Ph.D Program
Translational and Molecular Medicine

From understanding the molecular basis of Graft-*versus*-Host Disease (GvHD), to new diagnostic tools and innovative treatments for improving the management of patients undergoing allogeneic Hematopoietic Stem Cell Transplantation (HSCT)

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Chapter 1

INTRODUCTION

HEMATOPOIETIC STEM CELL TRANSPLANTATION

Hematopoietic Stem Cell Transplantation (HSCT) is the treatment of choice for many malignant and non-malignant disorders. The principal aim of an effective protocol is to eliminate or suppress host immunity, through the administration of a conditioning regimen including cytotoxic drugs and whole body irradiation, and to replace the hematopoietic system of the patient with one of a healthy individual[1].

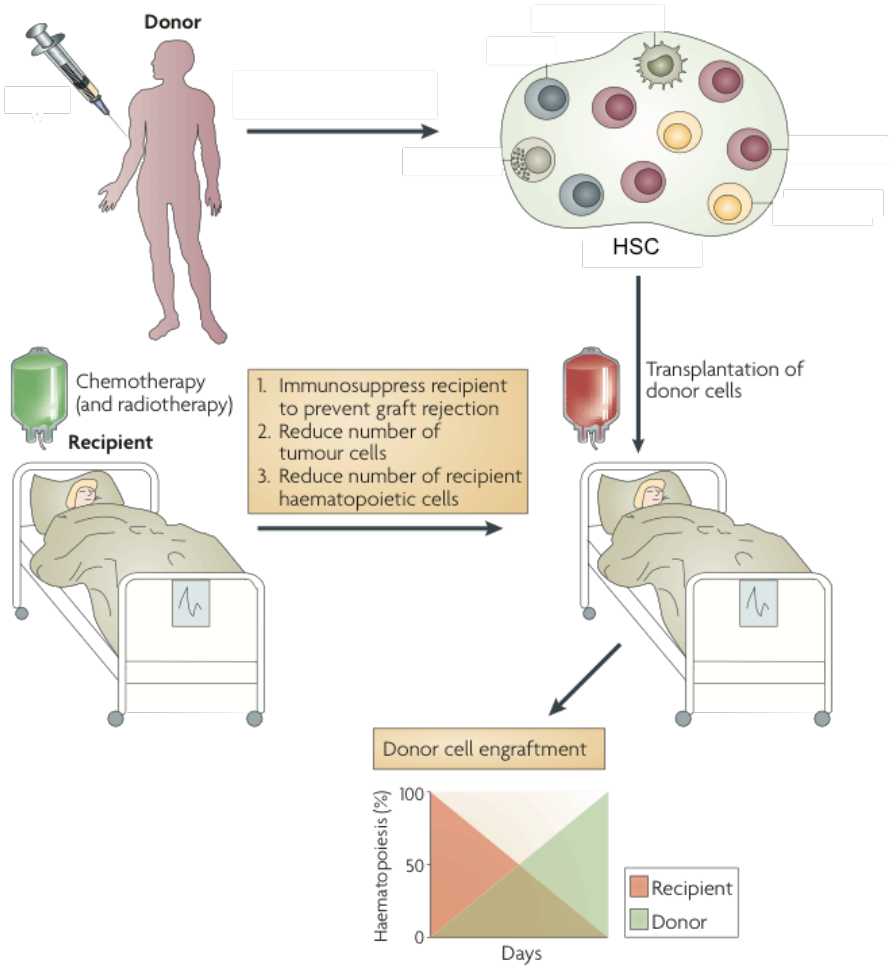

Fig.1. Allogeneic Hematopoietic Stem Cell Transplantation (HSCT)[2]
Hematopoietic stem cells are collected from donors from different source, such as bone marrow, peripheral blood and cord blood. Patients (recipients) receive chemotherapy (and radiotherapy), which is designed to prevent immunological graft rejection, reduce the number of tumour cells (when allogeneic HSCT is used to treat cancer) and to create niches for HSC engraftment. Donor HSC is then infused intravenously into the recipient. Engraftment of donor neutrophils and platelets typically occurs in the first month post-transplantation, but engraftment of other cell lineages, such as T cells, B cells, macrophages and dendritic cells may take longer.

Different types of HSCT can be classified based on the source of stem cells, the choice of the donor and the conditioning regimen used to prepare the recipient.

1.1 HSCT DONOR

The selection of the type of transplantation, autologous (from the patient itself), allogeneic (from an HLA matched unrelated donor), or haploidentical (from a parent of the patient with only one identical HLA haplotype) depends on the type of malignancy, age of the recipient, availability of a suitable donor, the ability to collect a tumour-free graft, the stage and status of disease.

Autologous transplantation is readily available, and there is no need to identify an HLA-matched donor. Autologous transplants have a lower risk of life-threatening complications, because there is no risk of GvHD and no need for immunosuppressive therapy to prevent GvHD and graft rejection. Immune reconstitution is more rapid than an allogeneic transplant, the risk of opportunistic infections's lower and graft failure occurs rarely. Treatment-related mortality is lower than 5% in most studies, and elderly patients can tolerate treatment relatively well[3, 4].

The donor of an allogeneic HSCT can be a sibling, a family member or a non-family member. Autologous hematopoietic stem cell transplantation is based upon the reinfusion of the patient own hematopoietic stem cells following chemotherapy or radiation therapy. For this reason the risk for disease recurrence is lower after allogeneic compared to autologous transplantation. However, allogeneic transplants may be associated with several complications such as regimen-related organ toxicity, graft failure, and Graft-*versus*-Host Disease (GvHD). Immune reconstitution is slower and opportunistic infections are more frequent. Treatment-related mortality is significantly higher compared to autologous transplantation and it increases in case of mismatched or unrelated donors compared to an HLA-identical sibling donor[5].

The transplantation of stem cells from a parent, sibling or child of a patient with only one identical HLA haplotype (haploidentical) was initially associated with high rate of engraftment failure and GvHD. In the past decade, technical advances have improved the outcome of this approach[6]. The success of this type of transplantation depends on the activity of alloreactive natural killer cells (NK), which express combination of activating and inhibitory killer-cell immunoglobulin-like receptors that interact with class I HLA epitopes. The balance of signals determines the cytolitic activity of the natural killer cells. Alloreactivity improves the chances of engraftment and reduces the risk of GvHD[7].

1.2 CONDITIONING REGIMEN

The conditioning treatment of the recipient is essential for the success of the therapy. The aim of the myeloablative conditioning regimen (MAC) before transplantation is to eliminate malignant cells, to prevent graft rejection and to create niche to permit hematopoietic stem cell engraftment. The preparative regimen can also induce an immune response against tumours by causing the death of tumour cells, which results in a flood of tumour antigens into antigen-presenting cells. This process can lead to the proliferation of T cells, which develop a response against malignant cells[8]. Total body irradiation (TBI) is myeloablative and immunosuppressive, is not associated with cross-resistance to chemotherapy, and reaches sites that are not affected by chemotherapy. The toxicity of TBI and the scarcity of facilities for the procedure have resulted in the development of radiation-free regimens.

The reduced-toxicity regimen (RTC) consists in the administration of busulfan combined with high doses of cyclophosphamide[9]. With this regimen, adverse effects are associated with high plasma levels of busulfan[10] and of metabolites of cyclophosphamide[11]. Toxicity can be reduced by adjusting the busulfan dose according to the drug plasma levels[12] or by using intravenous, instead of oral, busulfan[13].

In the late 1990s, a better understanding of the graft-versus tumour biology, led to the development of reduced-intensity preparative regimen (RIC). Unlike MAC, this regimen is primarily immunosuppressive and depends on the graft to eradicate cancer. This preparative regimen consists in the administration of low-dose TBI, with the addition of immunosuppressive drugs after transplantation to permit engraftment and to prevent GvHD[14]. This regimen is characterised by mild neutropenia and thrombocytopenia and minimal toxic effects. HSCT after the receipt of RIC is most effective in treating slow-growing cancers, but, for patients with hematologic cancer, the low mortality rate associated with reduced-intensity preparative regimens may be affected by high relapse rate[15].

In any case, after allogeneic transplantation, patients need to be treated with an immunosuppressive therapy in order to prevent graft rejection and Graft-*versus*-Host Disease GvHD.

1.3 SOURCE OF HSCT

The source of hematopoietic stem cells for transplant can be: bone marrow, peripheral blood and cord blood.

Bone marrow was the first source of hematopoietic stem cells. It can be obtained from the puncture of the posterior iliac crest while the donor is under a local anesthesia[1].

Since hematopoietic stem cells are able to migrate from the bone marrow to the periphery, they can also be obtained also from peripheral blood. When using peripheral blood as the source of transplant, the reconstitution is more rapid compared to bone marrow. On the other side, the use of peripheral blood for transplantation increases the incidence of GvHD[16]. The number of hematopoietic stem cells ($CD34^+$) can be increased in the peripheral blood by mobilizing them from the bone marrow with the administration of granulocyte colony-stimulating factors (G-CSF) in combination with AMD3100, a small molecule, which is a reversible inhibitor of the CXC chemokine receptor 4 (CXCR4)[17].

Recently, cord blood has been identified as a good source of hematopoietic stem cells because umbilical cord and placenta are rich of hematopoietic progenitors. They can be easily and safely collected but they are limited in volume. Because hematologic and immunologic reconstitution is slow, patients transplanted with cord blood are more susceptible to infections. Cord blood transplantation require less stringent HLA matching than does the transplantation of adult peripheral blood or marrow, because mismatched cord blood cells are less likely to cause GvHD, without loosing the graft versus leukemia effect[18].

In the last years the HSCT protocols have been optimised in order to improve the efficacy of this therapy. The procedure developed to obtained these goals is characterized by three phases:

1. Preparative (conditioning) treatment of the recipient before the infusion of the graft;
2. Manipulation of the graft to minimize side effects;
3. Post-transplant immunosuppressive treatment to prevent graft rejection and GvHD.

Despite the improvement in HLA matching technique, about 50% of HSCT recipients experience acute GvHD, which represent the major cause of mortality and morbidity after allogeneic HSCT[5].

GRAFT-*versus*-HOST DISEASE

Graft-*versus*-Host Disease represents a major cause of mortality and morbidity after allogeneic HSCT. Despite the improvement in HLA matching technique, about 50% of HSCT recipients experience acute GvHD. GvHD is a systemic pathology, which involves different organs such as skin, liver, lung, mucosae and gut. The development and severity of GvHD in transplant recipients depend on different factors such as recipient age, toxicity of the preparative regimen, hematopoietic graft source and GvHD prophylaxis schedule[19]. There are two different types of GvHD: ACUTE or CHRONIC. Epidemiological studies, suggested that acute GvHD develops before day +100 post-transplant, whereas chronic GvHD develop after day +100 post transplant. However, investigators currently believe that a pathological classification is more useful, because histological analysis demonstrated that acute GvHD can occur after day +100. Indeed, the NIH Consensus Conference recently proposed a new classification for acute and chronic GvHD (Tab.1)[20].

Category	Time of Symptoms after HCT or DLI	Presence of Acute GVHD Features*	Presence of Chronic GVHD Features*
Acute GVHD			
Classic acute GVHD	**≤100 d**	**Yes**	**No**
Persistent, recurrent, or late-onset acute GVHD	**>100 d**	**Yes**	**No**
Chronic GVHD			
Classic chronic GVHD	**No time limit**	**No**	**Yes**
Overlap syndrome	**No time limit**	**Yes**	**Yes**

GvHD indicates Graft-*versus*-Host Disease; HCT, haematopoietic cell transplantation; DLI, donor lymphocytes infusion

Tab.1 Categories of acute and chronic GvHD[20]

Acute GvHD targets skin, liver, intestine, lung, thymus and secondary lymphoid organs and is driven by T helper 1 (T_H-1) and T_H-17-type immune response and associated B cell lymphopenia. On the contrary, chronic GvHD can target skin and mucosa, but it also seriously involves membranes and exocrine glands. Chronic GvHD develops as an autoimmune disease. Experimental models showed that a Th2-type response is mainly involved, leading to autoantibody formation. The pathophysiology of chronic GvHD is less understood than acute GvHD, in part due to a lack of good animal models, able to represent that represent the full pathological spectrum for this disease[19].

Acute GvHD is characterized by inflammatory events induced by cytokines storms, which firstly activate donor T lymphocytes reactive against the recipient tissues. Pro-inflammatory cytokines produced by activated cells (both innate and adaptive immune cells) enhance the inflammatory reaction and increase the GvHD response.

The three fundamental features to develop a GvHD reaction were described by Prof. Billingham more than thirty years ago[21]:

1. the graft must contain immunologically competent cells;
2. the recipient must be incapable of mounting an effective response to eliminate the transplanted cells;
3. the recipient must express tissue antigens that are not present in the transplant donor.

2.1 GENETICS OF GvHD

Genetic variation across the human genome, can impact HSCT outcome by causing genetic disparity between patient and donor, and modifying gene function. Single nucleotide polymorphisms (SNP) and functional variation can results in mismatching for cellular peptides known as histocompatibility antigens (HA). About 25-30 polymorphic genes are known to encode functional HA in mismatched individuals, but their individual contribution to clinical GvHD is unclear. HSCT outcome can also be related to polymorphisms in donor or recipient. Association studies have implicated several genes with GvHD severity and mortality[22, 23, 24].

Genetic analysis has been an essential feature of HSCT for more than 40 years. The understanding of the genetics of the human major histocompatibility (MHC) permits the success of HSCT and refinement in donor selection. The MHC is a 7 megabase gene-rich region on chromosome 6p21. The MHC is encoded by class I and class II HLA genes. HLA mismatching has an adverse effect on HSCT outcome and increases the risk of developing severe GvHD, graft rejection and mortality. The optimization of high resolution typing and donor-recipient HLA matching at allele-level has improved HSCT outcome[25].

Gene polimorphisms of the HLA locus can be associated with survival after HSCT. Every HLA locus has been associated with different outcome of unrelated donor HSCT. Single mismatch of HLA-A, -B, -C, DRB1 or DQB1 was associated with significant decrement in survival, although did not increase the risk of acute GvHD. The presence of multiple mismatches was worse for survival and severe acute GvHD (grade III-IV)[26]. Since HLA class I molecules are crucial in both T cell and NK-mediated immune responses, there is a great interest in understanding the molecular mechanisms of GvHD. Different data about the role of KIR mismatching and missing ligands on transplant outcome has been recently produced[6, 7, 27]. KIR receptors can have inhibitory or activated potential. HLA-C serves as ligand for both

inhibitory KIR receptors (KIR2DL1, KIR2DL2, KIR2DL3), as well as selected activating receptors (KIR2DS1, KIR2DS4). The HLA-Bw4 motif is encoded by select HLA-A and HLA-B molecules, and is a ligand for inhibitory KIR (KIR3DL1). Therefore, when HLA-A, B and C polimorphisms are evaluated together, is it possible to have a number of information for evaluating the clinical importance of ligand mismatching and missing ligands on transplant outcome.

Although the risk of GvHD occurrence is higher in case of HLA mismatching, clinically significant GvHD can arise also in the case of HLA identical transplants, suggesting that other genes should be involved in the process of graft compatibility[28] (Tab 2). The response against these non-HLA or minor HA-Ag may be weak characterised by a few T cell activation, but, if several mismatched is present in minor HA, that are encoded through the genome, a polyclonal T cell response can be induced causing a severe and life-threatening GvHD[29].

Gene	*Polymorphism*	*Recipient/donor*	*Donor type*	*aGvHD outcome*
TNFα	d3/d3	Recipient	Identical sibling	↑ grade II–IV, GvHD, increased mortality
	TNF-863, TNF-857	Donor and/or recipient	MUD	Increased
	TNFα-238, TNFβ-252	Donor and/or recipient	Unrelated donor	Increased grade II–IV, increased mortality
	TNFd4, TNFα-1031C and TNFa5	Donor and/or recipient	Unrelated donor	Increased mortality
	TNFd4	Recipient	Identical sibling	↑ moderate aGvHD
	TNFRII-196R	Donor	MUD	↑ grade severe aGvHD
	TNFRII-196M	Homozygous donor	MUD	Reduced risk aGvHD
	TNFRII-196R	Recipient	Identical sibling	↑ grade severe aGvHD
IL-10	Low ACC producer	Recipient	Identical sibling	↑ grade severe aGvHD
	Intermediate ATA	Recipient	Identical sibling	↑ grade severe aGvHD, increased mortality
	R3-GCC	Recipient	MUD	Reduced aGvHD and mortality
IL-6	Il-6-174	Recipient	MUD	↑ grade severe aGvHD
INFγ	INFγ 2/2	Recipient	Identical sibling	Reduced aGvHD
	INFγ 3/3	Recipient	Identical sibling	↑ aGvHD
IL-1 family	IL-Ra	Donor	Identical sibling	Reduced aGvHD
		Recipient	Identical sibling	↑ Chronic GvHD
	IL-1α 889 (pediatrics)	Donor and recipient	MUD	Improved survival, less TRM
TGFβ	TGFβ-509	Donor and recipient	Identical sibling	No effects
	TGFβ codon 10 (pediatric)	Donor	Identical sibling	↑ aGvHD
	TGFβ codon receptor II (pediatric)	Recipient	Identical sibling	↑ aGvHD

Abbreviations: MUD = matched unrelated donor; TGF = transforming growth factor.

Tab. 2 GvHD risck correlating with cytokine genes donor/recipient polimorphisms[29].

2.2 PATHOPHYSIOLOGY OF ACUTE GvHD

The development and severity of GvHD in transplanted recipients depends on different factors, such as recipient age, source of stem cells, toxicity of the conditioning regimen and GvHD prophylaxis approach[19].

GvHD consist in an exaggerated but conventional inflammatory response of donor lymphocytes that target/destroy the recipient tissues recognised as non-self.

The pathophysiology of GvHD can be described in three different phases (Fig. 2)[30]:

Phase 1: Activation of Antigen Presenting Cells (APCs)

Phase 2: Activation of donor T cells

Phase 3: Effector phase

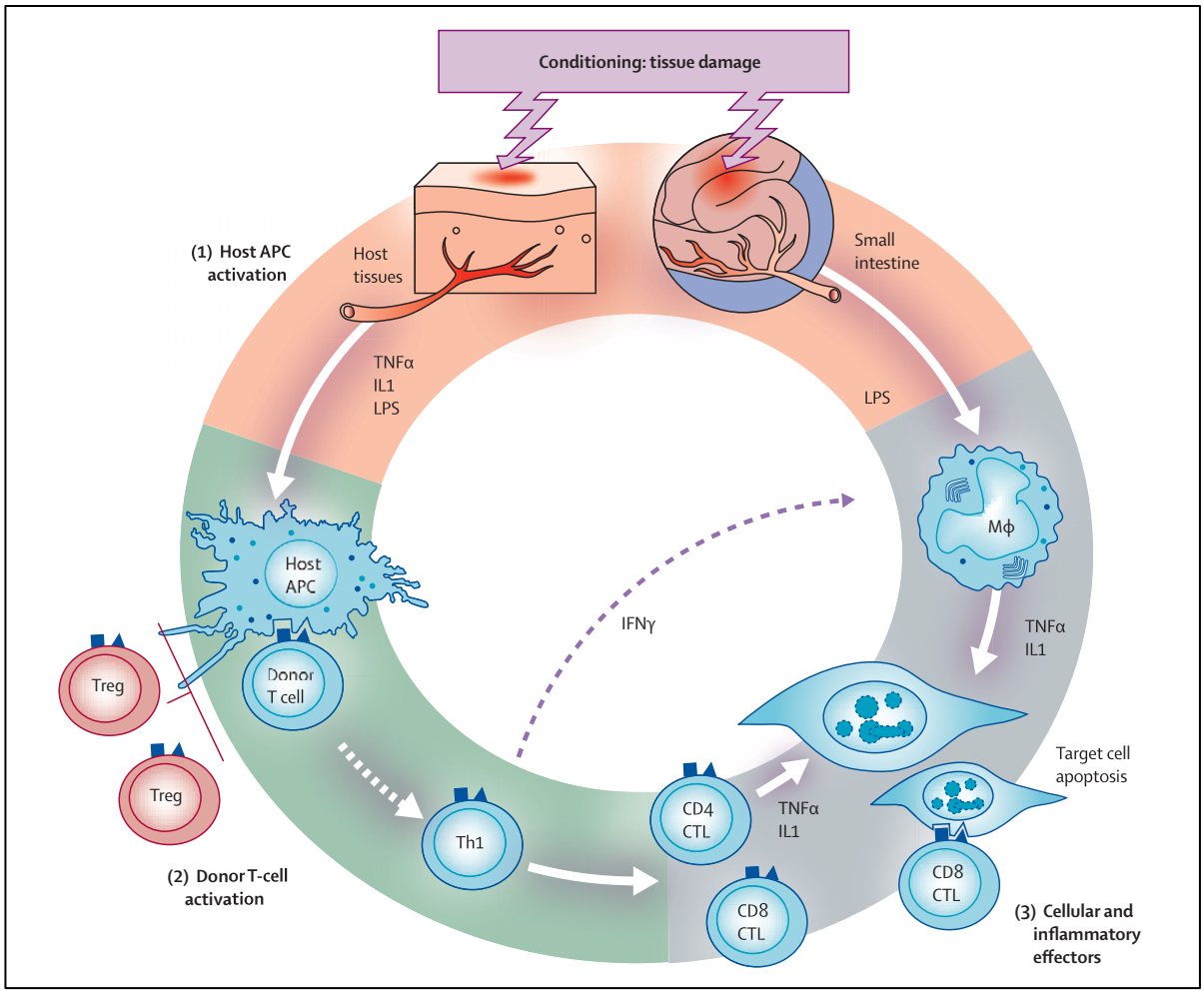

Fig. 2. Pathophysiology of GvHD[30]
IL1=interleukin 1; IFNγ=interferon γ; LPS=lipopolysaccharide; Treg=regulatory T cell; CTL=cytotoxic T lymphocytes

2.2.1 PHASE I: ACTIVATION OF ANTIGEN PRESENTING CELLS

The first phase of GvHD is characterized by the priming of the immune response. The underlying disease and the recipient conditioning represent key factors in GvHD pathogenesis.

Damaged host tissues respond to conditioning regimen by producing danger signals, including proinflammatory cytokines (eg. TNFα, IL-1 and IL-6), chemokines and by upregulating the expression of adhesion molecules and MHC antigens. Moreover, the conditioning regimen increases the expression of costimulatory molecules on host APCs[31, 32, 33]. In addition, damage of the gastrointestinal tract causes the systemic translocation of inflammatory signals including lipopolysaccharide and pathogen-associate molecular patterns (PAMPs), which enhance the activation of APCs. The release of microbial products that are produced by intestinal flora, as well as the release of inflammatory mediators by damaged host tissues, lead to the activation of innate immune cells through the engagement of pathogen recognition receptors, such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs)[33]. The secondary lymphoid tissue in the gastrointestinal tract is probably the first site of interaction between activated APCs and donor T cells[34].

For this reason, manipulating the activation of different types of APCs can represent a promising strategy to ameliorate acute GvHD[35, 36].

2.2.2 PHASE II: ACTIVATION OF T CELLS

This phase represents the core of GvHD pathogenesis. Activated APCs are able to present recipient allo-antigens to T cells, inducing their activation, proliferation and migration towards target organs. After HSCT, donor T cells are able to recognise antigens presented by both recipient (direct presentation) and donor (indirect presentation) APCs. In the case of donor-recipient are HLA-mismatch, donor T cells recognise non self-MHC recipient antigens inducing severe graft-*versus*-host reaction. On the contrary, in case of MHC-matching between the donor and the recipient, the GVH reaction is induced by the recognition of minor histocompatibility antigens (miH)[2].

2.2.3 PHASE III: EFFECTOR PHASE

The effector phase is a complex cascade of cellular mediators (i.e. cytotoxic T lymphocytes and Natural Killer Cells (NK)) and soluble molecules (eg TNF-α, IFN-γ, IL-1 and nitric oxide). All these cells and molecules work together to promote inflammation and local tissues damage.

2.3 ROLE OF IMMUNE CELL SUBSETS IN GVHD PATHOGENESIS

2.3.1 ROLE OF INNATE IMMUNITY

Cells and mediators of innate immunity are responsible for initiating and amplifying the graft-versus-host reaction. In particular, molecules, such as lipopolysaccharide (LPS), produced by the damaged intestine after the conditioning regimen, activate innate immune receptors, including TLRs and cause a cytokines storm, which induces acute GvHD. Several SNPs in the gene encoding the LPS receptor TLR4, have been shown, in mice and patients, to reduce the incidence of acute GvHD[37]. On the other hand, ligation of TLR9 by bacterial DNA can increase the incidence of acute GvHD[38, 39].

Gene polimorphisms encoding TLR4 and NLRs, such as nucleotide-binding oligomerization domain protein 2 (NOD2), are associated with a higher GvHD incidence. NOD2 and the TLR5 ligand flagellin have been shown to have an inhibitory effect on GvHD, by suppressing the function of APCs and favouring the generation of regulatory T cells (T_{reg})[40, 41]. Since TLR7 represents a key role in the development of GvHD, the application of a TLR7 activator on mice skin before inducing GvHD results in a very high T cell-infiltrate[42]. A role for TLR9 and its downstream signalling adaptor MyD88 (myeloid differentiation primary-response protein 88) was also observed in an intestinal acute GvHD model[39].

Manipulation of the gut flora, in order to inhibit the induction of an immune reaction might be a promising strategy to ameliorate GvHD, as suggested by the decreased GvHD severity and improved mice survival following the administration of probiotic bacteria[43]. Together these data suggest that MyD88 inhibitors might be useful in reducing the innate and adaptive immunity triggered by TLRs during GvHD[44].

Other molecules, known as damage-associate molecular patterns (DAMPs), which are released from the damaged tissues after conditioning regimen, can also have a role in GvHD induction. For example, apoptotic cells in the gut of mice or in peritoneal fluids of GvHD patients, induce the release of ATP, which binds to its receptor P2X7 on host APCs, in turn activating the inflammasome. This process induces the expression of co-stimulatory molecules on the surface of APCs. The blockade of P2X7 reduces the incidence of GvHD and increases the number of infiltrating T_{reg}[45]. Along with this data, polymorphisms in the gene encoding P2X7 are associated with survival differences among patients who receive allogeneic HSCT, which support the theory that blocking P2X7 can be a useful strategy to prevent or treat GvHD[46].

Despite the prominent role of the innate immune system in the pathogenesis of GvHD, T cells can be activated and GvHD can still occur in the absence of an appropriate TLR signalling.

2.3.2 ROLE OF ANTIGEN PRESENTING CELLS

During the early period after HSCT, the recipient is chimeric since donor and recipient APCs that resisted to the conditioning regimen, are equally present. Therefore, in this phase, both donor and recipient APCs can contribute to the development of a graft-*versus*-host response. Data from literature, support the importance of T cell recognise through their TCR receptor of recipient APCs in inducing MHC-mismatched allogeneic HSCT (direct recognition)[47, 48].

Further support to the role of recipient APCs in inducing GvHD, came from an experimental model showing that alloreactive donor NK cells could kill recipient Dendritic Cells (DCs) that lack the inhibitory MHC class I molecule thereby protecting mice from GvHD[7].

Even if a the large number of donor APCs is infused within the graft in the allogeneic HSCT, these cells are not involved in the early phase of acute GVHD, because they need to differentiate from their progenitors into mature cells. For this reason donor APCs might participate to GvHD pathogenesis in a later time than donor APCs. However, donor APCs, in particular DCs, are able to cross-present acquired antigens on MHC class I molecules. Therefore, in a setting of marked apoptosis that is induced by conditioning regimen, donor-derived APCs might be sufficiently activated to induce GvHD[2].

The presentation of minor histocompatibility antigens by MHC class I molecules on recipient APCs is important, although not required for a $CD8^+$ T cell-mediated GvHD. Donor APCs can augment this response[49, 50]. MHC class II-bearing host non-professional hematopoietic APCs were previously thought to be essential for the induction of CD4+ T cell-dependent acute GvHD, but this has been called into question[35, 48, 51, 52]. Recent studies have shown that host hematopoietic APCs in lymphoid organs may have only a limited capacity to induce GvHD, and host dendritic cells (DCs) may not be required. For example parenchymal tissue cells can acquire APC function and have been shown to promote the expansion of alloreactive donor T cells in the intestine. In the absence of functional host hematopoietic APCs, the presentation of minor histocompatibility antigens by donor hematopoietic APCs or host non-hematopoietic APCs is sufficient for GvHD induction[53, 54, 55].

Because donor and host hematopoietic APCs and host non-hematopoietic APCs can each contribute to GvHD, approaches that selectively deplete a single type of APCs may be inefficient for the prevention of acute GvHD.

Concerning the subset of APCs mainly required for GvHD induction, DCs are the most important since they are capable of priming naïve T cells, which alone can induce GvHD[56, 57, 58].

Langerhans cells have also been shown to be sufficient for the induction of GvHD when all other APCs are not able to prime donor T cells, although the role for Langerhans cells when all APCs are intact is unknown[59]. The role of DCs maturation in GvHD induction still needs to be elucidated. The impairment of CD40, CD28 or both CD80 and CD86 costimulatory molecules, that are upregulated along with DC maturation, decreases GvHD, suggesting that DC maturation has an important role in GvHD development[51, 60].

DCs have also been proposed to contribute to the maintenance of peripheral tolerance. In fact, infusion of cultured DCs with suppressive activity can inhibit GvHD, by inducing T_{reg} cells[36, 61].

On the overall, these data suggest that conditioning regimen resistant recipient APCs are essential for initiating MHC-mismatched GvHD. Targeting recipient APCs would not be predicted to have a long-lasting impact on pathogen specific immune responses that can be initiated by donor APCs, and these donor cells would also be targets for treating established GvHD.

2.3.3 ROLE OF B CELLS

Data about the role of B cells in acute GvHD pathogenesis are controversial.

Animal models showed that the depletion of B cells from the graft resulted in a decrease of GvHD incidence[62]. On the other hand, IL-10 secreted B cells, can have a protective role by controlling the differentiation of naïve T cells into effector T cells and by inhibiting the proliferation of alloantigen- specific effector T cells through the induction of T_{reg} cells[63].

In the clinical setting, rituximab, an anti CD20 monoclonal antibody, specifically deplete B lymphocytes and when is used as conditioning regimen is able to reduce the incidence and the severity of acute GvHD.

2.3.4 ROLE OF T CELLS AND T CELL SUBSETS

After HSCT, activated both donor and recipient APCs activate alloreactive effector T cells. These effector T cells are able to migrate to the GvHD target tissues, where they mediate

tissue damage by a direct cytotoxic activity. Moreover, after reaching GvHD target organs, activated alloreactive T cells are able to release soluble factors that can induce the expansion of other donor T cells in a sort of inflammatory cascade, thus perpetuating the damage to the host tissues. This mechanism does not require cell to cell contact, but it is based on the release of soluble cytokines. Indeed, in MHC-mismatched transplants, $CD4^+$ T cells can induce GvHD without such direct interactions and, the development of disease is, at least, in part due to the action of cytokines, including TNF and/or IL1, produced by activated high-frequency alloreactive T cells. Targeting these pathways has been studied as potential strategies to prevent or treat GvHD.

In GvHD resulting from MHC-matched transplant, direct contact of CD4+ cells with recipient parenchymal tissues is not required. Donor $CD4^+$ T cells can interact with MHC class II expressing donor cells, such as DCs and macrophages, which indirectly present recipient antigens. Tissue DC can also induce donor $CD4^+$ T cells to produce inflammatory mediators, whereas $CD4^+$ T cells can activate macrophages to induce tissue damage. This study does not exclude $CD4^+$ indirect cytotoxic activity due to cell-cell independent activation of cytotoxic CD8 effector cells. On the other hand, in MHC-matched $CD8^+$ T cells-mediated GvHD interaction with target tissue is absolutely required. (Shlomchik WD et al Blood (2005) ASH abstract 580).

Cytotoxic T lymphocytes that prefer to use Fas and FasL pathway of target lysis seem to predominate in GvHD liver damage (hepatocytes express large amount of Fas), whereas cells that use the perforine and granzyme pathways are more important in gut and skin[5, 64].

T_H-1 and T_H-2 cell response

T_H1 cells and pro-inflammatory molecules, such as IL-1, IL-6, IL-12, TNF and nitric oxide have been shown to be involved in the induction of GvHD[65, 66]. These pro-inflammatory molecules, contribute to a systemic syndrome with variable involvement of the skin, weight loss, diarrhoea and mortality rate. Although the role of Th1-associate cytokines IFNγ IL-2 and TNFα have been involved in the pathogenesis of acute GvHD[67], some studies have reported an opposite effect. IFNγ can both regulate immune suppression and support cellular cytotoxicity[68]. The impact of IFNγ on acute GvHD may depend on the timing of its production. In fact IFNγ, can have an immunosuppressive effects when it is present immediately after HSCT, but can be protective via its pro-inflammatory properties at later stage[69]. In rodents, the neutralization of TNFα has been associated with variable benefits in reducing acute GvHD, and a phase II randomised clinical trial based on TNF neutralization, in

patients with steroid refractory GvHD, demonstrated a relative low response rate compared with other second line strategies for GvHD[70].

Th2-type cytokines, such as IL-4, can reduce acute GvHD, but, as in the case of IFNγ, its effect depend on the time of release[71, 72]. Mice receiving donor T cells unable to secrete the classical T_H2-type cytokines (IL-4, IL-5, IL-9 and IL-13) showed enhanced T cell-proliferation and increased GvHD severity[73]. However, studies involving the transfer of donor T cell populations lacking T_H1 or T_H2 cells, taking advantage of *Stat4*$^{-/-}$ or *Stat6*$^{-/-}$ mice, respectively, showed a crucial role fro both CD4 subsets in acute GvHD pathophysiology, although with different target organ involvement[74]. The lack of conclusive and reproducible data supporting the role of Th1 and Th2 subsets in GvHD, suggests that other cell types could be involved in this reaction.

T_H-17 cell response

T_H-17 cells, which are characterized by the production of IL-17A, IL-17F, IL-21 and IL-22, have been also shown to play a direct role in GvHD. Initial studies observed that the lack of donor T_H-17 cells induced T_H-1 T cell differentiation amplifying GvHD reaction[75]. Other studies have shown that the absence of IL-17 production by donor cells could impair the development of $CD4^+$ T cell-mediated GvHD, although this effect was not observed for GvHD mediated by $CD8^+$ T cells[76]. Adoptive transfer of in vitro-differentiated T_H-17 cells resulted in lethal acute GvHD[77], whereas GvHD was not affected when T_H-17 cells differentiation was abrogated through deletion of the gene encoding the T_H-17 cell-specific transcription factor RoRγt (retinoic acid receptor-related orphan receptor-γt)[78]. These findings suggested that T_H-17 cells are sufficient but not necessary to induce GvHD. In patients with acute GvHD, IL-17-producing cells can be found in high number in biopsy samples from the gut[79]. For this reason, IL-17-producing cells can be a promising target for acute GvHD with gut involvement. Our group have recently published a work demonstrating that patients with acute GvHD or with an active chronic GvHD showed a higher number of T_H-17 cells. In contrast, the percentage of T_H-17 cells drastically decreased in patients with inactive chronic GVHD. Interestingly, $IFN\gamma^+$- T_H-17 cells were able to infiltrate GVHD lesions as observed in liver and skin sections. Moreover, the proportion of T_H-17 cells was inversely correlated with the proportion of regulatory T cells observed in the peripheral blood and tissues affected by GVHD[80].

The T_H-17-type cytokine IL-21 is another potential target, giving its role in promoting the activation, differentiation, maturation or expansion of NK cell, B cell, T cells and APCs. It

can also increase T_H-17 cell activity along with[81] inhibiting T_{reg} cells[82, 83]. Inhibition of IL-21/IL-21 receptor *in vivo* reduced acute GvHD reaction in the gastrointestinal tract, and this effect was associated with decrease of T_H-17 cells and increase of T_{reg} cell number in the gut mucosa[84]. Similar results were observed using a neutralising antibody for human IL-21 in a humanised-mouse model of gut GvHD[85]. Preclinical data suggest that the neutralisation of IL-21 is an attractive strategy for preventing and treating acute GvHD. An alternative approach to manipulate T_H-17 cell response is targeting the cytokines involved in the induction of T_H17 cells differentiation, such as IL-6 which is able to promote in combination with transforming growth factor β (TGF-β) the naïve T cells polarisation to the subset T_H-17, blocking T_{reg} cells induction[86, 87]. Accordingly, high serum levels of IL-6 can be predictive of severe acute GvHD[88], and IL-6 gene polymorphisms have been associated with acute and chronic GvHD in patients[89, 90]. Infusion of an IL-6 receptor specific blocking monoclonal antibody, in a model of acute GvHD led to increased T_{reg} cell numbers and to reduced tissue damaged, particularly in the gut[91]. Moreover, in preliminary studies, IL-6 inhibition has been recently translated to a clinical setting, but showed a modest protection from GvHD[87, 88].

Regulatory T cells

T_{reg} cells are a subset of T helper cells, specialized in suppression of T cell-mediated immune responses, that specifically express the forkhead box P3 (FoxP3) transcription factor. T_{reg} cells can be divided in two main subsets: naturally occurring $FoxP3^+$ T_{reg}, which are thymus derived and specific for self antigens and induced or adaptive T_{reg} that are derived from mature $CD4^+$ $CD25^+$ $FoxP3^+$ precursors in the periphery following inflammatory stimuli[92].

In vitro-expanded and freshly isolated CD4+CD25+ regulatory T cells (T_{reg}) are able to suppress GvHD[93, 94]. In preclinical models, administration of T_{reg} cells is able to improve immune recovery and is most effective in suppressing GvHD if infused early after transplantation. On the other hand, depletion of T_{reg} from the graft, or from in the recipient immediately after HSCT, promotes acute GvHD in different mouse models[95, 96], without affecting the graft-versus-tumour effect. CD62L expression on infused T_{reg} cells is important for the trafficking of these cells to secondary lymphoid organs, where they can suppress the expansion of alloreactive T cells[97]. T_{reg} cells activity at the target site is also important. In fact CCR5-deficient T_{reg} cells, which are unable to migrate into GvHD target organs, are less effective in suppressing GvHD[98]. Clinical trials based on T_{reg} infusion are ongoing.

T cell trafficking

Unlike in pathogen-specific response, during HSCT, T cells are infused intravenously. Therefore, the ability of infused T cells to migrate to specific sites could be an essential feature for the initiation of GvHD. A unique characteristic of immune cell-trafficking in GvHD is that there is no specific pathogen-induced tissue inflammation to create the vascular endothelial changes and the chemokine gradient that support T cell migration into tissues. The mechanisms of T cells recruitment to different target organs could be crucial for understanding the involvement of gut, skin and liver in GvHD. During GvHD, migratory signals derive from the damaged tissue by conditioning regimen and chemotherapy. However, GvHD can develop without irradiation or conditioning regimen, late after HSCT. For these reasons, other mechanisms should be involved in the recruitment of T cells to target organs. In particular, when high T cells activation is present, IFNs can act in an endocrine manner inducing chemokine production in GvHD target tissues[99]. Moreover, those target organs may have an increased ability to recruit activated T cells, either owing to basal synthesis of T cell chemo-attractants or owing to contact with pathogens or allergens that induce sufficient inflammatory signals to support T cell migration[100].

Modulating the trafficking patterns of alloreactive T cells has been identified as an effective method to ameliorate experimental GvHD[98]. Inhibition of T cell homing to inflamed tissues can be obtain by blocking one of the key stages necessary for T cell migration: tethering and rolling on the endothelium; chemokine ligand-receptor interactions; adhesion to the endothelium and migration in response to sphingosine-1-phosphate (S1P).

P-selectin is one member of a family of three glycosylated lectins (E-selectin, P-selectin, L-selectin), which is expressed in the vascular endothelium of the skin and bone marrow, and is expressed by other endothelial cells during inflammation. P-selectin is a key molecule for the tethering and rolling of T cells on the endothelium. The levels of mRNA encoding P-selectin glycoprotein ligand 1 (PSGL1) are upregulated during GvHD[101]. P-selectin-deficient recipient mice exhibit decrease severity of GvHD in the skin, liver and small bowel, and increased numbers of donor T cells in the spleen and in secondary lymphoid organs[102]. Blockade of selectin-ligand interaction can be used to inhibit alloreactive T cell homing to GvHD target organs.

Distinct chemokine ligand-receptor interactions mediate the homing of effector T cells to different tissues. The expression of CC-chemokine receptor 9 (CCR9) by alloreactive T cells facilitates their recruitment into the gut and skin. CCR4 and CCR10 are important for skin homing, and CXC-chemokine receptor 3 (CXCR3) has been shown to attract T_H-1 cells to

sites of tissue injury. Mice that received a CCR2-deficient $CD8^+$ T cell transplant presented less damage in the gut and liver compared to mice that received wild type $CD8^+$ T cells, but the GVT effect was preserved[103]. Another study showed that inhibition of CXCR3 reduced the severity of GvHD in mice. Although there is no evidence about the role of CCR9 in experimental GvHD, CCR9 polymorphisms have been associated to GvHD severity in patients[104].

The importance of high affinity integrins in inflammatory diseases has been recently investigated. Concerning GvHD, a recent study demonstrated that α4β7 has a crucial role in the homing of alloreactive cells in the gut and, its inhibition can reduce the severity of GvHD in this organ[105].

2.3.5 ROLE OF NATURAL KILLER CELLS

After HSCT, donor-derived NK cells are able to promote engraftment, suppress GvHD and promote GVT, whereas host-derived NK cells can mediate graft rejection and affect GvHD by eliminating donor HSCs or activated T cells. Adoptive transfer of activated NK cells early after transplant inhibits GvHD and promotes GVT in a murine model. Although the mechanisms by which NK cells are able to inhibit GvHD has not been fully understood, TGF-β could be a mediator[106]. Another possible mechanism is that NK cells can be able to deplete host APCs[7].

Additional studies are needed to better understand the benefits of potential infusing of NK cells in the contest of allogeneic HSCT.

TREATMENT OF GRAFT-*versus*-HOST DISEASE

3.1 PROPHYLAXIS OF GvHD

The primary pharmacological strategy to prevent GvHD is the inhibition of the cytoplasmic enzyme calcineurin, which is important for the activation of T cells. *Cyclosporine* and *tacrolimus* are two calcineurin inhibitors, which have similar mechanism of action, effectiveness and toxic effects, including hypomagnesaemia, hyperkalaemia, hypertension and nephrotoxicity.

Calcineurin inhibitors are usually administrated in combination with other immunosuppressive drugs, such as *methotrexate*, which is given at low doses in the early post-transplant period[107, 108]. The toxic effects of methotrexate, such as neutropenia and mucositis, have suggested to some investigators to replace it with *mycophenolate mofetil*. In a prospective randomised trial, patients who received mycophenolate mofetil as part of GvHD prophylaxis had significantly less severe mucositis and more rapid neutrophil engraftment than did those who received methotrexate. Frequency and severity of acute GvHD was similar between the two groupsl[109]. Due to the fast neutrophil engraftment, mycophenolate mofetil is used for umbilical-cord blood transplantation for which graft failure is a major concern[110]. For the same reason, this drug is sometimes administrated after reduce-intensity conditioning regimen[111, 112].

Sirolimus is an immunosuppressive drug that is structurally similar to tacrolimus, but does not inhibit calcineurin. In phase II trials, sirolimus resulted very effective in combination with tacrolimus, however, the drug damages endothelial cells and it might increase transplant-associated thrombotic microangiopathy, which is associated with calcineurin inhibitors[113, 114, 115]. The combination of tacrolimus and sirolimus is currently investigated in clinical trials.

Based on data obtained from animal models, which demonstrated the central role of T cells in the initiation of GvHD, many studies on the depletion of T cells from the graft as prophylaxis. Three main depletion strategies were studied: 1) *ex vivo* negative selection of T cells; 2) *ex vivo* positive selection of $CD34^+$ stem cells; 3) *in vivo* antibodies against T cells. Most of these approaches showed substantial limitation of acute GvHD[116, 117]. Unfortunatly, the lowest frequency of severe GvHD was offset by high rates of graft failure, relapse of malignant disease, infections, and Epstein-Barr virus-associate lympho-proliferative diseases. Several works have investigated partial T cell depletion, either by elimination of specific T cell subsets or by titration of the dose of T cell present in the graft[118, 119]. None of these

approaches, however, has been shown convincingly to be the best strategy to enhance long-term survival.

Alemtuzumab is a monoclonal antibody that binds CD52, a protein expressed on a broad range of leukocytes including lymphocytes, monocytes and dendritic cells. Its use in a phase II clinical trial of GvHD prophylaxis, decreased the incidence of acute GvHD after reduced-intensity transplant. In a perspective study, patients who received alemtuzumab rather than methotrexate, showed significantly lower rates of acute GvHD, but they presented more infections and higher rate of relapse. Indeed, no overall survival benefits were observed[120].

In vivo administration of antibodies against T cell in vivo as GvHD prophylaxis, has also been tested extensively. In particular, several studies, focused their attention on *anti-thymocyte globulin* or *anti-lymphocyte globulin* preparation. These serum samples are prepared by immunising horses or rabbit to thymocyte or lymphocyte, respectively. The in vivo effect of these preparations is greatly variable, since it was observed that even different brands show different biological effects[121]. However the different side effects of anti-thymocytes globulin and anti-lymphocytes globulin are similar across different preparations and include fever, chills, headache and thrombocytopenia. In retrospective studies, rabbit anti-thymocytes globulin, reduced the frequency of GvHD in related-donor HSCT recipients without enhancing survival[122, 123]. In patients receiving unrelated-donor HSCT, addition of anti-lymphocite globulin to GvHD prophylaxis, prevented severe GvHD, but did not result in better survival because of increased infections[124].

3.2 TREATMENT OF GvHD

3.2.1 FIRST LINE THERAPY

Steroids, with their strong immunosuppressive activity, are the gold standard for the treatment of GvHD. Many centres treat mild GvHD of the skin (grade I) only with a prolonged prophylaxis, or with topical steroids alone, but for more severe disease with the involvement of other visceral sites, high-dose systemic steroids are administered. Administration of steroids results in a complete response in less than 50% of patients[125], and more severe GvHD is less likely to respond to second line therapies[126].

3.2.2 SECOND LINE THERAPY

Management of steroid-refractory acute GVHD poses one of the most vexing and difficult problems faced by transplant physicians. Several studies have been focused their attention to evaluate different approaches for secondary treatment of acute GVHD. To date, no consensus has been reached regarding the optimal choice of agents for secondary treatment, and clinical management is generally approached through empirical trial.

One of the strategies for treating steroid-refractory GvHD is the blockade of the inflammatory cytokine TNFα (*etanercept*). TNFα can activate APCs, recruit effector cells, and cause direct tissue damage[127]. Data from a phase II trial taking advantage of etanercept (solubilised TNFα receptor 2) showed significant effectiveness of the drug when added to steroids as primary treatment for acute GvHD. 70% of patients had complete resolution of all GvHD symptoms within 1 month, with 80% complete responses in gut and skin[128].

An increasingly treatment for GvHD is *extracorporeal photopheresis*. During this procedure, the patients white blood cells are gathered by apheresis, incubated with the DNA-intercalating agent 8-methoxypsoralen, exposed to ultraviolet light, and return to the patient.

Extracorporeal photopheresis (ECP) is known to induce cellular apoptosis, which has strong anti-inflammatory effects in several systems, including prevention of rejection of solid organ grafts[129]. Animal models showed that extracorporeal photopheresis is able to reverse acute GvHD by increasing the number of T_{reg} cells[130]. Our group confirmed that GvHD patients treating with ECP were accompanied by a significantly increased of T_{reg} cells in peripheral blood[131]. A phase II clinical trial using ECP to treat steroid refractory GvHD, showed resolution of disease in most patients, with 50% long-term survival in the high risk group[132].

3.2.3 IMMUNO-MODULATORY THERAPIES

- T_{reg} cells and tolerogeneic DCs

As mentioned before, natural T_{reg} cells are able to suppress alloreactive T cells and control innate and adaptive immunity. In particular, animal models showed that adoptive transfer of natural T_{reg} cells was highly effective in suppressing acute GvHD, improving immune recovery. In the last years, several clinical trials using T_{reg} cells for the prevention of acute GvHD have been reported[133]. Human T_{reg} cell population that were expanded from umbilical cord blood before transplant substantially reduce the incidence of acute GvHD[134].

Improvement of *ex vivo* production of natural T_{reg} cells should permit the expansion of large numbers of natural T_{reg}[135]. In another preclinical study, an adoptive therapy with freshly

isolated T_{reg} cells from haploidentical donors almost completely prevented acute GvHD[136]. New methodologies to generate antigen specific T_{reg} cells will be tested in future trials, in order to restricting the immunosuppressive activity of these cells to acute GvHD, while maintain a GVT response.

Recent studies have shown that induced T_{reg} cells can reduce GvHD in rodent models. This effect has been demonstrated using rodent-antigen specific induced T_{reg} cells, generated from $CD4^+CD25^+$ T cells in presence of TGF-β or induced *in vivo* by tolerogeneic DCs. Special subsets of DCs, instead of promoting immune responses, have tolerogeneic functions and are able to inhibit GvHD in mice[137, 138, 139]. Tolerogeneic or regulatory DCs can be obtained by exposing bone marrow derived cells to GM-CSF, IL-10, TGF-β, and LPS or can be isolated from a mixed lymphocytes reaction supplemented with TGF-β and retinoic acid. Infusion of tolerogeneic DCs has been shown to rescue animals from lethal acute GvHD, and this was associated with the generation of induced T_{reg} cells[36].

- NK cells and NKT cells

Donor NK cells has been shown to be able to inhibit acute GvHD. Preclinical studies suggest that donor NK cells can suppress acute GvHD while maintain GVT responses. Subsequent studies, have shown that donor T cells exhibit less proliferation, lower CD25 expression and decreased IFNγ production in the presence of donor NK cells[106]. Clinical studies showed that infusion of NK cells within the graft was associated with a decreased GvHD occurrence and severity, compared to HSCT alone.

NKT cells, a cell subset co-expressing both NK and T cell markers, has also been shown to control GvHD in mice in an IFNγ- and IL-4-dependent manner[72]. In mouse models, the *in vivo* activation of NKT cells with glycosphingolipids such as α-galactosylceramide has been shown to be able to inhibit GvHD. However, a recent murine study demonstrated that an early administration of a syntetic form of α-galactosylceramide (KRN7000), can result in hyperacute GvHD[140]. Thus, it remains to be determined whether NKT cell-based therapy will be useful.

- Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs are a heterogeneous cell population of myeloid origin that consist of progenitors and mature macrophages, granulocytes and DCs. MDSCs are defined as $CD11b^+Gr1^+$ cells in mice, as LIN^-HLA-DR-$CD33^+$ or $CD11b^+CD14^-CD33^+$ cells in humans, although they have also been defined within $CD15^+$ peripheral blood cell population[141, 142]. MDSCs can be

expanded *in vitro* and can suppress T cell function by expressing enzyme that regulate essential amino acid metabolism, such as arginase 1 and indoleamine 2, 3-dioxygenase (IDO), by releasing soluble mediators, such as IL-10, reactive oxygen species or nitric oxide[143].

Animal models have shown that MDSCs can suppress acute GvHD[144]. Moreover, in one study, in vivo arginine depletion could also be accomplished through the use of a drug, pegylated arginase 1, suggesting a new pharmacological approach to acute GvHD prevention[145].

- Mesenchymal Stromal Cells (MSC)

A very promising strategy for treating steroid-refractory GvHD is represented by mesenchymal stromal cells administration.

Bone marrow-derived MSC are a group of fibroblast-like cells with the ability to differentiate *in vitro* into osteoblast, adipocytes, and chondroblasts. MSC have a wide range of immunomodulating effects on both innate and adaptive immune cells[146]. MSC have a protective effect on GvHD but results in clinical trials are confused, as early trials showed a substantial benefits, whereas two Phase III clinical trials, with at least one source of MSC did not shown any benefit[147, 148]. Differences in manufacturing and defining MSC, in their expression of homing receptors and in the type of GvHD injury may all contribute to the difficulty in comparing results and clinical outcome.

MESENCHYMAL STROMAL CELLS (MSC) FOR TREATING GvHD

MSC are defined as a heterogeneous population of cells that proliferate *in vitro* as plastic adherent cells with a fibroblast-like morphology and are able to differentiate into tissue of mesodermal origin, such as adipocytes, osteoblasts, chondrocytes[149, 150].

First identified and isolated from the bone marrow (BM), MSC can now be expanded from a variety of other tissues including adipose, umbilical cord blood, skin, tendon, muscle and dental pulp[151, 152, 153].

MSC can't be defined by a specific unique antigen, but are defined by a panel of positive and negative antigens. Human MSC do not expressed the hematopoietic markers CD45, CD34 and CD14, the co-stimulatory molecules CD80, CD86 and CD40, whereas they express variable levels of CD105, CD73, CD44, CD90, CD71 and STRO-1. In particular, MSC are characterized by the expression of low levels of class I MHC and they do not express class II MHC. Due to this particular antigen profile MSC are able to escape the immune response.

In addition to their stem/progenitors properties, MSC are also able to modulate the immune response, interacting with both innate and adaptive immunity.

Recent findings have demonstrated that MSC actively interact with component of the innate immune response and that, through these interactions, they display both anti-inflammatory and pro-inflammatory effects[154, 155, 156].

4.1 MSC-MEDIATED IMMUNOSUPPRESSION

Several recent data indicate that MSC possess immunomodulatory properties, and may play specific roles as immunomodulators in maintenance of peripheral tolerance, transplantation tolerance, autoimmunity, tumour evasion, as well as fetal-maternal tolerance.

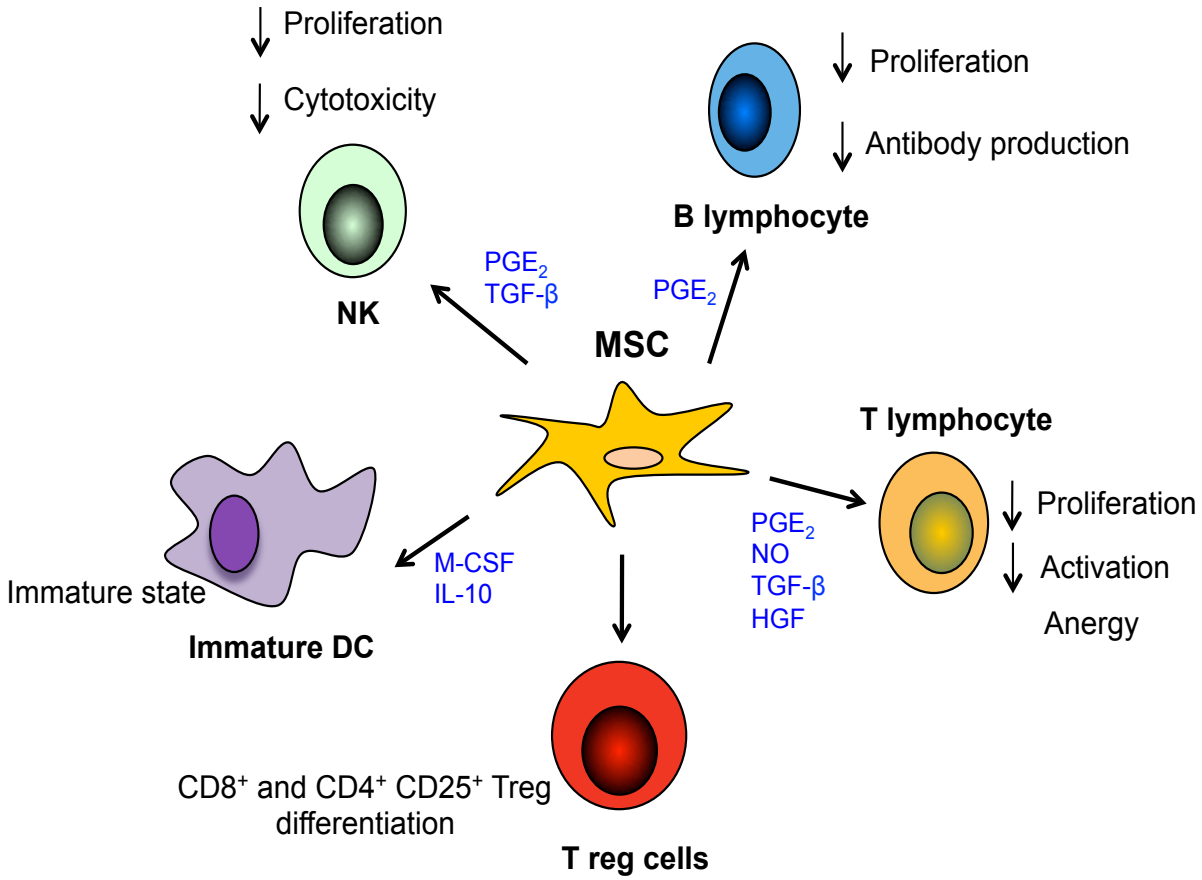

Fig.3 Immunomodulatory effects of MSC

4.1.1.MSC AND INNATE IMMUNITY

Human and mouse MSC express in culture a number of distinct and overlapping TLRs, and *in vitro* stimulation of specific TLRs influence the immune-modulation activity of MSC[157, 158, 159]. Under hypoxic culture conditions, stimulation of MSC with inflammatory cytokines, such as IFNγ, TNFα, IL-1β and IFNα upregulate the expression of TLRs, increasing the sensitivity of MSC toward inflammation[160]. However, prolonged stimulation with TLR ligands induces down-regulation of TLR2 and TLR4[161]. Following specific TLR stimulation, MSC are able to polarized towards two different phenotypes, each characterised

by distinct secretome and immune-modulatory activity. For example, TLR4 induce MSC to acquire a pro-inflammatory phenotype (MSC1), whereas TLR3 induce MSC towards an anti-inflammatory phenotype[162].

The inflammatory microenvironment influences the differentiation of monocytes arriving at the inflammatory sites, into M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotype. The production of pro-inflammatory cytokines by M1 macrophages or T cells can activate MSC and induce the release of soluble mediators towards an anti-inflammatory profile and a M2 phenotype[155]. The interaction between MSC and macrophages enhance the anti-inflammatory effect of MSC in a feedback manner.

These data suggest that the microenvironment is able to switch the role of MSC between a pro-inflammatory effect and an anti-inflammatory effect. In both cases, the switch mechanisms rely on the production of soluble mediators, such as inducible NO synthase (iNOS, in mice) and IDO (for humans), which are induced by pro-inflammatory cytokines. The concentrations of these factors may be critical in triggering the switch between pro-inflammatory and anti-inflammatory MSC[163].

Anti-inflammatory activity of MSC

After stimulation with sufficient levels of pro-inflammatory cytokines, MSC are able to acquire an anti-inflammatory activity and promote tissue homeostasis. Co-culture of monocytes with human or mouse BM-MSC promotes the formation of M2 macrophages[164]and this is mediated by both cellular contact and soluble factors, including prostaglandin E2 (PGE2) and catabolites of IDO activity[164, 165]. Moreover, activation of MSC with IFNγ, TNFα and LPS increases the expression of cyclooxygenase 2 (COX2) and IDO in BM-MSC, thereby further promoting a homeostatic response toward M2 macrophages polarization[165, 166]. Mouse and human MSC are also able to induce the migration of macrophages and monocytes to the site of inflammation through the production of chemokine (C-C motif) ligands CCL2, CCL3, and CCL12, promoting wound repair[167].

The effect of MSC to polarised M2 differentiation of macrophages is closely linked with the ability of MSC to favour the generation of T_{reg} cells, which are involved in immune-suppression. TGFβ is the key factor in the induction of T_{reg} by MSC in a monocyte-depending manner. Indeed, M2 macrophages produce IL-10, which has an anti-inflammatory activity alone, and produce CCL18, which in addition to TGFβ promotes the generation of T_{reg}[168]. The MSC-derived factors, that mediate the differentiation of M2 macrophages, are still unknown.

4.1.2 MSC AND ADAPTIVE IMMUNITY

The interaction between MSC and T cells

MSC have the specific characteristic of *in vitro* suppressing the proliferation of T lymphocytes induced by cellular and non specific mitogenic stimuli[169] through the secretion of soluble factors which include TGFβ, Hepatocytes Growth Factor (HGF), PGE2, IDO, NO and hemoxygenase (HO)[170] Stimulation of MSC with IFNγ and TNFα increase the production of these mediators, but unstimulated MSC are also produce these molecules. In human cells, IDO promotes the degradation of tryptophan, into kynurenine and other catabolites that have shown to suppress T cell proliferation while induce T_{reg} differentiation[146, 171]. Murine MSC are able to inhibit T cell proliferation due to the production of NO, mechanism that is supported by the observation that *in vitro* proliferation of murine T cells is boosted by the addition of the NO inhibitor L-NMMA[172].

MSC are able to exert their anti-inflammatory activity, polarizing T cells towards a regulatory phenotype[173]. In vitro co-culture of human MSC with peripheral blood mononuclear cells (PBMCs) induce the differentiation of $CD4^+$ T cells into induced T_{reg} cells, mechanism which involves both cellular contact and the production of TGFβ[174, 175]. The generation of T_{reg} cells by MSC may be monocyte dependent, because, it was not observed in co-culture of MSC with $CD4^+$ T cells, or monocyte depleted PBMCs, but it could be restored by the addition of monocytes[168]. Following the addition of mitogen-stimulated T cells, MSC-induced T_{reg} cells are able to suppress T cell response. Production of HLA-G5 by MSC has also been shown to promote MSC induced T_{reg} generation[176].

These results indicate that MSC are able to re-establish the balance between inflammatory effector T cells and anti-inflammatory T_{reg} cells. By linking together cytokine-mediated immunosuppressive activity and the induction of T_{reg} cells, an enhancement of anti-inflammatory response is obtained[162].

The interaction between MSC and APCs

Dendritic cells (DCs) play a key role in the induction of immunity and tolerance, depending on the activation and maturation stage and, the cytokine milieu at sites of inflammation[177]. MSC have been demonstrated to interfere with DC differentiation, maturation and function. Addition of MSC results in inhibition of differentiation of both monocytes and $CD34^+$ progenitors into $CD1a^+$-DCs, skewing their differentiation toward cells with features of macrophages. DCs generated in the presence of MSC were impaired in their response to maturation signals and exhibited no expression of CD83 or up-regulation of HLA-

DR and costimulatory molecules[178, 179, 180]. Consistent with these findings, immature DCs generated in the presence of MSC were strongly hampered in their ability to induce activation of T cells. In addition, an altered cytokine production pattern, for example decreased production of proinflammatory cytokines such as TNFα, IFNγ, and IL-12, and increased production of the anti-inflammatory cytokine IL-10 in MSC/monocyte culture, was also observed[146, 178, 181]. Taken together, these results suggest that MSC suppress the differentiation of dendritic cells, resulting in the formation of immature DCs that exhibit a suppressor or inhibitory phenotype.

The interaction between MSC and B cells

In murine studies, MSC have been reported to inhibit the proliferation of B cells, stimulated with anti-CD40L and IL-4[182], or with pokeweed mitogen[183]. Consistent with the murine studies, human MSC have been shown to inhibit proliferation of B cells activated with anti-Ig antibodies, soluble CD40 ligand and cytokines[184]. In addition, differentiation, antibody production and chemotactic behaviour of B cells was affected by MSC[184]. Krampera et al showed that MSC only reduced the proliferation of B cells in the presence of IFNγ. The suppressive effect of IFNγ was possibly related to its ability to stimulate the production of IDO by MSC, which in turn suppresses the proliferative response of effector cells through the tryptophan pathway[185]. Although the mechanisms involved in these activities are not yet fully understood, transwell experiments indicated that soluble factors released by MSC were sufficient to inhibit proliferation of B cells[184]. In contrast, culture supernatant from MSC had no effect, suggesting that the release of inhibitory factors requires paracrine signals from B cells.

The interaction between MSC and NK cells

Natural killer (NK) cells exhibit spontaneous cytolytic activity that mainly targets cells that lack expression of HLA class I molecules. Killing by NK cells is regulated by a balance of signals transmitted by activating and inhibitory receptors interacting with HLA molecules on target cells. It has been suggested that MSC suppress IL-2 or IL-15 driven NK-cell proliferation and IFN-γ production[146, 174, 186, 187]. MSC do not inhibit the lysis of freshly isolated NK cells[186], whereas NK cells cultured for 4 to 5 days with IL-2 in the presence of MSC have a reduced cytotoxic potential against K562 target cells[185]. Furthermore, Sotiropoulou et al demonstrated that short-term culture with MSC only affect NK-cell cytotoxicity against HLA class I-positive tumour cells but not against HLA class I-negative

targets[187]. These data indicate that MSC exert an inhibitory effect on the NK-cell cytotoxicity against HLA class I-positive targets that are less susceptible to NK-mediated lysis than HLA class I-negative cells.

Experiments with transwell culture systems have indicated that MSC are able to suppress the proliferation and cytokine production of IL-15 stimulated NK cells via soluble factors. In contrast, the inhibitory effect of MSC on NK-cell cytotoxicity required cell-cell contact, suggesting the existence of different mechanisms for MSC-mediated NK-cell suppression[187]. PGE2 secretion by MSC was demonstrated to partially affect NK-cell proliferation, CD56 expression and cytotoxicity, but did not interfere with cytokine production or expression of activating receptors[187]. Inhibition of TGF-β partially restores NK-cell proliferation, whereas blocking both PGE2 and TGF-β completely restored the proliferation capacity of NK cells, indicating that these factors suppress NK-cell activity by different mechanisms.

Until recently, MSC were considered immune-privileged and previous studies reported that MSC were not lysed by freshly isolated NK cells[186, 188]. However, recent data indicate that activated NK cell are capable of effectively lysing MSC[187, 189]. Although MSC express normal levels of MHC class I that should protect against NK-mediated killing, MSC express different ligands that are recognized by activating NK receptors that trigger NK alloreactivity[189]. Treatment of MSC with IFN-γ decreased their susceptibility to NK cell–mediated lysis due to up-regulation of HLA class I molecules[189].

Taken together, numerous studies convincingly demonstrate that MSC are able to modulate the function of different immune cells in vitro, particularly involving the suppression of T cell proliferation and the inhibition of DC differentiation. Despite several mediators have been described, the mechanisms underlying the immunosuppressive effects of MSC are still unclear.

4.1.3 MSC IMMUNO-MODULATORY ACTIVITY: FROM THE MOUSE MODEL TO THE BEDSIDE

The absent or low expression of class II MHC and absence of expression of costimolatory molecules suggested that MSC are immune privileged cells, avoiding the needs of autologous MSC for clinical purposes[190]. In fact, several studies suggest that under appropriate conditions, MSC are able to create a tolerogenic environment, permitting them to escape the immune response[191]. This ability to escape the immune system coupled to their easy expansion, makes them ideal as a potential cell therapy.

The immunomodulatory function of MSC suggests that these cells can be used as a cellular

therapy for autoimmune disease, such as Chron Disease, systemic lupus erythematosus or multiple sclerosis.

The infusion of MSC in patients with steroid refractory acute GvHD, developed after allogeneic HSCT is one of the most extensively clinical application investigated. Several studies in animal models have reported positive results, both in the reduction or prevention of GvHD after allogeneic HSCT[192]. In 2004 Le Blanc and colleagues first reported the complete recovery of a 9-year old boy affected by a grade IV steroid refractory acute GvHD treated with third-party BM-MSC[193]. Following this first report, several human trials both autologous and allogeneic MSC in the treatment of GvHD have been performed[194]. Kebriaei and colleagues, presented the first prospective trial of third-party, unmatched MSC, for the treatment of de novo acute GVHD. The results of this study provide evidence that MSC can effectively induce a response in a high percentage of GVHD cases, and when used in combination with existing therapy, may improve overall outcome. Seventy-seven percentage of patients had an initial complete response following the initiation of steroids and MSC therapy[195]. Concerning the usage of MSC as a profilaxis therapy for GvHD, Kuzmina and co-workers performed a clinical study in which 19 patients received the standard GvHD prophylaxis with immunosuppressive drugs in combination with the infusion of MSC expanded from the HSCT donor during leukocyte recovery by activation of hematopoietic transplant[196]. This group of patients was compared to 19 patients who were treated with the conventional profilaxis alone. In the MSC group only one patient developed acute GvHD, while in the standard group 6 patients developed the disease. No differences in the graft rejection rates or in the incidence of infections were observed in both groups. The overall mortality was 22.2% in the standard profilaxis compared to 5.3% in the MSC treated group. A phase II multicentre clinical trial showed a clinical response in the majority of patients (55 adults and children) with steroid resistant acute GvHD, treated with intravenous infusions of *third-party* MSC. Moreover, this response to the therapy correlated with the differences in terms of overall survivals between the patients[197]. These results have been extended in a cohort of paediatric patients treated with multiple infusions of MSC[198]. Similar results have been reported from our group in a smaller cohort of paediatric patients treated with platelet lysate (PL)-expanded MSC[199]. This work addresses the particular issue of GvHD treatment in children. Although GvHD is less common in children than adults, long-term site effects of prolonged immunosuppressive treatment is a major issue in the paediatric setting. It is therefore of the utmost importance to propose a treatment strategy that may be able to reduce the burden of conventional immunosuppression. Not all clinical trials have reported positive

results. The Phase III Clinical Trial of the human MSC preparation Prochymal showed no significant improvement of GvHD compared to controls[200]. The same Prochymal product, which was declared ineffective for skin GvHD treatment, obtained a 47% rate of response in skin GvHD in a paediatric cohort of patients. Younger patients may be, therefore, more prone to respond to this kind of treatment, either for peculiarities in their immunologic setting or for higher infused MSC dosage.

All these data suggest that much work needs to be done for standardising the usage of MSC as treatment for GvHD. One of the aspects that need to be investigated is the timing of the infusions of MSC. A lot of studies are investigating the ideal infusion protocol, including pre-transplant infusion[201] and infusion at the time of transplantation[192]. Another key factor that can affect the results obtained in clinical trials is the differences in the source of expanded cells used in different studies. The original source of MSC was the bone marrow, but in the last years several other source of MSC have also been investigated, including umbilical cord blood[202], placenta[203] and adipose tissue[42, 192, 204]. Further studies are needed to better understand the differences between MSC obtained from different sources in order to improve patient treatment. Moreover, more information about the inflammatory environment to which MSC are exposed and the effect of it on the "licencing" will be crucial to improve the efficacy of this therapy[205].

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SCOPE OF THE THESIS

Allogeneic haematopoietic-stem-cell transplantation (HSCT) is the treatment of choice for many malignant and non-malignant disorders. The development of novel strategies such as donor leukocyte infusion, nonmyeloablative HSCT, and cord blood transplantation allowed expanding the indications for allogeneic HSCT over the last several years, especially among older patients. However, the major toxicity of allogeneic HSCT, Graft-versus-Host Disease (GvHD), remains a complication that limits its wider application. Despite advances in post-transplantation immunosuppressive therapy, GvHD remains a major life-threatening post-HSCT complication, developing in a substantial number of patients and resulting in poor outcome. Although in the last three decades the risk of GvHD has been reduced by modifying the transplant program and the stem cell source, yet significant challenges remain. The best hope for continued progress lies in the development of innovative treatments, thanks to a better understanding of GvHD pathogenesis, and in the identification of new easily measurable disease markers able to predict GvHD onset and therapy response. Along these hypotheses, the project comprises two lines of research.

1) The first one is focused on the potential role of chemerin/chemerin receptors axis in the pathogenesis of GvHD, with the aim to define new diagnostic tools and therapeutic targets for improving the management of post-transplant GvHD.
2) The second line of research is focused on the immunosuppressive factors produced by mesenchymal stromal cells (MSC), a novel very promising therapy for steroid-resistant GvHD.

PART I

STUDY OF GRAFT-*versus*-HOST DISEASE PATHOGENESIS

Chapter 2

Potential role of the chemerin/ChemR23 CCRL2 chemokine receptors axis in a mouse model of acute Graft-*versus*-Host Disease

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ABSTRACT

Graft-*versus*-Host Disease (GvHD), represents the major cause of mortality and morbidity after allogeneic Hematopoietic Stem Cell Transplantation (HSCT). The infiltration of different cell subsets into target organs is an important step in GvHD pathogenesis and modulation of cell trafficking could represent a promising strategy for GvHD prophylaxis and treatment. We report in this paper that chemerin, a novel chemotactic protein, and its receptors ChemR23 and CCRL2 are involved in GvHD pathogenesis, particularly in the development of gut GvHD. The allogeneic transplantation of ChemR23 knock out (KO) bone marrow and splenocytes induced high mortality and severe GvHD in terms of weight loss and gut score in wild type (WT) transplant recipients. Adoptive transfer of WT CD11c+ dendritic cells (DCs) or plasmacytoid (pDCs) suggesting that these cell subsets are not able to improve GvHD. In addition, experiment performed using ChemR23 mice as recipients showed that KO mice developed a severe GvHD early after transplantation. As well as in donor experiments, KO recipients showed increased mortality, weight loss and gut score, compared to WT mice. In order to fully understand the role of chemerin in GvHD, we also performed GvHD experiments taking advantage of CCRL2 KO mice. GvHD observed after transplanting bone marrow and splenocytes obtained from CCRL2 KO mice showed a survival rate comparable to WT-transplanted mice, otherwise associated with an increased weight loss and gastrointestinal score in KO animals. Indeed CCRL2 recipients developed a severe GvHD early after HSCT, with significantly high mortality, weight loss, without any difference in gut score. All this data suggested that chemerin/chemerin receptors axis can be involved in GvHD pathophysiology.

INTRODUCTION

Hematopoietic Stem Cell Transplantation (HSCT) is the treatment of choice for many malignant and non-malignant disorders[1]. Although it is widely used, the occurrence of Graft-*versus*-Host-Disease (GvHD) severely limits its efficacy[2]. Acute GvHD consists in an immunological reaction of allo-reactive donor T cells against recipient antigens, with specific involvement of skin, liver, lung and gastrointestinal tract (GI)[3]. Among the different manifestations, gut GvHD represents one of the major causes of morbidity and mortality after HSCT because its diagnosis is particularly protean. On the contrary, skin GvHD is clinically easier to recognize and can be often controlled by the administration of local or systemic immunosuppressive therapies, while liver GvHD is much less frequent[4]. The involvement of chemokines in recruiting leukocytes to the inflammation sites has designed a number of chemokine receptors as attractive targets for therapeutic applications in the field of inflammatory diseases[5, 6, 7]. The identification of new chemkines/chemokine receptors, involved in GvHD pathogenesis, represents a potential strategy for the development of novel therapeutic approach for treating this life-threatening disease.

Chemerin has been recently identified as a chemotactic protein involved in both the initiation and resolution of inflammation[8]. Originally isolated from inflamed biological fluids, such as ovarian cancer ascites and rheumatoid arthritis synovial fluids[9], chemerin is secreted as a precursor. After secretion, it is converted in its full agonist through the proteolytic cleavage of the last six-seven amino acids by extracellular serine and cysteine proteases of the coagulation, fibrinolytic and inflammatory cascades, suggesting that the processing take place at site of inflammation[10, 11]. Active chemerin binds the G protein-coupled receptor ChemR23, expressed by immature myeloid Dendritic Cells (iDCs)[9], plasmacytoid Dendritic Cells (pDCs)[12], macrophages[13] and natural killer cells (NK)[14]. Chemerin is also able to bind to two atypical G protein-coupled receptors CCRL2, which is expressed by neutrophils, monocytes, DCs, mast cells[15], NK cells, T cells, $CD34^+$ cells[16, 17] and endothelial cells[18] and GPR1, which was described by few papers that act as a scavenger receptor, but the exactly function is still unknown[19]. The interaction of chemerin with the CCRL2 receptor, does not induce any intracellular signalling, however, recent studies reported that this receptor works as a regulator of chemerin concentrations. High amounts of active chemerin were detected in inflammatory diseases, such as lupus erythematosus and oral lichen planus, on high endothelial venules and in pathological peripheral tissues. Its role in these diseases has been

linked to an increased recruitment of ChemR23-expressing cells to inflamed peripheral tissues[12, 14].

The aim of this study was to evaluate the possible role of the chemerin/chemerin receptors axis in GvHD pathogenesis in order to identify disease-specific pathways exploitable for developing new potential therapeutic targets. With this aim, we took advantage of a murine model of allogeneic hematopoietic stem cell transplantation and acute GVHD by using ChemR23 and CCRL2 KO mice. The transplantation of donor derived ChemR23 KO or CCRL2 KO cells into WT animals, showed a more severe pathology in term of survival and clinical score especially of the gastrointestinal tract, compared to WT donor cells. Along with these data, when using ChemR23 KO and CCRL2 KO mice as transplant recipients, we observed an earlier and more severe GVHD occurrence, compared to WT mice. On the overall, these results suggest that the chemerin/chemerin receptors axis should have an important role in inducing acute GvHD, particularly in gastrointestinal tract. Further studies are needed to better comprehend the mechanisms by which this chemokine/chemokine receptor axis can be involved in GvHD pathogenesis.

METHODS

Mice:

C57BL/6 and Balb/c wild type mice were obtained from Charles River Laboratories. ChemR23-deficient (ChemR23 KO) and CCRL2-deficient (CCRL2 KO) mice, both in C57BL/6 strain, were kindly provided by Prof. Silvano Sozzani (University of Brescia). These mice are fertile, present a normal lifespan and do not show any overt phenotype under steady state conditions[15, 20]

Acute Graft-*versus*-Host Disease mouse model:

To induce acute GVHD, bone marrow transplantation was performed as previously described[21]. In detail, C57BL/6 wild type, ChemR23 KO and CCRL2 KO mice were lethally irradiated with 900 cGy (RADGIL, Ghilardoni) split in two doses with 2 hours interval, and received $10x10^6$ bone marrow cells and $20x10^6$ splenocytes harvested from Balb/c mice. Balb/c recipient mice received 700 cGy total body irradiation split in two doses and transplanted with $10x10^6$ bone marrow cells and $5x10^6$ splenocytes harvested from C57BL/6 mice. Bone marrow cell suspensions were obtained by flushing femurs and tibiae with RPMI 1640 medium added with 10% Fetal Bovine Serum (FBS). Splenocytes were obtained by gently crushing the spleen in the presence of complete medium. Red blood cells were lysed by incubation with ammonium chloride lysing solution (Voden Instruments) for 10 minutes, filtered to remove debris and re-suspended in PBS-/- for administration. All mice were male 8-10 weeks old. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of both Ministero della Salute and University of Milano-Bicocca.

Monitoring of GvHD score:

Transplanted mice were daily monitored for acute GvHD clinical signs. In particular, weight loss, diarrhoea, posture hunching, mobility, fur texture, skin integrity and mobility were evaluated at different time-points after transplantation and a score 0-2 was assigned for each parameter as follows: score 0=absence of GVHD signs; score 1=mild GVHD signs; score 2=severe GVHD signs. Skin clinical score was calculated as the sum of single scores from fur texture and skin integrity, while the overall clinical score was calculated as the sum of the clinical scores from all the single monitored organs.

Adoptive transfer of CD11c$^+$DCs and pDCs:

Plasmacytoid Dendritic Cells (pDCs) and CD11c$^+$ Dendritic Cells (DCs) for adoptive therapy experiments were obtained for transplantation by harvesting donor splenocytes (C57BL/6) and were purified with mouse Plasmacytoid Dendritic Cells isolation kit II (Miltenyi Biotec MACS) and mouse CD11c Microbeads (Miltenyi Biotec MACS).

Chemerin plasma levels in GvHD mouse model:

Peripheral blood samples were collected the day before total body irradiation (TBI) and starting 24 hours after transplantation every three days. Plasma was separated from cell fraction by centrifugation and cryopreserved before the use. Chemerin plasma levels were measured using mouse-chemerin ELISA assays (R&D) following the manufacturer instructions.

Statistical analysis:

Survival data were compared using GraphPad Prism 6.0d and groups were analysed with the Mantel-Cox long-rank test. Non-survival data were compared using Student Test. P-value ≤ 0.05 was considered to be statistically significant (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

RESULTS

Chemerin plasma levels increase in mice during acute GvHD

In order to evaluate chemerin plasma levels in Balb/c transplanted mice, we collected blood samples of transplanted mice at different time points. Chemerin plasma levels were evaluated by ELISA assay. We first observed that the total body irradiation (TBI) induced a significantly decrease of chemerin plasma concentrations in all transplanted mice (mean levels before TBI = 79,9 ng/ml, range=71,4-88,6 ng/ml; mean levels 24h after TBI = 52,79, range=39,4-85,29 ng/ml) (Fig 1A). Comparing allogeneic transplanted mice to syngeneic transplanted mice, we observed that at GvHD onset, we observed a significant increase in chemerin plasma levels in mice developing GvHD (allogeneic-transplanted mice), compared to the syngeneic group (allo-transplant mean=86,84 ng/ml, range=80,28-91,96 ng/ml versus syn-transplant mean=62,80 ng/ml, range=49,19-82,55 ng/ml) (Fig 1B). Interestingly, chemerin plasma levels resulted significantly higher starting from day + 10 after HSCT, until day +18 (p-value<0,001), because later time-points were influenced by the high mortality of GvHD mice. (Fig 1B).

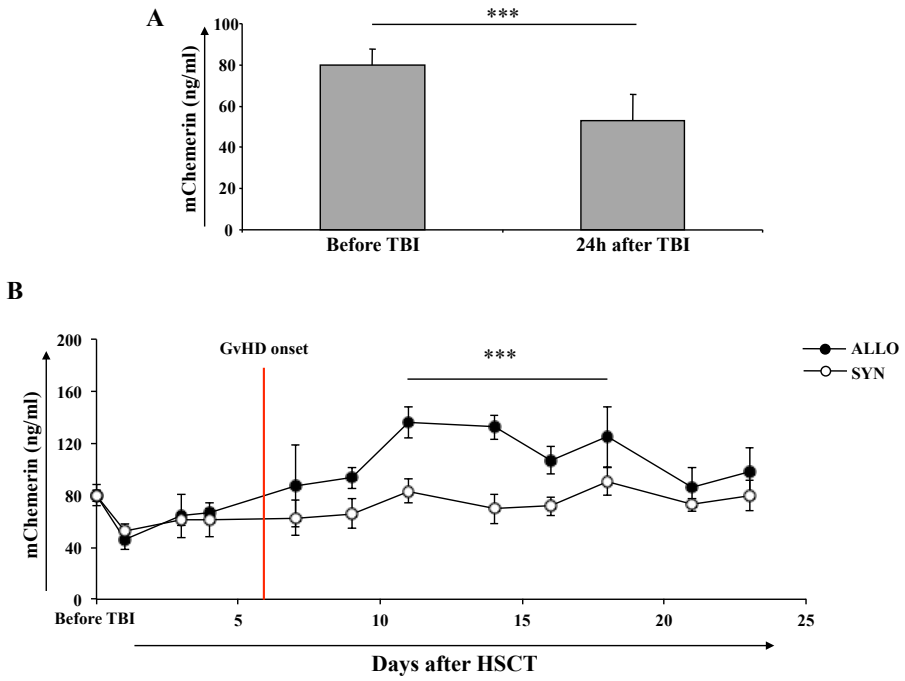

Fig. 1 Chemerin plasma levels significantly decrease after administration of TBI and increase during GvHD. Chemerin plasma levels were monitored before the administration of conditioning regimen (TBI) and 24h after the irradiation. A) Chemerin plasma concentration significantly decrease after TBI in all the mice tested. n=12/group B) Moreover chemerin plasma concentrations were monitored every three day, starting from 24h after the graft. Compared to syngeneic transplanted mice, allogeneic transplanted mice showed an increase of chemerin plasma levels during GvHD, starting from day +10, until day +18 n=6/group. Data are mean ± SEM of two independent experiments; *** p≤0,001

The role of Chemerin/ChemR23 axis in GvHD pathogenesis

With the aim of studying the role of Chemerin/ChemR23 axis in GvHD pathogenesis, we took advantage of ChemR23 knock out (KO) mice as donors or recipient of a model of acute GVHD.

Induction of acute GVHD in WT mice allo-transplanted with ChemR23 KO cells

After demonstrating the increase of chemerin plasma levels at GVHD onset, we started to investigate the role of chemerin in GvHD pathogenesis In this attempt, we performed allogeneic transplantation experiments by using ChemR23-deficient mice. When Balb/c mice were transplanted with $10x10^6$ ChemR23 KO BM cells and $5x10^6$ ChemR23 KO splenocytes, we observed a more severe GvHD compared to mice transplanted with WT cells (Fig 2), as underlined by a worse survival curve (p-value=0,0004) (Fig 2A) and overall GVHD score (Fig 2B). In particular, a strong difference between the two groups could be observed in terms of weight loss and manifestation of diarrhoea both more severe in the KO-transplanted mice (Fig 2B). On the contrary, no differences could be observed between the two groups in terms of skin integrity (data not shown).

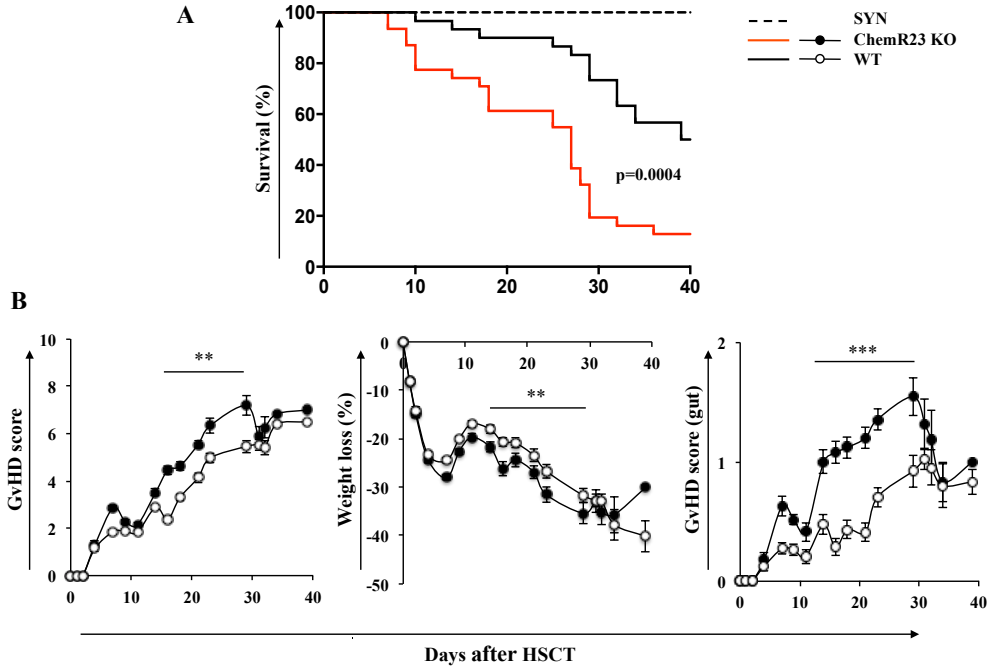

Fig. 2 ChemR23 KO-transplanted mice developed a more severe GvHD compared to wild type-transplanted mice

Lethally irradiated Balb/c mice received both 10x106 bone marrow cells and 5x106 splenocytes, obtained from ChemR23 KO mice or C57BL/6 wild type mice. Survival rate and overall score were measured daily. A) Survival rate analysis showed that ChemR23 KO-transplanted mice significantly increased mortality compared to wild-type transplanted mice. B) Results of the overall score showed that KO-transplanted mice developed a more severe GvHD compared to wild type mice, especially in terms od weight loss and gut score (measure with daily diarrhoea rate) n=45/group. Data are mean ± SEM of three independent experiments; **p≤0,01; *** p≤0,001

Adoptive cell transfer of WT CD11c$^+$ DCs and pDCs in mice transplanted with ChemR23 KO cells

Since intestinal DCs have been proved to be pivotal in the balance between tolerance and active immunity and, in particular, pDCs have been reported to be potent suppressors of antigen-specific immune responses including inhibiting acute GVHD[22], we investigated if these ChemR23$^+$ cell subsets could impact on the disease phenotype observed in our murine model. For this reason, we performed adoptive transfer experiments, in which CD11c$^+$ DCs and sorted pDCs obtained from C57BL/6 WT mice were co-transplanted along with ChemR23 KO bone marrow cells and splenocytes. As shown in figure 3A and 3B, the administration of WT CD11c$^+$ DCs as well as pDCs did not induce any significant change in the survival and in the overall disease activity score observed in ChemR23 KO-transplanted mice. In detail, ChemR23 KO-transplanted mice infused or not with both CD11c+ DCs or pDCs, starting from day +18 after transplant, developed acute GvHD more severe in terms of weight loss and diarrhea compared to WT transplanted mice (Fig 3B).

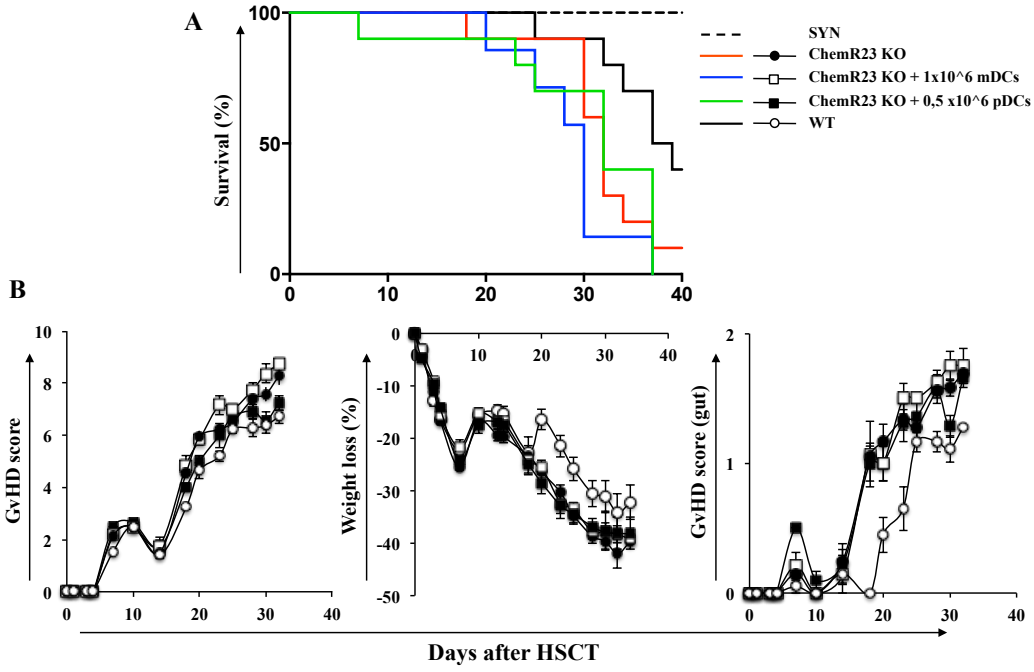

Fig. 3 Adoptive transfer of CD11c$^+$DCs or pDCs do not improve GvHD in ChemR23 KO-transplanted mice

Adoptive transfer experiments were performed transplanting ChemR23 KO cells into lethally irradiated Balb/c mice with in addition 1x10^6 CD11c$^+$ DCs or 0,5x10^6 pDCs obtained from wild type mice. A) Survival rate analysis showed that ChemR23 KO-transplanted mice added with both CD11c$^+$ DCs (red line) or pDCs (green line) did not improve GvHD in ChemR23-deficient transplanted mice. B) Similar results were obtained by overall score analysis, weight loss and gut GvHD. n=10/group

Data are mean ± SEM of two independent experiments.

Induction of acute GVHD in ChemR23 KO mice allo-transplanted with WT cells

To study the effect of ChemR23^{+} KO cells remaining in the recipient mice after condition regimen, we next transplanted donor cells obtained from Balb/c mice into WT or ChemR23 KO C57BL/6 recipients. In this setting, WT recipient mice started to die twenty-six days after transplantation (Fig 4A). On the contrary, ChemR23-deficient recipients developed severe GvHD early after transplantation (starting from day +7 after transplantation), resulting in a rapid mice death (before day +20). GVHD scoring confirmed the survival data, with ChemR23 KO mice reaching a high overall GvHD grade in few days (Fig. 4), with a prevalent involvement of the GI tract (Fig 4B).

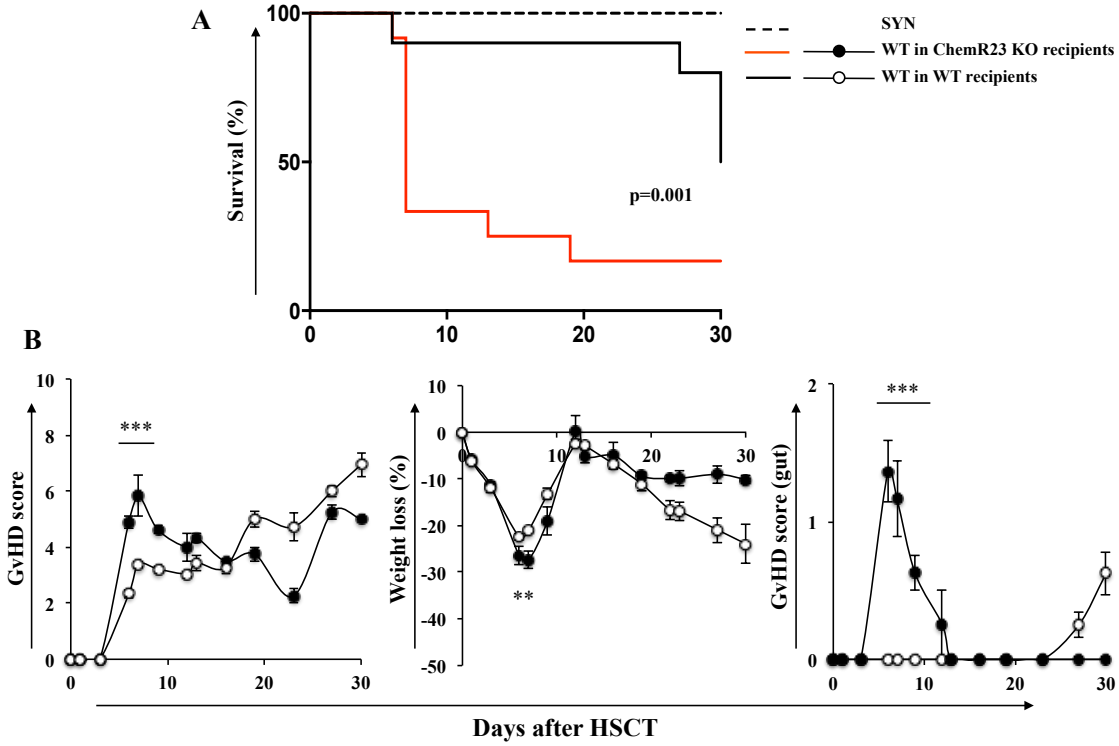

Fig. 4 ChemR23 KO recipients developed a severe GvHD early after transplantation
Lethally irradiated C57BL/6 mice and ChemR23 KO mice received both 10x10^{6} bone marrow cells and 20x10^{6} splenocytes, obtained from Balb/c wild type mice. Survival rate and overall score were measured daily. A) Survival rate analysis showed that ChemR23 KO recipient mice significantly increased mortality rate compared to wild-type mice and GvHD occurred early after transplantation. B) Overall score showed that KO mice developed a more severe GvHD compared to wild type mice, with a significantly increased of weight loss and gastrointestinal score (measure with daily diarrhoea rate) n=10/group.
**p≤0,01; *** p≤0,001

The role of Chemerin/CCRL2 axis in GvHD pathogenesis

Induction of acute GVHD in WT mice allo-transplanted with CCRL2 KO cells

To further investigate the role of chemerin in GvHD pathogenesis, we then focused on the chemerin/CCRL2 axis, taking advantage of CCRL2-deficient mice as allogeneic BM and splenocytes donors. In this set of experiments we did not observe any difference in terms of survival, in Balb/c mice allogenically transplanted with C57BL/6 WT compared to CCRL2 KO cells (Fig 5A). On the contrary, the analysis of GVHD activity revealed a more severe pathology in the CCRL2 KO-transplanted group compared to WT, with a higher GVHD score in CCRL2 KO transplanted mice especially in the GI tract (Fig 5B).

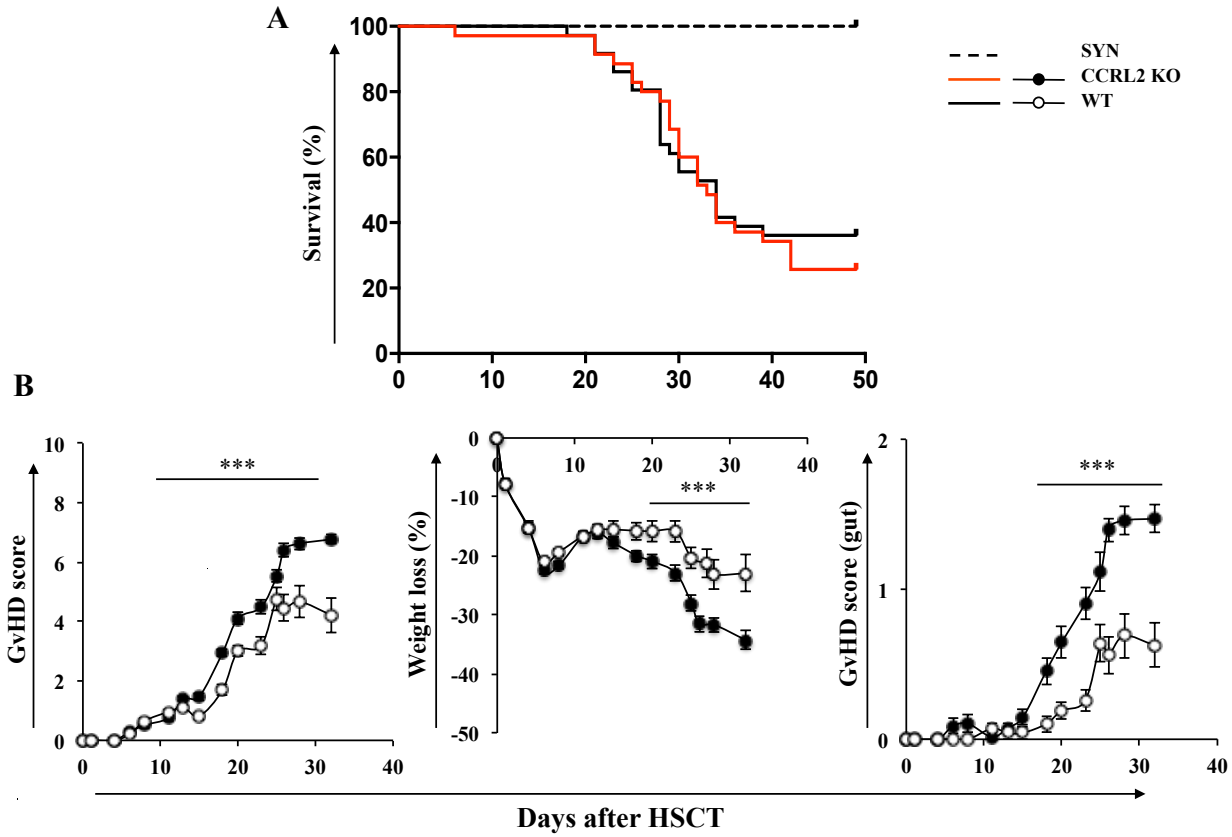

Fig. 5 CCRL2 KO-transplanted mice developed a more severe GvHD compared to WT transplanted mice
Lethally irradiated Balb/c mice received both 10x106 bone marrow cells and 5x106 splenocytes obtained from CCRL2 KO mice or C57BL/6 wild type mice. Survival rate and overall score were measured daily.
A) Survival rate analysis showed that the mortality rate of CCRL2 KO-transplanted mice was similar to WT transplanted mice. B) Results obtained from the overall score analysis showed that KO transplanted mice developed a more severe GvHD compared to WT mice, confirming by weight loss and gut score (measured with daily diarrhoea rate) n=35/group
Data are mean ± SEM of three independent experiments; *** p≤0,001

Induction of acute GVHD in CCRL2 KO mice allo-transplanted with WT cells

To study the effect of CCRL2, which is expressed at high levels on the recipient endothelial cells where it has been recently described to act as a chemokine concentrator, we transplanted donor cells obtained from Balb/c mice into WT or CCRL2 KO C57BL/6 recipients. GVHD evaluation in this transplantation setting showed that CCRL2 recipient mice, as well as in the case of ChemR23 KO recipients, developed GvHD very early after transplantation (during the first week) and showed a significantly higher mortality compared to WT transplanted mice (Fig 6). Due to the high and rapid mortality observed, it was not possible to statistically compare GVHD activity between the two experimental groups. Since an influence of CCRL2 deficiency on chemerin plasma levels has been recently described in different mice models of inflammatory diseases, we evaluated chemerin levels in WT and CCRL2 KO allotransplanted recipient mice. This analysis showed that, as we previously observed, chemerin significantly decreased 24h after conditioning regimen in WT recipients (mean before TBI= 61,89 ng/ml, range=47,01-73,2 ng/ml; mean 24h after TBI=47,29, range=39,6-54,3 ng/ml; p=0,0004) (Fig 7A). On the contrary, chemerin plasma levels in CCRL2 KO recipients did not show any decrease after TBI (mean after TBI=74,02 ng/ml, range=62,75-88,26 ng/ml) (Fig 7A). Moreover, chemerin plasma levels resulted significantly higher along all the monitored time frame in CCRL2 KO compared to WT recipient mice ($p \leq 0,01$) (Fig 7B).

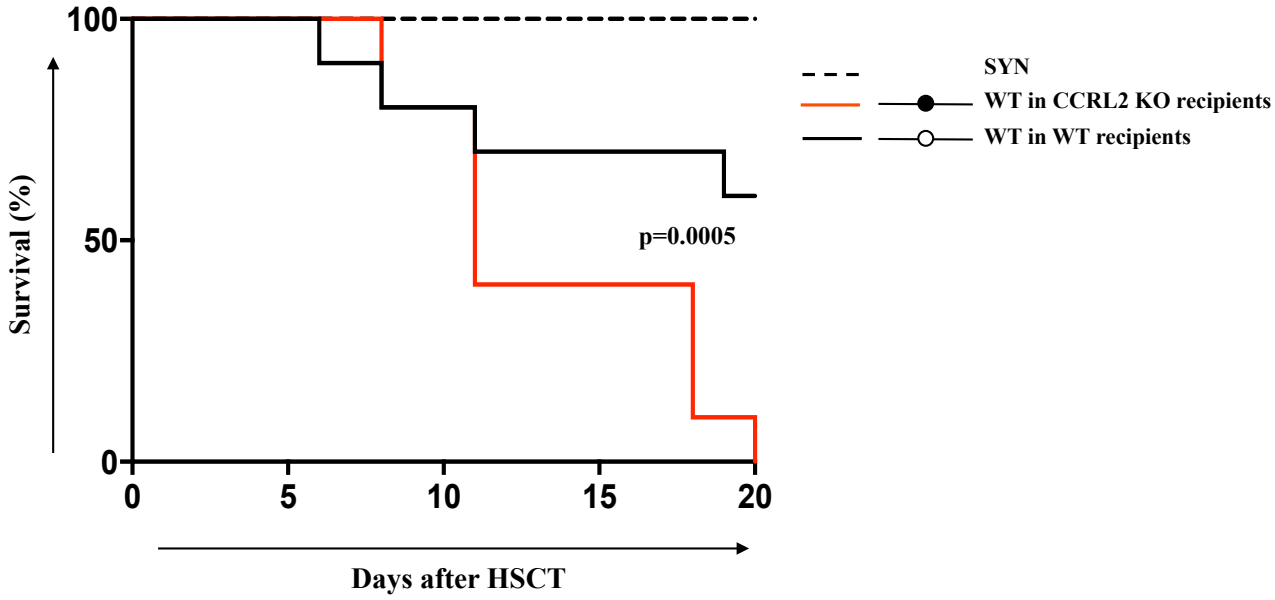

Fig. 6 CCRL2 KO recipients developed a severe GvHD early after transplantation
Lethally irradiated C57BL/6 mice and CCRL2-deficient recipients received both $10x10^6$ bone marrow cells and $20x10^6$ splenocytes, obtained from Balb/c wild type mice. Survival rate and overall score were measured daily. A) Survival rate analysis showed that CCRL2 KO recipient mice significantly increased mortality compared to wild-type mice and GvHD occurred early after transplantation. n=10/group.

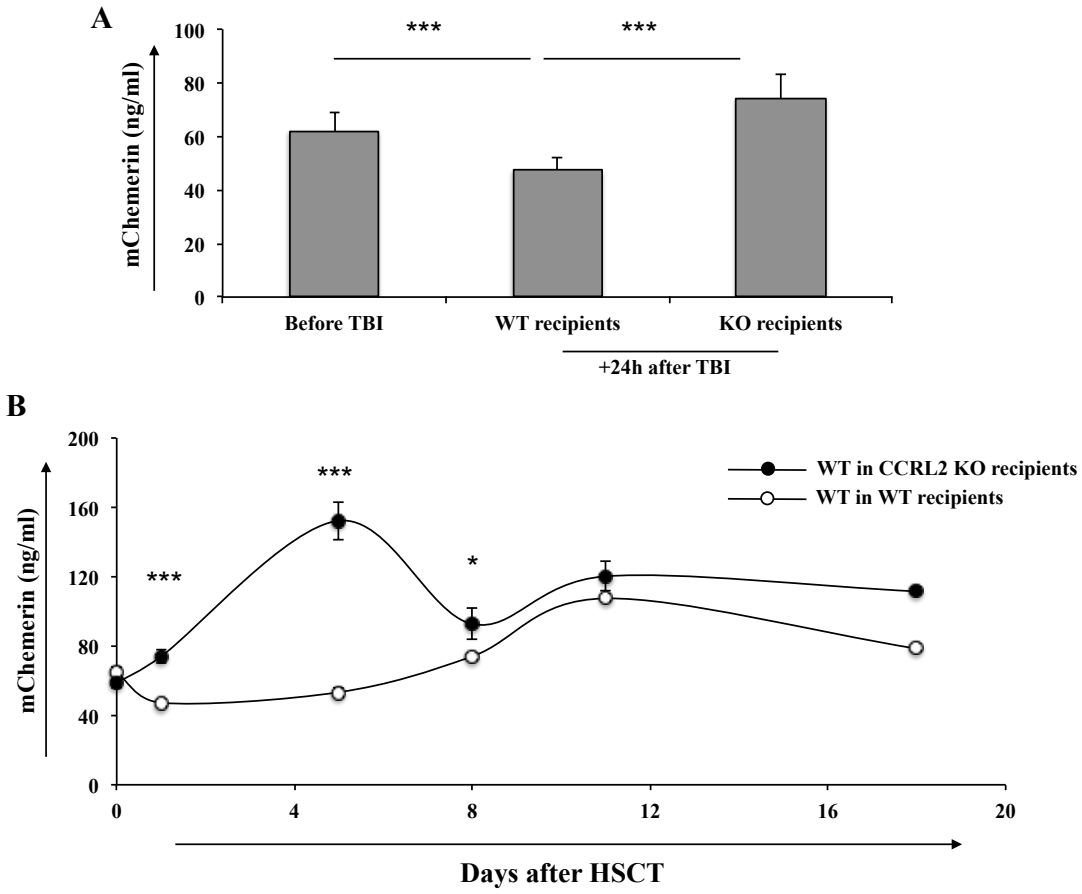

Fig. 7 Chemerin plasma levels do not decrease after administration of TBI in CCRL2 deficient recipients and remain higher during GvHD compared to WT mice.

Chemerin plasma levels were monitored before the administration of conditioning regimen (TBI) and 24h after the TBI. A) Chemerin plasma concentration significantly decreased after TBI in WT recipients mice but did not decrease in CCRL2 recipients. n=3/group B) Moreover chemerin plasma concentrations were monitored every three day, starting from 24h after HSCT. Compared to allogeneic WT, CCRL2 KO recipients showed an increased of chemerin plasma levels early after HSCT, which remain higher during all the time-frame monitored. n=3/group.

Data are mean ± SEM of two independent experiments; * $p \leq 0,05$ *** $p \leq 0,001$

DISCUSSION

HSCT represents the treatment of choice for many malignant and non-malignant disorders.

However, its efficacy is often impaired due to the development of post-transplant complications, such as graft rejection, disease relapse, infections occurrence and GvHD. In particular, GvHD represents the major cause of morbidity and mortality after HSCT. Corticosteroids which are used as first line therapy in patients experiencing GvHD, are only partially effective and the mortality rate is still high in the case of steroid-refractory GVHD, since a second line of therapy has not been established yet[23, 24]. For this reason, a better understanding of GvHD pathogenesis can lead to the development of innovative therapeutic strategies. In particular, the identification of the molecular mechanisms involved in controlling the expression of chemokines and their receptors in GvHD may provide efficient strategies to improve disease management. Chemerin has been recently identified as a chemotactic protein involved in both the initiation and resolution of inflammation and is able to modulate the migration of ChemR23-expressing cells, such as DC, pDC, macrophages and NK cells, which play a crucial role in GvHD pathogenesis[25, 26, 27, 28, 29].

In order to evaluate the role of chemerin/chemerin receptors axis in GvHD pathogenesis, we performed a murine model of transplantation and acute GVHD. In particular, since chemerin deficient mice are not commercially available, we taking advantage of mice lacking the chemerin receptors, ChemR23 and CCRL2, used both as transplant donors or recipients. Data obtained using ChemR23 KO animals as donors, showed a more severe GvHD compared to WT transplanted mice with a significantly higher overall score, weight loss percentage and gastrointestinal tract involvement. These results suggested that ChemR23-expressing cells present in the graft could have a role in GvHD pathogenesis. In order to understand which cell subset could be responsible of the GvHD phenotype observed transplanting ChemR23 KO cells, we performed adoptive transfer experiments with WT $CD11c^+$ DCs or pDCs, which are both involved in gut tolerance during GvHD[25]. Both DC subsets seemed not to be involved in the observed GvHD phenotype, since the addition of WT DCs to the transplanted graft did not succeed in decreasing GVHD severity. Other experiments are needed to better understand if different ChemR23-expressing cell subsets could mediate this protective effect. In this attempt, adoptive transfer of NK cells and macrophages, which have been described to be able to ameliorate GvHD, will be performed[26, 27 28, 29]. Moreover, we also evaluated the role of recipient ChemR23-expressing cells, in GVHD induction. The survival rate and the overall GVHD score showed that KO recipients

developed GvHD earlier and in a more severe way compared to WT mice. Even in this case, gastrointestinal tract was the most involved GVHD target organ. On the overall, these data suggest that ChemR23 KO mice, used as graft donors or recipients, are characterised by a more severe GvHD, confirming that chemerin/ChemR23 axis could have a role in GvHD pathogenesis, especially in the gut.

Importantly, recent findings showed that ChemR23 receptor is able to heterodimerize with the CXCR4 and CCR7 receptors, which are crucial in mediating leukocyte infiltration in GvHD target organs. The ChemR23/CXCR4 or CCR7 heterodimerization can significantly affect the migration of $CXCR4^+$ and $CCR7^+$ cells[30]. Our results obtained with ChemR23 KO donors suggest that the observed GvHD phenotype could be partly attributed to the ability of ChemR23 to affect other chemokine pathways, thus increasing the cellular response to the inflammatory microenvironment.

In the attempt to evaluate the involvement of the chemerin/CCRL2 axis in GvHD pathogenesis, we performed allogeneic transplantations using CCRL2-deficient mice. When CCRL2 KO mice were used as donors, we did not observe any significant difference in terms of survival rate, while we observed a more severe GvHD in terms of overall disease score due to an higher weight loss and GVHD score in the gut, compared to WT mice. Data obtained from the use of CCRL2 KO mice as transplant recipients showed that, CCRL2-KO mice developed a severe GvHD, with an increased mortality, compared to WT mice. Data obtained from CCRL2 KO mice revealed that similarly to the ChemR23, also chemerin/CCRL2 axis is involved in GvHD pathogenesis. CCRL2 is a member of the atypical G protein-coupled chemokine receptor which do not directly induce cell migration, but is able to indirectly control leukocyte recruitment by shaping chemokine gradients in tissues through degradation, transcytosis or local concentration of their cognate ligand[31, 32]. In particular, CCRL2 receptor, expressed by activated endothelial cells, is able to bind and present chemerin to $ChemR23^+$ cells, consequently decreasing chemerin concentration in the bloodstream[15, 18, 33]. Therefore, we evaluated chemerin plasma levels in our GvHD model, performed in CCRL2 recipient mice. Interestingly, while in WT mice chemerin plasma levels significantly decreased after irradiation, in CCRL2 deficient mice chemerin plasma levels remained elevated after TBI. Higher chemerin plasma levels were observed in CCRL2 recipient mice also during GvHD course. Interestingly, all these data suggest that the absence of CCRL2, can induce an increase in chemerin plasma concentration, perhaps due to a decreased binding of chemerin to CCRL2-expressing activated endothelium, affecting the migration of ChemR23-expressing

cells. However, CCRL2 receptor function has not been fully understood yet, and, for this reason, other migration mechanisms can be affected by the deficiency of the CCRL2 receptor.

All together these results suggest that, chemerin/chemerin receptors axis can be involved in GvHD pathogenesis, but further experiments are needed to better characterised the mechanisms underlying their activity in order to understand if their modulation can represent a good strategy to improve the management of GVHD in HSCT patients.

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PART 2

ELUCIDATING THE PROPERTIES OF MESENCHYMAL STROMAL CELLS: FROM THE CLINICAL EVIDENCE TO THE IDENTIFICATION OF NOVEL MOLECULES UNDERLYING THEIR IMMUNOMODULATORY ACTIVITY

Chapter 3

Mesenchymal stromal cells for the treatment of graft-*versus*-host disease: understanding the *in vivo* biological effect through patient immune monitoring

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Letter to the editor

Dear editor,

mesenchymal stem cells (MSC) are emerging as a helpful therapeutic tool for the treatment of Graft *versus*-Host Disease (GvHD) (1-2). Nevertheless, efficacy studies have been scarcely corroborated by immune monitoring of patients' response after cell infusion. The analysis of clinical samples from GVHD patients treated by MSC represents an unique possibility to understand the events leading *in vivo* to GVHD improvement. With the aim to provide the scientific basis for novel and better-tailored protocols for managing steroid-resistant GvHD, we investigated at a cellular and molecular level the disease course, before and after MSC infusion. Ten patients with post-transplant GvHD were enrolled in the study. Details of patients characteristics are reported in Table 1. Six patients were enrolled for aGvHD and 4 for overlap syndrome or active cGvHD resistant to first line steroid-based therapy.

All patients were treated with multiple infusions of third-party BM-derived MSC. Patients received from 2 to 5 cell infusions. The median dose of cells infused was $1x10^6$/kg (range=0.9-$2.9x10^6$/Kg). Consistently with our previous study [1], we confirmed a response rate of around 70% overall, with a complete response in 30% of the patients. Moreover, it is very important to underline that even patients presenting a partial response to MSC, could taper ongoing treatment without the need of additional lines of treatment, thus lowering the burden of immunosuppression in a very critical phase after transplantation with high risk of infections.

To corroborate clinical observations about the response of enrolled patients to MSC therapy, we monitored GvHD course at a molecular level, taking advantage of two biomarkers for aGvHD: IL-2Rα, and TNFRI. These markers, recently identified and validated by the Ferrara's group [2], present high differential expression between transplanted patients developing or not GvHD.

ELISA monitoring of TNFRI (Figure 1A) showed that mean plasma levels of aGvHD patients before MSC infusions were 3.89 ng/ml (range=0.91-10.58, n=6), significantly higher than Healthy Donors (HD) (mean level=0.74 ng/ml, range=0.58-0.95, n=12, $p<0.01$). Interestingly, also TNFRI plasma concentrations of patients with active cGvHD resulted, before MSC infusions, higher than HD (mean level=4.2 ng/ml, range=1.21-8.53, n=4, $p<0.01$). After MSC infusions, patients with steroid-resistant GvHD completely responding (CR) to therapy (n=2, Figure 1B) showed a strong and persistent decrease of TNFRI plasma levels at day +7, +14, which decreased even more significantly at day +28. On the contrary, 4/5 GvHD patients responding partially (PR) to MSC-based therapy (Figure 1B) showed a transient decrease of TNFRI, which however never

reached values typical of HD (mean level at day 7=2.07 ng/ml, range=1.59-2.47; mean at day 14=1.8 ng/ml, range=1.26-2.9; mean at day 28=2.59 ng/ml, range=2.07-3.45, n=4). 1/5 partial responder patient (unique patient number, UPN#10), did not present any decrease of TNFRI levels even if the GvHD clinical score slightly improved (data not shown). Non responder (NR) patients (n=3, Figure 1B), showed stable or even increasing levels of TNFRI compared to pre-MSC values. Consistent with TNFRI, IL2Rα plasma levels showed the same trend. More in detail, aGvHD patients presented levels of IL2Rα before MSC infusion (mean level=1.58 ng/ml, range=0.72-2.82) significantly higher (p=0.001) than HD (mean level=0.39 ng/ml, range=0.28-0.51, n=12) (Figure 1A). Moreover, IL2Rα resulted a suitable GvHD marker also in the case of active cGvHD, since cGvHD patients showed pre-infusion plasma levels strongly increased (mean level=2.44 ng/ml, range=0.5-6.03, p=0.02) compared to HD. After MSC therapy, IL2Rα plasma levels stably decreased at day 7, 14, 28 in both CR patients with steroid-resistant GvHD (Figure 1B). In all PR patients, we observed a partial decrease at day 7 and 14 post infusion of IL2Rα plasma levels, however holding steady above HD values (mean level at day 7=1.06 ng/ml, range=0.45-1.94, mean level at day 14=0.93 ng/ml, range=0.52-1.66). IL2Rα levels raised again at day 28 in case of GvHD reactivation (mean level at day 28=1266, range=589-3315). In accordance with TNFRI monitoring, NR patients showed stable or increasing IL2Rα plasma levels after MSC infusion compared to pre-therapy (Figure 1B). To further evaluate GvHD course after MSC infusion in patients affected by GvHD with skin involvement, elafin plasma levels were monitored. Elafin is an epidermal proteinase inhibitor, induced by TNF-α, which significantly increases in the plasma of patients with severe skin GvHD, as previously reported [3]. Pre-MSC levels of elafin resulted increased in patients with moderate to severe skin GvHD (>stage2; UPN#1;2;4,6,8,9) (mean level=56.23 ng/ml, range=13.31-199.21), compared to HD (mean level=10.30 ng/ml, range=6.79-12.7, n=8) (Figure 1A). In accordance with GvHD clinical course, elafin plasma levels in UPN#1 and 2, who responded completely to MSC infusion (Figure 1B), decreased at day 7, 14, 28 post therapy, below HD mean values (mean FD=2.7). PR patients (UPN#4,6) showed decreasing elafin levels at day 7 and 14 post-MSC (level range at day 7=9.37-10.23, level range at day 14=5.39-11.70), which raised up at day 28, upon reactivation of skin GvHD in UPN#4. On the contrary, in UPN#6, who experienced, at day 28 after therapy, a recurrence of GvHD with involvement of the sole gastro-intestinal tract, elafin levels remained persistently under the mean levels of HD. In NR patients (UPN#8,9) elafin levels stayed stably above mean HD values (mean elafin values post MSC=147.22 ng/ml, range=38.25-200). Overall, TNFRI, IL2Rα and elafin proved to be reliable

and easily measurable GvHD markers, strongly correlating with disease activity after MSC infusions. Moreover, these molecules seem to be very specific, since they are not apparently influenced by the occurrence of concomitant viral infections such as in the case of UPN#5 and 6 (data not shown). The monitoring of these markers after MSC infusion could represent a crucial tool to identify patients that are not responding to therapy or those that, following a temporary GvHD remission, experience disease reactivation. Interestingly, we noted that NR patients showed pre-MSC levels of TNFRI, IL2Rα and elafin higher than responding patients, thus confirming the prognostic value of these markers [2, 3]. It is interesting to note that patients with similar GVHD clinical score present different pre-MSC levels of all the three biomarkers, and that these levels could be more reliable sensors of the ongoing inflammatory process and predictive of patients response to GVHD therapy. In order to clarify this point, our future purpose is to increase the number of treated patients thus possibly establishing a correlation between biomarker plasma levels and GVHD severity/degree in patients with the same GVHD clinical score.

Moreover, in order to investigate the effect of MSC infusions on lymphocytes circulating in the PB, we analyzed the ratio between the pro-inflammatory, GvHD-promoting TH1 and TH17 subsets (6-7) and the anti-inflammatory Treg population [4, 5]. In CR patients we observed a change in CD4+ T-cell subsets after therapy: Tregs increased while Th1 and Th17 populations decreased. These variations resulted in a modification of TH1/Treg and TH17/Treg ratios, in particular, Th1/Treg ratio decreased up to 4.2 times and Th17/Treg ratio decreased up to 6 times (see supplementary). This observation is in accordance with recent reports from *in vitro* studies describing the ability of MSC to induce Tregs *in vitro* (10-11) and *in vivo* after infusion in patients with severe and treatment-refractory systemic lupus erythematosus [6]. In PR patients we noticed a transient increase of Treg cells after MSC infusion, which, unlike CR patients, was associated with stable or increasing proportions of TH1 and TH17 in the PB. Interestingly, NR patients showed TH1/Treg and TH17/Treg ratios significantly higher than those of CR and PR patients before and after MSC infusion. However, the levels of CD4+ T cells are strongly influenced by other causes of inflammation such as post-transplant opportunistic infections. This was clearly shown by monitoring of UPN#6, a partial responder patient, who experienced a viral infection few days after MSC infusion. In this patient, concomitantly with clinical improvement of GvHD a strong decrease of GvHD plasma markers was observed. However, at day 7 after therapy, unless GvHD improved, TH1/Treg and TH17/Treg ratios strongly increased, probably due to the ongoing infection. The fact that CD4+ T cells subsets could be severely influenced by other inflammatory events, frequently

observed in the early post-transplant period, and the high inter-patients variability of their percentages in the PB, suggests caution in using these parameters to monitor patients' response to MSC therapy.

Overall, MSC represent an effective therapeutic tool for the treatment of steroid-resistant GvHD. To support this clinical observation, we demonstrated that MSC, upon infusion, are able to convert an inflammatory environment to a more physiological one, both at the cellular level, promoting the increase of Treg circulating in the peripheral blood, and at the molecular level, diminishing the concentration of inflammatory molecules. In attempt to clarify the mechanisms underlying this process, we analysed the plasma levels of several immunosuppressive MSC-secreted mediators such as TSG6, IL-10, PGE2, VEGF and TGFβ1 (13-14), without nonetheless observing any significant variation that could be correlated with patients response to MSC-therapy (data not shown). This observation does not exclude a possible role of these molecules in tuning inflammation in GvHD involved tissues.

Only a very close clinical and immunological monitoring of GvHD patients infused with MSC will clarify the immunomodulatory properties and the *in vivo* activity of MSC in regulating alloreactions for treating GvHD. These observations in larger cohorts of patients will provide the rational to design better clinical protocols aiming at maximizing MSC-based therapies. Moreover, we suggest TNFRI, IL2Rα and elafin as biomarkers for monitoring patients' response to MSC infusions giving precious indications to design patient-tailored anti-GVHD therapy. This issue represents an urgent clinical need since, at the moment, there is no consensus about the stratification of patients not responding to first-line treatments.

Patients characteristics

UPN	Age (Years) and sex	Diagnosis	Type of Transplantation HLA matching source	Conditioning regimen	GvHD at MSC infusion	N° of MSC Infusions and cell Dose/Kg	IS at MSC infusion	IS at day +28	Response to MSC	Response duration (days)
1	6M	ALL2°CR	Unrelated 9/10 BM	TBI+VP16	Acute Skin grade II	3 $1.25x10^6$	Steroid	Steroid	Complete	95
2	4M	ALL2°CR	Related 10/10 BM	TBI+VP16	Chronic (overlap syndrome) Skin+mucosae grade II	2 $1.5x10^6$	Steroid	Steroid+ MMF	Complete	49
3	16M	ALL1°CR	Unrelated 8/10 PB	TBI+VP16	Acute Skin+liver grade III	2 $0.94x10^6$	Steroid	Steroid	Partial	25
4	33M	SAA	Unrelated 10/10 PB	CY	Acute Skin+gut grade III	2 $1x10^6$	Steroid+ etanercept	Steroid+ MMF+ CSA+ etanercept	Partial	28
5	23M	MDS	Unrelated 10/10 BM	BU+CY+MEL	Acute Gut grade III	3 $1.1x10^6$	Steroid	Steroid+ etanercept	Partial	13
6	35F	MNGIE	Unrelated 9/10 BM	BU+FLU	Chronic (overlap syndrome) Skin+gut grade III	5 $1.1x10^6$	Steroid+ etanercept	Steroid	Partial	17
7	6M	ALL3°CR	Related 8/8 PB	FLU+TREO	Chronic Liver+mucosae grade III	2 $0.96x10^6$	Steroid+ sirolimus	Steroid+ sirolimus	None	/
8	24M	AML	Unrelated 8/10 PB	FLU+MEL	Chronic Skin+mucosae grade III	2 $1x10^6$	Steroid+ imatinib+ ECP	Steroid+ CSA+ pentostatin	None	/
9†	26F	ALL1°CR	Unrelated 10/10 PB	TBI+CY	Acute Gut+liver grade IV	2 $1.9x10^6$	Steroid+ FK506+ MMF+ ECP	Steroid+ MMF+ pentostatin	None	/
10	19F	ALL1°CR	Unrelated 9/10 PB	TBI+CY	Acute Gut+liver grade IV	3 $1x10^6$	Steroid+ FK506	Steroid+ FK506	Partial	14

† Died before the end of follow up

UPN, unique patient number; BM, bone marrow; PB, peripheral blood; ALL, acute lymphoblastic leucemia; SAA, severe aplastic anemia; MDS, myelodysplastic syndromes; MNGIE, mitochondrial neurogastrointestinal encephalopathy; AML, acute myeloblastic leukemia; TBI, total body irradiation; VP-16, etoposide; CY, cyclophosphamide; BU, busulfan; MEL, melphalan; FLU, fludarabine; TREO, treosulfan ECP, extracorporeal photochemotherapy; FK506, tacrolimus; MMF, mycophenolate mofetil; CSA, cyclosporine.

Table 1

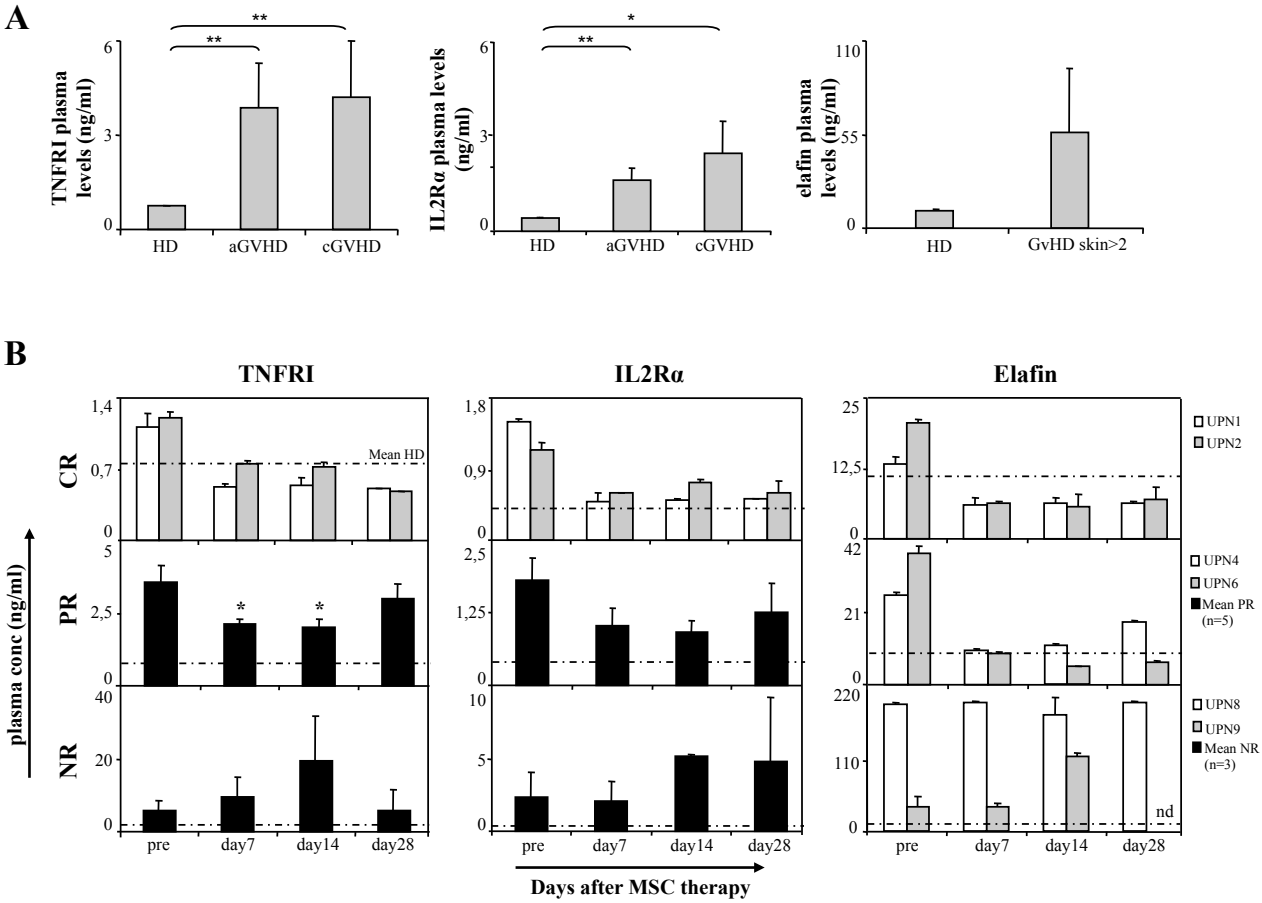

Figure 1

Figure 1. Monitoring of TNFRI IL2Rα and elafin in the plasma of GvHD patients before and after MSC treatment. (A) Plasma marker levels were detected in patients with aGvHD and cGvHD before MSC infusions (**p<0.01 and *p<0.05 vs. HD). Mean levels and SEM are represented. **(B)** Plasma marker concentrations were monitored by ELISA assay before (pre) MSC infusions and at day 7, 14 and 28 after treatment in complete responder patients (CR), partial responder patients (PR) and non responder patients (NR). Mean marker concentration in healthy donors (HD) was represented in each graph as dotted line. UPN=unique patient number.

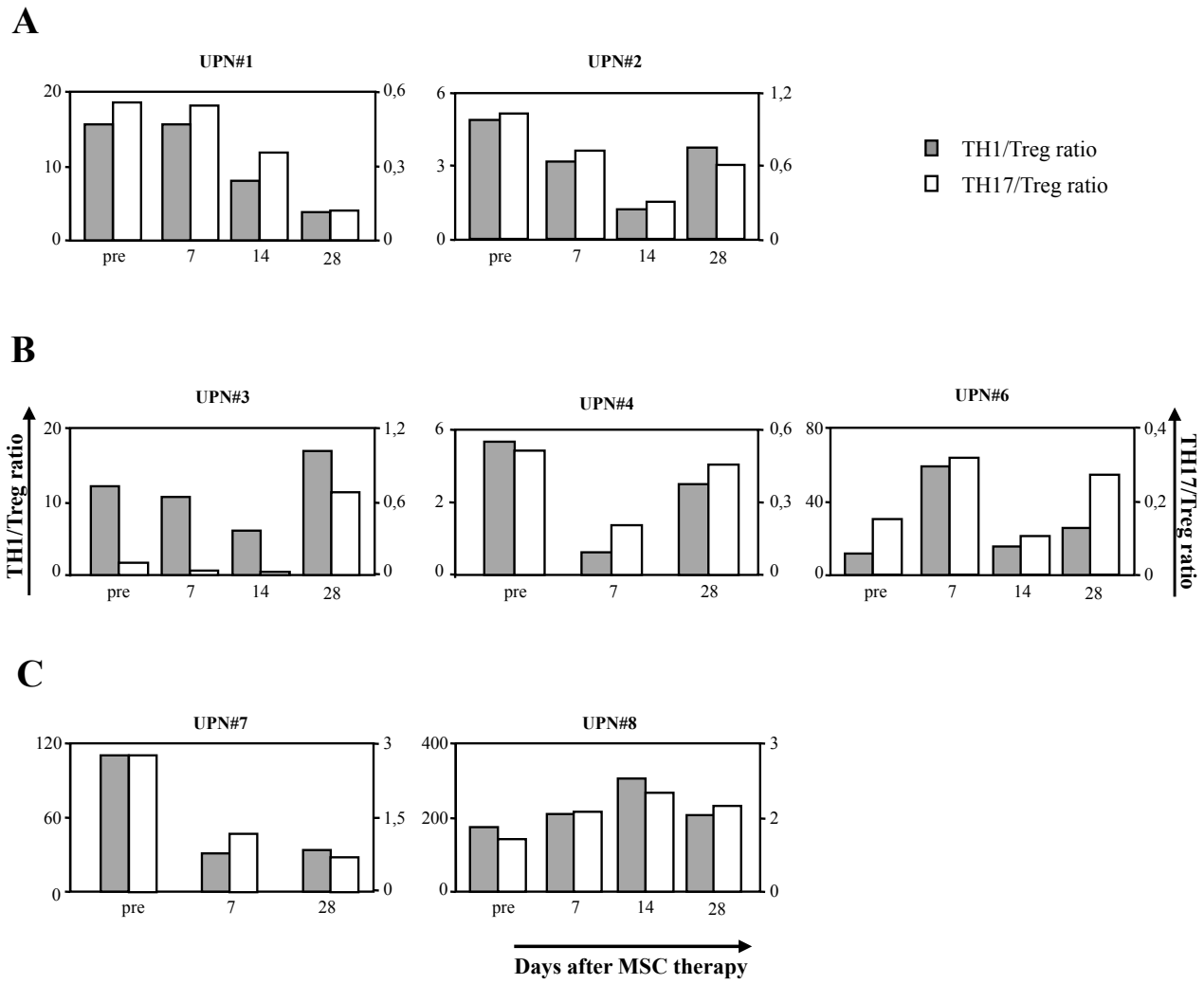

Figure 1S

Supplementary Figure 1. Monitoring of TH1/Treg and TH17/Treg ratios in the peripheral blood of GvHD patients treated with MSC. TH1/Treg ratio (grey columns) and TH17/Treg ratio (white columns) were calculated in the peripheral blood of GvHD patients before and at day 7, 14, 28 after MSC infusion. Measurements from 2 responding patients (**A**), 3 partially responding patients (**B**) and 2 non responding patients (**C**) are shown. UPN=unique patient number.

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Chapter 4

Chemerin production by Mesenchymal Stromal Cells (MSC) is influenced by culture conditions

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ABSTRACT

The involvement of chemokines in recruiting leukocytes to the inflammatory sites has designated a number of chemokine receptors as attractive targets for therapeutic applications in the field of inflammatory diseases. Chemerin is a chemotactic protein that, in its active form, binds to the G-protein coupled receptors ChemR23, CCRL2 and GPR1, and promotes chemotaxis. Different studies have demonstrated that chemerin is involved in both initiation and resolution of inflammation. Therefore, manipulation of the chemerin/ChemR23 axis may represent a novel therapeutic approach for treating several inflammatory pathologies, such as Graft-*versus*-Host Disease (GvHD).

MSC are multipotent cells, widely used for treating inflammatory diseases with various efficacy depending on culture conditions and treatment schedules. In particular, up to now MSC are cultured for clinical purposes both with 5% of Platelet Lysate and 10% of FBS. At the moment the biology and the mechanisms of action of MSC have been not fully understood. The aim of our study was to evaluate chemerin production by MSC under different culture conditions.

MSC cultured with FBS (FBS-MSC) are able to produce chemerin under basal conditions and their production is enhanced after stimulation for 72 hours with inflammatory cytokines. Interestingly, MSC cultured with platelet lysate (PL-MSC), which are currently used for the treatment of patients with inflammatory disorders, produced high amount of chemerin under basal conditions and its production is strongly increased after stimulation with inflammatory cytokines. Moreover, FBS-MSC, as well as PL-MSC, basally express chemerin receptors ChemR23, CCRL2 and GPR1. Chemerin is secreted by different cell subsets as a precursor and is converted into its active form through the proteolitic cleavage of the last six amino acids at the C-terminal domain. Chemerin produced by MSC was isolated from cell culture after stimulation with inflammatory cytokines and purified chemerin was used to perform biochemical and functional analysis. Migration assays showed that MSC-derived Chemerin (MSC-Chem) is able to induce the migration of ChemR23-expressing cells.

All these data suggest that when infused *in vivo*, during an inflammatory event, MSC are able to produce chemerin, which, could be activated through the proteolitic cleaveage by serine and cysteine proteases, highly expressed in an inflammatory microenvironment.

INTRODUCTION

Mesenchymal Stromal Cells (MSC) are multipotent stem cells characterised by a fibroblast-like morphology and the ability to differentiate into the mesengenic lineages, such as adipocytes, osteocytes and chondrocytes[1, 2]. Due to their ability to modulate the immune response [2, 3, 4, 5], MSC are widely used for treating many inflammatory disorders, such as Systemic Lupus Erithematosus[6], diabetes[7], Crohn Disease[8] and Graft-*versus*-Host Disease (GvHD)[9, 10, 11]. Several soluble molecules are involved in suppression of immunity mediated by MSC, such as indoleamine-2,3-dioxygenase (IDO)[12], heme oxygenase-1[13], prostaglandin E2 (PGE2)[14], and human leukocyte antigen G (HLA-G5)[15], among others. Although, in the last years, many works described several mechanisms by which MSC are able to modulate the immune response, all the mediators identified are not able to fully describe their immunosuppressive activity. Although many clinical trials infusing MSC as treatment of inflammatory diseases are ongoing, data obtained from different centre are still controversial and there is an urgent need to standardise protocols in terms of cell preparation and infusion conditions[16]. For example, in our centre, a phase I/II clinical trial has been recently closed, in which 40 steroid-refractory GvHD patients were treated with PL-MSC[11, 17]. Platelet lysate which is obtained from the lysis of platelets is rich of growth factors and can be used for cell culture instead of fetal bovine serum. The use of PL for clinical purpose reduces the risk of immune reactions against xenogeneic proteins and the eventual transmission of prions. Moreover, PL-MSC can be early available for administration since 2-3 passages are sufficient to obtain a purified cell line, compared to FBS-MSC that need at least 4-5 passages of culture. Despite differences between these culture systems, both FBS and PL are currently used to prepare MSC and additional studies are needed to understand the different immunomodulatory features of PL and FBS-MSC. Up to now, both PL and FBS-MSC are used as patients therapy regardless their potential differences in terms of immunomodulatory activity [9, 18].

Chemerin is synthesized as a secreted precursor, prochemerin, which is poorly active, but converted into a full agonist of ChemR23 through the proteolitic removal of the last six-seven amino acids at the C-terminal domain by serine and cysteine proteases of coagulation, fibrinolytic and inflammatory cascade[19]. Prochemerin is produced from many tissues, including the spleen, the lymph nodes, and the epithelia. Interestingly, chemerin is locally produced at high levels in lesions from autoimmune pathologies[20, 21]. Activated chemerin was reported to bind with high affinity one G protein-coupled receptor, with seven trans-

membrane domains, namely ChemR23. Recent data demonstrated that chemerin is also able to bind other two receptors, CCRL2 [22]and GPR1[23], which were recently classified as members of the atypical G protein-coupled receptors family due to their inability to induce migration in response to chemerin. ChemR23 receptor exhibits a unique expression pattern among leukocyte populations since it is expressed specifically in macrophages[24], natural killer cells (NK)[25], immature dendritic cells (iDCs) and plasmacytoid dendritic cells (pDCs)[26, 27]. The serine proteases plasmin and mast cell tryptase cleave prochemerin ($Chem_{163}$) into $Chem_{158}$, a weak activator of ChemR23. Sequential removal of the C-terminal lysine from $Chem_{158}$ by plasma carboxypeptidase N or B forms $Chem_{157}$, the product with the highest activity on the ChemR23. In contrast, neutrophil elastase and cathepsin G, K and L cleave 6 or 7 amino acids from prochemerin to produce $Chem_{157}$ and $Chem_{156}$, two potent ChemR23 agonists[19, 28].

Starting from these observations the aims of this study were: 1) to understand if MSC are able to produce chemerin under basal conditions and 2) if chemerin production can be influenced by different culture conditions.

In this study we demonstrated that both PL-MSC and FBS-MSC are able to produce Chemerin under basal conditions and its production is increased by stimulation with inflammatory cytokines. However PL-MSC produce a higher amount of chemerin compared to FBS-MSC. Moreover both FBS-MSC and PL-MSC express ChemR23, CCRL2 and GPR1 receptors under basal conditions. Moreover, MSC express the mRNA of cysteine and serine proteases, neutrophil elastase and cathepsin K, which are able to activate chemerin. Chemerin purified by MSC (MSC-Chem) is able to induce the migration of ChemR23-expressing cells, but these data suggest that only a fraction of chemerin is activated by MSC themselves. Starting from these data we speculate that, after *in vivo* administration, MSC are able to produce chemerin into the inflammatory microenvironment, where, serine and cysteine proteases can convert prochemerin produced by MSC into its active form. In this environment, MSC can shape chemerin concentration attracting ChemR23-expressing cells, which, in this way, are available for direct immunomodulation. Data obtained by mass spectrometry showed that the major part of MSC-Chem is produced as a precursor, ready for activation by inflammatory tissue proteases.

METHODS

Bone Marrow-derived MSC isolation:

Mononuclear cells were isolated from the washouts of sealed bone marrow collection bags and filters, and cells were plated, without further separation in DMEM low glucose complete medium supplemented with 5% freshly thawed Platelet Lysate (PL) or with 10% of Fetal Bovine serum at 800.000 cells/cm^2. As the culture reached around 80% of confluence, cells were trypsinized and split. PL-MSC were used at passage 3 (P3) and FBS-MSC were used at passage 4 (P4). The MSC phenotipical markers were evaluated by flow cytometry. MSC were tested for the following markers: CD11b APC (Biolegend), CD14 PE (eBioscience), CD19 PE (BD Bioscience), CD34 PE (BD Bioscience), CD45 PE (BD Bioscience), CD73 PE (BD Bioscience), CD90 PE (eBioscience), CD105 PE (eBioscience), HLA-ABC FITC (BD Bioscience) and HLA-DR PE (BD Bioscience). MSC lines were cultured with a conditioned medium in order to evaluate its ability to differentiate into the mesengenic lineages (adipogenic and osteogenic lineages). To fully characterized our MSC was tested their ability to inhibit leukocytes proliferation. Irradiated MSC were cultured with PBMCs, obtained from the buffy coats of healthy donors, at different MSC: PBMCs ratio. PBMCs were treated with phytohaemagglutinin (PHA) prior to add to MSC culture. After 48 hours of co-culture [3H] thymidine was added for 16 hours.

Stimulation of PL and FBS-MSC with inflammatory cytokines.

After reaching confluence, PL-MSC (at passage 2) and FBS-MSC (at passage 3) were both cultured in DMEM 2% FBS with or without inflammatory stimuli (IL-1β 50 ng/ml, IL-6 40 ng/ml and TNFα 100 ng/ml (Immunotools)) for 72 hours. At the end of stimulation, chemerin production was evaluated by ELISA assay in culture supernatants. Chemerin culture medium concentrations were measured by a commercial ELISA assay (Duoset, R&D) accordingly with the manufacturing instructions. The supernatants were harvested and then frozen until used.

Analysis of the expression of Chemerin Receptors and serine cysteine proteases

After 24h, 48h, and 72h of stimulation, MSC were digested with Trizol Reagent (Invitrogen). After extraction, total RNA was reverse transcribed, using a cDNA reverse transcription kit from Applied Biosystems (Invitrogen). The presence of ChemR23 (forward 5'-TTC TAG CTG TGT ACA GGG ACT GAT; reverse 5'-TGT AAT CTT CAT CCT CCA TTC TCA T),

CCRL2 (forward 5'-CAC ATA ACT AGG AAG TGG CAG AAC; reverse 5'-AGC GTA GGC TCT GAG CAA AT), neutrophil elastase (forward 5'- TTC CTC GCC TGT GTC CTG; reverse 5'-CTG CAG GGA CAC CAT GAA), cathepsin K (forward 5'-GCC AGA CAA CAG ATT TCC ATC; reverse 5'-CAG AGC AAA GCT CAC CAC AG), mast cell tryptase (forward 5'-GCG ATG TGG ACA ATG ATG AG; reverse 5'-TCC ATT ATG GGG ACC TTC AC), carboxypeptidase N (forward 5'-ATG AAC CCC GAC GGC TAC; reverse 5'-GCA TTG TTC CTG CCA ACT AGA), were evaluate with a RT-PCR using UPL-Light Cycler Technology (Roche). GPR1 (forward 5'-AAC TTT GGC CAC GCA CTT T; reverse 5'-TCA TTC CAC TTT TAT CTG GCT CT) was evaluated with SybrGreen Reagent (Invitrogen) using Light Cycler Technology (Roche). The Ct values for GAPDH (forward 5'-AGC CAC ATC GCT CAG ACA C; reverse 5'-GCC CAA TAC GAC CAA ATC C) were used to normalize the expression level of the gene of interest using the ΔΔCt method.

Purification of Chemerin from MSC supernatant

For immune-purfication, 1mg of monoclonal mouse IgG_{2B} human chemerin antibody (R&D System) was resuspend in 1 ml PBS-/- and incubated with 1ml of Protein G Sepharose 4 Fast Flow (GE Healthcare Life Sciences) for 1h, at room temperature. The Ab-sepharose resin was washed three times with 10ml of 0.2 M sodium borate pH 9.0. Resin was then resuspended with 10 ml of 0.2 M sodium borate, pH 9.0 and incubated with 20 mM dimethyl pimelimidate (final concentration)(Sigma-Aldrich). After 30 min at room temperature, the reaction was stopped by washing the Ab-coated resin twice with 10 ml of 0.2 M ethanolamine, pH 8.0 and incubated again for 2 h in 10 ml of 0.2 M ethanolamine, at room temperature. Resin was washed twice with PBS and was deposited into the column. After 4 days of stimulation with inflammatory cytokines (IL-4+IL1β+TNFα), () supernatants of MSC were collected and loaded onto the column for chemerin purification. MSC-derived chemerin was eluted with Glycine/HCl 0.1 M, pH 2.8. The amount of MSC-derived chemerin was quantified with an ELISA assay.

Migration assay

After purification, MSC-derived Chemerin was tested for an in vitro chemotaxis assay, using murine pre-B lymphoma L1.2 cells stably transfected with human CMKLR1 (ChemR23 receptor). Cell suspensions and recombinant human chemerin (R&D) or purified chemerin from MSC supernatant (MSC-Chem) were prepared and diluited in chemotaxis medium (RPMI 1% FBS). A total of 100 µl cells (0,5x 10^6 cells/well) was added to the top well of 5-

μm pore transwell inserts (Costar), and test samples were added to the bottom well in a 600-μl volume. Migration was assayed for 4 hours at 37°C. The insert were then removed and migrated cells through the filter to the lower chamber were collected and counted with the use of Trucount beads (BD Bioscience) by flow cytometry (FACS Canto, BD Bioscience). The results are presented as migration index compared to negative control (RPMI 1% FBS).

Mass Spectrometry

Before MS analysis, MSC-Chem or rh-chemerin (R&D) was reduced with 10 mM of DTT at 56 °C for 30 min, and was alkylated with 55 mM iodoacetamide 20 min, at room temperature in the dark. MSC-Chem was digested with trypsin or GluC in ammonium bicarbonate 0,1M in a ratio 1:20 (Chem:enz; w/w), at 37 °C, overnight in presence of 5% acetonitrile. Digested samples were finally analysed with LC/MS using the LTQ Orbitrap XL (Termo Scientific, Waltham, MA), interfaced with a capillary HPLC equipped with C18 capillary column (Termo Scientific, Waltham, MA).

Statistical analysis

To evaluate statistical significance Student's T test was used. P-value ≤0.05 was considered to be statistically significant (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

RESULTS

PL-MSC and FBS-MSC showed comparable phenotypic and functional features

In order to compare PL and FBS-MSC, we established 10 lines of bone marrow-derived MSC, cultured with both PL or FBS that were analysed phenotipically and functionally. For the phenotipical analysis, both PL and FBS cells were analysed for the expression of the typical MSC antigen-panel. As described in literature, both PL-MSC and FBS-MSC expressed the stem cell markers and adhesion molecules CD73, CD90, CD105, and low levels of class I MHC, and did not express the hematopoietic markers CD45, CD34, CD11b and class II MHC (Fig.1A). Moreover, Oil Red O and Alizarin Red stainings (which stain adipocytes and osteocytes, respectively) showed that, after 14-21 days of culture, both PL-MSC and FBS-MSC were able to differentiate into the adypogenic and osteogenic lineages (Fig.1B). In addition, *in vitro* proliferation assays showed that PL-MSC as well as FBS-MSC are able to inhibit T cell proliferation in a dose dependent manner (Fig.1 C).

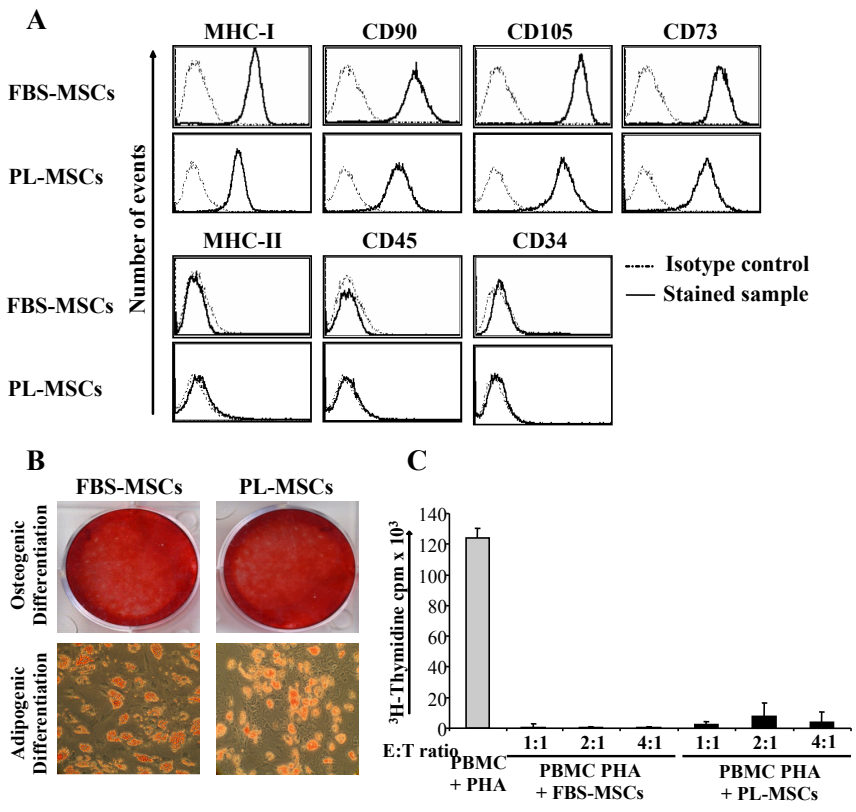


Fig. 1 PL-MSC and FBS-MSC showed the same phenotipic and functional features. (A) Flow cytometry analysis showed that PL-MSC, as well as FBS-MSC, were negative for hematopoietic markers (e.g. CD11b, CD45, CD117) and positive for stromal cell-associated markers (CD105, CD73, CD90 and HLA-ABC). (B) Moreover both PL-MSC and FBS-MSC are able to differentiate into the adipogenic and the osteogenic lineages. (C) The ability of MSC to inhibit leukocytes proliferation was evaluate with a co-coltured between MSC and human PBMCs. The results showed that PL-MSC inhibit leukocytes proliferation as well as FBS-MSC in a dose dependent manner.

MSC produce Chemerin and its production is influenced by culture conditions

In order to evaluate if MSC are able to produce chemerin under basal conditions, 10 lines of FBS-MSC were cultured without any stimulus for 72 hours. The production of chemerin was measured at three different time points (24h, 48h, 72h). Data obtained from ELISA assays showed that under basal culture conditions, FBS-MSC are able to produce chemerin (mean after 24h= 29 pg/ml, range=15-46.6 pg/ml) and its production increases after 48 and 72 hours (mean=62 pg/ml, range=15-154.7 pg/ml; mean=67 pg/ml, range=16.4-143.5 pg/ml; respectively). FBS-MSC were also cultured in presence of inflammatory cytokines (IL-1+IL-6+TNF-α) for 24, 48 and 72 hours. Stimulated cells show an increase in chemerin production compared to unstimulated cells (mean=34 pg/ml range=15-112.04 pg/ml; mean=79 pg/ml, range=15-334.55 pg/ml; mean=223 pg/ml, range= 23.9-869.7 pg/ml, respectively) (Fig.2A).
Interestingly, we observed that, the same cell lines, cultured in presence of PL produced a high amount of chemerin under basal conditions (after 24h mean= 558 pg/ml, range= 15-2772.9 pg/ml) and this production increased after 48 and 72 hours of culture without any stimulus (mean=1104 pg/ml, range= 31.8-5589 pg/ml; mean= 1883 pg/ml, range= 104.5-11912 pg/ml, respectively). Moreover, chemerin production by PL-MSC strongly increased after stimulation with inflammatory cytokines for all the time points evaluated (for 24h mean= 810 pg/ml, range= 19.3-4212 pg/ml; for 48h mean= 3078 pg/ml, range= 152.6-18850 pg/ml; for 72h mean=5771 pg/ml, range=794-25360 pg/ml) (Fig.2B). The comparison between FBS-MSC and PL-MSC showed that, PL-MSC are able to produce a higher amount of chemerin compared to FBS-MSC both under basal conditions and after stimulation with inflammatory cytokines (Fig.2).

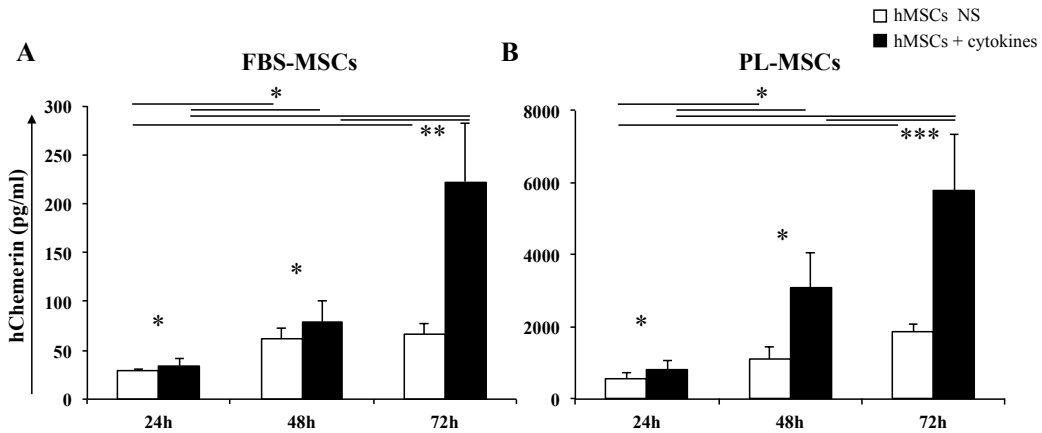


Fig. 2 PL-MSC and FBS-MSC produce chemerin under basal condition and its production increase after stimulation with inflammatory cytokines. Chemerin concentration was analysed in cell media after 24, 48 and 72 hours of culture by ELISAs assays. Both PL-MSC and FBS-MSC were able to produce chemerin under basal conditions and after stimulation with inflammatory cytokines, its production increased in both culture conditions. Moreover the results showed that PL-MSC produced a higher amount of chemerin compared with FBS-MSC. Data are mean ± SEM of ten independent experiments. * p≤0,05; ** p≤0,01; ***p≤0,001.

PL-MSC and FBS-MSC express the chemerin receptors ChemR23, CCRL2 and GPR1

As described in literature, MSC express the chemokine receptor ChemR23 and its expression increases after the induction of adipogeneic differentiation. Moreover, the absence of chemerin or ChemR23 has been reported to affect the ability of MSC to differentiate into adypocytes[29, 30]. In order to evaluate if, under basal conditions, MSC can autocrinnally respond to the chemerin that they secrete, we analysed the expression of chemerin receptors by RT-PCR after 24h of culture without any stimulus. The chemerin receptor ChemR23, highly expressed by immature Dendritic Cells (iDCs) (positive control), was expressed at low levels in both PL-MSC and FBS-MSC compared to iDCs levels (mean $2^{-\Delta\Delta Ct}$=0,005, range=0,002-0,01; mean $2^{-\Delta\Delta Ct}$=0,01 range=0,003-0,01; n=2 respectively) (Fig.3 A). In addition, PL-MSC, as well as FBS-MSC, expressed low levels of the atypical chemokine receptor CCRL2, (mean $2^{-\Delta\Delta Ct}$=0,015, range=0,01-0,02; mean $2^{-\Delta\Delta Ct}$=0,01, range=0,003-0,02; n=2, respectively), compared to iDCs (Fig.3 B). On the contrary, both PL-MSC and FBS-MSC expressed high levels of the atypical chemokine receptor GPR1 compared to freshly isolated PBMCs, used as negative control (mean $2^{-\Delta\Delta Ct}$=16,34, range=15,10-17,11 and mean $2^{-\Delta\Delta Ct}$=16,26, range=15,10-18,08, respectively, n=4) (Fig.3 C).

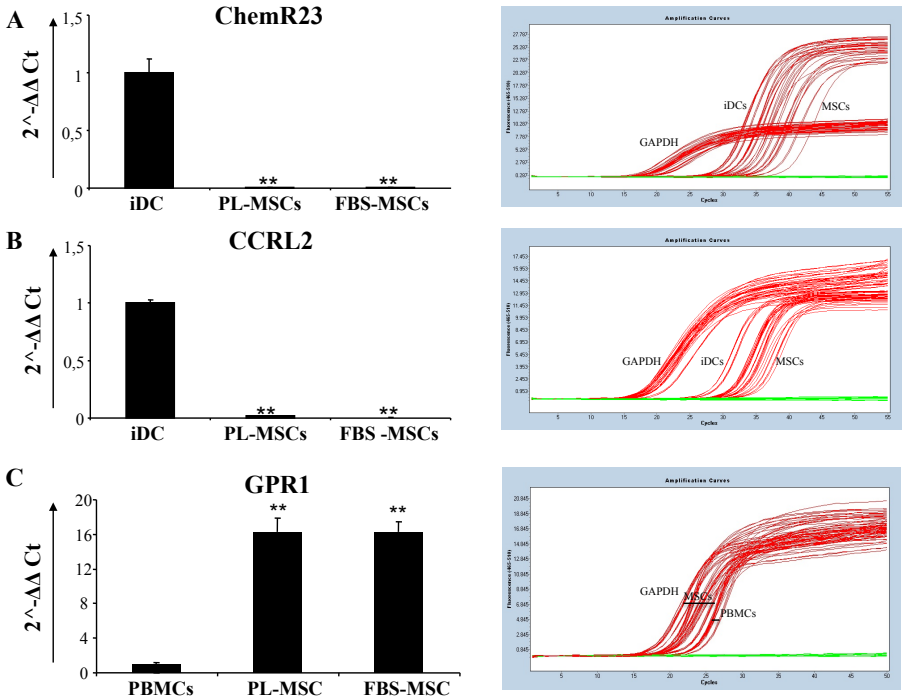
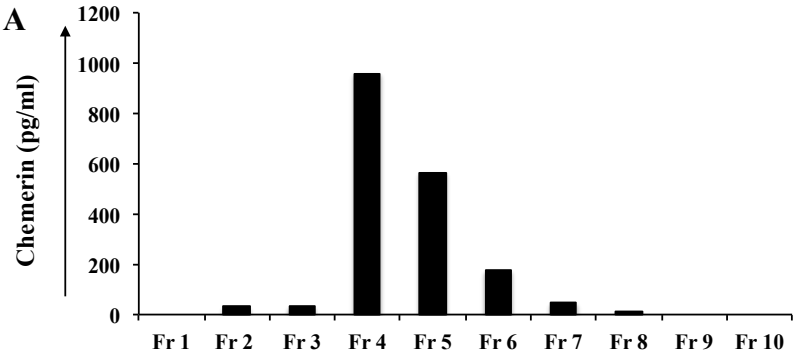


Fig. 3 MSC express the chemerin receptors ChemR23, CCRL2 and GPR1. (A) RT-PCR analysis showed that MSC express low levels of ChemR23 and (B) CCRL2 compared with the positive control (immature Dendritic Cells). (C) Although both FBS-MSC and PL-MSC express the chemerin receptor GPR1, compared with the negative control (PBMCs). Data are mean ± SEM of three independent experiments; ** p≤0,01.

Purification of Chemerin produced by MSC

In order to evaluate if chemerin produced by MSC is active, in view of their high chemerin production, chemerin was purified from the culture supernatant of PL-MSC stimulated with inflammatory cytokines for 72 hours, as previously described. Chemerin produced by PL-MSC was purified by immune-affinity chromatography and was eluted in 20 fractions of 270µl/each, which were quantified by ELISA assay. Through this technique we succeeded in purifying about 50% of loaded chemerin. Fractions 4 and 5, which contain the major concentration of chemerin, were used for biochemical and functional assays (Fig 4). Mass spectrometry analysis, and research in Mascot database identified Chemerin in both rh-chemerin (positive control) and MSC-Chem (Fig 5).

A



B

Match to: **RARR2_HUMAN** Score: **354**
Retinoic acid receptor responder protein 2 OS=Homo sapiens GN=RARRES2 PE=1 SV=1
Found in search of \\Ambshare\share\tossicologia_molecolare\Laura Share\MSC.mgf

Nominal mass (M_r): **18948**; Calculated pI value: **9.30**
NCBI BLAST search of RARR2_HUMAN against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Deamidated (NQ),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **31%**

Matched peptides shown in **Bold Red**

```
    1 MRRLLIPLAL WLGAVGVGVA ELTEAQRRGL QVALEEFHKH PPVQWAFQET
   51 SVESAVDTPF PAGIFVRLEF KLQQTSCRKR DWKKPECKVR PNGRKRKCLA
  101 CIKLGSEDKV LGRLVHCPIE TQVLREAEEH QETQCLRVQR AGEDPHSFYF
  151 PGQFAFSKAL PRS
```

C

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence	
104 - 113	537.3012	1072.5879	1072.5877	0	1	K.LGSEDKVLGR.L	(Ions score 89)
114 - 125	488.9381	1463.7925	1463.7919	0	0	R.LVHCPIETQVLR.E	(Ions score 38)
114 - 125	732.9040	1463.7935	1463.7919	1	0	R.LVHCPIETQVLR.E	(Ions score 40)
114 - 137	992.4868	2974.4387	2974.4389	-0	1	R.LVHCPIETQVLREAEEHQETQCLR.V	(Ions score 51)
114 - 137	744.6174	2974.4404	2974.4389	0	1	R.LVHCPIETQVLREAEEHQETQCLR.V	(Ions score 28)
114 - 137	744.6177	2974.4418	2974.4389	1	1	R.LVHCPIETQVLREAEEHQETQCLR.V	(Ions score 25)
114 - 137	595.8957	2974.4419	2974.4389	1	1	R.LVHCPIETQVLREAEEHQETQCLR.V	(Ions score 8)
141 - 158	1016.4660	2030.9174	2030.9163	1	0	R.AGEDPHSFYFPGQFAFSK.A	(Ions score 78)
141 - 158	677.9798	2030.9175	2030.9163	1	0	R.AGEDPHSFYFPGQFAFSK.A	(Ions score 70)
141 - 158	677.9799	2030.9178	2030.9163	1	0	R.AGEDPHSFYFPGQFAFSK.A	(Ions score 57)
141 - 158	677.9802	2030.9186	2030.9163	1	0	R.AGEDPHSFYFPGQFAFSK.A	(Ions score 29)

Fig. 4 Identification of chemerin purified from PL-MSC supernatant. Chemerin was purified using an immune-affinity column. A) Data obtained from an ELISA assay, performed on eluted fractions showed that the higher amount of chemerin (MSC-Chem) was eluted in fraction 4 and 5, which were used for the follow experiment. B) MS analysis were analysed using Mascot, which identified human chemerin (RARRES 2) in fraction 4 and 5. C) Bold red, marked chemerin peptides find by MS, used for identification.

Mass Spectrometry analysis of Chemerin produced by MSC

Since the cleavage rule for trypsin is: after arginine (R) or lysine (K), but not before proline (P) and the amino acid in position 158 is a lysine (and the last peptide with 5 amino acids is not detectable), we could not distinguish between $Chem_{158}$ and $Chem_{163}$ by trypsin digestion. In order to discriminate the two chemerin isoforms, MSC-Chem was analysed by Glu-C digestion, whose cleavage site is glutamate (E). After purification, MSC-Chem was digested with GluC for mass spectrometry analysis and loaded on a LC/MS spectrometer (Fig. 5). The Mascot analysis recognised recombinant human chemerin (rh-chemerin, positive control) as active form, because the peptide $Chem_{144}$-$Chem_{157}$, corresponding to the cleavage of the last six amino acids, was found (Fig 5A). On the contrary, data obtained from the analysis of MSC-Chem did not show the presence of the $Chem_{144}$-$Chem_{157}$ peptide, and only the $Chem_{144}$-$Chem_{163}$ prochemerin (precursor) could be identified in our samples (Fig 5A). These results suggested that the majority of MSC-Chem is inactive, but we could not exclude the presence of a minor fraction of active chemerin, undetectable in these experimental conditions.

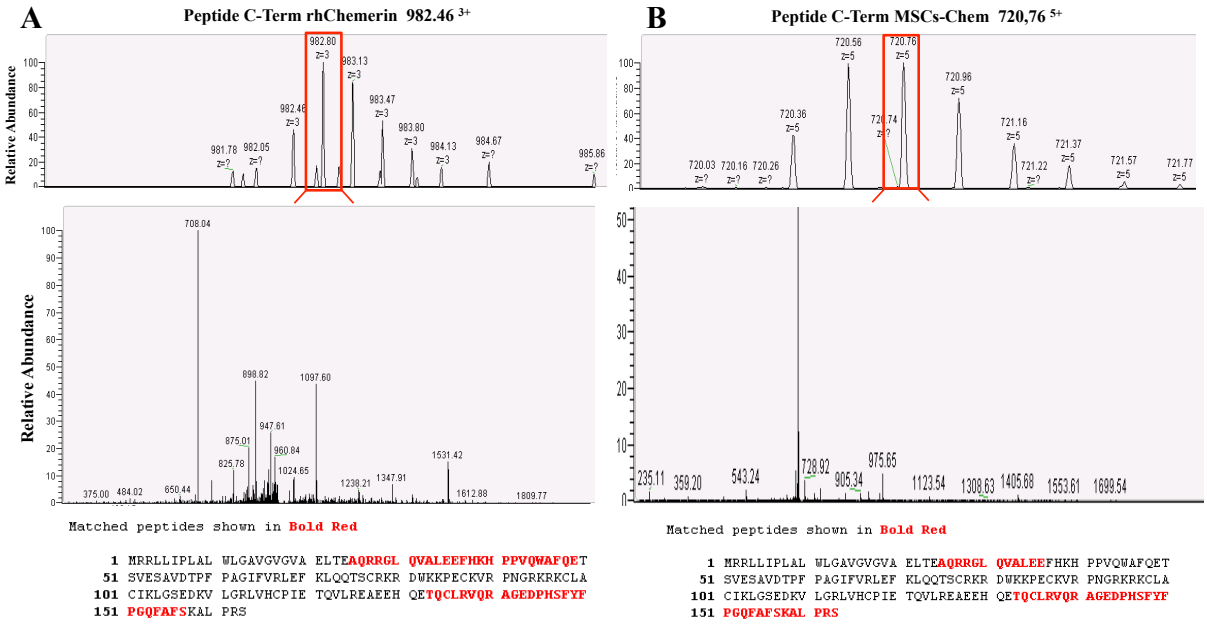


Fig. 5 The major fraction of MSC-Chem is produced in its inactive form (prochemerin)
Purified Chemerin (MSC-Chem) and commercial recombinant human chemerin (rh-chemerin) were analysed with LC/MS mass spectrometry. A) Mass spectrometry analysis of rh-chemerin showed that the C-terminal peptide correspond to chemerin active form ($Chem_{144}$-$Chem_{157}$), which amino acids sequence is marked in boil red. B) On the other hand, MSC-Chem analysis did not found the active C terminal peptide, but the unprocessed one ($Chem_{144}$-$Chem_{163}$), which amino acids sequence is marked in boil red.

MSC-Chem induce the migration of ChemR23-expressing cells

In order to evaluate the activity of chemerin produced by MSC, we performed migration assays using a pre-B cell line expressing the human ChemR23 receptor (L1.2-ChemR23). L1.2-ChemR23 cells are able to migrate in response to rh-chemerin in a dose depend manner until the concentration of 5nM (at 0,2 nM MI=2472, range=2201-2743; at 1 nM MI=9392, range=8902-9882; at 5nM MI=11737, range=11665-11809, at 10 nM MI=2904, range=3261-2548) (data not shown). L1.2-ChemR23 cells were then tested in chemotaxis assays using MSC-Chem as chemiotactic stimulus. Interestingly, data obtained showed that MSC-Chem induces the migration of L1.2-ChemR23 cells at 1nM, 5nM and 10nM (MI=85; 480; 1131; respectively) (Fig 6). However, the comparison between rh-chemerin and MSC-Chem showed that, at equivalent concentrations, rh-chemerin is able to induce a higher L1.2-ChemR23 migration, suggesting that in MSC supernatant only a fraction of the protein is in the active form.

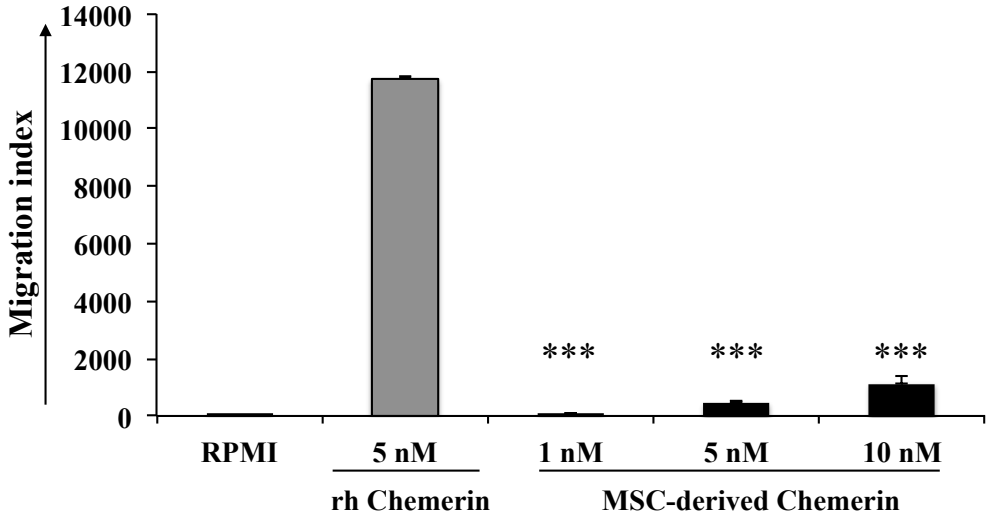


Fig. 6 MSC-Chem induce *in vitro* migration of L1.2 ChemR23 expressing cells
After purification MSC-Chem of fraction 4 and 5 was used to perform migration assays in order to understand if MSC are able to produce chemerin and to activate it. Migration assays' results showed that MSC-Chem is able to induce *in vitro* the migration of ChemR23 expressing cells in a dose dependent manner (compared to RPMI 1%FBS alone). However, migration index of MSC-Chem is significantly lower compared to rh-chemerin, suggesting that only a fraction of MSC-Chem is activated by MSC themselves.
Data are mean ± SEM of two indipendent experiments; *** $p \leq 0{,}001$.

MSC produce chemerin-activating serine and cysteine proteases

Chemerin has been reported to be cleaved by several serine and cysteine proteases, which are able to activate or inactivate chemerin, depending on the cleavage site. For this reason, we analysed the expression of chemerin serine-cysteine proteases by MSC both under basal conditions and after stimulation with inflammatory cytokines. RT-PCR showed that MSC express low levels of neutrophil elastase (mean $2^{-\Delta\Delta Ct}$=1, range=0,55-1,38 n=3) compared to PBMCs (positive control) (mean $2^{-\Delta\Delta Ct}$=234,48, range=201,32-284,71 n=3) and its expression does not significantly increase after 24h, 48h or 72h of stimulation with inflammatory cytokines (mean $2^{-\Delta\Delta Ct}$ = 5, range=3,08-5,83; mean $2^{-\Delta\Delta Ct}$ = 3,04, range=2,56-3,74; mean $2^{-\Delta\Delta Ct}$ = 2,10, range= 1,86-2,43; respectively, n=3) (Fig 7A). MSC also express cathepsin K (mean $2^{-\Delta\Delta Ct}$ = 7,41, range= 4,4-10,17, n=2), and its levels do not increase after stimulation with inflammatory cytokines (after 24h mean $2^{-\Delta\Delta Ct}$ = 8,47, range= 3,75-19,12; after 48h mean $2^{-\Delta\Delta Ct}$ = 8,83, range=4,98-12,61 and after 72h mean $2^{-\Delta\Delta Ct}$ = 10,48, range=9,43-11,77; n=2) (Fig 7B). On the contrary, mast cell tryptase and carboxypeptidase N are not expressed by MSC both under basal conditions and after stimulation with IL-6, IL1-β and TNF-α (data not shown).

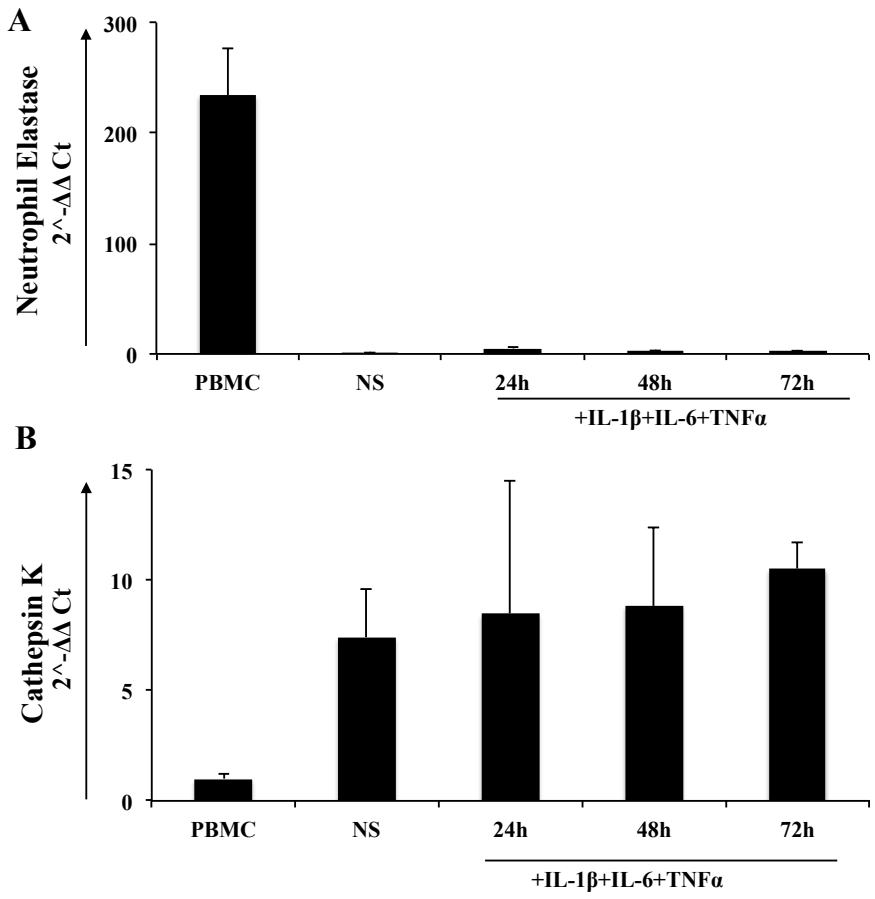


Fig. 7 MSC express the cysteine and serine proteases neutrophil elastase and cathepsin K.
Due to evaluate if MSC are able to produce chemerin and to activate it, RT-PCR were performed to test the presence of mRNA for cysteine and serine proteases. (A) RT-PCR showed that MSC express low levels of neutrophil elastase, which does not increase after stimulation with inflammatory cytokines at any time point. (B) Cathepsin K is also express by MSC and as well as neutrophil elastase its expression does not increase after stimulation. (C) Mast cell tryptase and (D) carboxypeptidase N are not detectable in MSC mRNA. Data are mean ± SEM of two independent experiments.

DISCUSSION

MSC are multipotent stem cells widely used for treating several inflammatory disorders[31], due to their ability to modulate the immune response. In the last years many groups focused their attention on the identification of different molecules involved in MSC immunosuppressive activity, but, until now, the mechanisms by which MSC are able to *in vivo* modulate immune responses are not fully understood. The identification of new molecules involved in MSC immunosuppressive activity, will represent a fundamental step to improve their usage in the clinical setting.

Chemerin has been recently identified as a chemotactic protein, which is able to induce the migration of cells that express the G-protein coupled receptor ChemR23, such as macrophages, iDCs, pDCs and NK cells. Modulating the infiltration of these cell subsets, chemerin can acquire a pro-inflammatory[32] as well as an anti-inflammatory role[33]. Chemerin is also able to bind to the receptors CCRL2 and GPR1, which are classified as atypical G protein-coupled receptors, because, after binding to their ligand, are not able to induce any intracellular signalling, but can act as scavenger or concentrator, shaping chemokine gradient[34]. Recently Muruganandan and colleagues demonstrated that chemerin is produced by MSC and has a crucial role in MSC-differentiation into the adipogeneic lineage through the binding of ChemR23 receptor [30].

This work was aimed to evaluate if chemerin, produced by MSC during adipogeneic differentiation, is also produced by MSC under basal conditions and in presence of inflammatory stimuli, in order to evaluate if chemerin could have a role in MSC immunosuppressive activity.

In order to evaluate the influence of different culture conditions on MSC immunomodulatory properties, we expanded ten MSC lines in presence of FBS or PL in their culture medium. As described in literature, phenotypical analysis showed that FBS and PL-MSC are comparable in terms of expression of the typical MSC markers. Moreover, functional analysis confirmed that cells cultured with different medium are similar in terms of differentiation potential and ability to *in vitro* inhibit T cell proliferation. Our data confirmed that, human MSC are able to produce chemerin, not only along the adipocyte differentiation process, but also under basal culture conditions. In particular, we demonstrate that both FBS and PL-MSC are able to produce chemerin and its production is increased after stimulation with inflammatory cytokines, such as IL-1β, IL-6 and TNF-α. Interestingly the production of chemerin results different between the two culture conditions: PL-MSC produce a higher amount of chemerin

compared to cells cultured in presence of FBS. Since PL-MSC are widely used for treating several inflammatory diseases, the higher production of chemerin could represent a characteristic feature responsible for their *in vivo* action.

Recent data showed that MSC increase the expression of ChemR23 during adypogeneic differentiation[30]. In order to evaluate if, under basal conditions, chemerin produced by MSC could have an autocrine role on MSC themselves, we analysed the expression of chemerin receptors by RT-PCR. Data obtained from RT-PCR analysis, confirmed the presence of ChemR23 mRNA, but the expression is lower compared to iDCs, used as positive control. Similar results were obtained analysing CCRL2 expression by MSC. Interestingly, we also detect that MSC express the atypical G protein-coupled receptor GPR1, whose function is still unknown[23].

Chemerin is produced by different cell types during inflammation as a precursor and is converted in its active form through the proteolitic cleavage of the last six or seven amino acids at the C-terminal domain by different serine and cysteine proteases which are present at high concentration during inflammation[19]. Unfortunately, the active and inactive isoforms of chemerin differ only for few amino acids, and are not distinguishable by ELISA assay. In order to fully characterise if MSC produce chemerin in its active or inactive form, we purified chemerin from MSC supernatant in order to perform further functional and biochemical analysis. Chemerin was purified by immune-affinity chromatography. Migration assays were performed using ChemR23-trasfected cells (L1.2-ChemR23) and results demonstrate that MSC-Chem is able to induce the migration of ChemR23–expressing cells in a dose-depend manner. The comparison between migration results performed with rh-chemerin and MSC-Chem suggest that only a part of MSC-Chem is converted in its active form and the major part of the protein is still inactive (prochemerin). In order to fully characterise the potential ability of PL-MSC to produce and activate chemerin, we evaluated the expression of cysteine and serine proteases that are able to convert chemerin in its active form. In particular, during adipogenic differentiation, pre-adipocytes increase the expression of neutrophil elastase, mast cell tryptase, cathepsin K, tPA, uPA and angiotensine converting enzyme, proteases which are able to convert chemerin in its active form[30]. Neutrophil elastase and cathepsin K mRNA were detected by RT-PCR, while mast cell tryptase and carboxypeptidase N were undetectable. In addition, we showed that the levels of these proteases do not increase during stimulation with inflammatory cytokines. Moreover, the biochemical analysis obtained from the LC/MS mass spectrometry detected the active form of chemerin (with the last peptide $Chem_{144}$-$Chem_{147}$) only in rh-chemerin but not in MSC-Chem ($Chem_{144}$-$Chem_{147}$),

confirming that the major part of chemerin produced by MSC is prochemerin. Unfortunatly, mass spectrometry is a very sensitive technique, but high protein levels are required to detect scarcely represented proteins, such as the active form of chemerin, which represents a small fraction in the MSC supernatant.

Starting from these data, we suggest that chemerin production by MSC could be involved in their immunomodulatory activity. We speculate that, when infused *in vivo* during a inflammation, MSC produce chemerin as precursor, and cysteine and serine proteases, which are present at high levels at peripheral inflamed tissues, convert MSC-Chem in its active form, inducing ChemR23-expressing cells migration towards MSC, which can better exert their anti-inflammatory activity.

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Chapter 5

CONCLUSIONS AND FUTURE PERSPECTIVES

Allogeneic haematopoietic-stem-cell transplantation (HSCT) is the treatment of choice for many malignant and non-malignant disorders. The development of novel strategies such as donor leukocyte infusion, non-myeloablative HSCT, and cord blood transplantation allowed expanding the indications for allogeneic HSCT over the last several years[1]. However several complications, such as pathology relapse, occurrence of opportunist infections, graft rejection and Graft-*versus*-Host Disease (GvHD), limits its wider application[2]. Despite the improvement in HLA matching techniques, transplantation protocols and donor/stem cell source selection, acute GvHD still remains a major life-threatening post-HSCT complication, developing in about 50% of HSCT patients. It has been estimated that about 30% - 50% of GVHD patients benefit from first line treatment, which is based on steroid administration. On the overall, the outcome of the pathology is poor, especially in the case of steroid-resistency[3]. In case of steroid-resistant GVHD, second-line treatment is not univocally established, and varies according to GVHD clinical patterns and patients underlying disease[4]. So far, a great percentage of HSCT patients suffering from steroid-resistant GVHD, especially with gut involvement, have no therapeutic options. The best hope to improve GVHD management in these patients lies on the better understanding of GvHD pathogenesis, in order to identify new highly specific molecular targets and novel therapeutic strategy.

PART I: UNDERSTANDING THE ROLE OF CHEMERIN/CHEMERIN RECEPTORS AXIS IN GvHD PATHOGENESIS

The first part of the project was focused on the potential role of chemerin/ChemR23 CCRL2, chemerin receptors axis in the pathogenesis of GvHD, with the aim to define new therapeutic targets for improving the management of post-transplant GvHD.

In order to address this point, a good tool is represented by preclinical mouse models of allogeneic stem cell transplantation and acute GVHD[5]. In particular, the usage of knockout and transgenic mice can facilitate the mechanistic dissection of the immunological processes underlying GVHD pathogenesis, otherwise impossible in HSCT patients[6]. Indeed, we established an allogeneic GvHD mouse model, taking advantage by the MHC-mismatched existing between Balb/c mice and C57BL/6 mice. In this experimental setting, we observed that chemerin plasma concentrations were significantly higher in allogeneic transplanted mice compared to syngeneic controls at GVHD onset. Very interestingly, chemerin levels resulted persistently increased along all the monitored disease course. These observations are consistent with recent findings about the dual pro/anti inflammatory role of chemerin, which could participate with different roles in subsequent GVHD phases.

Starting from these observations, in order to evaluate the role of chemerin in GVHD pathogenesis, we performed allogeneic hematopoietic transplants using mice lacking the chemerin receptors ChemR23 and CCRL2 as graft donors or recipients. In particular, transplanting ChemR23 or CCRL2 KO cells into Balb/c recipients resulted in the development of more severe GvHD compared to wild type donor cells. The transplant of both KO cells was associated with severe weight loss and a worst GvHD overall score, with a strong involvement of gut. In terms of survival, only ChemR23-deficient transplanted cells induced a significant increase in the mortality rate, compared to wild type transplanted mice. Interestingly, the evaluation of GvHD development in ChemR23 and CCRL2 KO recipients showed that, even in this case, KO mice developed more severe GvHD, characterised by high rate of mortality and weight loss, with an involvement of gut only in ChemR23-deficient mice. In addition, recipient KO mice, develop GvHD early after HSCT compared to experiments performed using KO cells as donors.

In order to better characterise ***the role of chemerin/chemerin receptors in GvHD pathogenesis***:

- we will analyse chemerin production in GVHD target tissues by different techniques such as immune-histocheminstry on paraffin embedded sections, RT-PCR and ELISA assays on digested tissues. In particular, we will focus our attention on two organs, liver and lung, whose involvement in GvHD cannot be studied by observing GvHD phenotipical manifestation. In addition, we will characterise by flow cytometry analysis different cell subsets infiltrating GvHD target organs, such as antigen presenting cells, M1 and M2 polarized macrophages, NK cells, CD8 and CD4 T cells, with a special focus on T_{regs}, B cells, with special attention on IL-10 producing cells.
- since chemerin represents a chemotactic factor for different hematopoietic cell subsets, we will study post-transplant hematopoietic reconstitution in allogeneic transplant experiments using ChemR23 and CCRL2 KO mice as graft donors or recipients. With this particular aim, we will analyse by flow cytometry the composition of bone marrow, lymph nodes and spleen at different time points after transplantation.
- we will perform adoptive transfer experiments to understand which cell subset is responsible for the severe GvHD phenotype observed using ChemR23 and CCRL2 KO mice as transplant donors. Data so far obtained seemed to indicate that CD11c+ DCs and pDCs are not the main actors in this scenario. Indeed, we will now focus on other possible $ChemR23^+$ cell subsets, performing adoptive transfer of for example NK cells, which were described to have a protective role in GvHD pathogenesis[7, 8].

Reliable biomarkers facilitating the early and accurate recognition of GVHD are highly warranted to improve the management of this invalidating disease. So far, the diagnosis of gut and liver GvHD is particularly difficult because it is based on the observation of clinical parameters such as diarrhoea and bilirubinemia, which are sometimes not satisfactories. To address this point we will try to understand ***if chemerin could represent a disease marker for GvHD monitoring***, especially in the case of gut and liver involvement. Observations obtained from chemerin plasma levels in mouse models of acute GVHD, seem to indicate the potential use of chemerin as GvHD marker. To further investigate this point, we already collected plasma samples from 100 paediatric patients who, in the last years, underwent HSCT at the Clinica Pediatrica, San Gerardo Hospital. Cryopreserved plasma samples are banked at Tettamanti Research Centre and are available for the determination of human chemerin concentration by ELISA assays. To evaluate if chemerin could represent a GVHD marker in HSCT patients, we will correlate chemerin plasma levels with patients clinical information about the type of transplant, GVHD occurrence (date, grading, organ involvement) and patients response to the adopted anti-GVHD therapy.

PART II: CHARACTERIZATION OF MSC IMMUNOSUPPRESSIVE ACTIVITY

Since MSC are a widely used for treating steroid-refractory GvHD, the better comprehension of their immune-suppressive activity, will provide the scientific basis for novel and better-tailored protocols for managing steroid-resistant GvHD.

1. MSC for the treatment of GvHD: understanding the in vivo biological effect through patients immune monitoring.

In order to provide the scientific bases for improving MSC treatment protocols for steroid–refractory GvHD patients, we investigated at cellular and molecular level the disease course, of MSC treated patients before and after MSC infusion. In our work we monitored GvHD course at a molecular level, taking advantage of three validated biomarkers GvHD marker, TNFRI, IL2Rα and elafin[9, 10]. We observed a response rate of 70% overall, with a complete response in 30% of the patients, confirming that MSC represent an effective therapeutic tool for the treatment of steroid-refractory GvHD. Supporting these clinical observations, after MSC infusions we observed that MSC are able to convert an inflammatory environment to a more physiological one, both at cellular level, promoting an increase of T_{reg} circulating in peripheral blood, and at molecular level, diminishing the concentration of inflammatory molecules.

An immunological monitoring on a larger cohort of patients infused with MSC will help to understand the immune-modulatory properties and the *in vivo* activity of MSC towards GvHD.

2. Characterization of chemerin produced by MSC

Despite MSC are widely used for treating several inflammatory disease and the identification of many soluble molecules involved in their immune-suppressive activity, their mechanisms of action have not been fully understood. In particular, this part of the thesis was aimed to discover new molecules that can be involved in MSC-mediated immunosuppressive activity. Chemerin is produced by MSC under basal conditions and represents a key molecule for their adipogeneic differentiation[11, 12]. Interestingly, we noticed that, inflammatory stimulation increased chemerin production by MSC, thus candidating chemerin as a new possible molecule with a crucial role in MSC immunomodulatory activity. As mentioned before, platelet lysate–expanded MSC (PL-MSC), are currently used in our Centre to treat HSCT patients experiencing steroid-refractory GVHD. On the other hand fetal bovine serum-expanded MSC (FBS-MSC) are used in other clinical protocols to treat different inflammatory diseases, including in some cases also GVHD. For this reason the effect of the culture conditions adopted on the immunosuppressive properties of MSC represents a crucial issue to define which MSC could be the ideal therapy in different disease settings. Understanding if different culture conditions are able to influence MSC features, will help clinicians to design better clinical protocols for improving MSC-based cure of inflammatory diseases. With this aim we evaluated chemerin production in FBS and PL-based culture systems.

Interestingly, we observed that PL-MSC produce higher amounts of chemerin compared to FBS-MSC both under basal conditions and after inflammatory stimulation. In order to understand if MSC-derived chemerin is functional, we purified this chemokine by immune-affinity techniques and analysed it by mass spectrometry. Tryptic map obtained from the digestion of purified chemerin showed that the most abundant part of MSC-Chem is prochemerin ($Chem_{163}$). On the other hand functional assays showed that MSC-Chem is able to induce *in vitro* migration of L1.2 ChemR23+ cells. All together, these data suggest that MSC are able to produce chemerin and that a part is active. Starting from these observations we hypothesize that MCS through chemerin production are able to attract ChemR23-expressing cells and to immunosuppress them.

In order to better characterize ***the role of chemerin in MSC-mediated immunomodulatory activity***:

- we will perform migration assays using ChemR23-expressing primary cells, such as iDC, pDC and NK cells.
- we will evaluate if MSC-derived prochemerin could be activated at inflammed peripheral tissues by digesting it with inflammation induced proteases.
- we will study the role of chemerin in the *in vivo* MSC-mediated anti-GVHD action. With this purpose, chemerin-silenced MSC will be characterised phenotypically and functionally *in vitro* and will be infused *in vivo* in an acute GvHD mouse model.

The better understanding of GvHD pathogenetic mechanisms and the optimisation of MSC-based therapy, will represent a crucial step for improving the treatment of HSCT patients experiencing severe post transplant GvHD.

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12. Muruganandan S, Roman AA, Sinal CJ. Role of chemerin/CMKLR1 signaling in adipogenesis and osteoblastogenesis of bone marrow stem cells. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 2010, **25**(2)**:** 222-234.

Chapter 6

APPENDIX

Manuscripts not included in the thesis

G. Lucchini, M. Introna, E. Dander, A. Rovelli, A. Balduzzi, S. Bonanomi, A. Salvade`, C. Capelli, D. Belotti, G. Gaipa, P. Perseghin, **P. Vinci**, E. Lanino, P. Chiusolo, M.G. Orofino, S. Marktel, J. Golay, A. Rambaldi, A. Biondi, G. D'Amico,* E. Biagi.*

Platelet-lysate-Expanded Mesenchymal Stromal Cells as a Salvage Therapy for Severe Resistant Graft versus- Host Disease in a Pediatric Population.

Biol Blood Marrow Transplant 16: 1293-1301, 2010

ER. Zanier, F. Pischiutta, L. Riganti, F. Marchesi, E. Turola, S. Fumagalli, C. Perego, E. Parotto, **P. Vinci**, P. Veglianese, G. D'Amico, C. Verderio and MG De Simoni.

Bone Marrow mesenchymal stromal cells drive protective M2 microglia polarization after brain trauma.

Under revision.