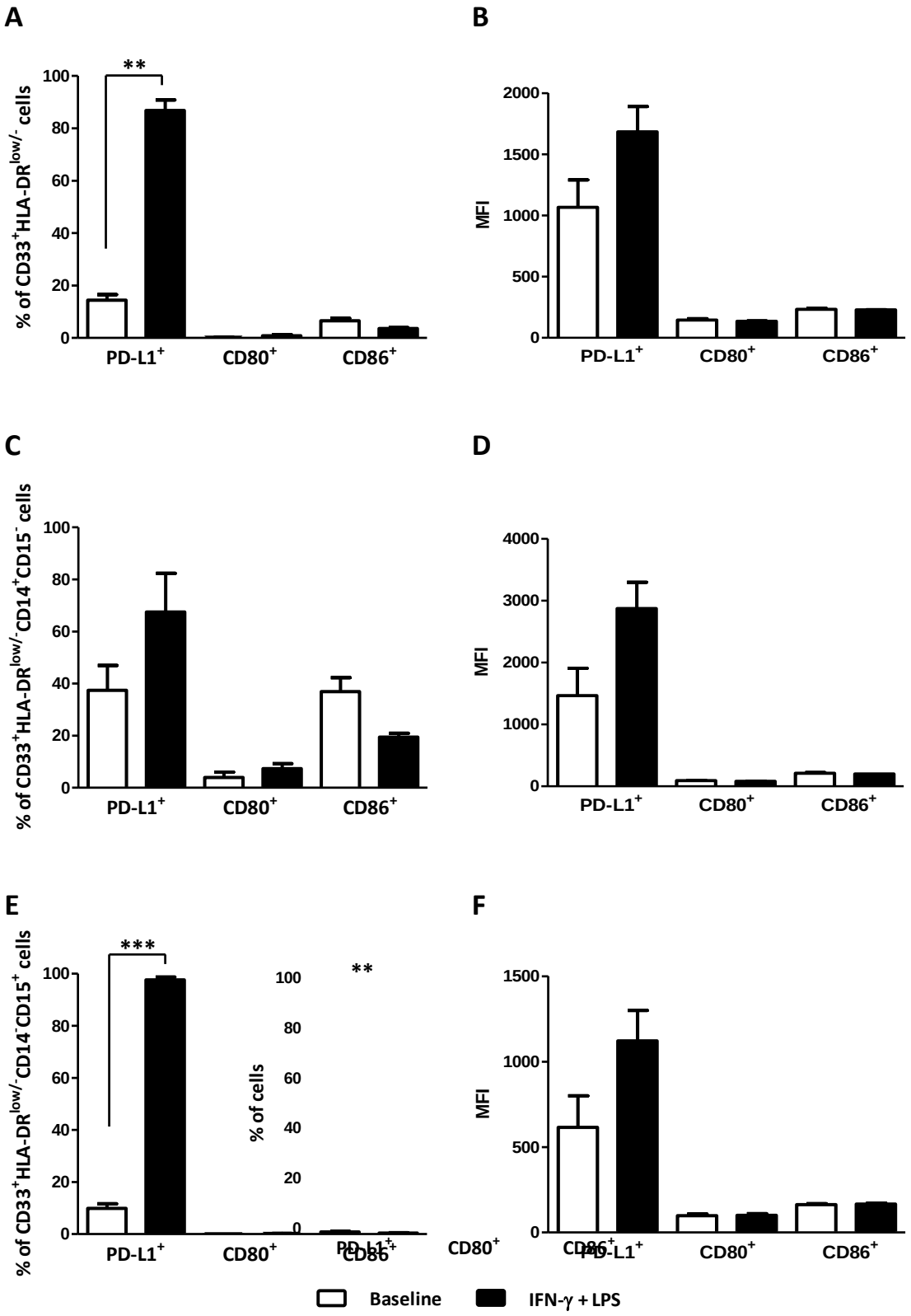


Figure 50. Phenotypic characterization of the MDSCs generated in cultures of 20 days. (A) Percentage of total positive MDSCs for PD-L1, CD80 and CD86. (B) MFI of PD-L1, CD80 and CD86 of total MDSCs. (C) Percentage of positive M-MDSCs for PD-L1, CD80 and CD86. (D) MFI of PD-L1, CD80 and CD86 of M-MDSCs. (E) Percentage of positive G-MDSCs for PD-L1, CD80 and CD86. (F) MFI of PD-L1, CD80 and CD86 of G-MDSCs. Data are represented as the mean ± SEM. ** p<0.01, *** p<0.001; (n=3).

3. SUPPRESSION OF ALLOGENEIC PBMCs PROLIFERATION

To analyze the suppressive effect of the *in vitro* generated cells with a MDSC phenotype ($CD33^{+}HLA\text{-}DR^{low/-}$ cells), so that they could be considered functional MDSCs, we decided to study the ability of these cells to inhibit T-cell responses. $CD33^{+}HLA\text{-}DR^{low/-}$ cells from cultures of 14 and 20 days were sorted and irradiated at 25 Gy. Because it was not feasible to use MDSCs and PBMCs from the same donor, we studied the ability of these cells to suppress the proliferation of allogeneic PBMCs. First of all, we analyzed the intrinsic alloreactivity of the *in vitro* generated MDSCs. To this end, PBMCs from blood samples of healthy donors were cultured for 96 h with the same ratio of sorted $CD33^{+}HLA\text{-}DR^{low/-}$ MDSCs or with $CD33^{+}HLA\text{-}DR^{+}$ cells, that were used as a positive control of an allogeneic proliferative response.

As shown in Figure 51, *in vitro* generated MDSCs in cultures of 14 and 20 days showed a weak stimulatory ability compared to their $HLA\text{-}DR^{+}$ counterparts, which triggered a strong allogeneic proliferative response.

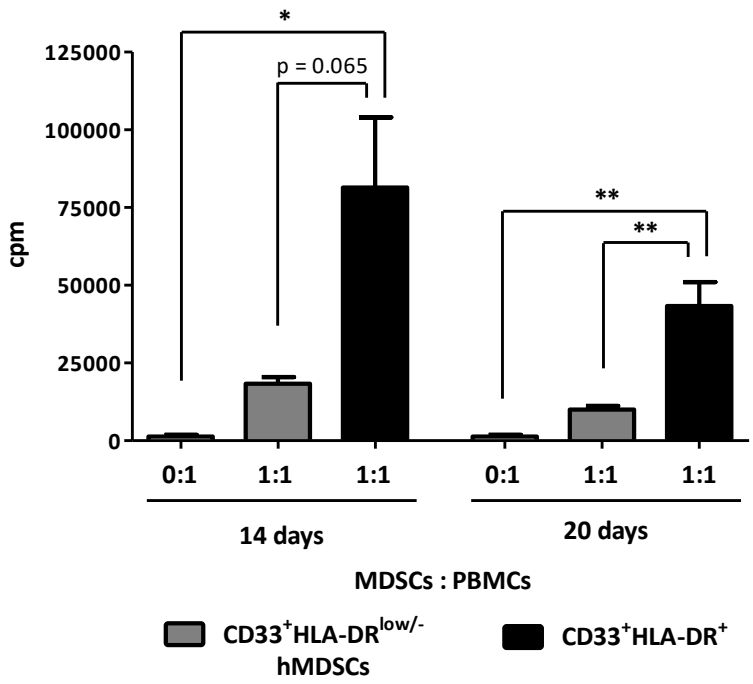


Figure 51. Proliferation of PBMCs co-cultured with allogeneic $CD33^{-}HLA\text{-}DR^{low/-}$ MDSCs or with $CD33^{+}HLA\text{-}DR^{+}$ cells. Data are expressed as cpm. Data are represented as the mean ± SEM. * $p<0.05$, ** $p<0.01$; (n = 3-4).

Moreover, we studied the capacity of our *in vitro* generated MDSCs to inhibit PHA-L-induced T-cell proliferation. To this end, PBMCs were cultured with different proportions of MDSCs generated in 14 and 20 day cultures with PHA-L. The results obtained in these suppression assays show that both types of MDSCs were able to suppress PBMC proliferation in a dose-dependent manner (Figure 53). No differences were observed between the MDSCs from cultures of 14 and 20 days, suggesting that they do not lose their functionality over time and that they preserve their immature state, which is an inherent characteristic of MDSCs.

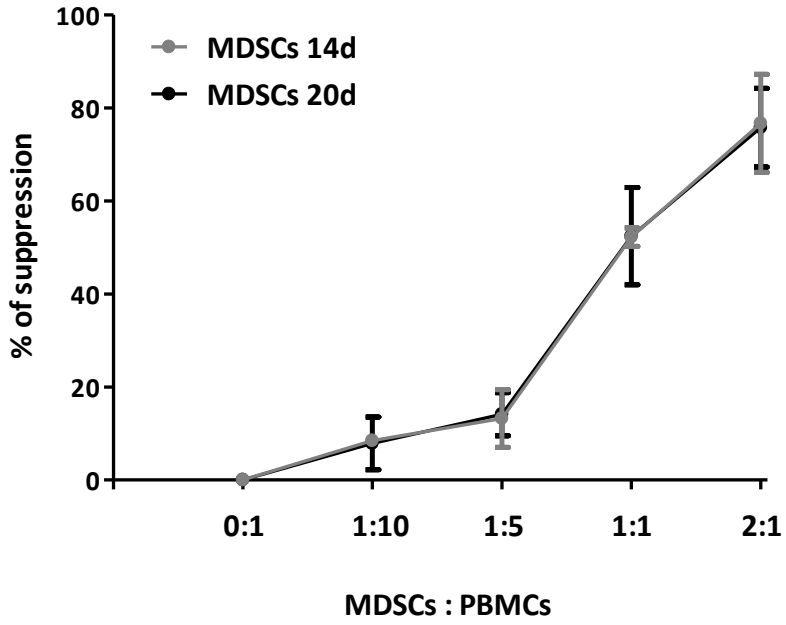


Figure 53. *In vitro* generated MDSCs suppress PHA-L-induced PBMC proliferation. The chart shows the percentage of suppression of PHA-L-induced PBMC proliferation by irradiated MDSCs from 14 (grey) and 20 (black) days cultures. Data are represented as the mean ± SEM; (n=3).

3. *IN VITRO* GENERATED MDSCs DECREASE THE LEVELS OF PROINFLAMMATORY CYTOKINES AND INCREASE THE LEVELS OF IL-10

After studying the capacity of the *in vitro* generated MDSCs to suppress T-cell proliferation, the secreted cytokines in the supernatants of the suppression assays of PHA-L-induced T-cell proliferation were quantified to determine whether the MDSCs generated in 14 days and 20 days cultures were able to modify the Th1, Th2 or Th17 cytokine secretion profile.

As shown in Figure 54, the MDSCs generated in both 14 and 20 days cultures significantly decreased the levels of the proinflammatory cytokines TNF-α and GM-CSF in a dose-dependent manner and, in the case of the 14 days generated MDSCs, the levels of IFN-γ were also significantly reduced. The levels of IL-2, IL-5 and IL-13 were also reduced in the higher ratios although the differences did not reach statistical significance. In the case of the 20 days generated MDSCs the proportions of IL-17A were significantly increased in the highest ratio, which was not observed in 14 days generated MDSCs. Moreover, the levels of the immunomodulatory cytokine IL-10 were increased in a dose-dependent manner although the differences only reached statistical significance in the case of the MDSCs generated in 14 days cultures.

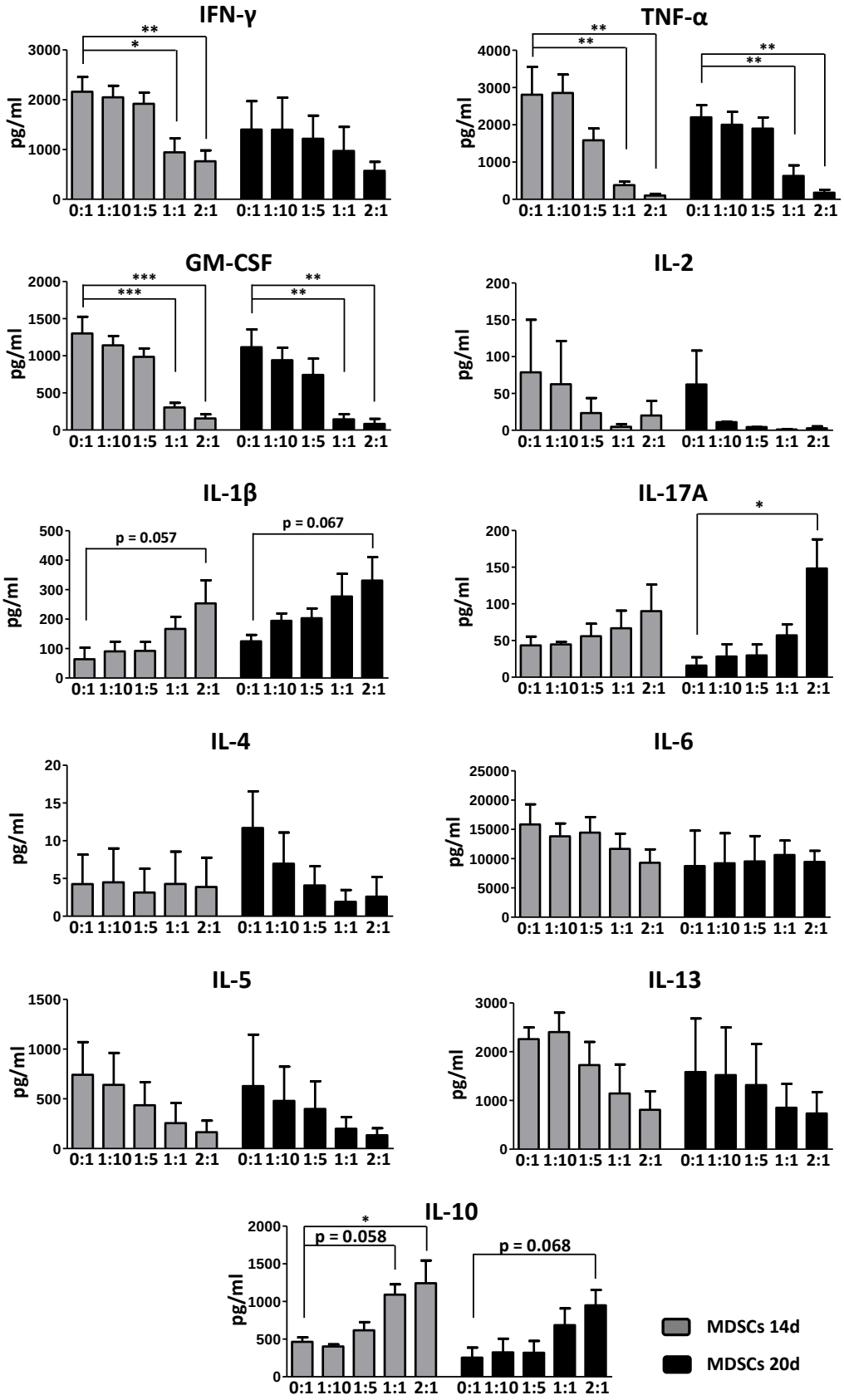


Figure 54. Th1/Th2/Th17 cytokines secreted in the culture supernatants of the suppression assays of T-cell proliferation. Secreted cytokines levels were measured in the culture supernatants of the suppression assays of PHA-L-induced PBMC proliferation by 14 days (grey bars) and 20 days (black bars) MDSCs. Data are represented as the mean ± SEM. * p<0.05, ** p<0.01 and *** p<0.001; (n=3).

Let the future tell the truth, and evaluate each one according to his work and accomplishments. The present is theirs; the future, for which I have really worked, is mine.

Nikola Tesla

DISCUSSION

PART 1: ANTIGEN-SPECIFIC MDSCs INDUCE IMMUNOLOGICAL TOLERANCE IN THE EAE IN BOTH PREVENTIVE AND THERAPEUTIC APPROACHES

In previous studies conducted at our laboratory we demonstrated that infusion of BM cells transduced with a self-antigen (MOG_{40-55}) induced immunological tolerance in MOG-induced EAE, both in a partially myeloablative and non-myeloablative context (Eixarch, Espejo et al. 2009). In the preventive approach, the generation of stable molecular chimerism was associated with a robust tolerance and a high resistance to the disease, even in partially myeloablated animals displaying relatively low levels of chimerism. On the other hand, when transduced BM cells were given therapeutically, the disease improved very rapidly after the infusion of MOG-expressing BM cells. However, despite the fact that mice were partially myeloablated and that donor engraftment was present in all the transplanted animals, molecular chimerism was only observed in control mice that received mock-transduced cells, but not in those infused with cells transduced with the self-antigen. This result was somehow anticipated given that the animals are immunized with this antigen to induce the disease, so that when the cells are infused the animals are already sensitized and have an ongoing immune response to MOG and MOG-expressing cells, fact that eventually leads to rejection of these cells but not of non-transduced or control cells not expressing the MOG peptide. Since the therapeutic effect occured in the absence of engraftment, we postulated and confirmed that myeloablation was not required to induce immunological tolerance in subsequent non-myeloablative experiments. Moreover, the absence of molecular chimerism and the rapid recovery of the animals made us hypothesize that the therapeutic effect observed was not mediated by central tolerance but rather by mechanisms of peripheral tolerance. These observations led us to reconsider our initial ideas and we postulated that the therapeutic effect observed was not mediated by cells with engrafting potential and their progeny, but rather by a more mature cell type that was necessarily present in BM transduction cultures and that could present the self-antigen to T cells via MHC-II (due to our particularly strategy and the vector design) in a tolerogenic manner. More recently, we found that the most abundant cell types in these cultures were of myeloid lineage and that these

cells fulfilled the morphologic, phenotypic and functional criteria of MDSCs of both subtypes, monocytic and granulocytic. Moreover, these cells displayed arginase-1 and iNOS activities and strongly suppressed MOG-induced proliferation of splenocytes from EAE mice (Gomez, Espejo et al. 2014). Therefore, this project was initiated with the purpose of determining whether the MDSCs generated in BM transduction cultures were responsible for the induction of the immunological tolerance and the therapeutic effect observed in the EAE model.

Due to the elapsed time between the previous experiments and this project, immunophenotypic analyses of BM cells were performed before and after retroviral transduction to confirm that the subsets of MDSCs were generated. As we expected and in agreement with our previous results (Gomez, Espejo et al. 2014), the vast majority of cells that were generated in BM retroviral transduction cultures consisted of two main myeloid cell populations, $CD11b^{+}$ $Gr\text{-}1^{low}$ and $CD11b^{+}$ $Gr\text{-}1^{high}$, which correspond to the phenotypes described for M- and G-MDSCs respectively (Youn, Nagaraj et al. 2008). Moreover, these cells were the ones in which the transduction efficiency was higher, especially in the M-MDSC subset, which was also the subpopulation that expanded to a greater extent in relative terms. Since the therapeutic effect observed in EAE only occurred when the transplanted cells expressed the self-antigen and not with the control cells (Eixarch, Espejo et al. 2009), the cells responsible for the beneficial effect must be transduced. For this reason, MDSCs represent the best candidates for mediating the induction of immunological tolerance in the EAE model.

Since in the previous study we had already demonstrated that the generated MDSCs expressed several markers that define these cells, such as CD124, F4/80 and MHC-I (Gomez, Espejo et al. 2014), we decided to study the expression of several molecules functionally associated with the inhibition of T-cell responses in both M- and G-MDSCs subsets. As MDSCs need to be activated to exert their immunosuppressive functions, the expression of these molecules was studied both at baseline and in the presence of inflammatory stimuli. We found that the majority of both types of the MDSCs expressed PD-L1 and that upon activation with IFN-γ, LPS and IFN-γ plus LPS there was a significant increase in these proportions and in their expression levels, especially with

the combination of both stimuli. The importance of the PD-1/PD-L1 pathway in cancer has been recognized for many years (Iwai, Ishida et al. 2002) and more recently also for the maintenance of immune tolerance [reviewed in (Francisco, Sage et al. 2010; Fife and Pauken 2011; Gianchecchi, Delfino et al. 2013)]. Regarding MDSCs, PD-L1 expression was shown to be induced in these cells (as well as in macrophages, DCs and tumor cells) by hypoxia in the tumor sites (Noman, Desantis et al. 2014). Moreover, it has been demonstrated that PD-1/PD-L1 blockade and inhibition of MDSCs can synergize to revert or inhibit immunosuppression in cancer. Inhibition of MDSC trafficking increased the efficacy of anti-PD-1 therapy in a murine model of rabdomyosarcoma (Highfill, Cui et al. 2014), and PD-L1 blockade was shown to neutralize liver MDSC-mediated immunosuppression and restore the therapeutic activity of an anti-carcinoembryonic antigen chimeric antigen receptor-modified T cell (CAR-T) in a murine model of cancer (Burga, Thorn et al. 2015). Regarding the role of MDSCs and the PD-1/PD-L1 pathway in the pathogenesis of autoimmune diseases, published data establishes a crucial role of PD-1/PD-L1 engagement in the negative regulation of lymphocyte activation and consequently in the regulation of autoimmune responses (Freeman, Long et al. 2000). PD-1 pathway limits autoimmunity by inhibiting the expansion of self-reactive T cells through the induction of T-cell anergy or apoptosis. Ligation of PD-L1 with PD-1 also blocks reactivation, expansion and the effector function of autoreactive T cells in tissues (Joller, Peters et al. 2012). Genetically modified DCs that expressed high levels of PD-L1 and presented the MOG_{35-55} peptide through MHC-II protected mice against EAE when administered preventively and ameliorated the disease when administered therapeutically (Hirata, Senju et al. 2005). Another study showed that adoptive transfer of G-MDSCs from PD-L1 deficient mice failed to suppress EAE pathology in comparison to wild-type G-MDSCs, which ameliorated the disease and constrained autoreactive T cells, suggesting a PD-L1-dependent mediated regulation of the autoimmune response in EAE (Ioannou, Alissafi et al. 2011). All these data suggest that apart from the broadly defined suppressive mechanisms of MDSCs (arginase-1 and iNOS activities among others) PD-1/PD-L1 pathway may constitute a pivotal mechanism for the inhibition of self-reactive T cells and for the induction of immune tolerance by MDSCs.

Apart from expressing PD-L1, the majority of M-MDSCs but only a minority of G-MDSCs expressed CD80 and CD86 and their expression levels were significantly increased upon stimulation with IFN-γ plus LPS. CD80 and CD86 molecules are the ligands of CD28 and CTLA-4, the latter is the first described molecule with inhibitory properties that is expressed on T cells upon activation (Brunet, Denizot et al. 1987). The binding of CTLA-4 to CD80 or CD86 inhibits T-cell proliferation and activation, diminishes cytokine production and induces T-cell unresponsiveness and anergy leading to tolerance (Perez, Van Parijs et al. 1997). There are very few published studies regarding the expression of CD80 and CD86 by MDSCs. Ioannou *et al* did not find an increased expression of CD86 by G-MDSCs upon IFN-γ plus LPS stimulation, although they found expression of this molecule at baseline. No expression of CD80 at baseline or after stimulation was found (Ioannou, Alissafi et al. 2011). In addition, MDSCs from tumor-bearing mice failed to overexpress CD80 and CD86 under hypoxia conditions (Noman, Desantis et al. 2014). On the contrary, hepatic MDSCs from tumor-bearing mice showed up-regulation of CD80 and CD86 after treatment with the mitogen lectin concanavalin A, although this up-regulation was associated with the loss of the immunosuppressive functions (Kapanadze, Medina-Echeverz et al. 2015). In other tumor models CD80 expression has been reported to be increased in splenic MDSCs and inhibition of this molecule partially abrogated their suppressive function, highlighting the importance of CD80 in the MDSC-mediated suppression (Yang, Cai et al. 2006; Maenhout, Thielemans et al. 2014). The disparity in these results could be due to the heterogeneity of MDSCs populations and is consistent with the idea that MDSCs phenotype and function may depend on many factors including their level of activation, their localization and environmental milieu, the experimental model used or their clinical or pathological context. Our data show expression of PD-L1 along with CD80 and CD86 on the surface of both M- and G-MDSCs, fact that can contribute to the immunosuppressive function of these cells. While both CTLA-4 and PD-1 receptors are involved in dampening immune responses, there are considerable differences between them. CTLA-4 is displayed on the surface of naïve T cells upon activation in the lymph node whereas PD-1 is expressed on effector T cells after activation in the periphery. For this reason, antigen-specific suppression of T cells by MDSCs could be

mediated through ligation of CD80 or CD86 to CTLA-4 or through PD-L1 to PD-1 depending on the time and localization.

Regarding MHC-II expression, we found that half of the generated M-MDSCs expressed MHC-II, which was nearly absent in G-MDSCs, and that activation increased its level of expression only in M-MDSCs. In contrast to human MDSCs, which are extensively described as HLA-DR$^{low/-}$ cells (Serafini 2013; Gantt, Gervassi et al. 2014), murine MDSCs were reported to have variable expression of the MHC-II molecule depending on the type of MDSC and the environmental milieu. The importance of MHC-II expression by murine MDSCs was demonstrated by the fact that MDSCs from MHC-II-deficient mice could induce tolerance to CD8$^+$ T cells but not to CD4$^+$ T cells. Moreover, antigen-specific CD4$^+$ T cells, but not CD8$^+$ T cells, could boost the immune suppressive activity of MDSCs through the cross-linking of MHC-II molecules on MDSCs (Nagaraj, Nelson et al. 2012). This highlighted the importance of a crosstalk between these two immune cell populations (Nagaraj, Youn et al. 2013). Furthermore, expression of MHC-II was found to be induced in MDSCs upon activation and that expression of this molecule was required for mediating the generation of Treg cells *in vivo* and for the induction of immune tolerance (Pan, Ma et al. 2010).

As mentioned above, in our previous study we demonstrated that MDSCs generated in BM transduction cultures displayed arginase-1 and iNOS activities (Gomez, Espejo et al. 2014). Therefore, we considered interesting to study other immunosuppressive mechanisms described for these cells, specifically the production of ROS by both subsets of MDSCs. We found that both M- and G-MDSC subsets produced high levels of ROS. In particular, M-MDSCs were the ones which showed a higher ROS production in comparison with G-MDSCs and unfractionated BM cells. ROS production is an important immunosuppressive mechanism extensively described for MDSCs. It causes conformational changes in the TCR resulting in an impaired TCR/MHC-peptide recognition, rendering T cells unresponsive to the antigen (Gabrilovich and Nagaraj 2009; Serafini 2013; Gantt, Gervassi et al. 2014). Published data indicate that ROS production may be essential for the immunosuppressive function of MDSCs. For example, norepinephrine-induced MDSCs inhibited T-cell responses *in vitro* and this inhibition was dependent on ROS production as blocking of ROS activity partly

abrogated the inhibitory effects of MDSCs on T-cell proliferation (Liu, Wei et al. 2015). Moreover, it was suggested that ROS production could be responsible for the antigen-specific MDSC-mediated suppression of T-cell responses. Tumor-derived MDSCs producing high levels of ROS suppressed $CD8^+$ T-cell proliferation in an antigen-specific manner and inhibition of these molecules completely abrogated the suppressive effect of MDSCs on T cells, indicating that ROS production is a crucial mechanism for MDSC-mediated suppression (Kusmartsev, Nefedova et al. 2004). Apart from being a potent suppressive mechanism of MDSCs, high levels of ROS were reported to block the differentiation of MDSCs into mature myeloid cells (Kusmartsev and Gabrilovich 2003; Nefedova, Fishman et al. 2007).

In previous experiments we already demonstrated that transduced BM cells, M- and G-MDSCs were able to suppress T-cell proliferation *in vitro* (Gomez, Espejo et al. 2014). Prior to the *in vivo* experiments, due to the elapsed time and to the changes we made in our BM culture conditions, we decided to verify the ability of the generated MDSCs to suppress T-cell proliferation. As expected, the isolated MDSCs were able to suppress MOG-induced splenocyte proliferation in a dose-dependent manner to a similar extent as unfractionated BM cells, indicating that MDSCs are the major contributors to the suppressive effect.

Taking into account that the generated MDSCs potently suppressed T-cell proliferation and all the immunosuppressive mechanisms that present our MDSCs, as the expression of the inhibitory molecules PD-L1, CD80 and CD86, the production of ROS together with arginase-1 and iNOS activities, make these cells very good candidates to mediate the tolerogenic effect previously observed after the infusion of MOG-expressing BM cells (Eixarch, Espejo et al. 2009). Therefore, to evaluate whether the MDSCs present in the transduced BM cells were the cell type responsible for the beneficial effect observed in the EAE model, we decided to isolate total MDSCs (M- and G-MDSCs together) and infuse them into EAE mice both in a preventive and therapeutic approaches. In the preventive arm, animals were infused with $1x10^6$ unfractionated BM cells or with $0.5x10^6$ total MDSCs, either transduced with the control vector or with the therapeutic one seven days before EAE induction. We decided to use this dose of total MDSCs as we estimated that these cells represented

at least half of the content of unfractionated BM cells. The fact that only the groups treated with MOG-specific BM cells and MDSCs were protected against the disease indicated an antigen-specific effect, which was manifested as a significant reduction in both cumulative and maximum clinical scores compared to controls. A single infusion of MOG-specific MDSCs ameliorated the outcome of the disease to a similar extend as unfractionated MOG-specific BM cells, indicating that MDSCs were the main contributors to the therapeutic effect. Consistently, the reduction in the clinical score was accompanied by a lower weight loss compared to controls. As the results obtained in the preventive approach are of limited relevance in terms of their clinical applicability, we proceeded to apply the same strategy but therapeutically. In other words, the animals received the transduced cells once the majority of them (77.0%) had developed the first clinical signs of the disease. In this case, the challenge was far superior compared to the preventive approach since EAE mice, having been immunized with the self-antigen, already had an established immune response against the MOG_{40-55} peptide. First, we decided to use the same dose of MDSCs as the one used in the preventive approach, however, although this cell dose had worked preventively, we failed to observe a significant therapeutic effect. For this reason we decided to double the dose to $1x10^6$ cells per mouse. This time, infusion of $1x10^6$ IiMOG-MDSCs significantly ameliorated the clinical course of the disease compared to their counterparts and the therapeutic effect resembled that obtained with the infusion of $1x10^6$ IiMOG-BM cells. IiMOG-treated mice presented less cumulative and maximum clinical scores and this improvement was paralleled by a lower weight loss and by a better motor function in comparison with their respective controls. Interestingly, the MDSC dose used in our experiments is considerably lower compared to those used in other studies to treat murine autoimmune diseases. For example, Iannou *et al* transferred $2x10^6$ G-MDSCs at days 4 and 7 p.i. to treat EAE (Ioannou, Alissafi et al. 2011). To protect mice from collagen-induced arthritis (CIA), $2x10^6$ $CD11b^+GR\text{-}1^+$ MDSCs (Fujii, Ashihara et al. 2013) or $1.5x10^6$ M-MDSCs were infused every five days (Crook, Jin et al. 2015). Even higher doses have been administered, $5x10^6$ sorted MDSCs were infused twice to prevent murine T1D (Yin, Ma et al. 2010). What differentiates our strategy from the one used in these studies is the fact the therapeutic effect was observed with a low cell dose and that a relatively small

proportion of our MDSCs is antigen-specific and express the self-peptide by MHC-II molecules. Replacement of the CLIP region of the Ii molecule by the MOG_{40-55} peptide in the vector used promotes the binding of the peptide to the MHC-II groove, making the system efficient and avoiding the need to obtain high levels of expression of the transgene in the target cells, important fact to induce tolerance without the need to infuse large numbers of cells. The use of relatively small cell doses makes the procedure safer and closer to a potential clinical application.

In both preventive and therapeutic approaches, clinical improvement was correlated with a reduced neuropathology. Animals treated with MOG-expressing cells presented significantly less infiltrating T cells, demyelination, microglia activation, reactive astrogliosis and axonal damage in the CNS compared to animals treated with controls cells, which showed huge demyelination areas with moderate to severe inflammatory infiltration in the spinal cord white matter. On the contrary, all mice developed low levels of anti-MOG_{40-55} antibodies and no differences were found between groups. This observation confirms our previous results (Eixarch, Espejo et al. 2009) and is in line with other studies that support the notion of a little role, if any, of these antibodies in the pathogenesis of this EAE model (Marin, Mecha et al. 2014) or at least in the therapeutic mechanisms of antigen-specific MDSCs. One possible explanation for this fact could be that when the T-cell response generated after the immunization is strong enough, like the one triggered by the rat MOG_{35-55} peptide, the anti-MOG antibodies do not appear to be pathogenic, while if the generated T-cell response is milder, like the one triggered by the human peptide, the contribution of B cells and anti-MOG antibodies acquire a crucial role in the development of the disease (Marta, Oliver et al. 2005). The difference between the human and the rat peptides resides only in the change of an amino acid at position 42 (Oliver, Lyon et al. 2003; Marta, Oliver et al. 2005), therefore we can assume that the peptide used in this work (rat MOG_{40-55}) triggers the same pathogenic mechanism that the rat MOG_{35-55}, which is independent of the humoral response.

Considering the significant improvement in the EAE clinical course and CNS pathology observed in animals treated with antigen-specific cells, we decided to study different T- and B-cell populations, relevant to the pathogenesis of EAE and MS, in order to

better characterize the potential mechanisms by which MOG-specific BM cells and MDSCs produce their beneficial effect in EAE, in both the preventive and the therapeutic approaches. We did not find any differences in the proportions of total splenic $CD4^+$ and $CD8^+$ T lymphocytes between animals treated with MOG-specific cells and their respective controls. This result is not surprising as these populations were found to be similar in both EAE resistant and susceptible mice (Marin, Mecha et al. 2014). It seems that the total proportions of $CD4^+$ or $CD8^+$ T cells in the spleen is a poor indicator of what is occurring at the clonal (antigen-specific) level. On the other hand, the rapid recovery of the animals suggests that the mechanism mediating the induction of immunological tolerance is peripheral rather than central, which would require longer periods of time to act. Peripheral tolerance mechanisms include anergy induction, deletion by apoptosis and suppression of self-reactive lymphocytes by Treg cells. We believe that the mechanism constraining the autoimmune response is the induction of anergy and/or apoptosis of autoreactive T cells mediated by the adoptively transferred MDSCs in an antigen-specific manner. This observation is supported by the rapid recovery of the animals and by the reduction in the proportions of activated $CD4^+CD25^+FoxP3^-$ T cells in the spleens of mice treated with the MOG-specific cells in comparison with their controls. There is ample evidence that MDSCs can suppress activated T cells through many mechanisms such as cell surface inhibitory receptors or through the release of short-lived soluble factors such as arginase-1, NO and ROS (Gabrilovich and Nagaraj 2009; Youn and Gabrilovich 2010). It has also been described that MDSCs can inhibit T-cell activation by decreasing the expression of L-selectin (CD62L) on T cells, a molecule necessary for naïve T lymphocytes to migrate to the lymph nodes where they are normally activated by antigens (Hanson, Clements et al. 2009). In addition, MDSCs can block the activation of T cells by depleting their microenvironment of cysteine, an essential amino acid that T cells need for activation (Srivastava, Sinha et al. 2010). Antigen-specific $CD8^+$ T-cell suppression by MDSCs is considered to be fundamentally exerted by G-MDSCs, as they provide cell-to-cell contact and produce high levels of ROS, which can nitrosylate and alter the specificity of TCRs and the functionality of the CD8 molecule in these cells. On the other hand, M-MDSCs can induce both antigen-specific and non-specific suppression, which are mainly mediated by arginase-1 and NO and do not require cell-to-cell contact

(Gabrilovich and Nagaraj 2009). MDSCs can induce antigen-specific tolerance to $CD8^+$ T cells via MHC-I and to $CD4^+$ T cells via MHC-II (Nagaraj, Youn et al. 2013). Gr-1^+ cells from tumor-bearing mice inhibited antigen-specific $CD8^+$ T cells through cell-to-cell contact in a MHC-I dependent manner as inhibition of MHC-I molecules restored lymphocyte proliferation (Gabrilovich, Velders et al. 2001). The same group also demonstrated that only tumor-primed Gr-1^+ cells could block antigen-specific cytotoxic T lymphocyte activity while maintaining the ability to respond to non-specific stimuli, demonstrating that these cells are able to capture soluble proteins and present the antigenic peptides on their surface to induce T-cell anergy in an antigen-specific manner (Kusmartsev, Nagaraj et al. 2005). However, on the $CD4^+$ T cell compartment, antigen-specific suppression by MDSCs is still controversial, although recent studies demonstrate this hypothesis (Solito, Bronte et al. 2011). MDSCs isolated from tumor-bearing mice were able to suppress splenic antigen-specific $CD8^+$ and $CD4^+$ T cells (Chalmin, Ladoire et al. 2010). In murine tumor models MHC-II expression by MDSCs is usually low. However, MDSCs expressing MHC-II were found to suppress antigen-specific $CD4^+$ T cell responses in tumor-bearing mice (Nagaraj, Nelson et al. 2012). In addition, activated antigen-specific $CD4^+$ T cells enhanced MDSCs suppressive activity and also converted them into non-specific suppressor cells. This highlights the importance of a crosstalk between activated lymphocytes and MDSCs (Nagaraj, Youn et al. 2013). The mechanisms by which MDSCs exert antigen-specific or non-specific suppression remain elusive. It has been proposed that in peripheral lymphoid organs MDSCs mediate immune suppression by only antigen-specific mechanisms, while at the tumor site MDSCs became more potent and are able to abrogate T-cell responses in both antigen-specific and non-specific manners (Solito, Bronte et al. 2011). Taking all this into account, it seems that the mechanisms by which MDSCs exert their immunosuppressive function depend on many factors such as the MDSC nature, their localization, the type of the tumor, the inflammatory microenvironment and the level of T-cell activation. It is conceivable that the rapid therapeutic effect observed after the infusion of IiMOG-specific cells is a consequence, at least in part, of the suppression of antigen-specific effector T cell responses through the induction of anergy and/or apoptosis via PD-1/PD-L1 engagement in conjunction with the production of ROS (mechanisms described to be antigen-specific), as reflected by the

reduction of activated $CD4^+CD25^+FoxP3^-$ T cells in the spleens of IiMOG-treated mice. However, we cannot rule out the potential contribution of other regulatory cell types.

On the other hand, we did not find increased proportions of $CD4^+CD25^+FoxP3^+$ Treg cells, which include natural and inducible Treg cells, in the spleens of IiMOG-treated animals. This fact was somehow unexpected as it has been described that MDSCs can induce the generation and expansion of Treg cells in cancer (Huang, Pan et al. 2006; Serafini, Mgebroff et al. 2008), transplantation (Garcia, Ledgerwood et al. 2010; Chou, Hsieh et al. 2012) and autoimmunity (Yin, Ma et al. 2010; Zoso, Mazza et al. 2014). This can be related to the relatively long period elapsed between the cell infusions and the analyses, especially in the preventive experiments. It has been reported that Treg cells can suppress autoimmune responses both during the induction and the effector phases. In the induction phase, naïve Treg cells are recruited in lymphoid tissues (thymus, spleen and lymph nodes) to suppress early immune responses by inhibiting the induction and expansion of primed autoreactive T cells. On the other hand, during the effector phase of an autoimmune response, these cells become activated and express chemokine receptors and adhesion molecules which allow them to migrate from the lymphoid tissues to the site of inflammation, controlling the later stages of the response directly at the inflamed site (Huehn and Hamann 2005; Chow, Banerjee et al. 2015). We performed the studies of T- and B-cell populations at the end of the experiments, which corresponds with the effector phase of the disease. At this point, effector Treg cells could have been recruited into the areas of inflammation in the CNS to locally suppress autoreactive T cells, which would explain why we did not find any differences in splenic Treg cell proportions between the experimental groups. Moreover, it has been described that the generation of Treg cells by MDSCs is CD40 dependent as $CD40^{-/-}$ MDSCs failed to induce Treg cells and immune tolerance *in vivo* while the wild-type ones did (Pan, Ma et al. 2010). In our previous study we found very low levels of this molecule in both M- and G-MDSCs (Gomez, Espejo et al. 2014), fact that could also explain why we did not find increased percentages of these cells. However, we cannot rule out a potential contribution of other types of Treg cells, like Tr-1 or $CD8^+$ T cell populations, in the maintenance of immune tolerance. Tr-1 cells are distinguished from other Treg cells by their unique cytokine expression profile. Upon

activation, Tr-1 cells secrete high levels of IL-10, TGF-β and IL-5, low amounts of IFN-γ and IL-2 and no IL-4 (IL-10^{++}TGF-β^{+}IL-5^{+}IFN-γ^{+}IL-$2^{low/-}$IL-4^{-}). In 2013, Roncarolo *et al.* proposed for the first time a unique panel of cell surface markers to selectively identify human and murine Tr-1 cells ($CD4^{+}CD49b^{+}$LAG-$3^{+}CD226^{+}$) (Gagliani, Magnani et al. 2013). The same group demonstrated that both types of Treg cells synergize in the induction and maintenance of immune tolerance in a murine model of T1D (Battaglia, Stabilini et al. 2006). In this work, the authors elegantly demonstrate that the suppressor activity of Treg and Tr-1 cells does not overlap and that each cell population has a different suppressor function. In this experimental model of diabetes, Tr-1 cells were not found in the pancreatic infiltrates but in the spleen, where the levels of IL-10, TGF-β and IL-5 were significantly higher in tolerized mice. The levels of these cytokines, which are characteristic of Tr-1 cells, were not found increased neither in the lymph nodes nor in the pancreatic infiltrates. On the other hand, the percentages of Treg cells were far superior in the pancreatic infiltrates and not in the lymph nodes or the spleen. Considering these findings, the authors postulated that once the tolerance is established Tr-1 cells would leave the areas of inflammation and they would be recruited into the spleen where their role would be to prevent migration of effector T cells. On the other hand, Treg cells would be recruited to the site of inflammation to locally block the immune response. In our previous study, in which mice were given partial myeloablation before cell infusion, we found increased proportions of IL-10 and IL-5 in the spleens of mice treated with MOG-specific BM cells suggesting a potential role of Tr-1 cells in the maintenance of immune tolerance (Eixarch, Espejo et al. 2009). However, in the present study, splenocytes from both Ii- and IiMOG-treated mice presented an altered proliferative response and an altered cytokine secretion profile compared to NT mice, which could have masked the results and prevented us to elucidate the potential involvement of these cells in the induction or maintenance of the observed immune tolerance. As mentioned before and in line with the results obtained in the murine model of T1D, at the end of the experiments we did not find increased percentages of Treg cells in the spleens of the tolerized mice because these cells could have been recruited to the areas of inflammation in the CNS to locally suppress the activity of the effector autoreactive T cells while Tr-1 cells could have been recruited into the spleen to avoid the migration of effector T cells to the

areas of inflammation in the CNS. Both mechanisms could explain the significant decrease in the proportions of T cells in the inflammatory infiltrates of the CNS of mice treated with MOG-specific cells.

In relation to B cells, we also observed no differences in the percentages of total B cells, B1 and B2 cells between the animals from the different experimental groups. However, we found that the proportions of $B220^{+}CD1d^{high}CD5^{+}$ B cells were increased in the spleens of mice treated with IiMOG-MDSCs in comparison with their controls. This phenotype includes a type of murine Breg cells, also known as B10, which represent 1-3% of B cells in the spleen of normal adult mice. About 15-20% of the $CD1d^{high}CD5^{+}$ B cell subpopulation are B10 cells and up to 50% are B10 plus B10 progenitor cells (Tedder 2015). These regulatory cells are functionally defined by their ability to secrete IL-10, which is the cytokine that mediates virtually all their immunosuppressive effects. This occurs upon T-cell activation by the antigen and the release of IL-21 (Yoshizaki and Tedder 2015). B10 cells can modulate antigen presentation by DCs, inhibit T-cell responses and induce Treg cells in an antigen-specific manner (Tedder 2015). In our experiments, the relative increase of a cell population with this phenotype in the spleen of animals treated with MDSCs expressing the self-antigen does not prove, but strongly suggests that B10 cells could be involved in the tolerogenic effect observed. There is solid evidence that Breg cells play a crucial role in down-regulating the autoimmune response in various mouse models of autoimmune diseases such as CIA, SLE and EAE [reviewed in (Goode, Xu et al. 2013)]. Regarding the EAE model, specific depletion of Breg cells prior to EAE induction worsened EAE pathology and adoptive transfer of antigen-sensitized Breg cells into naïve mice protected them against the disease (Matsushita, Horikawa et al. 2010). Moreover, in accordance with our results, the same group also demonstrated that Breg numbers determined the outcome of the disease. Particularly, mice with increased Breg numbers developed a milder EAE compared to mice with reduced Breg numbers, which showed an exacerbated disease (Matsushita, Horikawa et al. 2010). This notion has recently been supported by the finding that, upon MOG_{35-55} immunization, CD1 resistant mice showed increased percentages of $CD1d^{high}CD5^{+}$ cells while C57BL/6J susceptible mice that developed the disease presented reduced

percentages of these cells, suggesting a pivotal role of Breg cells in EAE resistance (Marin, Mecha et al. 2014). Our results are also in agreement with a previous study in which the infusion of human mesenchymal stem cells into EAE mice led to decreased disease severity and reduced CNS inflammation and demyelination that was paralleled by up-regulation of Breg cells and IL-10 production in the spleens of treated mice (Guo, Chan et al. 2013). Furthermore, it has been suggested that B cells induce the migration of Treg cells into the CNS of EAE mice through the production of IL-10 (Mann, Maresz et al. 2007), fact that could also explain why we did not find increased percentages of Treg cells in the spleens of mice treated with MOG-specific cells. Taken together, these data indicate that the immunomodulatory effects of antigen-specific MDSCs also involve the induction of $CD1d^{high}CD5^{+}$ Breg cells and that these cells could be responsible, at least in part, for the maintenance of long-term immune tolerance. However, as functional Breg cells can only be defined by IL-10 production, further assays assessing the production of this cytokine by $CD1d^{high}CD5^{+}$ cell subpopulation from treated mice are needed to definitively confirm these results.

Even though the infusion of MOG-specific MDSCs has a clear tolerogenic effect in EAE mice, it should be noted that, despite the fact that the infused cells expressing the self-antigen are rejected makes the therapy safer for the recipient, we cannot rule out the possibility that the therapy loses effectiveness at long-term. However, in the previous experiments in which we did a long-term follow-up, the clinical benefit was maintained in the majority of the animals that had clinically improved after BM transplantation (Eixarch, Espejo et al. 2009), suggesting that, at least in this model, the immunological tolerance could be maintained over time. In order to address this issue, it would be interesting to study the susceptibility to EAE in these animals partially or totally tolerized after reimmunization with the encephalitogenic peptide or to induce EAE to naïve mice by passive transfer of T lymphocytes from treated mice in future experiments. On the other hand, the fact that the transferred cells are eventually rejected reduces the risk of malignancies associated with the integration of the retroviral vector due to insertional oncogenesis (Hacein-Bey-Abina, Garrigue et al. 2008; Boztug, Schmidt et al. 2010), since the MOG-expressing cells are literally cleared out from the organism in a relatively short period of time. The low toxicity of the

process and the reduction of risks associated with the treatment make this protocol closer to a possible clinical application for the treatment of human autoimmune diseases. In the case of MS, one of the major drawbacks of this therapeutic strategy lies in the lack of knowledge of the antigen or antigens responsible for triggering the autoimmune response. The treatment of MS patients with this type of therapy should probably be aimed at inducing tolerance to all or several of the candidate antigens (mainly MOG, PLP and MBP), either by transducing these cells with a vector encoding some or all of them or by loading these cells with cocktails of these proteins or peptides. In this way, the induction of immunological tolerance to multiple peptides would overcome this limitation and, moreover, the therapy would also anticipate the so-called phenomenon of epitope spreading. One example of a successful clinical trial using this strategy is the case of the ETIMS trial conducted by Dr. Roland Martin's team, in which nine MS patients received a single injection of autologous lymphocytes chemically coupled to a cocktail of seven myelin peptide antigens (derived from MBP, MOG and PLP proteins) in escalating doses. Results showed that the four patients receiving the highest doses presented a reduction in the proliferation response to some or all of the antigens tested (Lutterotti, Yousef et al. 2013).

In summary, in this work we have demonstrated that tolerogenic MDSCs are generated in BM retroviral transduction cultures and that these cells are the main contributors to the therapeutic effect previously observed in the EAE model. First and foremost, the infusion of these MDSCs induces specific immunological tolerance to the transgene product *in vivo* and is able to prevent and ameliorate established EAE in an antigen-specific manner. Moreover, these cells express inhibitory molecules such as PD-L1 and produce ROS, mechanisms associated with the inhibition of autoreactive T lymphocytes and with the induction of immunological tolerance. In addition, the fact that the antigen-expressing cells are rejected increases the safety of this therapeutic strategy. For these reasons, we believe that *ex vivo* generated MDSCs expressing self-antigens constitute a potential therapy for human autoimmune diseases, especially for those in which the disease-causing antigens are known.

PART 2: GENERATION OF HUMAN MDSCs FROM HEMATOPOIETIC PROGENITOR CELLS

In the previous part of this thesis we have successfully generated murine MDSCs able to suppress antigen-specific T-cell responses *in vivo* in an experimental model of MS. After achieving this, we decided to move one step further and try to generate human MDSCs from hematopoietic progenitor cells for its potential clinical application. It has been reported that MDSCs can have therapeutic effects by suppressing alloimmune and autoimmune responses. While endogenous MDSCs appeared to be pathogenic in some autoimmune diseases, exogenous MDSCs could efficiently inhibit T-cell responses and ameliorate the disease [reviewed in (Cripps and Gorham 2011; Zhang, Fujino et al. 2015)]. Therefore, *in vitro* generation of functionally suppressive MDSCs could be used as a potential cellular therapy to treat human autoimmune diseases and to prevent GVHD and promote graft survival after transplantation.

For the generation of human MDSCs, we have used $CD34^+$ progenitor cells from apheresis products as a source of HSCs. By using this type of cells we found that it is feasible to *in vitro* generate MDSCs for their potential clinical application. We cultured the $CD34^+$ cells for 9, 14 and 20 days in the presence of different combinations of cytokines (SCF, TPO, FLT3-L, IL-3, GM-CSF and IL-6) and at the end of the culture the generated cells were phenotypically characterized. In contrast to murine MDSCs, which can be clearly defined by their phenotype, being M-MDSCs $CD11b^+Gr\text{-}1^{low}$ and G-MDSCs $CD11b^+Gr\text{-}1^{high}$, the phenotype to clearly identify human MDSCs still remains elusive. Phenotypically identifying human MDSCs is complicated due to the lack of a human homologue of the Gr-1 molecule and to the absence of specific markers that define them. Although many different phenotypes have been proposed for human MDSCs, in general they are defined as $CD33^+CD11b^+HLA\text{-}DR^{low/-}$, with M-MDSCs being $CD14^+CD15^{low/-}$ and G-MDSCs being $CD14^-CD15^+CD66b^+$ (Serafini 2013; Gantt, Gervassi et al. 2014). However, this phenotype seems to vary depending on the disease, the type of cancer, the anatomic site, the environmental milieu and, to a further extent, on the stage of differentiation in which the MDSCs are and on the function they perform (Solito, Marigo et al. 2014; Dai, El Gazzar et al. 2015). Taking this into account, we

decided to phenotypically define the generated MDSCs as $CD33^{+}HLA\text{-}DR^{low/-}$, with M-MDSCs being $CD14^{+}CD15^{-}$ and G-MDSCs being $CD14^{-}CD15^{+}$.

Several groups have already demonstrated the *ex vivo* generation of different subsets of MDSCs, although all of them use different cell sources and different combinations of cytokines and growth factors. It has been reported that murine MDSCs can be generated *ex vivo* from BM cells using GM-CSF and G-CSF in combination with IL-13 (Highfill, Rodriguez et al. 2010) or using GM-CSF with IL-6 or G-CSF (Marigo, Bosio et al. 2010). These cells inhibited GVHD and allowed long-term acceptance of pancreatic islet allografts, respectively. More recently, BM-derived murine MDSCs were differentiated *in vitro* by culturing them with conditioned medium of GM-CSF-secreting tumor cells. Such cells strongly suppressed T-cell proliferation in an arginase-1 and iNOS dependent manner (Dufait, Schwarze et al. 2015). Murine MDSCs have also been generated from embryonic stem cells using a cocktail of various cytokines including SCF, IL-6, IL-3, TPO, VEGF, FLT3-L and M-CSF for ten days (Zhou, French et al. 2010). In line with these results, human MDSCs were generated from BM cells using GM-CSF with IL-6 or G-CSF, being the first combination of cytokines the most efficient in inducing MDSCs with the strongest tolerogenic activity (Marigo, Bosio et al. 2010). Human MDSCs were also generated from PBMCs cultured with GM-CSF in combination with IL-1β, IL-6, VEGF, TGF-β, TNF-α, or PGE2, although the MDSCs which showed the higher ability to suppress T-cell proliferation where those generated with GM-CSF + IL-6 and GM-CSF + IL-6 + VEGF (Lechner, Liebertz et al. 2010). The same group also reported the generation of functional MDSCs from PBMCs by co-culturing them with tumor cell lines secreting IL-1β, IL-6, TNF-α, VEGF, GM-CSF, FLT3-L and TGF-β (Lechner, Megiel et al. 2011). MDSCs were also produced from PB monocytes cultured with PGE2, factor that induced the production of indoleamine 2,3-dioxygenase (IDO), IL-10 and iNOS by these cells (Obermajer and Kalinski 2012). More recently, a population of fibrocytic MDSCs generated from umbilical CB progenitors cultured with GM-CSF and G-CSF was reported. This type of MDSCs was defined by the expression of MDSC-, DC-, and fibrocyte-associated markers, and by their ability to induce Treg cell expansion and normoglycemia in a xenogeneic murine model of T1D (Zoso, Mazza et al. 2014).

Taking all these data into account, we decided to test four different combinations of cytokines, which included some that are necessary for the survival and proliferation of immature progenitors (SCF, TPO and FLT3-L) (Ando, Yahata et al. 2006; Du, Jin et al. 2015) in combination with cytokines extensively reported to induce the generation and expansion of MDSCs (GM-CSF, IL-3 and IL-6). As GM-CSF seemed necessary but not sufficient to promote the generation and expansion of MDSCs, we decided to use this cytokine in combination with IL-6, another cytokine reported to play a crucial role in the induction of MDSCs. After nine days of culture, about 20% of the cells presented a MDSC phenotype ($CD33^{+}HLA\text{-}DR^{low/-}$) with all the combinations of cytokines. However, a reduction in the proportion of M-MDSCs was observed when cells were cultured without IL-3 and when GM-CSF and IL-6 were added at day 4 of culture. However, it was with this combination of cytokines where the highest percentage of G-MDSCs was observed, probably because this combination of cytokines skewed the differentiation of immature progenitors towards G-MDSCs. This could be due to the absence of IL-3 in the culture, as it was reported that this cytokine induces myeloid differentiation favoring monocytic instead of granulocytic differentiation, especially when used in combination with FLT3-L (Jacobsen, Okkenhaug et al. 1995). As the majority of cells still expressed the CD34 progenitor cell marker, indicating that they still had potential to further differentiate, we decided to extend the cultures to 14 and 20 days. Results showed that total percentages of MDSCs increased along with the days of culture, while the proportions of M-MDSCs remained constant those of G-MDSCs increased over time. However, a reduction in the proportion of G-MDSCs was observed when cells were cultivated in the presence of IL-3. As mentioned above, this result could be due to the fact that IL-3 may skew progenitor cell differentiation towards M-MDSCs (Jacobsen, Okkenhaug et al. 1995). For this reason we established that the best combination of cytokines to efficiently generate high numbers of MDSCs, including M- and G-MDSCs, was SCF + TPO + FLT3-L + GM-CSF + IL-6.

As we did with the murine MDSCs, we decided to further characterize the phenotype of these cells and study the expression of the inhibitory molecules PD-L1, CD80 and CD86 both at baseline and in an activated state after exposing them to inflammatory stimuli. At baseline, low levels of PD-L1 were observed on total MDSCs and the

expression of this molecule was significantly increased upon IFN-γ plus LPS stimulation. Although M-MDSCs showed a high expression of PD-L1 at baseline, and though its expression was further increased upon stimulation, were the G-MDSCs that presented the highest expression of PD-L1 after stimulation. Regarding to CD80 and CD86, only M-MDSCs showed some percentage of expression of these molecules, which did not increase upon stimulation. In contrast to murine cells, very little is known about the expression of these inhibitory molecules on human MDSCs and their potential relevance on MDSC-mediated suppression. Probably this is due to the fact that working with human MDSCs is considerably more difficult because, apart from the major discrepancies in the definition of their phenotype, obtaining enough tumor-infiltrating MDSCs for research is technically challenging, which explains why almost all the studies conducted to date with human MDSCs are performed with PB MDSCs. Whether the information obtained from these cells is also valid for the MDSCs acting within the tumor microenvironment remains to be determined (Maenhout, Thielemans et al. 2014). However, recent studies have reported an increased expression of PD-L1 on PB M-MDSCs from cancer patients compared to that of healthy controls (Huang, Zhang et al. 2015) or to that of patients who responded to immunotherapy (Gebhardt, Sevko et al. 2015), indicating a potential role for PD-L1 in human MDSC-mediated suppression.

In order to determine if our generated cells with a MDSC phenotype were truly MDSCs with suppressive activity, we studied the capacity of these cells to inhibit T-cell proliferation. Due to the inability to use MDSCs and PBMCs from the same donor, we co-cultured our MDSCs with allogeneic PBMCs in the presence or absence of a mitogenic stimulus (PHA-L). These experiments allowed us to study the ability of these cells to inhibit the proliferation of allogeneic T cells. In the absence of the mitogenic stimulus we studied if the generated MDSCs could suppress allogeneic-induced T-cell proliferation. Results showed that both 14- and 20-days generated MDSCs had a weaker alloreactivity compared to their $CD33^{+}HLA\text{-}DR^{+}$ counterparts, which triggered a strong allogeneic proliferative response. However, it should be noted that by using this experimental setting it is not possible to distinguish whether the low proliferative response of T cells in contact with these cells is due to the fact that MDSCs are not

immunogenic or that MDSCs are actually inhibiting T-cell allogeneic-induced proliferation. The fact that MDSCs can inhibit allogeneic T-cell proliferation is very promising, as these cells could be used to prevent GVHD after transplantation. In murine BM allogeneic transplantation models it has already been reported that MDSCs can suppress allogeneic T cells while preserving the GVT effect. This suppressive effect of MDSCs was associated with a decreased donor T-cell proliferation and production of proinflammatory cytokines (Highfill, Rodriguez et al. 2010) and with the induction of Th2 allogeneic T cells and the up-regulation of Th2-specific cytokine production (Messmann, Reisser et al. 2015).

Moreover, we also studied the capacity of the *in vitro* generated MDSCs to inhibit PHA-L-induced allogeneic T-cell proliferation. The results showed that both 14- and 20-day MDSCs were able to potently suppress PBMC proliferation in a dose-dependent manner, indicating that they do not lose their functionality over this period of time. However, although both types of MDSCs displayed a similar suppression pattern, the profile of the secreted cytokines in the culture supernatants was slightly different. In general, we observed a decrease in the proinflammatory cytokines IFN-γ, TNF-α, GM-CSF and IL-2 and an increase of the immunosuppressive cytokine IL-10, although the differences were more significant in the case of the MDSCs generated in 14 days. In addition, in the cultures with 14-day MDSCs the levels of IL-4 and IL-6 remained constant. It has extensively been reported that MDSCs suppress effector T cells and consequently inhibit the production of proinflammatory cytokines by these cells. Moreover, production of IL-10 by MDSCs is also another well reported suppressive mechanism of these cells (Gabrilovich, Ostrand-Rosenberg et al. 2012; Manjili, Wang et al. 2014). However, it should be noted that it is not possible to distinguish if the increase in IL-10 levels is due to the production of this cytokine by MDSCs, to Treg induction or to Th2 polarization. $CD4^+$ T cells are mainly classified into Th1, Th2 and Th17 types depending on the cytokines they produce. Th1 cells produce high levels of IFN-γ, TNF-α and IL-2, which activate macrophages and promote the proliferation of Th1 cells and cell-mediated immune responses. On the other hand, Th2 cells produce anti-inflammatory cytokines including IL-4, IL-5, IL-6, IL-10 and IL-13 which promote humoral responses and inhibit the differentiation of Th1 cells and the release of

proinflammatory cytokines (Opal and DePalo 2000). Our findings are in line with the idea that MDSCs, in addition to inhibit proliferation and cytokine production by Th1 cells, may skew the differentiation of T cells towards Th2 cells. The production of IL-10 by MDSCs resulted in an impaired production of IL-12 by macrophages which, in turn, decreased the levels of proinflammatory cytokines and predisposed to Th2-type immune responses (Sinha, Clements et al. 2007). In a murine model of influenza A virus infection, the accumulation of MDSCs correlated with an increased production of Th2 cytokines (Jeisy-Scott, Davis et al. 2011) and in a murine model of sepsis MDSCs inhibited T-cell proliferation and IFN-γ production while induced Th2 polarization (Delano, Scumpia et al. 2007). As mentioned above, adoptive transfer of MDSCs prevented GVHD by suppressing T-cell proliferation and inducing the differentiation of Th2 cells and Th2 cytokine production (Messmann, Reisser et al. 2015). The increase in the IL-10 levels could also be due to the induction and expansion of Treg cells by MDSCs. The interaction between MDSCs and Treg cells is well documented. Murine MDSCs induced the generation of Treg cells both *in vitro* and *in vivo* in an IL-10 dependent manner in a mouse model of colon carcinoma (Huang, Pan et al. 2006). Another study showed that *in vivo* depletion of MDSCs correlated with decreased numbers of tumor-infiltrating Treg cells (Zhang, Liu et al. 2009). Adoptive transfer of *ex vivo* generated MDSCs protected islet allografts from rejection through the induction of Treg cells via PD-L1 interactions (Chou, Hsieh et al. 2012). Studies with human MDSCs have also found induction of Treg cells by MDSCs. Human $CD33^{+}CD11b^{+}CD14^{-}$ cells inhibited allogeneic lymphocyte proliferation and induced the expansion of Treg cells (Yen, Yen et al. 2013) and fibrocytic MDSCs induced Treg cell expansion in a xenogeneic mouse model of T1D (Zoso, Mazza et al. 2014). Furthermore, M-MDSCs from renal transplant patients suppressed $CD4^{+}$ T-cell proliferation and expanded Treg cells *in vitro* and accumulation of these cells after transplantation correlated with increased numbers or Treg cells *in vivo* (Luan, Mosheir et al. 2013). Despite the fact that the MDSCs shifted the proinflammatory microenvironment to a more anti-inflammatory one, we found increased levels of IL-17A in the supernatants of the suppression assays with the 20-days MDSCs. Although the induction of Th17 cells by MDSCs is still controversial, some studies have demonstrated the relationship between these two cell populations. The Th17 polarization of naïve T cells by MDSCs seems to

depend on the cytokines released by the MDSCs (Chatterjee, Das et al. 2013). In the EAE model, Th17 cell differentiation was driven by MDSCs and *in vivo* depletion of these cells reduced the numbers of pathologic Th17 cells and ameliorated the disease (Yi, Guo et al. 2012). Similar results were observed in tumor models, where tumor-infiltrating Th17 cells were differentiated from $CD4^+$ T cells by MDSCs releasing IL-1β, IL-6, IL-23 and NO (Obermajer, Wong et al. 2013). The fact that the increase in IL-17A was found in the supernatants of the 20-days MDSCs and not in those from 14-days MDSCs suggests that the effect of the MDSCs on T-cell polarization may depend on the stage of maturation of MDSCs, the more mature MDSCs could drive Th17 polarization while the more immature ones could induce the differentiation of Th2 cells and the generation of Treg cells. This observation supports the idea of the multifaceted nature of MDSCs and the different roles they play in many different situations, as depending on their stage of maturation and level of activation, their localization, environmental milieu and pathological context the MDSCs can exacerbate or ameliorate different diseases.

In summary, in this work we have demonstrated that functional human MDSCs can be efficiently generated from $CD34^+$ hematopoietic progenitor cells under conditions suitable for their potential clinical application. The generated MDSCs contained both subsets of MDSCs, M- and G-MDSCs, and expressed the immunosuppressive molecule PD-L1. In addition, these cells had very little alloreactivity, suppressed PHA-L-induced T-cell proliferation in a dose-dependent manner and decreased the levels of proinflammatory cytokines while increasing the levels of the immunosuppressive cytokine IL-10. For these reasons, we believe that *in vitro* generated human MDSCs constitute a potential tool for the treatment of many immune disorders.

There’s an end to every storm. Once all the trees have been uprooted. Once all the houses have been ripped apart. The wind will hush. The clouds will part. The rain will stop. The sky will clear in an instant and only then, in those quiet moments after the storm, do we learn who was strong enough to survive it.

Grey's Anatomy

CONCLUSIONS

PART 1: ANTIGEN-SPECIFIC MDSCs INDUCE IMMUNOLOGICAL TOLERANCE IN THE EAE IN BOTH PREVENTIVE AND THERAPEUTIC APPROACHES

1. The vast majority of cells that are generated in BM retroviral transduction cultures consist of two myeloid cell populations, $CD11b^{+}Gr\text{-}1^{low}$ and $CD11b^{+}Gr\text{-}1^{high}$, which correspond, respectively, to M- and G-MDSCs. In turn, these populations are the ones which show greater transduction efficiency.

2. Both *ex vivo* generated M- and G-MDSCs express PD-L1 at baseline and its expression is further increased upon inflammatory stimulation. CD80 and CD86 are expressed in the majority of M-MDSCs but only in a reduced proportion of G-MSDCs. M-MDSCs also express MHC-II molecules and its expression also increases upon inflammatory stimulation.

3. Unfractionated BM cells and the two types of MDSCs produce high levels of ROS, being the M-MDSCs the major contributors of ROS production in unfractionated BM cells.

4. Total MDSCs potently suppress *in vitro* MOG-induced splenocyte proliferation in a dose-dependent manner to a similar extent as unfractionated BM cells.

5. A single infusion of MOG-specific MDSCs protects the animals against EAE (preventive approach) and ameliorates the ongoing disease (therapeutic approach) to a similar extent as BM cells, demonstrating that the MDSCs present in unfractionated BM cells are the main contributors to the therapeutic effect. The immunological tolerance is induced in an antigen-specific manner, since there is no clinical improvement in the control groups.

6. MOG-specific MDSCs are able to constrain activated T cells and induce the expansion of a population of B cells with a regulatory phenotype *in vivo*.

PART 2: GENERATION OF HUMAN MDSCs FROM HEMATOPOIETIC PROGENITOR CELLS

1. The culture of $CD34^+$ hematopoietic progenitor cells with SCF, TPO, FLT3-L, GM-CSF and IL-6 generates human $CD33^+HLA\text{-}DR^{low/-}$ MDSCs. The generated MDSCs contain both subsets of MDSCs, M- and G-MDSCs, and express the immunosuppressive molecule PD-L1, which is further increased upon inflammatory stimulation.

2. The *in vitro* generated MDSCs strongly suppress allogeneic- and polyclonal-induced T-cell proliferation in a dose-dependent manner. In addition, MDSCs decrease the levels of the proinflammatory cytokines IFN-γ, TNF-α, GM-CSF and IL-2 while increase the levels of the immunomodulatory cytokine IL-10.

It's time to say goodbye, but I think goodbyes are sad and I'd much rather say hello.

Hello to a new adventure.

Ernie Harwell

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