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# MODULATION OF THE NATURAL AND ADAPTIVE IMMUNE SYSTEM BY MULTISTEM®

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## List of abbreviations

1-MT: 1-methyl-tryptophan

Ag: antigen

ALP: alkaline phosphatase

ALS: amyotrophic lateral sclerosis

AMI: acute myocardial infarction

AML: acute myeloid leukemia

APC(s): antigen-presenting cell(s)

AT: adipose tissue

BM: bone marrow

CD: cluster of differentiation

CFU-F: colony-forming unit-fibroblast

cpm: counts per minute

Cr: chromium

CR: complete response

CsA: cyclosporin A

CTL(s): cytotoxic T lymphocyte(s)

(p)DC(s): (plasmacytoid) dendritic cell(s)

DNAM-1: DNAX accessory molecule-1

E:T ratio: effector:target ratio

EAE: experimental autoimmune encephalomyelitis

EBMT: European Society for Blood and Marrow Transplantation

EBV: Epstein-Barr virus

EGF: epidermal growth factor

ELISA: enzyme-linked immunosorbent assay

(m)(h)ESC(s): (murine) (human) embryonic stem cell(s)

FACS: fluorescence-activated cell sorting

FBS: fetal bovine serum

FCS: fetal calf serum

FITC: fluorescein isothiocyanate

FMO: fluorescence minus one

FoxP3: forkhead box P3

Gal-1: galectin-1

GMP: good manufacturing practice

(a/c)GvHD: (acute/chronic) graft-*versus*-host disease

GvL: graft-*versus*-leukemia

Gy: gray

HGF: hepatocyte growth factor

HLA: human leukocyte antigen

HO-1: heme oxygenase-1

HSC(s): hematopoietic stem cell(s)

HSCT: hematopoietic stem cell transplantation

HUVEC(s): human umbilical vein endothelial cell(s)

IBD: inflammatory bowel disease

ICAM-1: intercellular adhesion molecule-1

ICM: inner cell mass

IDO: indoleamine 2,3-dioxygenase

IFN: interferon

Ig: immunoglobulin

IL: interleukin

iNOS: inducible nitric oxide synthase

ISCT: International Society for Cellular Therapy

KDR: kinase insert domain receptor

KIR: killer-cell immunoglobulin-like receptor

LIF: leukemia inhibitory factor

LPS: lipopolysaccharide

mAb: monoclonal antibody

(m)(h)MAPC(s): (murine) (human) multipotent adult progenitor cell(s)

M-CSF: macrophage colony-stimulating factor

MDSC(s): myeloid-derived suppressor cell(s)

MFI: mean/median fluorescence intensity

MHC: major histocompatibility complex

MIC-A/B: MHC class I chain-related A/B

MLC: mixed-lymphocyte culture

MMF: mycophenolate mofetil

MS: MultiStem®

MS: multiple sclerosis

(m)(h)MSC(s): (murine) (human) mesenchymal stem cell(s)

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NK cell(s): natural killer cell(s)

NO: nitric oxide

NSC(s): neural stem cell(s)

PB: peripheral blood

PBMC(s): peripheral blood mononuclear cell(s)

PD: population doubling

PD-1: programmed death-1

PD-L1/2: programmed death ligand-1/2

PDGF: platelet-derived growth factor

PE: phycoerythrin

PGE$_2$: prostaglandin E$_2$

PHA: phytohaemagglutinin

PI: propidium iodide

PVR: poliovirus receptor

S:R ratio: stimulator:responder ratio – suppressor:responder ratio

SCIL: Stem Cell Institute Leuven

SEM: standard error of the mean

SLE: systemic lupus erythematosus

SR: specific release

TCR: T cell receptor

TGF: transforming growth factor

T$_h$1/2: T helper 1/2

TLR: Toll-like receptor

TNF: tumor necrosis factor

Treg(s): regulatory T cell(s)

TSG-6: TNF-stimulated gene-6

UCB: umbilical cord blood

ULBP: UL16-binding protein

VCAM-1: vascular cell adhesion molecule-1

VEGF(R): vascular endothelial growth factor (receptor)

# Chapter 1 - INTRODUCTION

## 1. Stem cells

### 1.1 Definition

Stem cells are the most primitive, unspecialized cells present in the body. During early embryonic and fetal development they play a crucial role in tissue and organ generation, whereas during adult life they serve as a sort of internal repair system to replace damaged tissue after disease, injury or upon ageing.

Stem cells can be defined by three important criteria: (1) long-term self-renewal ability, which means generating identical daughter cells for several symmetrical cell divisions to sustain the stem cell pool; (2) ability to differentiate at the single-cell level into numerous tissue- or organ-specific cells with specialized functions and (3) the ability to functionally reconstitute a given tissue when transplanted *in vivo*.[1,2] Based on these characteristics of self-renewal and multi-lineage differentiation, stem cells are excellently suited as cell-based therapies in the context of regenerative medicine and tissue repair.

### 1.2 Stem cell hierarchy

Stem cells are being classified based on their differentiation potential (**Fig. 1**). The potency of a stem cell is defined based on the number of different specialized cell types that can be generated. On top of the stem cell hierarchy is the totipotent zygote which can give rise to both embryonic and extraembryonic tissues. These totipotent stem cells will further differentiate into the 3- to 5-day-old blastocyst, consisting of an outer layer (trophectoderm) from which the supportive extra-embryonic tissues (e.g. placenta) develop and the inner layer, called the inner cell mass (ICM). Cells isolated from the ICM are defined pluripotent since they can give rise to all three somatic germ layers (ectoderm, mesoderm and endoderm) and to the germ cells of a multicellular organism, but not to extra-embryonic tissue.[3,4] During embryonic development, these pluripotent cells become increasingly restricted in their differentiation potential and generate multipotent stem cells, which are often named after the tissue from which they are derived. These cells will mature into finally differentiated cells from one specific tissue of one germ layer.
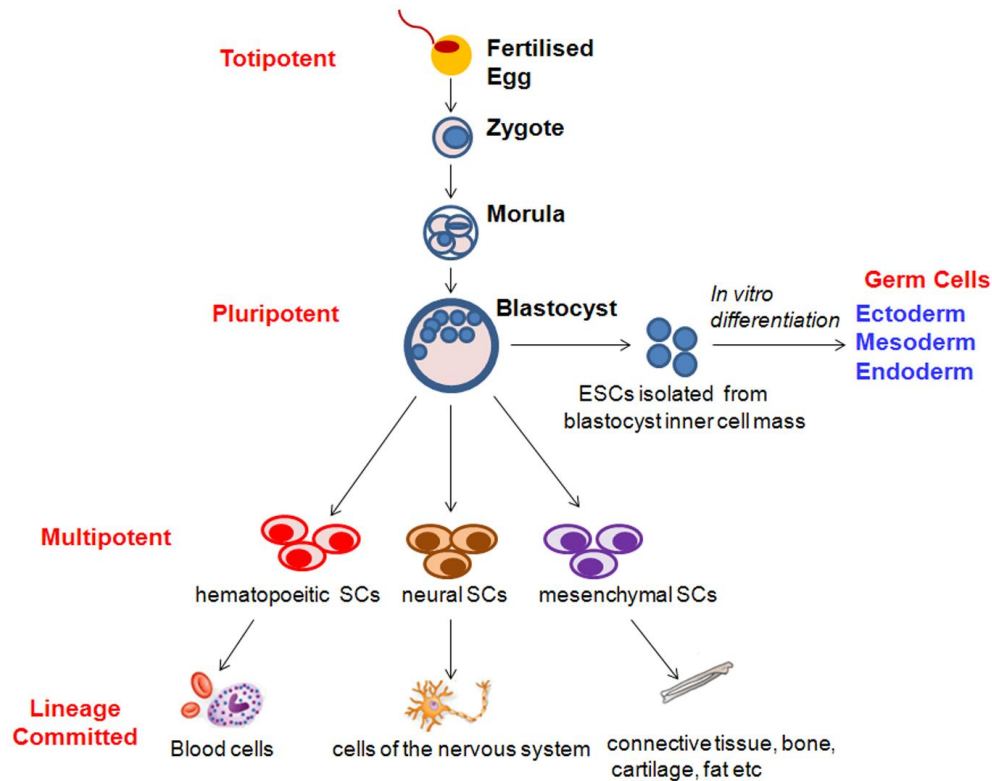
**Figure 1: Hierarchy of stem cells based on their differentiation capacity.**

The totipotent zygote develops from a fertilized egg. Cells from the ICM of a blastocyst can be maintained in culture as pluripotent ESCs. During embryonic development, stem cells become increasingly restricted in their differentiation potential and generate tissue-specific, multipotent stem cells.[5] SCs, stem cells.

## 1.3 Embryonic stem cells

Embryonic stem cells (ESCs) are derived from the ICM of early mammalian blastocysts (**Fig. 1**) and were first isolated from early pre-implantation-stage mouse embryos in 1981.[3,6] These cells are the most extensively characterized pluripotent cell type, and can be maintained *in vitro* with specific culture conditions to allow unlimited undifferentiated proliferation, due to high telomerase activity.[7] Remarkably, when murine ESCs (mESCs) are injected into blastocysts, they are able to contribute to competent chimeric mice and give rise to a wide range of tissues of all three somatic germ layers and to germ cells.[8,9] Because they do not generate extra-embryonic tissues, they are considered pluripotent and not totipotent. When mESCs are transplanted in ectopic sites, they form benign tumors containing derivatives of all three germ layers (teratomas).[10] Only a decade after the generation of mESCs, in 1998, Thomson *et al.* reported on the derivation and characterization of ESCs from human embryos.[4] hESCs are positive for known pluripotency genes (transcription factors *Oct4*, *Nanog* and *Sox2*) and also form teratomas upon transplantation under the skin of nude mice, which make them highly similar to mESCs.[11] However, due to ethical limitations, chimeric

contribution upon transfer in blastocysts cannot be tested.[12] The clinical use of hESCs is limited not only due to their tumorigenicity in their undifferentiated state but also due to ethical constraints. On the other hand, adult stem cells, which we will discuss below, have more limited differentiation capacity and thus also less tumorigenic potential and are therefore being considered for clinical use.

## 1.4 Adult stem cells

Adult or somatic stem cells are considered multipotent since they can only give rise to differentiated specialized cell types of the tissue of origin. In contrast to ESCs which can be grown relatively easily in culture, these tissue-specific stem cells have less self-renewal ability *ex vivo*, in part because of lower levels of telomerase.[13] Adult stem cells can be found in relatively low amounts in a specific tissue or organ in so-called 'stem cell niches', a complex microenvironment which is composed of differentiated somatic cells and extracellular matrix, as well as stem cells and their progeny.[14] Here, the stem cells can renew themselves to maintain homeostasis in tissues where large numbers of differentiated cells are needed on a daily base (e.g. epithelium of skin and gut). As already mentioned before, adult stem cells can also serve as an internal reserve for cellular replacement or repair following injury in other tissues with a lower cell turnover.[15] Nowadays, adult stem cells have been derived from a variety of post-natal tissues.

One of the first characterized and best known adult stem cell is the hematopoietic stem cell (HSC).[16] HSCs can be found in the bone marrow (BM) niche, in umbilical cord blood (UCB) and in peripheral blood (PB) after mobilization by growth factors or chemotherapy, where they undergo self-renewing cell divisions and eventually differentiate at the single-cell level to all mature blood cells.[17] The fate of HSCs, being self-renewing *versus* differentiation, is controlled by intrinsic signals from the stem cell niche.[14,18] Upon transplantation (HSCT), HSCs can functionally reconstitute the lymphohematopoietic system of a myeloablated individual, which led to the use of HSCs after high doses of chemo- and/or radiotherapy for the treatment of a variety of hematopoietic disorders and other malignancies.[17]

Other tissue-specific stem cell populations have been identified more recently and are for instance neural stem cells (NSCs), epidermal stem cells and intestinal stem cells. NSCs differentiate into the three main types of neural cells in the adult brain (neurons, astrocytes

and oligodendrocytes) and can be found in the subventricular zone and the hippocampus of the post-natal brain.[19,20] Epidermal and intestinal stem cells can be detected in tissues with high cell turnover being respectively the epidermis and the intestinal epithelium. Epidermal stem cells are able to maintain the different compartments of the skin, namely hair follicles, sebaceous glands and the interfollicular epidermis.[21] Intestinal stem cells reside in the base of the crypts of Lieberkühn and as they mature, they migrate upwards to the villi and differentiate into the specialized cells from the intestinal epithelium.[22] Both stem cell populations remain quiescent, until tissue homeostasis is disrupted, for example upon wounding, and will subsequently differentiate and replenish the damaged tissue-specific cells.

### 1.4.1   Mesenchymal stem cells

Mesenchymal stem cells (MSCs), also known as marrow stromal cells or mesenchymal stromal cells, were originally described in the early 1970s by Friedenstein and colleagues as a BM-derived plastic-adherent spindle-shaped fibroblast-like population and were named colony-forming unit fibroblasts (CFU-F).[23] Later, it was found that these cells are a rare distinct non-hematopoietic population in the stromal compartment of the BM, coexisting with and supporting the growth and differentiation of HSCs, and they were renamed by Caplan as MSCs.[24,25] Despite the low number of MSCs in the BM – approximately between 0.01 % and 0.001 % of the total nucleated cells, and even declining by age – isolation and expansion is relatively easy.[26] Although originally isolated from BM, MSCs have since been isolated from many other adult tissue sources, including adipose tissue (AT), umbilical cord tissue (Wharton's jelly), periosteum, dental pulp, synovial fluid and several fetal tissues.[27-33] These cells have the potential to differentiate in the presence of various factors towards mesenchymal cell lineages including bone, fat, cartilage, connective tissue, smooth muscle and hematopoietic supportive stroma.[34]

Three minimal criteria were provided by the International Society for Cellular therapy (ISCT) and are since being used to define multipotent human MSCs: (1) expression of a specific pattern of surface antigens being CD73, CD90 and CD105 and lack of expression of hematopoietic markers (CD14, CD34 and CD45), endothelial markers (CD31) and major histocompatibility (MHC) class II surface molecules; (2) plastic-adherence when maintained in standard culture conditions and (3) ability to differentiate *in vitro* into osteoblasts, adipocytes and chondrocytes.[35] Murine and human MSCs are somehow different regarding their expansion and functional properties. Murine MSCs grow much slower during *ex vivo*

expansion and are more prone to undergo malignant transformation.[36,37] Moreover, they fail to express indoleamine 2,3-dioxygenase (IDO) [38] and seem to display a lower immune suppressive capacity *in vitro*, compared to hMSCs.[36]

Although MSCs have been studied for some decades, the exact origin of the progenitor cells has only recently been discovered. When obtained by plastic-adherence, MSC populations are initially very heterogeneous and contain probably only a minor fraction of 'true' self-renewing long-lived progenitor cells.[39,40] Hence, the term mesenchymal 'stem' cells is being questioned and should theoretically be replaced by mesenchymal 'stromal' cells. MSCs may denote a mixture of diverse cell types with a large variability between MSC preparations due to the age and sex of the donor, the tissue source, the isolation technique used, variations in the expansion protocol and culture conditions (e.g. oxygen tension, temperature and medium composition), passage number and topographical specificities.[41] Furthermore, it remains uncertain whether culture-expanded MSCs differ phenotypically and functionally from their *in vivo* progeny from which they are obtained, because relatively little is known about their biological properties in an unmanipulated state. Until now, little agreement has been reached on the use of a specific universal marker to unequivocally identify and prospectively isolate MSCs *in vivo*. Although the exact identity of MSCs *in situ* is not entirely clear, recently, reports have suggested they may have a fibroblastic or pericytic origin. MSCs closely resemble fibroblasts, as both cell types are plastic-adherent, share similar cell morphology, surface marker phenotype and gene expression, and display tripotency and comparable immunoregulatory function, although less potent in case of fibroblasts.[42,43] Recently, it has been demonstrated that a CD146-positive population of self-renewing osteoprogenitors in human BM, in contrast to muscle or skin fibroblasts, were able to generate bone and stroma and to organize a hematopoietic microenvironment in immunocompromised mice.[44] These cells are located in the subendothelial layer of BM sinusoids and represent adventitial reticular cells, a subpopulation of pericytes. As pericytes are present in nearly every organ on the abluminal surface of endothelial cells in the microvasculature, it has been hypothesized that all MSCs found in different tissues could also be derived from this perivascular zone in blood vessels.[45,46] This may reflect the *in vivo* niche of MSCs, from which they can be readily released upon tissue damage to secrete immunoregulatory and trophic bioactive factors. This close relationship between MSCs and perivascular cells was confirmed by Covas *et al.* who reported low CD146 expression of fibroblasts and consistent clustering of MSCs with pericytes, rather than with fibroblasts, by means of gene expression analysis.[47]

### 1.4.2 Multipotent Adult Progenitor Cells

Only a decade ago, Verfaillie and her colleagues described the derivation of rare cells copurifying with MSCs from rat and mouse BM, termed Multipotent Adult Progenitor Cells (MAPCs).[48,49] MAPCs have characteristics different from most adult stem cells. In contrast to other adult stem cell populations, these progenitor cells proliferate extensively without obvious senescence or loss of differentiation potential and can differentiate *in vitro* at the single-cell level into cells of all three germ layers.[48,50] Comparable with mESCs, when injected into blastocysts, mMAPCs can (at a low level) contribute to most, if not all, somatic tissues.

Since the initial description of the isolation methods, improvements have been made to the culture system: isolation and maintenance of the cultures is done at 5% $O_2$, a different serum component is used and cells are maintained at higher densities for the first four weeks in culture.[51] Rodent MAPCs are cultured at low cell density and in the presence of leukemia inhibitory factor (LIF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). They are morphologically significantly smaller than their MSC counterpart, do not express CD45 or other mature hematopoietic markers and, in contrast to MSCs, do not express MHC class I and CD44 antigens.[52] Similar to rodent MAPCs, human (h)MAPCs are isolated from BM or from bone fragments and can be long-term [more than 70 population doublings (PD)] expanded *ex vivo*. They do not require LIF in their culture procedure to maintain their self-renewal and differentiate not only towards mesenchymal cell types, but also towards endothelium, skeletal muscle and hepatocyte-like cells.[53,54] In contrast to rodent MAPCs, hMAPCs do not express significant levels of *Oct4*, a transcription factor which is expressed during early mammalian development and which is essential to maintain pluripotency.[55]

A recent comparative analysis (**Table 1**) has shown that hMAPCs and hMSCs are two clearly distinguishable cell populations.[56] These stem cell populations differ in terms of their phenotype, expansion and differentiation capacity. MAPCs have substantial replication potential and can be expanded for a significantly longer time than MSCs (>70 PD *versus* 20-25 PD), which was consistent with a higher and more sustained telomerase activity in hMAPCs. Their surface marker expression is quite similar to that of hMSCs, lacking hematopoietic (CD34, CD45 and c-kit) and endothelial markers (VEGFR-2 and CD34) and expressing CD13, CD44, CD73, CD90 and CD105. In contrast to hMSCs, hMAPCs are

negative for CD140a and b, ALP and, importantly, express MHC class I molecules at lower levels. In addition, CD56, CD271 and CD146 (dimly positive) are not present on their surface membrane. Unlike hMSCs, hMAPCs can differentiate into functional endothelium *in vitro* and *in vivo*, next to differentiation into typical mesenchymal cell types. These phenotypic and functional differences between human MSCs and MAPCs were confirmed at the transcriptome level by using microarrays. Furthermore, it has been shown that the phenotype, the functional properties and the expressed gene profile of these cell populations may not be solely determined by cell intrinsic characteristics, but are also influenced by culture-mediated changes.[57] Therefore, until now, it is not known yet whether hMSCs and hMAPCs represent truly different cell types *in vivo*.

**Table 1: Comparative analysis of hMSCs and hMAPCs.**

| Stem cell | hMSCs | hMAPCs |
|---|---|---|
| *Isolation and culture* | | |
| Source | Bone marrow | Bone marrow/bone |
| % Serum in expansion medium | 10% | 2% |
| Extra growth factors in expansion medium | / | PDGF/EGF |
| Plating density | $5000\,cm^{-2}$ | $400\,cm^{-2}$ |
| % Oxygen | 21% | 5% |
| | | |
| *Cell surface phenotype* | | |
| CD34 | negative | negative |
| CD45 | negative | negative |
| c-kit | negative | negative |
| KDR | negative | negative |
| CD56 | negative | negative |
| CD271 | negative | negative |
| CD146 | low | low |
| CD44 | high | high |
| CD13 | high | high |
| CD73 | high | high |
| CD90 | high | high |
| CD105 | high | high |
| MHC class I | high | low |
| CD140a | low | negative |
| CD140b | high | negative |
| ALP | low | negative |
| | | |
| *Proliferation capacities* | | |
| Population doublings | 20–30 | 70 |
| Differentiation potential | | |
| Adipocytes/osteoblasts/chondrocytes | yes | yes |
| Smooth muscle cells | yes | yes |
| Endothelial cells | no | yes |

ALP, alkaline phosphatase; EGF, epidermal growth factor; hMAPC, human multipotent adult progenitor cell; hMSC, human mesenchymal stem cell; KDR, kinase insert domain receptor; MHC, major histocompatibility complex; PDGF, platelet-derived growth factor.[58]

### 1.4.3 MultiStem®

Adult stem cells are being explored as allogeneic cell products in regenerative medicine and immune-related disorders, as they can exert their restorative and immunoregulatory functions independent of MHC compatibility (see **Section 2**). Before safe application in HLA-unrelated patients, stem cells need to be quality tested and expanded in order to reach a sufficient amount of cells with therapeutic activity. This hampers the use of autologous cells for applications where cells are needed immediately. Furthermore, one of the major drawbacks of MSCs is their limited proliferative capacity *in vitro* (20-30 PD). On the other hand, MAPCs have the capacity to undergo extensive expansion doublings (> 70 PD) and can reach a sufficient number of cells, so that one specific clinical trial with several patients can be performed with the same cell product derived from a single healthy donor.

This extensive proliferation capacity and the specific MHC-independent and immune-priviliged properties of MAPCs (see **Section 2**) have led to large-scale manufacturing and banking of these cells according to good manufacturing practice (GMP) conditions, allowing the production of uniform well-characterized doses without the use of multiple donors.[59] This clinical-grade commercially available stem cell product (MultiStem®) is developed and patented by the biotech companies Athersys/ReGenesys (for more information: www.athersys.com - www.regenesys.eu).[60] MultiStem behaves in a drug-like fashion by expressing proteins and other factors involved in tissue repair and immune system regulation, and acts through multiple mechanisms such as protecting damaged or injured cells, reducing inflammation and promoting new blood vessel formation in areas of ischemic injury.[61-63] Compared to other stem cell populations with only limited proliferative capacity, these clinical-grade MAPCs might be a potentially more advantageous adoptive cellular therapy with widespread applicability. During its large-scale propagation, MultiStem retains its genomic stability as evaluated by Boozer *et al.*[59] Because of the fact that this proprietary stem cell product can be safely and stably cryopreserved for an extended period of time, MultiStem cells are being used as an allogeneic 'off-the-shelf' stem cell product at the time of need without patient matching for multiple disease indications in the areas of inflammatory or immune-related, neurological and cardiovascular diseases.

## 2. Immune modulation by adult stem cells

### 2.1 Mesenchymal stem cells

During the last two decades, MSCs have found their way into the clinic for several therapeutic purposes. To be successful in transplantation, certain criteria have to be fulfilled: (1) differentiation into a specialized cell; (2) survival in the host after transplantation; (3) homing and integration into the microenvironment of the tissue that is in need for repair and (4) lack of a graft reaction against the host tissue and fulfillment of an adequate function in the host.

Because of their potential to differentiate towards chondrocytes and osteocytes *in vivo*, MSCs are an important tool to create cartilage or bone tissue in cell replacement therapies.[64,65] A second domain of interest is revascularization of ischemic tissues (myocardium, brain and peripheral limb) as MSCs have the capacity to differentiate into vascular smooth muscle cells and secrete angiogenesis-promoting trophic factors.[66] Thirdly, undifferentiated MSCs produce several bioactive molecules like growth factors and cytokines that create a supportive and appropriate microenvironment for allogeneic grafts in the context of solid organ or HSC transplantation.[67] One of the very first *in vivo* studies demonstrated a prolonged survival of allogeneic skin grafts in primates that were given a systemic infusion with BM-derived allogeneic MSCs.[68] Another report showed that hematopoietic engraftment in immunodeficient mice could be ameliorated by cotransplanting MSCs.[69] Since then, several clinical trials have been performed to test the efficacy of MSCs to prevent rejection and enhance engraftment of HSCs upon transplantation.[70,71] Nowadays, as *in vitro* studies and preclinical animal models have demonstrated that MSCs display a remarkable set of immune regulatory, anti-inflammatory and trophic properties, MSCs gained interest in the context of autoimmune and autoinflammatory diseases [e.g. Crohn's disease and multiple sclerosis (MS)] and for the prevention and treatment of graft-*versus*-host disease (GvHD) after HSCT, and graft rejection after solid organ transplantation.

#### 2.1.1 Immune regulatory properties *in vitro*

##### 2.1.1.1 Adaptive immune system (**Fig. 2**)

*T cells*

Human mesenchymal stem cells have been shown to interact with a wide variety of immune cells of the innate and adaptive immune system. The interplay between MSCs and

allogeneic T cells has already been studied by many different groups. First, regarding the immunogenicity, MSCs have been proven to be poor stimulators of an *in vitro* allogeneic T cell response. They fail to induce activation of allogeneic T cells, measured as proliferation, interferon (IFN)-γ production or upregulation of activation-associated markers.[72] Two signals are required to adequately activate T cells: (1) the recognition of MHC molecules clustered with an antigen on the surface of an antigen-presenting cell (APC) by the T cell receptor (TCR) and (2) a co-stimulatory signal involving interaction of CD28 on the T cell with CD80 or CD86 (B7 superfamily) on the APC. It has been shown that MSCs express low levels of MHC class I molecules on its surface, and even lack MHC class II and co-stimulatory molecule (CD80, CD86 or CD40) expression. The expression of both MHC class I and class II can be upregulated upon stimulation with IFN-γ, but this increase was not sufficient to enhance the immunogenicity of MSCs.[72] In contrast, however, Stagg *et al.* reported that in case of syngeneic mMSCs, stimulation with low levels of IFN-γ rendered MSCs as conditional APCs which were able to activate antigen-specific immune responses.[73] Klyushnenkova *et al.* showed that the lack of T cell response was not due to a deficiency in co-stimulatory signals, since retroviral transduction of MSCs with B7-1 (CD80) or B7-2 (CD86) did not result in T cell proliferation.[72] Accordingly, Tse *et al.* described that IFN-γ-pretreated MSCs, even in combination with direct co-stimulation via an anti-CD28 antibody could not induce a T cell proliferative response.[74] Aside from that, they showed that the lack of proliferation was not due to MSC-induced T cell apoptosis.

Second, MSCs have been shown to suppress both naive and memory T lymphocyte proliferation and activation induced by alloantigens [75-77], mitogens [75,77,78], and anti-CD3/CD28 monoclonal antibodies (mAb).[76,79] This dose-dependent suppression is most marked if MSCs are added at the beginning of the activation period. MHC restriction is irrelevant as suppression can be mediated by both autologous and allogeneic stem cells.[76] Again, this reduced lymphocyte proliferation is not associated with the induction of tolerance, apoptosis or anergy and appears to be enhanced by IFN-γ pretreatment, suggesting the possible need for MSC licensing by proinflammatory cytokines.[72,80-83] MSCs also influence the antigen-presenting capacity of APCs, which will be discussed in detail below, and as a result, T cell responses skew away from a proinflammatory type I response [producing $T_h1$ cytokines IFN-γ and tumor necrosis factor (TNF)-α] towards an anti-inflammatory type 2 response ($T_h2$ cytokines IL-4 and IL-13) in the presence of MSCs.[77] Furthermore, MSCs also inhibit T cell responses by diminishing surface expression of early T cell activation markers CD25, CD38

and CD69.[78] Di Nicola *et al.* demonstrated that the suppressive effect of hMSCs on mitogen-induced allogeneic T cell proliferation is only transient, and disappears after MSC removal.[75] This is in contrast to the findings on mMSCs which induce a condition of anergy due to divisional arrest of T cells in the G0/G1 phase of the cell cycle.[80]

Most studies agree that a soluble factor is involved in MSC-mediated T cell proliferation suppression, as MSCs are still inhibitory – albeit at a lower level – when MSCs and T lymphocytes are separated by a permeable membrane. However, this suppressive factor is not constitutively secreted because supernatants from MSC cultures seemed not to have any inhibitory effect, implying that cross-talk between MSCs and target cells is mandatory. Several candidate mediators have been postulated, but the available data are often very contradictory, which could possibly be explained by the variable experimental designs. A role for transforming growth factor-β (TGF-β) and hepatocyte growth factor (HGF) has been suggested by Di Nicola *et al.*, who found that antibodies against TGF-β and HGF partially restored proliferation of purified T cells stimulated with allogeneic peripheral blood lymphocytes.[75] However, TGF-β and HGF were not involved in the case that T cells were stimulated with mitogens or when peripheral blood mononuclear cells (PBMCs) were used as responder cells.[74,78,84] Aggarwal *et al.* reported that inhibition of the synthesis of prostaglandin $E_2$ (PGE$_2$) mitigated MSC-mediated suppression.[77] PGE$_2$ was found to be constitutively produced by MSCs and the production was even enhanced upon coculture of MSCs with PBMCs.[74] The attribution of PGE$_2$ in the suppression by MSCs was confirmed by Rasmusson *et al.* who showed restoration of T cell proliferation suppressed by MSCs with indomethacin (an inhibitor of PGE$_2$ synthesis), but only when T cells were stimulated with phytohaemagglutinin (PHA), and not in a mixed lymphocyte culture (MLC) with allogeneic APCs.[85] Another possible mediator has been suggested by Meisel *et al.* who described a role for IDO.[86] IDO is important in the catabolic intracellular pathway, which catalyzes the degradation of the essential amino acid tryptophan into kynurenine and has been identified as a major immunosuppressive effector pathway that inhibits T cell responses to autoantigens and fetal alloantigens *in vivo*.[87] In MSCs, IDO is not constitutively being expressed, but can be induced by IFN-γ pretreatment. The group of Meisel *et al.* observed a restored T cell proliferation after tryptophan addition in the coculture of MSCs and T cells.[86] However, tryptophan depletion was not responsible for the immunosuppressive effect of MSCs when unfractionated PBMCs were used as responder cells.[74] It has become clear that

several factors in the experimental design contribute to all these discrepant results. The kind of responder population (unseparated mononuclear cells *versus* purified T cells), the exact stimulator used, as well as the origin of the MSCs might alter the mechanism of MSC-mediated suppression. More recently, other mediators in MSC-mediated immunosuppression have been suggested. Upregulation of stress response pathways such as inducible nitric oxide synthetase (iNOS) and heme-oxygenase-1 (HO-1) contribute to MSC-induced immune suppression.[88,89] The human leukocyte antigen HLA-G protein, which is a soluble non-classical HLA class I molecule, was also found to mediate the suppressive effect of MSCs through the induction of proliferation of regulatory T cells (Tregs).[90] Recently, TNF-stimulated gene-6 (TSG-6) was identified to play an important role in MSC-mediated cardioprotective effects in a mouse model of acute myocardial infarction (AMI).[91] MSCs have also been reported to induce the production of anti-inflammatory IL-10 by plasmacytoid dendritic cells (pDCs), which in turn triggers the generation of Tregs.[92] Additional immune regulatory cells that are induced by MSCs include regulatory DCs, alternatively activated anti-inflammatory M2 macrophages and myeloid-derived suppressor cells (MDSCs).[93-95] In addition, galectins are now emerging as a main regulator of MSC immunosuppressive function.[96]

However, published data do not exclude the possibility that a part of the immune suppressive effect exerted by human MSCs on alloantigen-induced T cell activation may be dependent on cell-to-cell contact mechanisms. Examples of MSC-related contact-dependent suppressive mechanisms are the programmed death ligand-1 (PD-L1)/ programmed death-1 (PD-1) signaling pathway, the apoptosis-inducing FasL/FasR interaction, the induction of adhesion molecules (ICAM-1/VCAM-1) and Toll-like receptors (TLR).[97-100] In summary, it is obvious that the mechanism(s) by which MSCs exert their immune suppressive function are pleiotropic and redundant.

Thirdly, MSCs have been reported to inhibit the cytotoxic effects of antigen-primed cytotoxic T cells (CTLs), only when added at the beginning of the MLC, suggesting that MSCs probably rather suppress CTL proliferation and development than directly target cytolytic activity.[101-103] At the same time, they were not susceptible to CTL-mediated lysis.[92,101,104] And finally, MSCs increase the proportion of $CD4^+CD25^+FoxP3^+$ Tregs in PBMC or T cell cultures.[77,92,105] However, another study showed that depletion of Tregs had no effect on the inhibition of T cell proliferation by MSCs.[106]

*B cells*

Studies on the effect of MSCs on B cell function have been performed, although less frequently and leading to conflicting results. *In vitro* experiments showed that B cell proliferation was inhibited by hMSCs through an arrest in the G0/G1 phase of the cell cycle. In addition, hMSCs inhibited B cell differentiation because antibody production (IgM, IgG, and IgA) was significantly impaired, as well as chemotaxis.[107,108] In contrast, however, others have reported stimulatory effects on *in vitro* activated B cells or plasma cells from healthy humans or patients with systemic lupus erythematosus (SLE).[109,110]
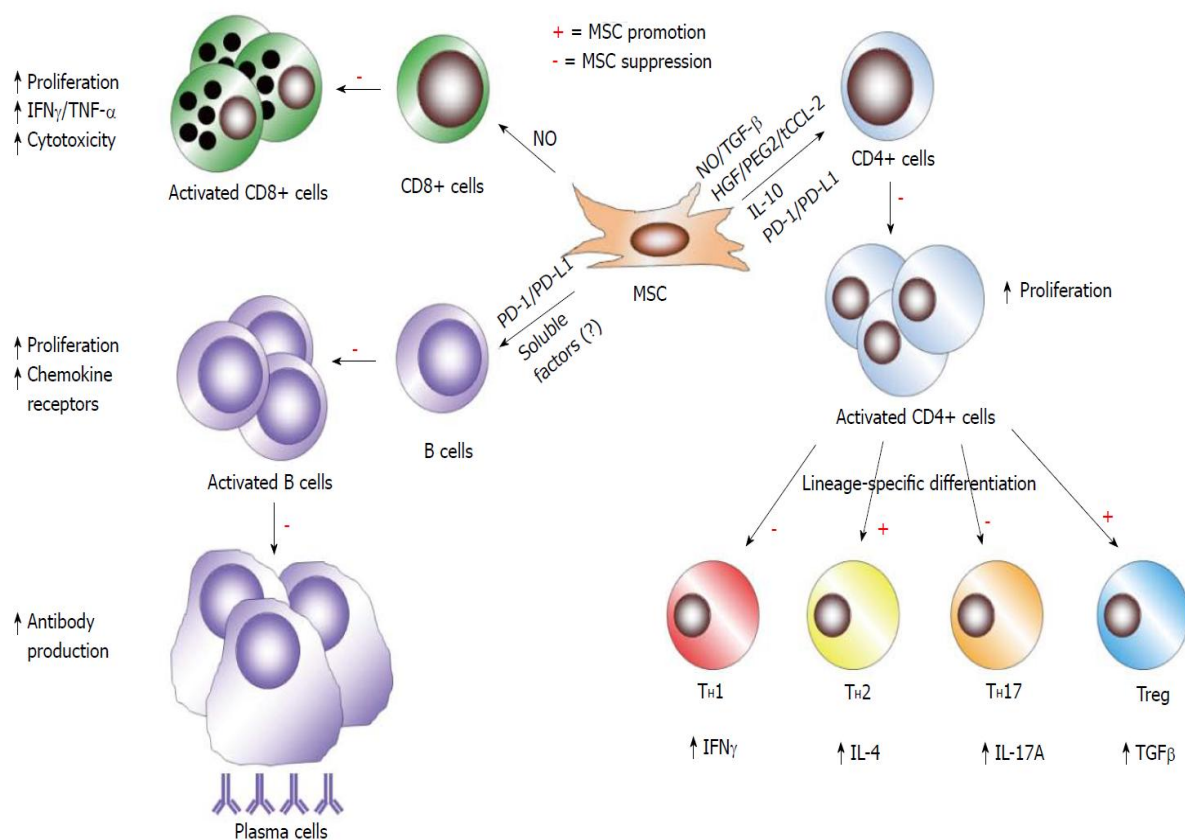


**Figure 2: Immunomodulatory properties of mesenchymal stem cells on <u>adaptive</u> immune cells.**

Mesenchymal stem cells (MSCs) suppress CD4$^+$ T cell proliferation and polarization (in response to specific cytokine milieus) towards $T_h1$ and $T_h17$ cells, while enhancing differentiation towards $T_h2$ and Treg effector populations. MSCs impair CD8$^+$ T cell proliferation, cytokine production and cytotoxicity, and inhibit various aspects of B cell activity, including activation, proliferation, chemokine receptor expression and differentiation to antibody-secreting plasma cells. MSCs mediate these effects by producing a variety of soluble factors and by membrane-bound molecules.[111]

### 2.1.1.2  Innate immune system (**Fig. 3**)

*Natural killer cells*

Natural killer (NK) cells are important effector cells of innate immunity. These cells display spontaneous cytolytic activity against cells that lack the expression of MHC class I molecules. The function of NK cells is regulated by the balance of the interaction between activating and inhibitory signals with their cell surface receptors.[112] MSCs are known to express low levels of MHC class I molecules, which makes them vulnerable for NK cell-mediated killing.[113] Nevertheless, MSCs are immunoprivileged and are not lysed by freshly isolated resting NK cells, even despite killer-cell immunoglobulin-like receptor (KIR) mismatch between MSC and NK cell donors.[101] On the other hand, both autologous and allogeneic MSCs can be successfully killed by activated NK cells.[114,115] This indicates that interactions between MHC class I-specific inhibitory receptors on NK cells and MSCs are not sufficient to protect MSCs from lysis. It is in fact also known that MSCs express ligands for activating NK cell receptors on its surface, like MIC-A and ULBPs (both ligands of NKG2D) as well as PVR and Nectin-2 (both ligands of DNAM-1), which triggers NK cell alloreactivity. In line with previously mentioned upregulation of HLA-ABC on MSCs upon culture with IFN-γ, IFN-γ-treated MSCs were less susceptible to NK cell-mediated lysis.[114] In addition, MSCs exert an inhibitory effect on NK cells affecting different aspects of NK cell function like proliferation, cytotoxic activity and cytokine production.[114,116] MSCs inhibit the cytokine (IL-2 and IL-15) driven proliferation of purified NK cells in a dose-dependent way. Even though MSCs did not inhibit the resting NK cell-mediated lysis of K562 cells, cytokine-stimulated NK cells cocultured with MSCs exhibit a reduced cytolytic function against K562 cells.[101] Sotiropoulou *et al.* could only demonstrate an impaired cytolytic function against HLA class-I positive tumor targets.[113] Together with the cytolytic activity, the IFN-γ production by NK cells is impaired after coculture with MSCs.[113,116] As for the effect on T cells, the mechanism of inhibition of MSCs on NK cells is not yet completely unraveled. Different soluble mediators like PGE$_2$, IDO, TGF-β and HLA-G have been proposed.[113,116,117]

*DCs*

DCs play a critical role in the induction of adaptive immunity and tolerance acting as the primary APCs to initiate a T cell response. This process is essential to initiate adaptive immunity against foreign antigens, but in case of allogeneic transplantation with a non-HLA-

identical donor, DCs can promote T cell alloreactivity leading to graft rejection. Therefore, the interaction between DCs and MSCs has been subject of intensive research. In addition to direct suppression of T cell proliferation, MSCs have been demonstrated to interfere also with DC differentiation, maturation and function, implying an equally important indirect suppression of T cell proliferation. First, MSCs inhibit the differentiation of monocytes or CD34^{+} progenitors to immature dendritic cells (iDCs). This effect is reversible and mediated by PGE$_2$.[118,119] Second, consistent data have shown that MSCs interfere with the DC maturation. It has been shown that DCs cocultured with MSCs and exposed to maturation factors such as lipopolysaccharide (LPS) or TNF-α, did not express CD83 and failed to show upregulation of maturation markers like MHC class II, CD40 or CD86.[118,120] In line with these findings, iDCs generated in the presence of MSCs were strongly hampered in their ability to induce activation of T cells. MSC cocultures induced an altered cytokine expression with reduced IL-12, IFN-γ and TNF-α production and increased IL-10 generation.[77,120] Additionally, DCs cultured with MSCs have been shown to induce indirect expansion of Tregs, as mentioned before. Collectively, MSCs impair DC differentiation, maturation, function and their migration to lymph nodes. Moreover, they promote the generation of tolerogenic iDCs that exhibit a suppressor or inhibitory anti-inflammatory phenotype, unable to maximally induce alloreactive T cell activation. Again, transwell experiments have shown that the suppressive effect of MSCs on DC differentiation is mediated by soluble factors, with possible roles for IL-6, M-CSF, PGE$_2$ and IL-10.[118-120]

*Macrophages*

Macrophages are key players in the innate immune system and are important in initiating and controlling inflammation.[121] In a proinflammatory context, MSCs may be activated to skew the differentiation of monocytes from classically activated proinflammatory M1 macrophages towards alternatively activated and IL-10-secreting anti-inflammatory M2 macrophages, possibly through PGE$_2$ and IDO.[122,123] Busch *et al.* showed similar findings for rat MAPCs.[124]
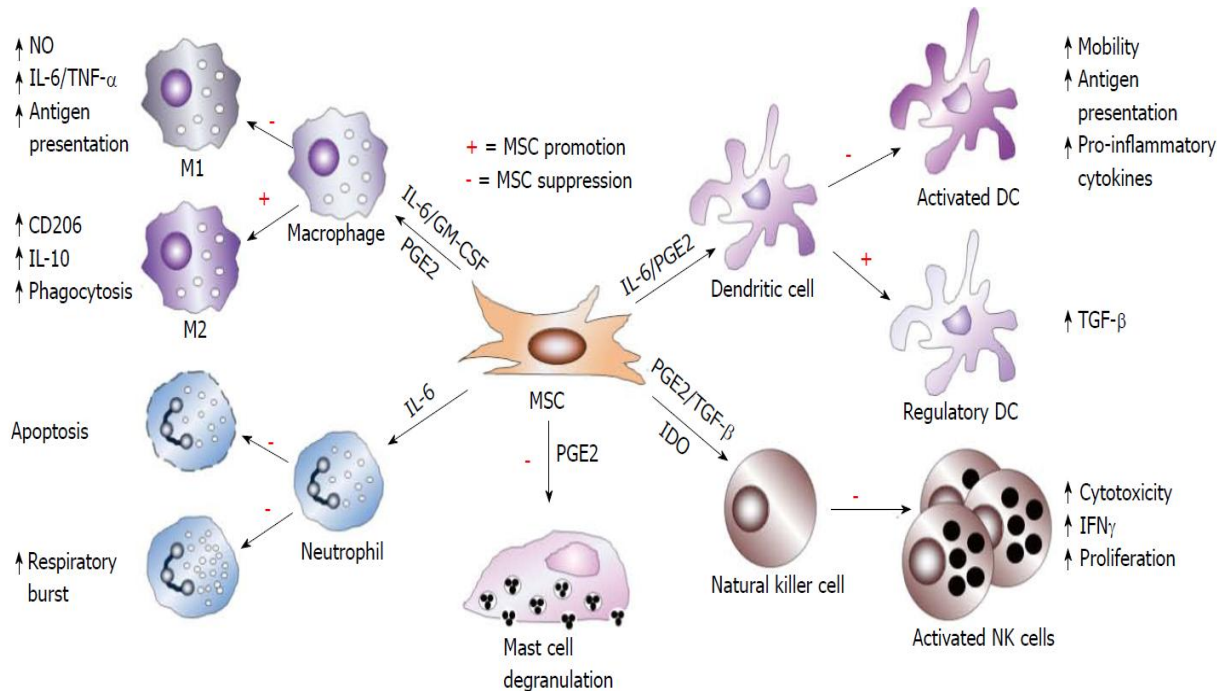
**Figure 3: Immunomodulatory properties of mesenchymal stem cells on <u>innate</u> immune cells.**

Mesenchymal stem cells (MSCs) utilize diverse molecular mechanisms to suppress innate immune cells. MSCs inhibit NK cell and DC activation, differentiation and effector functions, while the generation of regulatory DCs is supported by MSCs. MSCs also favor macrophage polarization towards M2, impair neutrophil apoptosis and respiratory burst, and mast cell degranulation of histamine-containing granules. MSC-derived PGE$_2$ and IL-6, amongst other factors, mediate most of these suppressive effects.[111]

## 2.1.2 Clinical experience on immune modulation *in vivo*

### 2.1.2.1 Safety assessment

Some decades ago, the very first clinical studies were performed to investigate the safety profile of intravenous administration of autologous human MSCs.[125,126] Back then, only patients with severe disorders like hematological malignancies and breast cancer were included, because of the fear for adverse effects and tumor formation. Autologous culture-expanded MSC infusion at the time of HSCT was feasible, well-tolerated and safe. Many studies followed and so far no reports of any infusion-related toxicity, immediate adverse outcomes or ectopic tissue formation are described.

### 2.1.2.2 HSC engraftment

Several factors have limited the clinical impact of traditional BM or HSC transplants, such as the need to tissue-match donor and recipient, the need for coadministration of immunosuppressive drugs to reduce the risk of rejection or other immunological

complications (GvHD). Many research groups have analyzed the function of MSCs as an adjunctive treatment for stromal support on the engraftment of HSCs and subsequent hematopoietic recovery after BM transplantation in patients with hematological malignancies. For such patients, treatment typically involves radiation therapy or chemotherapy, alone or in combination. This can substantially deplete the cells of the blood and the immune system, by reducing the number of stem cells in the BM from which they arise. One strategy for treating the depletion of BM is to perform a peripheral blood stem cell transplant or a BM transplant. This approach may augment the patient's ability to form new blood and immune cells and provides a significant survival advantage. However, finding a closely matched donor is frequently difficult or even impossible. Even when such a donor is found, in many cases there are immunological complications, such as GvHD, which may result in serious disability or death.

Experimental animal models have already shown that MSCs promote hematopoietic cell engraftment.[127,128] In 2000, Koç and co-workers were the first to report on infusion of autologous MSCs with a therapeutic intent.[125] Twenty-eight advanced breast cancer patients received high-dose chemotherapy and autologous peripheral-blood derived progenitor cells together with culture-expanded MSCs. They showed rapid hematopoietic recovery, suggesting that coinfusion of autologous MSCs after myeloablative therapy has a positive impact on hematopoiesis. Two years later, Lee *et al.* were the first to use allogeneic MSCs (HLA-haploidentical donor) in a patient with high-risk acute myeloid leukemia (AML) who was transplanted with mobilized peripheral blood HSCs combined with BM-derived MSCs.[129] Rapid engraftment was observed without acute or chronic GvHD and, at 31 months after HSCT, no sign of relapse was noted. In a pilot study, the group of Katarina Le Blanc *et al.* cotransplanted seven patients together with HLA-identical (3 cases) or haploidentical (4 cases) MSCs to enhance engraftment.[130] Despite notable differences in the patient population, sources of HSCs and MSCs and HLA compatibility, all patients experienced stable hematopoietic engraftment and full donor chimerism within 100 days after transplantation. However, mild GvHD was seen in 6 out of 7 patients. Baron *et al.* cotransplanted 20 patients undergoing reduced-intensity treatment with HLA-mismatched HSCs and third-party HLA-disparate MSCs.[131] Compared to 16 historical controls, the HSC engraftment and incidence of relapse were similar in both groups, but the overall survival at one year was significantly higher in the MSC-treated patient population. Poloni and colleagues treated 26 patients with HLA-identical sibling BM or mobilized peripheral blood

cells after reduced intensity conditioning together with MSCs, and 23 patients (88%) achieved sustained engraftment.[132] In a study performed by Ball *et al.*, fourteen pediatric patients with hematological malignancies and immune deficiency or nonmalignant disorders received haploidentical HSCs together with donor-derived MSCs.[133] Although graft failure in 47 historical controls was 15%, all pediatric patients given MSCs showed sustained hematopoietic recovery without any adverse effect or increased number of infections. Furthermore, two case reports were described by Fouillard *et al.*, involving one patient suffering from end-stage severe aplastic anemia and another patient with graft failure secondary to incomplete engraftment after autologous HSCT for AML.[134,135] Despite back-up BM infusion, the graft failure persisted in the latter patient, and this patient was infused with MSCs three years after the initial HSCT. Hematopoietic recovery of polymorphonuclear cells and platelets was observed in the absence of any additional HSC support. After receiving haploidentical MSCs, the first patient showed histological improvement in the BM microenvironment without hematopoietic recovery after MSC administration. In a study by the group of Macmillan *et al.*, 8 pediatric patients with acute leukemia were transplanted with allogeneic MSCs at the time of UCB transplantation.[136] All patients showed neutrophil engraftment and probability of platelet engraftment was 75%. In a last exemplary study, Meuleman *et al.* transplanted 6 patients with graft failure secondary to allogeneic HSCT, with MSCs without HSC coinfusion.[137] Two of the patients, both transplanted in first complete remission, showed rapid hematopoietic recovery within several weeks, whereas other patients transplanted at later stages of their disease were unresponsive.

These observations imply an enhancement of HSC engraftment and thus a reduced risk of early HSC graft failure in case of HLA disparity after cotransplantation with MSCs in a majority of treated patients, but larger studies are still required.

### 2.1.2.3   GvHD prophylaxis/treatment

Based on the fact that MSCs also share important immune modulatory properties, clinical focus shifted to the prevention of GvHD in patients who had undergone HSCT. Until now, GvHD is the far most studied therapeutic application for MSCs.[138] GvHD is a frequent and severe complication of allogeneic HSCT and prevalence ranges from 35 to 80% depending on the degree of HLA-matching, the patient's age and the amount of donor T cells present in the graft.

Several animal studies have addressed the role of MSCs in the context of GvHD prevention after BM transplantation, but conflicting results were delivered.[36,139-141] Clinical benefits were shown in some (but not all) GvHD models, predominantly in the studies when (preactivated) MSCs were repeatedly administered at the time of and after transplantation. These results indicate the variability of experimental outcome, depending on the origin of MSCs and on the timing, dose and frequency of MSC injections. Only recently, in 2005, Lazarus *et al.* conducted a large multicenter clinical trial including 46 patients diagnosed with hematological malignancies, who received a myeloablative regimen and a cotransplantation of HLA-identical sibling-derived HSCs and MSCs in escalating doses from 1 to 5 x $10^6$/kg.[126] Infusions were well tolerated, without any adverse reactions, but stromal cell chimerism could only be demonstrated in 2 out of 19 examined patients at 6 and 18 months after transplantation. Moderate to severe acute GvHD (aGvHD) was seen in 28% of the patients, and 61 percent developed chronic GvHD (cGvHD). Next, in a similar set of patients, in a small open-label randomized clinical study, HLA-identical sibling HSCs were transplanted in the absence or presence of additional MSCs.[142] Compared to HSC transplantation, additional infusion of MSCs prevented GvHD development (11% in MSC group *versus* 53% in non-MSC group grades II-IV GvHD). However, on the other hand, the early relapse rate was significantly higher in the MSC group than in the control group (60% *versus* 20%), resulting in discontinuation of the trial. More recently, the group of Liu *et al.* performed a randomized controlled phase II study.[143] A total of 55 leukemic patients received MSCs at the same time of haploidentical HSCT. Platelet recovery within 100 days was faster in the treatment group than in the control group. A higher frequency of aGvHD was observed in the treatment group compared with the control group (respectively 51.8% *versus* 38.9%), whereas less cGvHD developed in the treatment group (respectively 51.4% *versus* 74.1%). The overall survival rate did not significantly differ. The authors claim that the heavily pretreated status of their patients together with the low dose of MSCs ($10^5$ cells/kilo) were responsible for the absence of a beneficial effect of MSCs on the prevention of GvHD. In conclusion, these results are a promising base for adult stem cell therapy in GvHD prophylaxis, but the number of clinical studies and evaluated patients remains limited, and additional studies are necessary to determine timing and frequency of administration, and optimal cell dose.

As it became clear that MSCs were able to suppress the proliferation of alloreactive T cells *in vitro* and could promote tissue repair in animal models, MSCs were not only used

prophylactically at the time of HSCT but were also tested in the treatment of acute GvHD. The most impressive results came from a landmark case report, in which Le Blanc and colleagues described the very successful therapy of a 9-year-old boy with steroid-resistant grade IV aGvHD using haploidentical third-party MSCs.[144] Prompt reversal of severe gut and liver GvHD was reported and sustained complete response (CR) occurred after the second infusion of MSCs. A subsequent study included 8 additional patients with similar treatment-refractory aGvHD.[145] Six of them showed CR after MSC infusion, and their survival rate was better compared to 16 controls. Some less impressive results were published by Muller *et al.* who saw amelioration of GvHD in only two out of seven pediatric patients receiving haploidentical parental MSCs.[146] Other groups followed and tried to reproduce earlier beneficial results. Von Bonin and co-workers described improving effects of third-party MSCs expanded in platelet lysate-containing medium in 2 of 13 adult patients with aGvHD.[147] Complete remission in five out of six steroid-refractory aGvHD patients was obtained by the group of Fang *et al.*, who published promising results by using AT-derived third-party MSCs.[148] All these reports indicate that the included patient population, the timing of MSC infusion, and even the origin, isolation and expansion methods of MSCs can largely influence clinical outcome. Therefore, the European Society for Blood and Marrow Transplantation (EBMT) MSC consortium initiated a large multicenter non-randomized phase II trial, using a standardized MSC expansion protocol and common reagents.[149] Fifty-five patients with steroid-resistant severe aGvHD were included, receiving one ($n = 27$) or more ($n = 28$) MSC infusions (HLA-identical, haploidentical or unrelated HLA-mismatched source) with a median dose of 1 x $10^6$ cells/kg. Results confirmed a beneficial effect: CR to MSC infusion was seen in 30 patients (55%), with 27 complete responders already after a single infusion. Complete responders had a higher overall survival 2 years after HSCT, compared to partial/non-responding patients. Responses were somewhat more frequent in children than in adults, although this difference was not statistically significant. Most interestingly, there was no difference in response rates with respect to the source of MSCs. These results were extended with a retrospective analysis of a cohort of 37 children treated with multiple MSC infusions for refractory grade III-IV aGvHD.[150] CR was seen in 24 patients (65%), especially when MSCs were employed early in the disease onset, and overall survival after a median follow-up of 2.9 years was larger in patients who achieved CR. This observation paved the way for the establishment of large uniform banks of MSCs enabling rapid availability of MSCs without the need for HLA typing. In 2010, the first randomized placebo-controlled multicenter phase III trial for the treatment of steroid-

resistant aGvHD was conducted using large-scale expanded MSCs derived from a healthy third-party donor (Prochymal®; Osiris Therapeutics). A total number of 244 patients were enrolled, from which 163 patients received eight infusions of Prochymal (dose 2 x $10^6$/kg) over a period of 4 weeks *versus* 81 patients who received placebo. Unpublished unreviewed results showed similar sustained complete remission between both study groups, implying the failure to reach primary endpoints. However, according to the sponsoring company, a more in-depth subgroup analysis showed that pediatric patients experienced larger benefit and that gut and liver involvement had better response rates with Prochymal compared to patients with skin involvement, which would be in accordance with other published results by Ringden *et al*.[151] Kebriaei *et al.* studied the use of this clinical-grade MSC product to treat *de novo* aGvHD (grade II-IV).[152] Patients were randomized to receive 2 infusions of either low-dose (2 x $10^6$ cells/kg) or high-dose (8 x $10^6$ cells/kg) third-party MSCs in combination with corticosteroids. Combination therapy resulted in complete and partial response in respectively 77% and 16% of cases. There was no difference with respect to safety or efficacy between the low and high dose of MSCs. Recently, Prochymal was used to treat 12 children with steroid-refractory grade III-IV aGvHD.[153] They received 2 or 8 x $10^6$ cells/kg twice a week for 4 weeks. Partial and mixed responders received subsequent therapy for 4 weeks. Clinical response, particularly in the gastro-intestinal system, was seen in the majority of children (58% complete response, 17% partial response). For the moment, conditional product approval of Prochymal – being the first approved stem cell drug worldwide – has been achieved for treatment of pediatric GvHD in Canada and New Zealand. Very recently, Zhao *et al.* enrolled 47 patients with refractory aGvHD in a study to evaluate the immune modulatory effects of third-party MSCs.[154] Twenty-eight patients received MSCs (median dose 1 x $10^6$ cells/kg) weekly until patients got CR or they received eight doses. Overall response rate was 75% in the MSC-treated population compared with 42% in the untreated group. Occurrence and severity of cGvHD were lower in the MSC group, and no increase of infections or tumor relapse were observed.

Taken together, complete or partial response was seen in a vast majority of patients in all these studies with varying numbers of patients treated for acute GvHD with MSC infusions. Pediatric patients tended to have a better response compared to adults.[149,151,153] This might be explained by a better healing capacity, implying that children can better tolerate severe aGvHD. Importantly, HLA compatibility between MSC donor and recipient does not seem to be of major importance. Furthermore, there seemed to be no difference regarding the dose of

MSCs and the number of MSC infusions. Whether a particular source of stem cells is superior, and whether the number of MSC passages is of relevance remains to be answered. Interestingly in this part is that the number of MSC expansion passages correlated with the clinical outcomes in the studies performed by the group of Le Blanc *et al.*; patients given MSCs with passage number 1 or 2 experienced a higher response rate and overall survival after one year (50% *versus* 8%) than those given MSCs from passage 3 or 4.[155]

As chronic GvHD still represents substantial morbidity and mortality after allogeneic HSCT, reports of MSCs to treat cGvHD are emerging.[145,156] Weng *et al.* reported on 19 patients with steroid-refractory cGVHD treated with MSCs.[157] Partial or complete response was seen in 74% of the patients. The question whether the established mechanism of graft-*versus*-leukemia (GvL) will remain in patients with cGvHD who have been successfully treated needs to be further addressed.

### 2.1.2.4 Autoimmune diseases

At the moment, GvHD is by far the most studied therapeutic application for MSCs. However, MSCs are also being used to treat other diseases like AMI, neurological diseases, Crohn's disease and other autoimmune diseases.[158-160] Murine studies have demonstrated that MSCs are able to ameliorate the signs and symptoms of experimental autoimmune encephalomyelitis (EAE; a model of human MS), diabetes and SLE.[161-163] Nowadays, preliminary results of clinical trials using MSCs in the context of autoimmune diseases are very promising. In the context of Crohn's disease, the results of two phase I trials were recently published. Duijvestein *et al.* treated nine patients twice with systemically administered MSCs ($1\text{-}2 \times 10^6$ cells/kg), without observing a clinical response.[164] In the second study, complete fistula closure was achieved in 7 out of 10 patients after local injection of MSCs ($15\text{-}30 \times 10^6$ cells) every four weeks.[160] A phase III study with Prochymal is being initiated. Karussis *et al.* reported on the outcome of a phase I/II trial in patients with amyotrophic lateral sclerosis (ALS) or MS, who were injected intravenously and intrathecally with autologous MSCs.[165] No unexpected pathology occurred and MSC treatment resulted in a significant improvement of the clinical score.

## 2.2 Multipotent Adult Progenitor Cells

### 2.2.1 Immune regulatory properties *in vitro*

Like hMSCs, hMAPCs lack expression of MHC class II and co-stimulatory molecules belonging to the B7-family (CD40, CD80 and CD86), and subsequently do not induce proliferation of allogeneic T cells *in vitro*.[166] Despite upregulation of MHC class I, class II and ICAM-1 (CD54) expression after pretreatment with IFN-γ, hMAPCs still failed to evoke alloreactive T cell proliferation. Although low levels of activation markers on responder T cells were induced by hMAPCs, they did not induce production of $T_h1$ or $T_h2$ cytokines upon coculture with allogeneic T cells. hMAPCs significantly suppress proliferation of T cells, of memory T cells upon stimulation with recall antigens and of effector T cells during a secondary MLC. Even delayed addition of hMAPCs to a MLC showed inhibitory effects, meaning that hMAPCs can also reduce ongoing immune responses and can possibly be applied not only to prevent but also to treat immune-mediated diseases. Similar as with hMSCs, suppressive effects of hMAPCs were independent from MHC compatibility and not abrogated by IFN-γ pretreatment. Hence, hMAPCs might retain their immune suppressive capacity when injected into an inflammatory microenvironment *in vivo*. Furthermore, hMAPCs do not induce T cell anergy and allow priming of T cells. As it is the case for hMSCs, hMAPC-mediated suppression of T cell proliferation is, at least in part, mediated by a soluble factor. In the case of hMAPCs, Jacobs *et al.* found a partial role for IDO.[166] However, the degree of inhibition is stronger when cell-to-cell contact is present, which points to a parallel cell contact-dependent suppressive mechanism. Overall, the immunosuppressive properties of hMAPCs are similar as those of hMSCs, at least on T cell alloreactivity *in vitro*.

### 2.2.2 Clinical experience on immune modulation *in vivo*

Tolar and colleagues were the first to study the immunological behavior of murine MAPCs (mMAPCs).[167] These cells were unable to stimulate allogeneic CD4^{+} and CD8^{+} T cells *in vitro*, and were susceptible to NK cell-mediated lysis. Luyckx *et al.* confirmed these findings and demonstrated a bimodal modulatory effect of mMAPCs on alloreactive T cell proliferation with immune stimulatory effect at low ratios and suppressive effect at high ratios.[168] Moreover, mMAPCs suppressed *in vivo* alloreactive T cell expansion in a local GvHD model, but failed to inhibit systemic GvHD. Similarly to these findings, Highfill *et al.* proved that mMAPCs had a prophylactic effect on GvHD and were able to prevent activation and proliferation of alloreactive CD4^{+} and CD8^{+} T cells, only when delivered

locally at the site of initial T cell priming.[169] Furthermore, a dose-dependent suppressive effect was observed *in vitro*, which was mediated by $PGE_2$. Kovacsovics-Bankowski *et al.* reported on the immunological behavior of clinical scale-expanded rat MAPCs.[170,171] Similar to their murine counterparts, rat MAPCs lacked *in vivo* immunogenicity and did not induce alloreactive T cell responses. Furthermore, rat MAPCs were not susceptible to NK cell-mediated lysis and they dose-dependently suppressed alloreactive T cell proliferation, which was mediated by IDO-associated tryptophan depletion. In addition, the authors demonstrated the safety of administering rat MAPCs in a myeloablative HSCT setting, during which they could even modulate GvHD.

## 2.3 MultiStem

Preclinical studies with MultiStem cells have been conducted in a range of animal models including cardiovascular and neurological diseases, and BM transplantation-associated GvHD.[170-174] Administration of repeated dosing regimens in these models was considered safe and well-tolerated. MultiStem has shown potent immunomodulatory properties, including the ability to reduce active inflammation through various modes of action, to stimulate tissue repair and to restore immune system balance. MultiStem infusion – even one week after onset of injury in the stroke model – corresponded with an inflammation reduction in the ischemic injury regions and with cytoprotective benefits, as well as vasculogenic and proangiogenic effects. Furthermore, MultiStem can play a role in reducing the incidence and severity of GvHD in BM transplant recipients.[175]

MultiStem is currently being evaluated in the clinic in multiple disease areas in the context of inflammatory and immune-related disorders, next to their regenerative role in cardiovascular and neurological diseases. Third-party MultiStem has been proven safe and well-tolerated in phase I studies in patients with AMI and as an adjuvant cell therapy to enhance engraftment of HSCs and as GvHD prophylaxis after allogeneic HSCT.[61,176] Moreover, a partial beneficial effect of MultiStem was seen in the AMI study, because of its ability to induce neovascularization through secretion of trophic factors such as VEGF, IL-8 and CXCL5.[62] MultiStem was delivered directly into the region of ischemic damage in the heart, and improved cardiovascular performance including left ventricular ejection fraction, left ventricular end systolic volume and wall motion.[61] The open-label, phase I, multicenter GvHD prevention trial by Maziarz *et al.* showed that single and repeated-dose intravenous

administrations were well-tolerated, and a reduced incidence and severity of aGvHD was observed.[176] The overall cumulative incidences of grade II-IV and grade III-IV aGvHD in 36 patients were 37% and 14%, respectively. The group that received the highest MAPC dose (10 x $10^6$ cells/kg) had an incidence of 11.1% (1 out of 9) of grade II-IV, with no reported cases of grade III-IV aGvHD. A phase II and phase II/III study for respectively AMI and HSCT support/GvHD prophylaxis are planned to initiate later in 2014. Moreover, phase I studies are started in the context of immune modulation after liver transplantation and phase II studies are recruiting patients with refractory ulcerative colitis or ischemic stroke.[177,178] Preliminary results of the colitis study demonstrated a favorable safety and tolerability profile for MultiStem through 8 weeks following treatment, but failed to show significant improvement compared to placebo in the primary efficacy endpoints.

## 2.4   *In vivo* mechanism

Despite the obtained results from *in vitro* immunoassays, the exact mechanism(s) by which adult stem cells and more specific MSCs display their immunosuppressive effects *in vivo* remains topic of further research. Initially, infused donor MSCs were thought to differentiate *in situ* and substitute damaged cells in injured tissues of the recipient. However, it has become increasingly clear that persistent MSC engraftment is not mandatory. Preclinical research in different animal models has demonstrated that MSCs distribute to a wide range of tissues (mainly lung, liver, kidney, spleen and bones) and are able to home and migrate to peripheral sites of injury and inflammation after systemic infusion in MHC-mismatched recipients.[179-181] Interestingly, MSCs could even exert their protective effects without long-term engraftment and with only limited levels of engraftment and transdifferentiation *in situ*, indicating that the multipotentiality of MSCs is not required for their therapeutic effect.[182] The complex coordinated multistep process of MSC migration into specific tissues is highly similar with the well-known leukocyte adhesion cascade, including selectin-mediated rolling on the blood vessel wall, cell activation by chemokines and cytokines, integrin-mediated adhesion on the endothelium and transendothelial migration into the extracellular matrix involving integrin-dependent interactions and matrix-degrading proteases.[183] It has been demonstrated that MSCs have chemotactic ability due to a restricted pattern of chemokine receptors (like CXCR4) allowing them to invade injured tissue, which expresses a chemokine density gradient and specific molecules to facilitate trafficking, adhesion and infiltration of MSCs.[184,185] Most of the favorable effects of MSCs could be explained by secretion of

therapeutic paracrine or endocrine factors – in response to the inflammatory microenvironment – that have multiple effects, including modulation of inflammatory and immune reactions, protection from cell death and stimulation of endogenous progenitor cells in productive tissue repair.[67] These findings suggest that even transient MSC engraftment ('touch-and-go effect') may exert beneficial effects.

## 2.5  Safety issues

Stem cell-based immunotherapy seems one of the most fascinating and promising areas of contemporary biology and can possibly fulfill major unmet needs, but it is important not to overestimate the potential therapeutic effects as it also raises important scientific questions. Some main concerns about the *in vivo* use of adult stem cells in general – hMSCs, hMAPCs and MultiStem among others – need to be further addressed.

Although a vast majority of *in vitro* studies has shown that adult stem cell-mediated immune suppression is MHC-independent, these observations are not directly translatable to the *in vivo* setting. In contrast to a number of preclinical studies, no toxicities or ectopic tissue formation have been noticed in the performed clinical trials with hMSC therapy, probably because of the potential alloimmune response directed against third-party MSCs resulting in their limited survival *in vivo*. A systematic review and meta-analysis of clinical trials examining the safety of systemic MSC administration only revealed a higher occurrence of transient fever and described no associations between MSC treatment and the development of acute infusional toxicity, organ system complications, infection, malignancy or death.[186] According to Moermans *et al.*, who examined the impact of MSC administration on human lung function for the first time, MSC coinfusion had no detrimental effect on pulmonary function over a period of one year after unrelated HSCT with nonmyeloablative conditioning.[187] Cumulative incidence of fungal infections appeared to be higher in the MSC-treated group. Collectively, these observations indicate that clinical MSC therapy appears to be safe overall. However, the clear requirement for strict and long-term follow-up in larger-scale controlled clinical trials with large cohorts of patients should not be neglected to exclude late complications and rare adverse events.

Because of their immune suppressive properties, adult stem cells might interfere with the normal protective immune system against foreign pathogens. For instance, hMSCs are able to

suppress T cell proliferation induced by recall antigens, implying that the adoptive treatment might render the host more vulnerable to infections.[166] However, several different data derived from clinical studies to prevent GvHD after allogeneic HSCT showed that anti-viral immune responses may still normally occur following systemic administration of hMSCs.[133] Furthermore, caution needs to be taken into account as there are no indications that administration of stem cells will selectively impair GvHD but not the desired GvL effect, resulting in a possibly increased risk of relapse.[142,188] In immunocompetent mice, local and systemic infusion of MSCs suppressed the host antitumor immune response and favored the allogeneic tumor formation.[189] In a randomized clinical trial in patients with hematological malignancies, Ning *et al.* observed that MSC therapy had a beneficial effect on the incidence of GvHD but was associated with a higher relapse rate.[142] On the other hand, Baron *et al.* showed that MSC coinfusion did not abrogate GvL effects in patients receiving HLA-mismatched HSCT following nonmyeloablative conditioning.[131] This highlights the relatively unknown influence of adult stem cells on the balance between GvHD and GvL in allogeneic HSCT. Another issue to remark is that MSCs and MAPCs are considered to be hypo-immunogenic, although evidence indicates that MSCs can act as APCs under appropriate conditions.[73] Nauta *et al.* described MSCs as immunogenic as they were able to induce memory T cell responses in naive immunocompetent mice.[190] These observations suggest the requirement of further studies regarding the immunogenicity of stem cells.

Thirdly, the interaction of adult stem cells with other immunosuppressive drugs should be investigated further. Standard immune suppressive therapy following allogeneic HSCT or organ transplantation includes the administration of calcineurin inhibitors [cyclosporine A (CsA), tacrolimus or mycophenolate mofetil (MMF)]. The group of Le Blanc *et al.* was the first to prove an *in vitro* synergistic effect of CsA on the hMSC-mediated immune suppression of T cell reactivity.[78] However, in contrast, Buron *et al.* observed that CsA, tacrolimus and rapamycin antagonized the inhibitory effects of hMSCs, whereas MMF promoted them.[191] Moreover, Eggenhofer *et al.* demonstrated in a rat model of heart transplantation that MSCs and MMF synergistically prevented the infiltration of antigen-presenting cells and T cells into the graft.[192] By contrast, calcineurin inhibitors have been shown to abrogate the immunosuppressive effect of rat MSC therapy.[193] These observations emphasize the need to study the appropriate drugs in combination with the adoptive stem cell-mediated immunotherapy.

Finally, some concerns have originated regarding *ex vivo* expansion of adult stem cells. First, cell culture is mainly performed in the presence of fetal bovine serum (FBS), which might be associated with the risk of transmitting zoonoses and eliciting potential immune responses in the host. Therefore, alternative animal-free media components or serum-free isolation and expansion procedure of adult stem cells should be taken into consideration. Second, the expanded stem cells should be subjected to extensive quality control testing before administration into patients, because culture expansion may alter the functional *in vivo* characteristics. During the *ex vivo* culture of MSCs, required for obtaining sufficient numbers of cells, it has been suggested that MSCs could undergo malignant transformation and accumulate chromosomal aberrations and epigenetic changes.[194] Murine MSCs seem to be more prone to this spontaneous maldifferentiation and immortalization in culture, compared to their less susceptible human counterpart.[37,195] Nevertheless, it has been described that human AT-derived MSCs may undergo spontaneous transformation into neoplastic cells upon prolonged expansion under stressful conditions.[196] Important to note is that MSCs used in the clinical setting are mostly cultured less extensively prolonged (number of passages ≤ 4-5), reducing the potential risk of malignant cell transformation. Given their proangiogenic, anti-apoptotic and immunomodulatory properties, MSCs may also promote and sustain tumor formation and growth. However, and even after long-term follow-up, no clinical reports have been described in patients with hematologic tumors or non-neoplastic disease, exposed to MSC therapy, about *de novo* neoplasias.[194]

# Chapter 2 – RESEARCH OBJECTIVES

Within our research unit, an appropriate methodology for the study of the induction and modulation of T cell proliferative responses by human MSCs, MAPCs and MultiStem® has been established in a former PhD project (***Immunological properties of Human Multipotent Adult Progenitor Cells - Sandra Jacobs, 2012***). Current project started as a continuation of this research line, and the data presented in Chapter 3 (*Mutual interaction between human multipotent adult progenitor cells and NK cells*) were gathered by collaboration and led to a publication with co-first authorship.

The overall objective of this research line is to study the immunological properties of hMAPCs and its clinical-grade counterpart MultiStem in the context of their widespread therapeutic applicability, which has the advantage over MSCs of having a significantly more extensive replication potential and a broader differentiation potential. While it is difficult in the case of MSCs to generate sufficient amounts from a single donor to treat several allogeneic patients, the proliferative properties of MAPCs allow banking and prompt availability in case of acute illness.

Despite its ongoing preclinical testing and comprehensive clinical evaluation as an off-the-shelf product for stem cell-based therapy in various allogeneic patients suffering from immune-related, cardiovascular and neurological diseases, data regarding the exact immunological behavior of MAPCs and its clinical-grade counterpart MultiStem are still limited. A detailed knowledge of the immunogenic and immune modulatory capacities and the accompanying mechanism of immune regulation *in vitro* of this clinical-grade MAPC-derived stem cell product is indispensable for designing, understanding and optimizing future clinical trials.

1. *To explore the bidirectional interactions of MultiStem with a functional human immune system*

   As has been described for MSCs, the influence of MultiStem on the phenotype and functionality of various immune effector cells *in vitro* will be evaluated. While the role of MAPCs has already been investigated in the regulation of proliferative responses of immune cells in the former PhD project, this work will focus on the induction and

modulation by (clinical-grade) MAPCs of cytotoxic immune responses, initiated by immune cells of the adaptive and innate immune system, being respectively cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. This extensive immunological characterization will be achieved by studying the interaction between (clinical-grade) hMAPCs and the specified immune cells during direct coculture, or upon addition as modulating cells during the activation or functioning of the two immune cell populations. Moreover, we will investigate whether the stem cells can persist and maintain their immune regulatory function in an inflammatory environment *in vitro*, long enough to modulate the inflammation.

2. *To further elaborate the mechanism by which immune modulation by MultiStem is established and to identify essential immune regulatory factors and pathways*

Stem cell-associated immune regulation is mediated by a coordinated action of contact-dependent signaling pathways in combination with a multitude of constitutively or contact-induced soluble factors. The exact immunomodulatory mechanism depends on the studied immune cell population, the specific experimental design and on the origin and expansion procedure of the stem cell product. This project will search for the responsible mechanism in case of cytotoxicity modulation by (clinical-grade) hMAPCs of CTLs and NK cells.

# Chapter 3 – Mutual interaction between human multipotent adult progenitor cells and NK cells

*Results described in this chapter have been adopted from:*

*Jacobs SA\*, Plessers J\*, Pinxteren J, Roobrouck VD, Verfaillie CM, Van Gool SW. Mutual interaction between human multipotent adult progenitor cells and NK cells. Cell Transplant. 2014;23(9):1099-1110.*

*\* equal contribution*

<u>Sandra Jacobs</u> performed the MTT experiments (section 4.2 and 4.3; **Figure 1A** and **3A**) and wrote the first version of the manuscript.

<u>Jeroen Plessers</u> performed the flow cytometry experiments on NK cell receptor ligand expression of hMAPCs (section 4.1; **Table 1 and 2**) and on the expansion of NK cells (section 4.4; **Figure 5B**); the [$^3$H]thymidine incorporation experiments to analyze the influence of soluble mediators (section 4.4; **Figure 6**); and the $^{51}$Cr-release experiments to investigate hMAPC-mediated modulation of resting/activated NK cell function (section 4.2 and 4.3; **Figure 2A-B** and **Figure 4**), to analyze the influence of IFN-γ pretreatment on hMAPC susceptibility to NK cell-mediated lysis (section 4.3; **Figure 3C**) and to test the influence of hMAPCs during the stimulation phase of PBMCs/NK cells (section 4.4; **Figure 7A-B**). He also wrote the revision of the manuscript.

Results obtained by <u>collaboration</u>: $^{51}$Cr-release experiments on hMAPC susceptibility to NK cell-mediated lysis (section 4.2 and 4.3; **Figure 1B and 3B**) and [$^3$H]thymidine incorporation experiments to analyze NK cell proliferation suppression by hMAPCs (section 4.4; **Figure 5A**).

# 1. Abstract

Human multipotent adult progenitor cells (hMAPCs) are isolated from bone marrow with a more extensive expansion capacity compared to human mesenchymal stem cells (hMSCs) and with the ability to differentiate into endothelium. Like hMSCs, hMAPCs inhibit T cell proliferation induced by alloantigens. In this study, we tested the interaction between hMAPCs and natural killer (NK) cells. We assessed the susceptibility of hMAPCs to NK cell-mediated lysis and the immunomodulation of hMAPCs on NK cell function during the IL-2-driven stimulation and the cytolytic effector phase. Human MAPCs express the ligands PVR and ULBP-2/5/6, which are recognized by activating NK cell receptors. However, they also express MHC class I molecules, which induce inhibitory signals in NK cells. Freshly isolated NK cells at different effector:target ratios did not kill hMAPCs as assessed by an MTT and $^{51}$Cr-release assay, while hMAPCs impaired the cytotoxic activity of resting NK cells against the NK-sensitive K562 leukemia cell line. By contrast, IL-2-stimulated NK cells were capable of killing hMAPCs, and preactivated NK cells were not influenced during their cytotoxic effector function against K562 cells by hMAPCs. When added during the 6-day preactivation phase with IL-2, hMAPCs dose-dependently reduced NK cell proliferation in an IDO-dependent manner, but they did not influence the induction of cytotoxic capacity by IL-2. This study indicates that human MAPCs mutually interact with NK cells.

## 2. Introduction

Stem cell-based therapy has become a promising tool to control immune responses. In the past decade, human mesenchymal stem cells (hMSCs) have been studied for their role as a cellular immunosuppressive cell population.[41,197] MSCs are bone marrow (BM)-derived cells, which are capable of differentiating into chondrocytes, osteoblasts and adipocytes.[23,198] Clinical trials wherein hMSCs are infused intravenously are currently under way to evaluate their ability to prevent or suppress graft-*versus*-host disease (GvHD) in patients who underwent HSCT or to treat autoimmune diseases.[144,149] The results of phase I and II clinical trials have demonstrated the feasibility and safety of *in vivo* use of these cells. The rationale for the use of hMSCs as an immunosuppressive cell population is based on a large number of studies that have shown that hMSCs inhibit T cell responses *in vitro* [72,75,77,86], aside from affecting B cells [107], dendritic cells [118,120], and natural killer (NK) cells *in vitro*.[113,114,116]

NK cells are part of the innate immune system and play a key role in the immune defense against viral infections and in anti-tumor immune responses, based on their cytolytic function and production of proinflammatory cytokines. NK cell function is regulated by the balance between activating and inhibitory signals transduced by multiple cell surface receptors on NK cells.[199] NK cell-mediated killing of a target cell requires the presence of activating ligands on the target cell interacting with activating receptors on the NK cells in combination with low or absent levels of major histocompatibility complex (MHC) class I molecules on the target cell, as the latter stimulate inhibitory receptors.[200,201] MSCs are known to express high levels of MHC class I molecules.[56] Both autologous and allogeneic MSCs can be lysed by activated NK cells. On the other hand, MSCs themselves can inhibit the cytotoxic activity and proliferation of NK cells.[113,114,116]

Human multipotent adult progenitor cells (hMAPCs) are also BM-derived stem cells.[56] Compared with human MSCs, hMAPCs can be expanded more extensively than hMSCs. In addition, hMAPCs express lower levels of MHC class I molecules and differentiate robustly into endothelium both *in vitro* and *in vivo* in Matrigel plug assays. Like hMSCs, hMAPCs suppress allogeneic T cell responses *in vitro* and block ongoing and secondary allogeneic T cell responses and responses of memory T cells.[166] Their immunosuppressive potency, combined with their extensive proliferation potential, means that hMAPCs are a preferable alternative source for cell-based immunotherapy because a large cohort of patients can be treated with one single and well-defined batch of cells. Clinical trials with MultiStem®, the clinical-grade product of MAPCs, are being performed to test their ability to prevent acute

GvHD, treat inflammatory bowel disease (IBD), and prevent rejection of liver grafts, aside from evaluating their ability to improve cardiac and neural function, when administered in the setting of acute myocardial infarction and stroke (www.ClinicalTrials.gov).

No systematic studies have been performed to address the interaction between hMAPCs and NK cells, so we here describe the NK cell function in the presence of hMAPCs and the hMAPC-mediated modulation of NK cell function during the interleukin (IL)-2-driven stimulation and cytolytic effector phase of NK cells.

## 3.  Materials and methods

*Isolation and culture of stem cells*

hMAPC ($n$ = 6) isolations were done by the Stem Cell Institute Leuven (SCIL, Leuven, Belgium) from bone fragments of four children (two male and two female between 5 and 15 years old; donors 1-4) undergoing orthopedic surgery or by ReGenesys (www.regenesys.eu; Heverlee, Belgium) from the bone marrow of two healthy volunteers [a 45-year-old male (donor 5) and a 30-year-old female (donor 6)], after obtaining informed consent in accordance with the guidelines of the Medical Ethics Committee of the University Hospitals Leuven. Isolation and culture of the cells were performed as previously described.[53] Briefly, hMAPCs were generated by plating the total cell fraction at $0.5 \times 10^6$ cells/cm$^2$ in medium consisting of 60% Dulbecco's modified Eagle's medium (DMEM) low-glucose (Gibco, Invitrogen, Carlsbad, CA, USA), 40% MCDB-201 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 50 nM dexamethasone, $10^{-4}$ M L-Ascorbic Acid, 1x insulin-transferrin-selenium (ITS), $0.5\times$ linoleic acid-bovine serum albumin (LA-BSA) (all from Sigma-Aldrich), 1% penicillin/streptomycin (Gibco, Invitrogen), along with 2% Serum Supreme (Lonza BioWhittaker, Basel, Switzerland) and 10 ng/ml human platelet-derived growth factor (PDGF)-BB (R&D Systems, Minneapolis, MN, USA) and epidermal growth factor (EGF; Sigma-Aldrich). MAPC cultures were maintained under hypoxic conditions (5% $O_2$) at a density of 400 cells/cm$^2$ and were split every 2 to 3 days. Clonal populations of hMAPCs isolated by SCIL were obtained through limiting dilution by plating five cells/well in a 96-well or 48-well plate (Corning, NY, USA) between passages 5 to 10. Cells were used at PD 25-30. In some experiments, hMAPCs were treated with 100 U/ml interferon (IFN)-γ (Roche Diagnostics, Vilvoorde, Belgium) for 48 h.

hMSCs were generated by ReGenesys from the bone marrow of the two adult hMAPC donors (donors 5 and 6) by plating the mononuclear fraction, obtained after Lymphoprep[TM] (Axis-Shield, Oslo, Norway) density gradient centrifugation, at $0.5 \times 10^6$ cells/cm² in MSC growth medium containing DMEM high-glucose, 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin (all from Lonza). MSC cultures were maintained at 5,000 cells/cm², at normal oxygen level (20% $O_2$), were split every 4 to 7 days, were not clonally derived and were used at PD 20-25.

*Isolation and culture of peripheral blood mononuclear cells and NK cells*

All subjects donating blood for these experiments were healthy volunteers of both sexes, aged 20 to 50 years. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque (Axis-Shield) density gradient centrifugation (specific gravity, 1.077 g/ml). Untouched NK cells were negatively selected from PBMCs using the human NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purity ranged from 88% to 98%.

To obtain activated NK cells, PBMCs or purified NK cells were cultured for 6 days in Roswell Park Memorial Institute (RPMI) 1640 with 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all Lonza), and 10% autologous serum supplemented with 100 U/ml recombinant human IL-2 (TECIN; Hoffmann-La Roche, Nutley, NJ, USA) in T25 culture flasks (Greiner Bio-One, Wemmel, Belgium). PBMCs were cultured at $10 \times 10^6$ cells in 10 ml, while purified NK cells were cultured at 1 to $3 \times 10^6$ cells in 10 ml. To these cultures, nonirradiated hMAPCs were added in a ratio of 1:3 PBMCs or purified NK cells.

*Flow cytometry*

hMAPCs, cultured PBMCs, or NK cells ($1 \times 10^5$ for each) per sample were suspended in 100 µl phosphate-buffered saline (PBS; Lonza) supplemented with 10% heat-inactivated human serum (Lonza) to block nonspecific staining. Cells were subsequently surface stained with 5 µl fluorescence-conjugated specific monoclonal antibodies. The specifications of the antibodies used for flow cytometry are described in **Table 1**. Isotype control staining was performed. Acquisition was done using a FACSort or FACSCanto (BD Biosciences, Erembodegem, Belgium). For analysis of the samples, CellQuest Pro or BD FACSDiva software was used.

**Table 1: List of monoclonal antibodies used for flow cytometry**

| Monoclonal Ab | Supplier (catalog No.) | Label | Isotype |
|---|---|---|---|
| CD3 | BD Pharmingen (Erembodegem, Belgium; 555332) | FITC | IgG1 |
| CD56 | BD Biosciences (Erembodegem, Belgium; 345810) | PE | IgG1 |
| CD3/CD16+CD56 | BD Simultest (342403) | FITC/PE | IgG1 |
| HLA-ABC | BD Pharmingen (555552) | FITC | IgG1 |
| HLA-E | eBioscience (Vienna, Austria; 12-9953) | PE | IgG1 |
| Nectin-2 (CD112) | BD Pharmingen (551057) | PE | IgG1 |
| PVR (CD155) | eBioscience (12-1550) | PE | IgG1 |
| MICA/B | BD Pharmingen (558352) | PE | IgG2a |
| ULBP-1 | R&D Systems (Abingdon, UK; FAB1380P) | PE | IgG2a |
| ULBP-2/5/6 | R&D Systems (FAB1298P) | PE | IgG2a |
| ULBP-3 | R&D Systems (FAB1517P) | PE | IgG2a |
| IgG1 | BD Biosciences (345815) | FITC | |
| IgG1 | BD Biosciences (345816) | PE | |
| IgG2a | BD Pharmingen (555574) | PE | |

CD, cluster of differentiation; HLA, human leukocyte antigen; PVR, poliovirus receptor; MICA/B, major histocompatibility complex (MHC) class I chain-related genes A and B; ULBP, UL-16 binding protein; Ig, immunoglobulin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

*Cytotoxicity assays*

The viability of hMAPC target cells was tested with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, Bornem, Belgium) assay as previously described with some modifications.[202] Briefly, target cells were seeded in a flat-bottomed 96-well cell culture plate (TPP, St. Louis, MO, USA) at a density of 5 x $10^4$ cells/well and cultured overnight to become adherent to the plate. The next day, NK cells were added at different effector:target (E:T) ratios and cocultured for 24 h, after which the medium, together with the nonadherent cells, was removed. Subsequently, the target cells were rinsed with PBS at room temperature, and 100 µl of a 0.5 mg/ml MTT solution was added to the residual adherent cells. After 2 h of incubation, the MTT solution was removed, and 100 µl dimethyl sulfoxide (Merck, Darmstadt, Germany) per well was added to resolve the formazan produced in the viable cells. After stirring the plate for 5 min, optical density was measured at 570 and 620 nm using an ELISA reader (Thermo Labsystems, Franklin, MA, USA). The $OD_{570-620\ nm}$ value was used as a measure for cell viability.

Cytotoxicity assays were also performed using a 4 h $^{51}$Cr-release method. Target cells were labeled with 100 µCi $^{51}$Cr/$10^6$ cells ($^{51}$Cr, Perkin Elmer Life Sciences, Inc., Zaventem, Belgium) and seeded at $10^4$ cells/well in round-bottomed 96-well plates (Greiner Bio-One). As control target cells, the human NK-sensitive and MHC class I-deficient K562 leukemia cell line was used (ATCC, Manassas, VA, USA). The lytic potential of NK cells was tested by coculturing cells at different E:T ratios. Saponin (Merck) was added to the target cells to measure the maximum release of $^{51}$Cr. Release of $^{51}$Cr was measured by a Topcount gamma counter (Packard Instrument Company, Meriden, CT, USA). The percentage cytotoxicity was calculated as [(experimental release − spontaneous release)/(maximal release − spontaneous release)] x 100.

To test the influence of hMAPCs during the lytic functioning of NK cells against K562 cells, nonirradiated hMAPCs were added at 1:2 ratio MAPC:NK at the beginning of the 4 h assay. We used the NK-resistant cell line KM-H2 (Hodgkin disease-derived cell line; kindly provided by Dr. S. Fukuhara , Kyoto University, Kyoto, Japan) as control cells for hMAPCs in this assay.

*Proliferation assay*

Responder purified NK cells (1 x $10^5$) were stimulated with 100 U/ml exogenous recombinant IL-2 (TECIN; Hoffmann-La Roche). Irradiated (30 Gy) allogeneic hMAPCs were added at

different suppressor:responder (S:R) ratios. NK cell proliferation was measured at day 6 by means of an 8 h pulse with $1\mu$Ci/well [3H]thymidine (MP Biomedicals Europe, Illkirch, France). [3H]Thymidine incorporation was measured by using a liquid scintillation counter (Tri-Carb® 2100TR Liquid Scintillation Counter, PerkinElmer). The data were analyzed as mean counts per minute (cpm) of quadruplicate wells. The results are expressed as percent response related to the control response in the absence of hMAPCs.

To analyze the involvement of indoleamine 2,3-dioxygenase (IDO), prostaglandin $E_2$ (PGE$_2$), transforming growth factor (TGF)-β and IL-10 as immunosuppressive mediators, we used their respective inhibitors: 200 µM/ml 1-methyl-trypthophan (1-MT; Sigma-Aldrich), 2 µg/ml indomethacin (Cayman Chemical Company, Ann Arbor, MI, USA), 50 µg/ml anti-TGF-β neutralizing mAb (R&D Systems, Abingdon, UK), and 2.5 µg/ml anti-IL-10 plus 2.5 µg/ml anti-IL-10 receptor mAb (both from R&D Systems).

*Statistical analysis*

Statistics were calculated with Prism software 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was calculated by paired or unpaired *t* tests for comparisons between two groups and by one-way ANOVA with Dunnett's post hoc test for comparisons between three or more groups. Values of $p < 0.05$ were considered significant.

# 4. Results

## 4.1 Human MAPCs express ligands of activating NK receptors

To assess whether hMAPCs might be susceptible for NK cell-mediated lysis, we first analyzed hMAPCs for the expression of ligands recognized by activating and inhibitory NK cell receptors. **Table 2** shows the expression level of ligands of NK cell receptor D (NKG2D) [UL16-binding proteins (ULBPs) and MHC class I chain-related genes A and B (MICA/B)] and DNAX accessory molecule-1 [DNAM-1; cluster of differentiation 226 (CD226)] [Nectin-2 and poliovirus receptor (PVR; CD155)] triggering receptors and of inhibitory MHC class I molecules (HLA-ABC and nonclassic HLA-E) in five hMAPC populations (donors 1-5). Flow cytometric analysis showed that all hMAPC populations are dimly positive for HLA-ABC [mean fluorescence intensity (MFI) $\pm$ SEM for the five hMAPC populations: 1,992 $\pm$ 246] and negative for HLA-E. hMAPCs expressed PVR (8,540 $\pm$ 150) and low levels of ULBP-2/5/6 (1,173 $\pm$ 123), but generally did not express MICA/B, Nectin-2, ULBP-1, and ULBP-3. Upon stimulation with IFN-$\gamma$, expression of MHC class I molecules was upregulated, whereas the expression of the activating ligands remained unchanged.

**Table 2: NK cell receptor ligand expression by hMAPCs**

| | | Donor | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | 4 | | 5 | |
| Marker | - | + | - | + | - | + | - | + | - | + |
| bare cells FITC | 336 | 266 | 276 | 309 | 443 | 506 | 245 | 301 | 391 | 416 |
| bare cells PE | 212 | 189 | 170 | 187 | 270 | 311 | 154 | 188 | 241 | 265 |
| isotype IgG1 FITC | 368 | 402 | 330 | 333 | 534 | 560 | 251 | 325 | 439 | 473 |
| isotype IgG1 PE | 455 | 481 | 403 | 474 | 537 | 615 | 348 | 413 | 555 | 594 |
| isotype IgG2a PE | 238 | 233 | 194 | 200 | 264 | 307 | 246 | 266 | 346 | 358 |
| HLA-ABC FITC | 1,826 | 12,737 | 3,470 | 13,363 | 1,843 | 7,469 | 1,440 | 8,488 | 1,381 | 10,134 |
| HLA-E PE | 619 | 2189 | 900 | 2450 | 640 | 1569 | 486 | 1850 | 708 | 2230 |
| MICA/B PE | 250 | 262 | 583 | 760 | 329 | 344 | 461 | 423 | 346 | 544 |
| Nectin-2 PE | 175 | 229 | 368 | 380 | 656 | 598 | 309 | 411 | 208 | 243 |
| PVR PE | 8114 | 8580 | 8137 | 8074 | 9269 | 9736 | 8907 | 10428 | 8275 | 8497 |
| ULBP-1 PE | 381 | 370 | 1023 | 982 | 399 | 369 | 423 | 468 | 616 | 666 |
| ULBP-2/5/6 PE | 756 | 710 | 1263 | 1093 | 867 | 890 | 1137 | 1114 | 1844 | 1831 |
| ULBP-3 PE | 247 | 239 | 305 | 325 | 238 | 292 | 258 | 275 | 404 | 330 |

Flow cytometric analysis of five human multipotent adult progenitor cell (hMAPC) donors (donors 1-5) for ligands of inhibitory (HLA-ABC and HLA-E) and activating natural killer (NK) cell receptors (MICA/B, Nectin-2, PVR, ULBP-1, ULBP-2/5/6 and ULBP-3) before and after treatment with 100 U/ml interferon (IFN)-γ for 48 h. Results are expressed as mean fluorescence intensity (MFI). Positive values are underlined. -, without IFN-γ pretreatment; +, with IFN-γ pretreatment.

## 4.2 Resting NK cells do not kill hMAPCs but are blocked in their cytolytic function by hMAPCs

The combination of expression of ligands of activating NK cell receptors together with low levels of MHC class I molecules on hMAPCs suggests that hMAPC target cells might be killed by allogeneic NK cells. To investigate whether hMAPCs are susceptible to NK cell-

mediated lysis, freshly isolated NK cells were cocultured with allogeneic hMAPCs at E:T ratios of 1:1 to 8:1 during 24 h. Coculture was followed by an assessment of hMAPC viability using the MTT assay. Human MAPCs were not killed by resting allogeneic NK cells (**Fig. 1A**). We confirmed these findings using a chromium release assay. This revealed that hMAPCs were only minimally lysed by resting allogeneic NK cells even at higher E:T ratios (**Fig. 1B**).
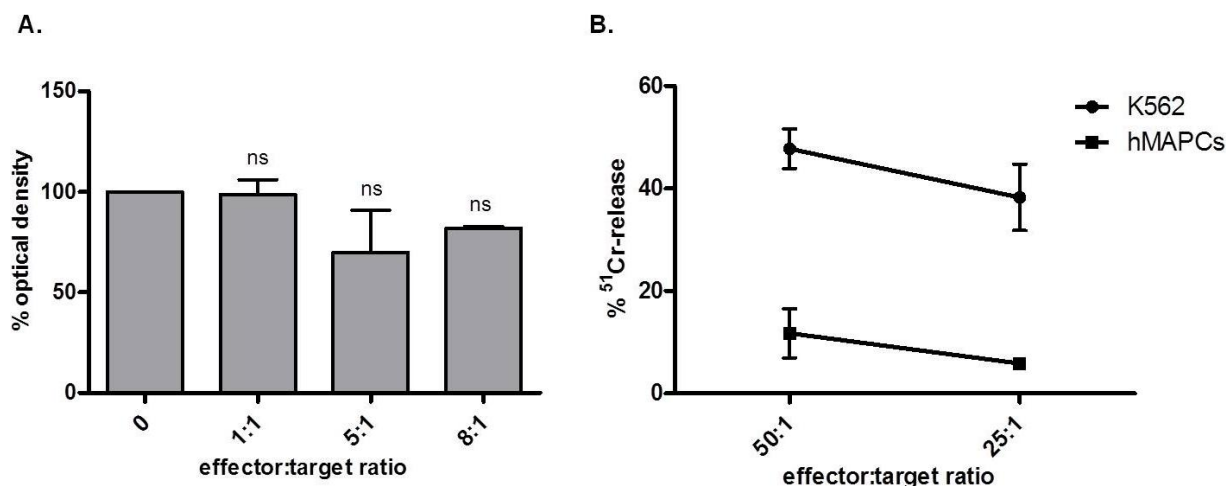


**Figure 1. Human MAPCs are not killed by resting NK cells.**

(A) Freshly isolated natural killer (NK) cells ($n = 3$) were cocultured with allogeneic human multipotent adult progenitor cell (hMAPC) target cells ($n = 2$ ; donors 5 and 6) at effector:target (E:T) ratios of 1:1 to 8:1 during 24 h. Coculture was followed by an assessment of hMAPC viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data are expressed as percentage (mean ± SEM) of target cell viability (optical density) with respect to target cell viability without coincubation of NK cells (100%). Statistical significance was tested when compared with control condition without NK cells. One-way ANOVA with Dunnett's post hoc test was used, ns = not significant.

(B) Freshly isolated NK cells were cocultured in a standard $^{51}$Cr-release assay with K562 cells or allogeneic hMAPCs at different E:T ratios of 50:1 to 25:1. Data are expressed as mean ± SEM percentage $^{51}$Cr-release of four different experiments with four different NK cell donors and three different hMAPC donors (donors 1, 5, 6).

Subsequently, to investigate whether hMAPCs could interfere with the effector function of resting NK cells, we added hMAPCs at the beginning of a $^{51}$Cr-release assay of freshly isolated NK cells against K562 target cells. As shown in **Figure 2A**, hMAPCs impaired the cytotoxic activity of resting NK cells (mean ± SEM % $^{51}$Cr-release: 44.81 ± 4.97 % *versus* 60.32 ± 3.40 %). To exclude the possibility of "cold target inhibition" by hMAPCs [i.e. lysis inhibition of $^{51}$Cr-labeled (hot) target cells by addition of unlabeled (cold) target cells], the NK-resistant cell line KM-H2 was used as a modulating cell line instead of hMAPCs. The

resistance of KM-H2 cells to NK cell-mediated killing was verified (data not shown). Addition of KM-H2 cells at the beginning of a $^{51}$Cr-release assay of freshly isolated NK cells against K562 target cells did not significantly influence the cytotoxic activity of the NK cells, in contrast to hMAPCs (**Fig. 2B**).
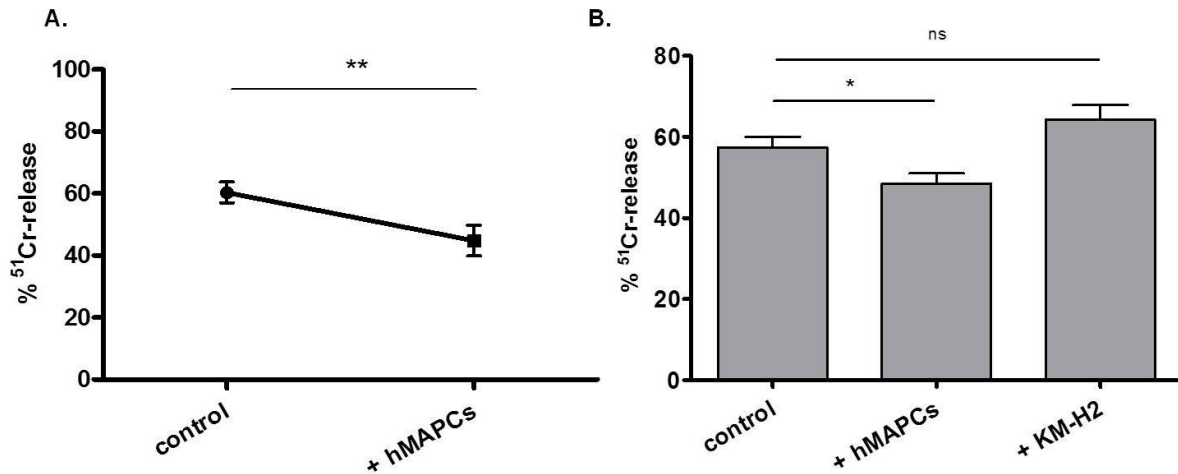


**Figure 2. Impaired cytotoxic activity of resting NK cells in the presence of hMAPCs.**

(A) Results of cytotoxic activity of freshly isolated NK cells against K562 target cells (E:T 25:1) in the absence (control) or presence (+ hMAPCs) of allogeneic hMAPCs at suppressor:responder (S:R) ratio of 1:2. Results are expressed as mean ± SEM percentage $^{51}$Cr-release of 10 experiments, in which six different NK cell donors and two different hMAPC donors (donors 5 and 6) were used. Statistical significance was calculated with the paired $t$ test. **$p < 0.01$.

(B) Results of cytotoxic activity of freshly isolated NK cells against K562 target cells (E:T 25:1) in the absence (control) or presence of allogeneic hMAPCs (+ hMAPCs) or NK-resistant KM-H2 cells (+ KM-H2) at S:R ratio of 1:2. Results are expressed as mean ± SEM percentage $^{51}$Cr-release of four experiments, in which four different NK cell donors and two different hMAPC donors (donors 5 and 6) were used. Statistical significance was calculated with ANOVA and Dunnett's post hoc test. ns = not significant, *$p < 0.05$.

## 4.3 Activated NK cells lyse allogeneic hMAPCs

The adoptive transfer of hMAPCs is aimed at controlling immune responses. The local inflammatory environment wherein hMAPCs reside upon injection will probably lead to activation of the regional NK cell population. To address the interaction between activated NK cells and allogeneic hMAPCs, we activated NK cells with rIL-2 for 6 days and tested their ability to kill allogeneic hMAPC target cells. The MTT viability assay revealed that preactivated NK cells were capable of killing allogeneic hMAPCs (**Fig. 3A**). We confirmed these findings using a chromium release assay, showing that the chromium release of hMAPCs as target cells for activated NK cells was similar to the control condition using K562

cells (**Fig. 3B**), indicating that hMAPCs can be killed by activated NK cells. However, pretreatment of hMAPCs with 100 U/ml IFN-γ for 48 h, leading to an upregulation of HLA-ABC and HLA-E expression (**Table 2**), rendered those IFN-γ-pretreated hMAPCs resistant to cytotoxic lysis by activated NK cells (6.59 ± 2.80 % $^{51}$Cr-release *versus* 21.29 ± 5.55 % $^{51}$Cr-release; **Fig. 3C**).
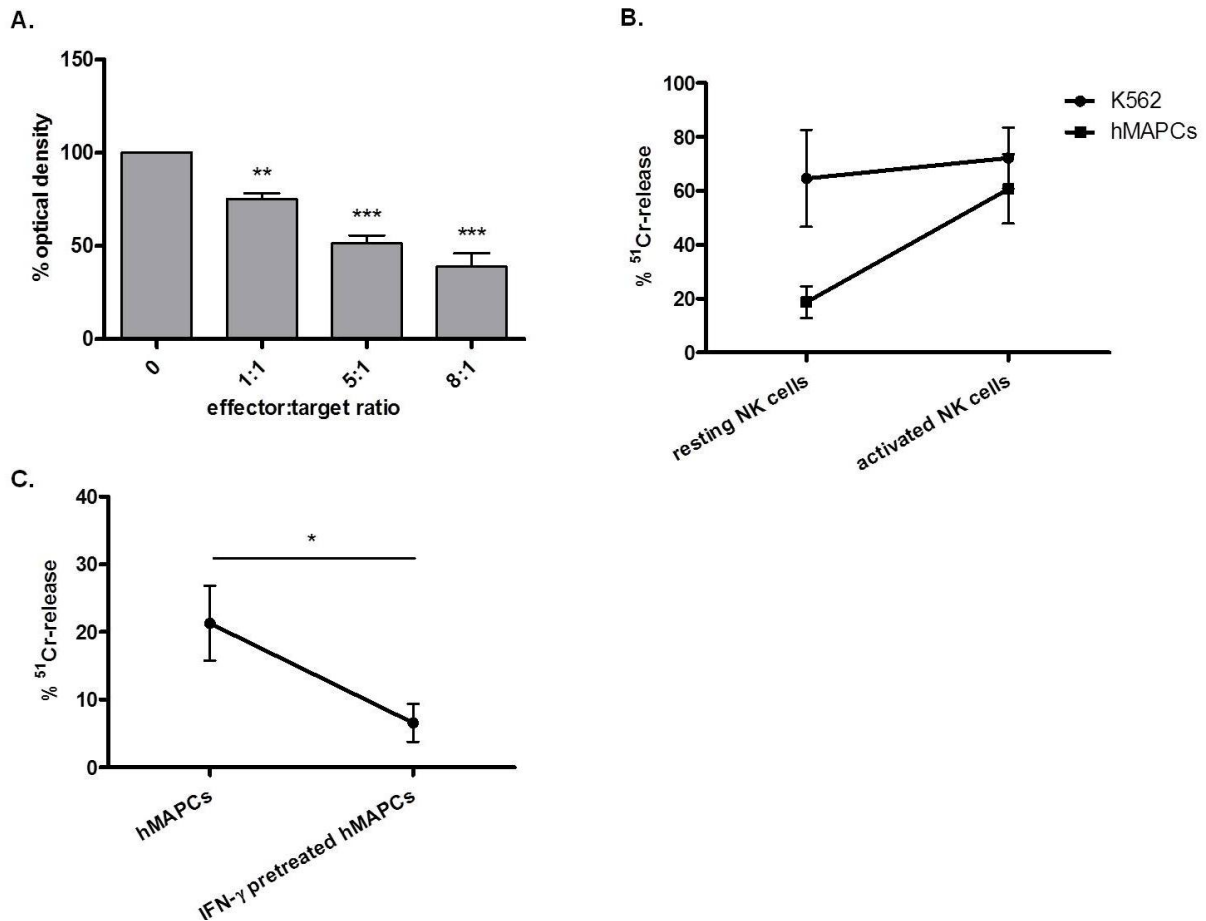


**Figure 3. Human MAPCs are killed by activated NK cells.**

(A) NK cell populations ($n = 2$) that were exposed to 100 U/ml recombinant interleukin (IL)-2 for 6 days were cocultured with allogeneic hMAPC ($n = 2$ ; donors 5 and 6) target cells at E:T ratios of 1:1 to 8:1. Coculture for 24 h was followed by an assessment of hMAPC viability using the MTT assay. Data are expressed as percentage (mean ± SEM) of target cell viability (optical density) with respect to target cell viability without coincubation of NK cells (100%). Statistical significance was tested when compared with control condition without NK cells. One-way ANOVA with Dunnett's post hoc test was used, ***$p < 0.001$, **$p < 0.01$.

(B) Cytotoxic activity measured by $^{51}$Cr-release assay of freshly isolated NK cells (resting NK cells) and NK cell populations cultured for 6 days with rIL-2 (activated NK cells) against K562 or allogeneic hMAPC target cells at an E:T ratio of 25:1. Data are expressed as mean ± SEM percentage $^{51}$Cr-release of five different experiments with three different NK cell donors and three different hMAPC donors (donors 1, 5, 6).

(C) Cytotoxic activity measured by $^{51}$Cr-release assay of activated NK cell populations cultured for 6 days with rIL-2, coincubated with allogeneic hMAPCs or interferon (IFN)-γ pretreated hMAPCs at an

E:T ratio of 25:1. Data are expressed as mean ± SEM percentage $^{51}$Cr-release of five different experiments with three different NK cell donors and three different hMAPC donors (donors 1, 5, 6). Statistical significance was calculated with the unpaired $t$ test, $*p < 0.05$.

In addition, we tested the ability of hMAPCs to influence the cytotoxic function of activated NK cells during their effector phase. Therefore, a standard $^{51}$Cr-release assay was performed using NK cells that were cultured for 6 days in the presence of 100 U/ml rIL-2, and cytotoxic activity against K562 target cells was tested at an E:T ratio of 25:1 in the presence or absence of allogeneic hMAPCs (ratio hMAPC:NK cell 1:2). We noticed that the presence of hMAPCs reduced the percentage of $^{51}$Cr-release (41.60 ± 3.12 %) compared to the conditions cultured in the absence of hMAPCs (64.80 ± 4.17 %) (**Fig. 4**). However, because activated NK cells have been shown to kill hMAPCs as well, we could not exclude that the reduction in $^{51}$Cr-release was due to competition between hMAPCs and K562 cells as both cell populations are known target cells for the activated NK cells. To test this hypothesis, a $^{51}$Cr-release assay was performed using rIL-2-activated NK cells against K562 cells (E:T 25:1) in the presence or absence of extra unlabeled (cold) or $^{51}$Cr-labeled (hot) K562 cells or hMAPCs (both 1:2 ratio to NK cells). In comparison to the control condition, the percentage $^{51}$Cr-release decreased with extra addition of unlabeled K562 cells or hMAPCs, while the percentage clearly increased with extra addition of labeled K562 cells or hMAPCs (data not shown). Addition of supernatant of cultured hMAPCs did not influence the cytotoxic function of rIL-2-activated NK cells. These observations imply a cold target inhibition effect.
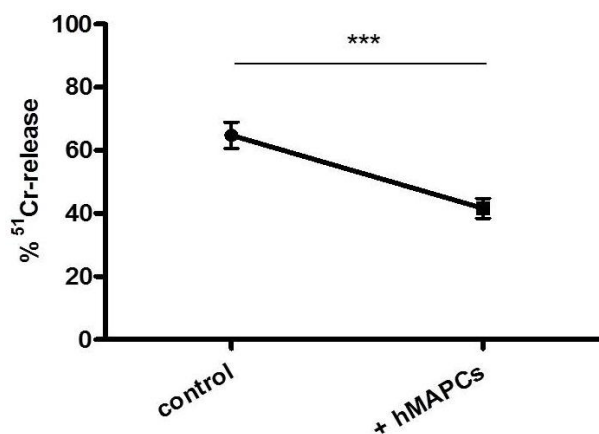


**Figure 4. Impaired cytotoxic activity of activated NK cells in the presence of hMAPCs.**
Results of cytotoxic activity of rIL-2-activated NK cells against K562 target cells (E:T 25:1) in the absence (control) or presence (+ hMAPCs) of allogeneic hMAPCs at S:R of 1:2. Results are expressed as mean ± SEM percentage $^{51}$Cr-release of four experiments, in which two different NK cell donors

and two different hMAPC donors (donors 5 and 6) were used. Statistical significance was calculated with the paired $t$ test. ***$p < 0.001$.

## 4.4 Human MAPCs inhibit IL-2-induced proliferation of allogeneic NK cells in an IDO-dependent manner

We finally assessed whether hMAPCs have a similar suppressive effect on NK cell proliferation as they have on T cell proliferation. NK cells were stimulated with rIL-2 (100 U/ml) in the presence or absence of irradiated allogeneic hMAPCs at different S:R ratios and evaluated with [$^3$H]thymidine incorporation after 6 days. As shown in **Figure 5A**, human MAPCs dose-dependently suppressed IL-2-induced proliferation of highly purified NK cells. The expansion of NK cells (defined as % CD3$^-$CD56$^+$ cells) was also blocked when total PBMC fractions were cultured in medium supplemented with exogenous rIL-2 for 6 days in the presence of hMAPCs (10.64 ± 2.77 % CD3$^-$CD56$^+$ cells) as compared to similar cultures of PBMCs in the absence of hMAPCs (37.57 ± 3.59 % CD3$^-$CD56$^+$ cells) (**Fig. 5B**). Of note, in this set of experiments, hMSCs from the same donor as the hMAPCs were available. The decrease in the expansion of NK cells was less pronounced when PBMCs were activated with rIL-2 in the presence of hMSCs (22.70 ± 8.76 % CD3$^-$CD56$^+$ cells), compared to the condition with hMAPCs.

**A.**



**B.**
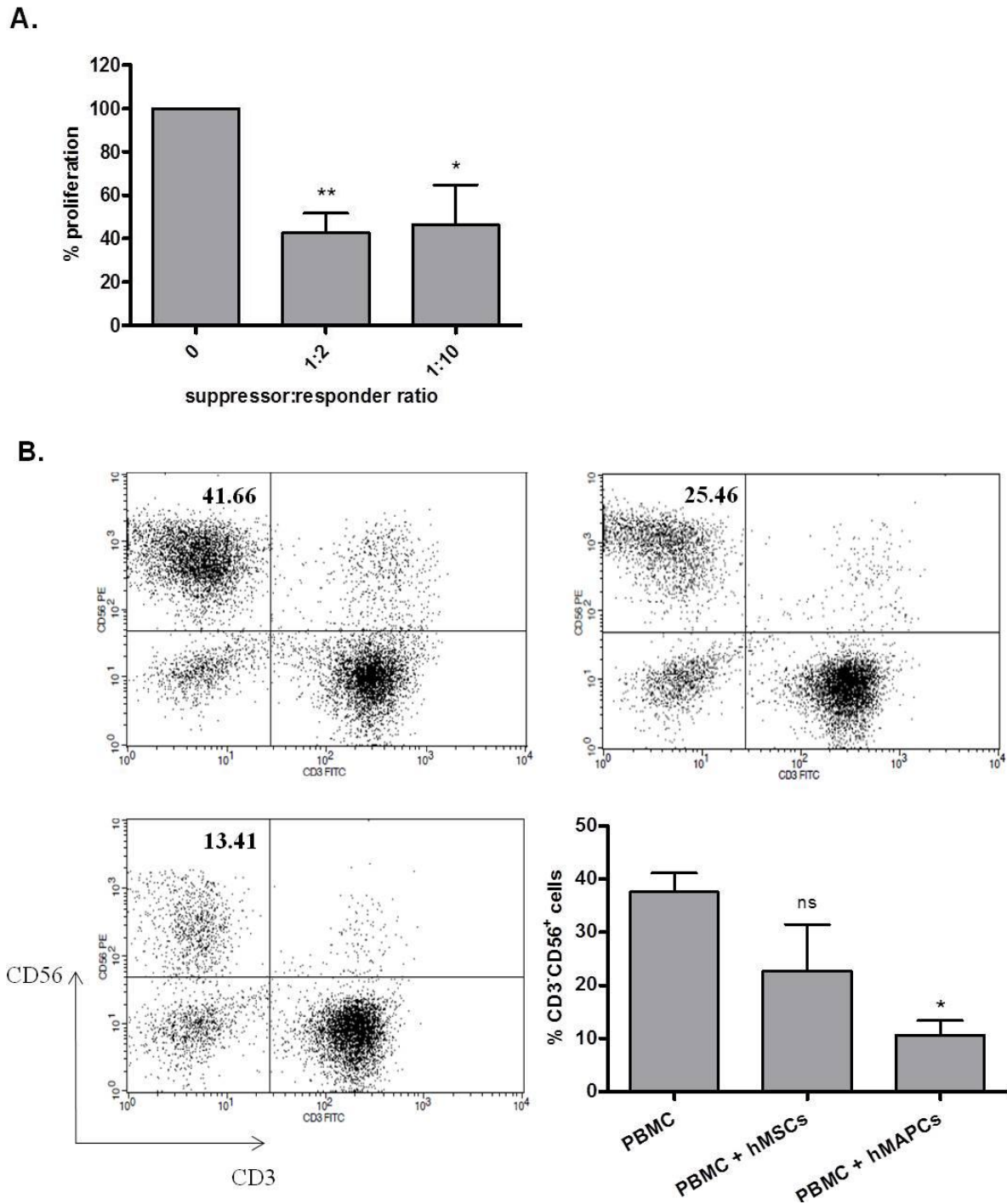


**Figure 5. hMAPCs dose-dependently suppress IL-2-induced NK cell proliferation.**

(A) Freshly isolated NK cells were cultured during 6 days in medium supplemented with 100 U/ml rIL-2 in the absence or presence of allogeneic irradiated (30 Gy) hMAPCs at different S:R ratios. The proliferative response was measured on day 6 by [$^3$H]thymidine incorporation. Results are expressed as mean ± SEM percentage proliferation relative to control cultures in the absence of hMAPCs of six experiments in which five different NK cell donors and three different hMAPC donors (donors 1, 5, 6) were used. Statistical significance was calculated with one-way ANOVA with Dunnett's post hoc test, **$p < 0.01$, *$p < 0.05$.

(B) Total peripheral blood mononuclear cell (PBMC) fractions were cultured in medium supplemented with exogenous rIL-2 for 6 days in the absence (upper left) or presence of human mesenchymal stem cells (hMSCs; upper right) or hMAPCs (lower left) from the same donor at S:R ratio of 1:3. The

expansion of NK cells [percentage cluster of differentiation 3 negative cluster of differentiation 56 positive (CD3⁻CD56⁺) cells] in the total PBMC fraction was afterwards analyzed by flow cytometry. One out of three representative experiments is shown in the flow cytometry plots. In the bar graph (lower right), results from three different experiments with three different PBMC donors and two hMSC/hMAPC donors (donors 5 and 6) were pooled and expressed as mean ± SEM percentage CD3⁻ CD56⁺ cells. Statistical significance was calculated with one-way ANOVA with Dunnett's post hoc test, ns = not significant, $*p < 0.05$.

The immune modulatory mechanism of hMAPCs is mediated at least in part via a soluble factor [166], so we performed some functional tests to identify the responsible mediator. Blocking IDO with 1-MT completely neutralized the inhibitory activity of hMAPCs on IL-2-induced NK cell proliferation, suggesting IDO as a mediator of the immune modulation by hMAPCs (**Fig. 6**). Addition of monoclonal antibodies neutralizing IL-10 plus IL-10R, or monoclonal antibodies to neutralize TGF-β, or addition of indomethacin to neutralize PGE$_2$ synthesis did not change the immunomodulatory properties of hMAPCs on IL-2-induced NK cell proliferation. None of these molecules mediated the immune modulatory role of hMAPCs on the cytotoxic activity of resting NK cells against K562 cells (data not shown).
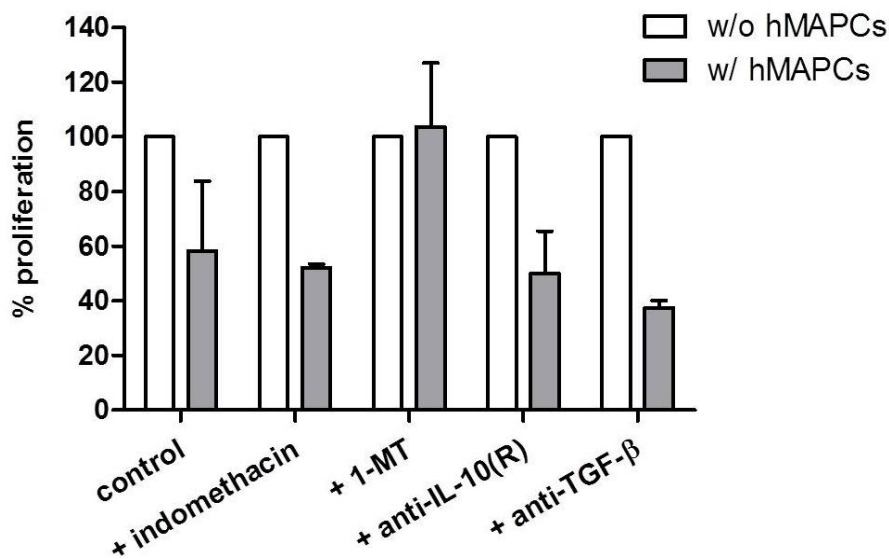


**Figure 6. The suppressive effect of hMAPCs on NK cell proliferation is dependent on IDO activity.**

Freshly isolated NK cells were cultured during 6 days in medium supplemented with 100 U/ml rIL-2 in the absence or presence of irradiated hMAPCs at S:R ratio of 1:2 without (control) or with addition of 2 µg/ml indomethacin, 200 µM/ml 1-methyl tryptophan (1-MT), 2.5 µg/ml anti-IL-10 plus 2.5 µg/ml anti-IL-10 receptor (R) mAb, or 50 µg/ml anti-transforming growth factor (TGF)-β neutralizing mAb. The proliferative response was measured on day 6 by [³H]thymidine incorporation. Data are expressed as mean ± SEM percentage proliferation relative to control cultures in the absence of hMAPCs (100 %) of three experiments in which two NK cell donors and two hMAPC donors (donors 5 and 6) were used. The average counts per minute (cpm) of the cultures without hMAPCs for all

conditions were all similar to the control condition without hMAPCs (control: 13855; indomethacin: 13949; 1-MT: 14258; anti-IL-10(R): 16161; anti-TGF-β: 14317). IDO, indoleamine 2,3-dioxygenase.

In the next set of experiments, total PBMC fractions were cultured for 6 days with rIL-2 in the presence or absence of nonirradiated hMAPCs, and the cytolytic function of the NK cells was subsequently measured against K562 cells. As shown in **Figure 7A**, the lytic activity was strongly diminished when the whole PBMC population was stimulated with rIL-2 in the presence of hMAPCs compared to the control condition without hMAPCs. We did not see any influence on the cytotoxic activity of the NK cells when PBMCs were activated with rIL-2 in the presence of hMSCs from the same hMAPC donors. The cytolytic activity in these experiments was influenced by the inhibition of NK cell proliferation, thereby influencing the net E:T ratio during the subsequent effector phase, so similar experiments were performed with purified NK cells as responder and effector cells. After stimulation of purified NK cells with rIL-2 in the presence of nonirradiated hMAPCs, the cells were adjusted prior to the cytotoxicity assay. In this condition, we could demonstrate that the cytolytic function of NK cells was retained after rIL-2 activation in the presence of hMAPCs, in spite of the blocked proliferative response (**Fig. 7B**). Of note, addition of irradiated hMAPCs in this experimental condition yielded similar results (data not shown).
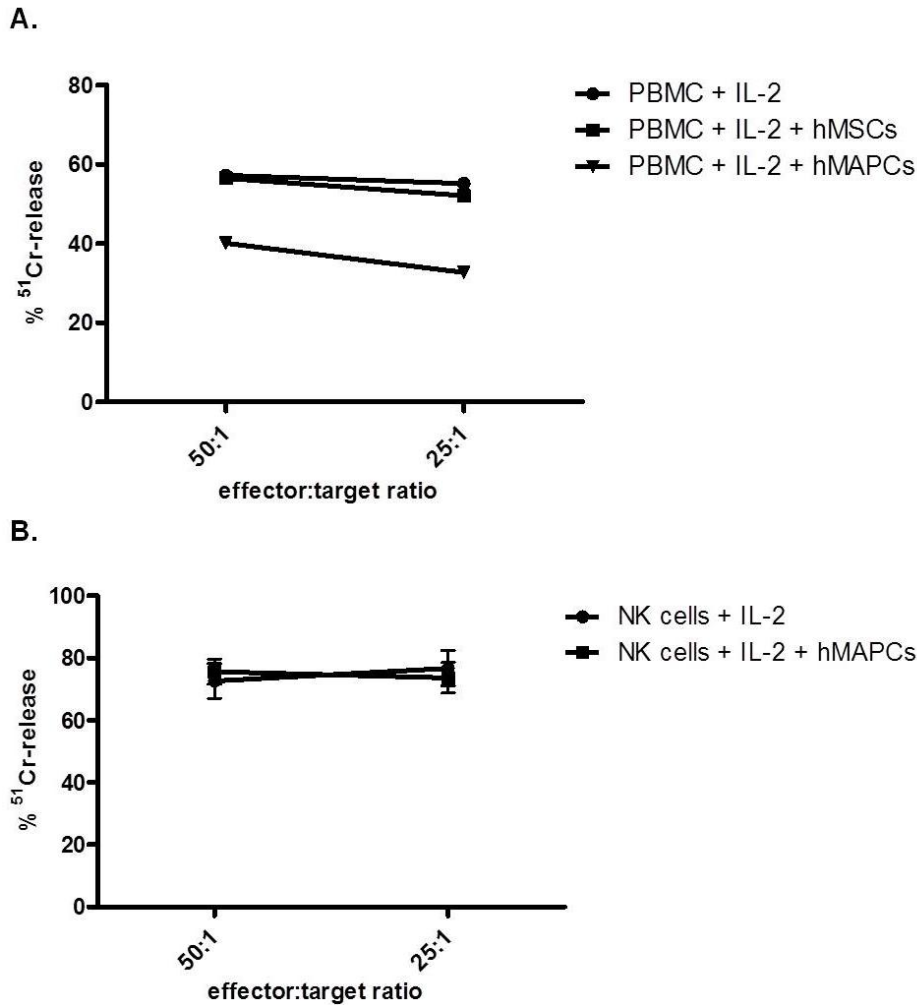
**Figure 7. Suppression of NK cells by hMAPCs during activation with rIL-2.**

(A) After 6-day culture with rIL-2 in the presence or absence of hMAPCs or hMSCs (S:R ratio of 1:3) during the activation phase, the total PBMC fraction was subsequently cocultured with K562 leukemia cell line at E:T ratios of 50:1 and 25:1 during the effector phase. Specific K562 lysis was measured with $^{51}$Cr-release. Data are expressed as mean percentage $^{51}$Cr-release of three independent experiments using three different PBMC donors and two different hMAPC/hMSC donors (donors 5 and 6).

(B) After culture of 6 days with rIL-2 in the presence or absence of hMAPCs (S:R ratio of 1:3) during the activation phase, purified NK cells were cocultured with K562 leukemia cell line at E:T ratios of 50:1 and 25:1 during the effector phase of NK cells. Specific K562 lysis was measured with $^{51}$Cr-release. Data are expressed as mean ± SEM percentage $^{51}$Cr-release of eight experiments using five different NK cell donors and three different hMAPC donors (donors 1, 5, 6).

## 5. Discussion

In the present study, we report on the interaction between hMAPCs and NK cells. Resting NK cells do not kill hMAPCs and are blocked in their cytolytic function by hMAPCs. hMAPCs block IL-2-induced NK cell proliferation but not their subsequent effector function. On the other hand, activated NK cells kill hMAPCs, unless the latter were preincubated with IFN-γ, and are no longer blocked by hMAPCs during their cytotoxic functioning. Hence, there is a balance in the mutual interaction between hMAPCs and NK cells depending on the activation state of the NK cells and the priming of hMAPCs.

Our data on the NK cell susceptibility of allogeneic hMAPCs are in accordance with published reports on hMSCs.[101,113,114] hMAPCs express lower levels of MHC class I molecules as compared to hMSCs [56,166], and also express ligands for activating NK cell receptors on its surface. To trigger a NK cell, failure to recognize appropriate inhibitory ligands on a target cell is mandatory. MSCs express high levels of MHC class I molecules, so it was hypothesized that MSCs would escape NK cell-mediated lysis. Indeed, published data showed that MSCs resist lysis of alloreactive resting NK cells. However, activated NK cells are capable of killing allogeneic and autologous MSCs.[113,114,203] The data suggest that the interaction between MHC class I-specific inhibitory receptors on NK cells and the MHC class I molecules on the MSCs is not sufficient to protect MSCs from lysis. The susceptibility of hMAPCs for activated NK cells may hamper their survival *in vivo*. hMAPCs will be used as an off-the-shelf stem cell product and will be consequently of third-party origin; it can therefore be hypothesized that in an inflammatory environment, which is the situation in the case of GvHD or ischemia, nearly all hMAPCs will be killed. This however might, at least in part, be counteracted by the inflammation-induced upregulation of MHC class I molecules on hMAPCs. Indeed, for hMSCs, Spaggiari *et al*. reported that the upregulation of MHC class I molecules on hMSCs due to stimulation with IFN-γ rendered these cells resistant to NK cell-mediated lysis.[114] We were able to document similar results for hMAPCs. Based on these findings, we can hypothesize that the final outcome of the interaction between hMAPCs and NK cells *in vivo* will depend on the local inflammatory environment.

The presence of hMAPCs impaired the cytolytic potential of resting NK cells *in vitro*. hMAPCs were not killed by resting NK cells, so the reduced cytotoxic effect of resting NK cells in the presence of hMAPCs was not due to competition of target cells. We confirmed this hypothesis by using NK-resistant KM-H2 cells instead of hMAPCs as modulating cells in some experiments. The question whether hMAPCs were able to block the cytotoxic function

of rIL-2-activated NK cells could not be answered in our experiments, as activated NK cells were killing both the K562 target cells and hMAPCs.

hMAPCs suppressed rIL-2-induced proliferation of NK cells during stimulation with rIL-2 for 6 days but not their cytotoxic function. The mechanism responsible for the immunosuppression of hMAPCs is not yet completely understood. Several candidate molecules have been proposed as the soluble immunosuppressive factor produced by hMSCs, although data are contradictory because of different experimental designs to study immunosuppression.[75,77,85,86] In our hands, blockage of TGF-β, IL-10, or PGE$_2$ synthesis did not influence the suppressive effect of human MAPCs on rIL-2-induced NK cell proliferation. Addition of 1-MT to cocultures of freshly isolated NK cells with hMAPCs resulted in a loss of the hMAPC-mediated suppressive effect on IL-2-mediated NK cell proliferation, confirming the role of IDO as one of the responsible mediators. None of these mediators was responsible for the modulation during the cytotoxic effector phase of resting NK cells. Our data are in accordance with previously published results on T cell alloreactivity.[166]

The present study was primarily aimed to study the mutual interaction between hMAPCs and NK cells. Related to sensitivity to NK cell-mediated killing, modulation of NK cell proliferation, and mechanism of immunomodulation, hMAPCs did not differ in function from hMSCs as described in literature. Nevertheless, some differences in immunomodulatory action between hMAPCs and hMSCs were found compared to literature and from own experiments. First of all, the modulatory effects of hMAPCs during the rIL-2-induced stimulation phase of NK cells in our study were different compared to hMSCs in the study by Spaggiari *et al*.[116] In the latter study, coculture of purified NK cells with irradiated hMSCs for 6 days in the presence of rIL-2 did not only strongly reduce the rIL-2-induced proliferation but also inhibited the cytotoxic activity of the purified NK cells. Next, in our hands, hMSCs became available from the same donor as hMAPCs only for the experiments on rIL-2-induced stimulation of PBMC populations. Enrichment of NK cells upon rIL-2 stimulation in the PBMC cultures was blocked more by hMAPCs than by hMSCs. As a consequence, the subsequent NK potency of these stimulated PBMCs was also reduced in the cultures in the presence of hMAPCs but less in the presence of hMSCs. Thirdly, Rasmusson *et al*. demonstrated that hMSCs did not influence the specific K562 lysis of resting NK cells [101], whereas hMAPCs in our study reduced the cytotoxic function of resting NK cells when cocultured during the effector phase.

The fate of hMAPCs after injection into patients is not yet understood. In this study, we demonstrated that hMAPCs interact with NK cells *in vitro*. The final outcome of this

interaction *in vivo* will depend on the inflammatory microenvironment both affecting the activation state of the NK cells but also the MHC expression on hMAPCs. Our data add to the understanding of clinical results of currently running trials using adoptive transfer of hMAPCs. Further *in vivo* studies with patients treated with hMAPCs should be done to better understand the fate and function of hMAPCs as an immune-modulating stem cell population.

# Chapter 4 – Influence of clinical-grade human multipotent adult progenitor cells on CD8$^+$ cytotoxic T lymphocytes

## 1. Abstract

MultiStem$^®$ (MS) cells are clinical-grade multipotent adult progenitor cells (MAPCs), which are bone marrow-derived progenitor cells with extensive replication potential and broader differentiation capacity compared to mesenchymal stem cells (MSCs). Human (h)MAPCs suppress T cell proliferative responses induced by alloantigens and mutually interact with allogeneic natural killer (NK) cells. In this study, we addressed the interaction between MultiStem and CD8$^+$ cytotoxic T lymphocytes (CTLs). In an *in vitro* setting, we investigated the immunogenicity of MultiStem, the susceptibility of these clinical-grade MAPCs towards CTL-mediated lysis and the effects of MultiStem on CTL function. MultiStem was nonimmunogenic for alloreactive CTL induction and was – even after MHC class I upregulation by IFN-γ pretreatment – insensitive to alloantigen-specific CTL-mediated lysis. Furthermore, MultiStem reduced CTL proliferation and significantly decreased the intracellular expression of perforin during the T cell activation phase. As a consequence, MultiStem dose-dependently impaired the induction of CTL function. These effects of MultiStem were mediated predominantly through contact-dependent mechanisms. MultiStem cells had a considerable influence on the expression pattern of T cell activation markers. Finally, the killer activity of activated antigen-specific CTLs during their cytolytic effector phase was also diminished in the presence of MultiStem. This study confirms that these clinical-grade MAPCs are an immune privileged population, which inhibits CTL activation and effector responses and are consequently a highly valuable cell population for adoptive immunosuppressive therapy in diseases where damage is induced by CTLs.

## 2. Introduction

During the last decade stem cell-based therapy has made an enormous progress in the treatment of various diseases. Stem cells are unspecialized self-renewing cells that can undergo multi-lineage differentiation and are classified according to their differentiation potential.[1] Besides their capacity to regenerate damaged or diseased tissues, multipotent or tissue-specific adult stem cells also possess a remarkably diverse array of immune modulatory characteristics. Mesenchymal stem cells (MSCs) are a prototype of adult nonhematopoietic stem cells, which can be isolated from various post-natal tissues and are able to differentiate into several cell types of the mesenchymal lineage.[23,25,33,34,204] Human (h)MSCs have been proven to be a non-major histocompatibility complex (MHC)-restricted immunosuppressive cell population *in vitro* as they suppress allogeneic T cell responses and impair differentiation and maturation of dendritic cells (DCs).[41,72,75,77,120] hMSCs also interfere with natural killer (NK) cell and B cell proliferation and function.[107,116] Clinical trials are ongoing to investigate the therapeutic use of hMSCs in several immune-related diseases [e.g. graft-*versus*-host disease (GvHD) and autoimmune diseases]. Several phase I and II studies have already shown the feasibility and safety of *in vivo* use of hMSCs, and phase III studies are initiated to explore the efficacy of hMSC therapy (www.ClinicalTrials.gov).

Cytotoxic T lymphocytes (CTLs) become capable of killing cancer cells or virus-infected cells after activation through the CD8-mediated recognition of specific antigens presented in a MHC class I-dependent way by professional antigen-presenting cells (APCs). This interaction between the MHC complex and the T cell receptor (TCR) leads to the activation and clonal expansion of antigen-specific CTLs, which – upon second encounter of infected cells – exert their killer effector function. Target cell death by activated CTLs is induced by the targeted release of granules containing the cytotoxins perforin and granzyme or via proapoptotic Fas ligand-receptor (FasL-FasR) clustering on the respective cell surfaces.[205] Both pathways eventually trigger apoptosis in the target cells via the caspase cascade. Despite the high expression of MHC class I molecules on their surface, MSCs escape CTL-mediated lysis.[104] On the other hand, MSCs inhibit CTL formation and prevent CTL-mediated lysis of target cells when added during the primary activation phase.[101]

Multipotent adult progenitor cells (MAPCs) are another population of adherent progenitor cells derived from adult bone marrow.[48,52] In contrast to hMSCs, hMAPCs can also differentiate into functional endothelium *in vitro* and *in vivo* and can be expanded *ex vivo* for a significantly longer time.[53,56] This extensive proliferation capacity has led to large-scale

manufacturing and banking of MAPCs on an industrial scale, allowing the production of uniform well-characterized clinical doses without the use of multiple donors.[59] Nowadays, such clinical-grade MAPCs are infused as an allogeneic off-the-shelf adoptive cell product (MultiStem[®]) resulting in a potentially more advantageous cellular therapy in the context of tissue regeneration, and cardiovascular, neurological and immune-related diseases. Third-party MultiStem has been proven safe and well-tolerated in phase I studies in patients with acute myocardial infarction (AMI) [61] and as an adjuvant cell therapy to enhance engraftment of HSCs and to reduce GvHD incidence after hematological stem cell transplantation (HSCT) in patients with hematological malignancies.[176] Partial beneficial effects were seen in these studies. Phase II and phase II/III trials are planned to be started soon. Moreover, phase I safety testing is also started in the context of immune modulation after liver transplantation [178] and phase II studies are recruiting patients with ischemic stroke [177] or refractory ulcerative colitis (www.ClinicalTrials.gov).

However, despite their ongoing clinical evaluation, data regarding the immune modulatory capacities of these clinical-grade MAPCs are still limited. A detailed knowledge of the exact immunological behavior and mechanism of immune regulation of this stem cell product is indispensable for designing and understanding the observations of future clinical trials. In our hands, hMAPCs are nonimmunogenic for alloreactive T cell proliferation, they impair allogeneic proliferative T cell responses and they mutually interact with allogeneic NK cells *in vitro*.[166,206] In this study, we assessed the influence of MultiStem cells on the cytotoxic function of CD8^{+} T cells by evaluating the immunogenicity of MultiStem and the susceptibility of the stem cell population towards CTL-mediated lysis and by analyzing the MultiStem-mediated modulation of CTL functioning.

## 3. Materials and methods

*Isolation and culture of MultiStem*

Research-grade human MAPCs (MultiStem) were isolated by Athersys/ReGenesys (www.athersys.com - www.regenesys.eu) from bone marrow (BM) of five different healthy volunteers (donors 1-5; **Table 1**). Informed consent for BM collection was obtained in accordance with the guidelines of the Medical Ethics Committee of the University Hospitals Leuven. Isolation and culture of the MultiStem clinical product were based on MAPC isolation and expansion protocols as previously described.[53,59] Briefly, MultiStem cells were generated by plating the total cell fraction of the isolated BM sample at $0.5 \times 10^6$ cells/cm$^2$ in medium consisting of 60% DMEM low-glucose (Lonza, Verviers, Belgium), 40% MCDB-201 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 50 nM dexamethasone, $10^{-4}$ M L-ascorbic acid, 0.5x linoleic acid-bovine serum albumin (LA-BSA) (all from Sigma-Aldrich), 1x insulin-transferrin-selenium (ITS), 100 U/ml penicillin/streptomycin, along with 18% fetal bovine serum (FBS; Serum Supreme) (all from Lonza), 10 ng/ml human platelet-derived growth factor (PDGF) and 10 ng/ml human epidermal growth factor (EGF) (both from R&D Systems, Minneapolis, MN, USA). MultiStem cultures were maintained under hypoxic conditions (5% O$_2$) at a density of 2000 cells/cm$^2$ per 1x fibronectin (FN)-coated (Sigma-Aldrich) T75 culture flask (Corning, NY, USA). The cells were split every two to three days, were not clonally derived and were used at PD 25-35. In some experiments, MultiStem cells were pretreated with 100 U/ml interferon (IFN)-γ (Roche Diagnostics, Vilvoorde, Belgium) for 48 h to upregulate MHC class I expression.

For all five stem cell donors, the MultiStem cellular product was also generated on an alternative larger-scale cell culture platform by means of an automated closed Quantum Cell Expansion System (CES; Terumo BCT, Lakewood, CO, USA). This system consists of a disposable synthetic hollow-fiber bioreactor of 2.1 m$^2$ surface area connected to a sterile closed-loop, computer-controlled media perfusion platform and gas exchangers.[207] Extensive characterization and quality control [expansion properties (growth, morphology and viability) by using the Cellavista image-based platform (Roche), characterization (flow cytometry, qPCR and ELISA), differentiation capacity (osteo-, adipo- and chondrogenic), karyotype analysis (SNP arrays and CNV analysis), telomerase activity, *in vitro* tube formation assay [62], high-throughput screening (transcriptome and epigenetic analysis) and immunosuppressive activity] confirmed phenotypic and functional equivalency of these cells.

**Table 1: List of MultiStem donors**

| Donor | Sex | Age | Cell line |
|-------|--------|-----|-----------|
| 1 | female | 30 | SJA |
| 2 | male | 45 | SVG/2 |
| 3 | male | 45 | OG2 |
| 4 | male | 28 | L08 |
| 5 | male | 25 | BMC134 |

*Epstein-Barr virus (EBV)-mediated transformation of B lymphocytes*

Peripheral blood mononuclear cells (PBMCs) were obtained from three MultiStem donors (donors 1 to 3) and from one healthy volunteer by density gradient centrifugation (ACCUSPIN[TM] System-Histopaque®-1077, Sigma-Aldrich). After washing, mononuclear cells were cultured in suspension in T25 culture flasks (Greiner Bio-One, Wemmel, Belgium) in 4 ml medium consisting of DMEM-F12 w/o Tes & Hepes supplemented with 2.5 mM L-glutamine, 10% FBS (all from Lonza), 2 µg/ml cyclosporin (Sigma-Aldrich) and infected with EBV supernatant [obtained from the growth of monkey B95-8 cell lines; American Type Culture Collection (ATCC), Manassas, VA, USA]. After one and two weeks, 2 ml medium was added and clump forming was regularly checked. The virally infected B cells were transferred to T75 culture flasks (Greiner Bio-One) and after 6 to 8 weeks, lymphoblastoid cell lines were obtained. These cell lines can be indefinitely grown in culture medium consisting of RPMI 1640 (Lonza) supplemented with 10% heat-inactivated FBS, 1 mM/ml sodium pyruvate, 1x nonessential amino acids (NEAA), 50 µg/ml gentamicin, 2 mM/ml L-glutamine (all from Lonza), 8 µg/ml levofloxacin (Tavanic; Sanofi-Aventis, Diegem, Belgium) and 100 µM 2-mercapto-ethanol (2-ME; Sigma-Aldrich). These immortalized B lymphocytes were used as a long-term continuous source of allogeneic stimulatory cells in mixed-lymphocyte cultures (MLC) to generate clonal expansion of antigen-specific T cells and as alloantigen-specific target cells during killing assays.

*Isolation and activation of (CD8$^+$ cytotoxic) T lymphocytes*

All subjects donating blood for these experiments were healthy volunteers of both sexes, aged 20 to 60 years. PBMCs were isolated from fresh blood samples by Ficoll-Hypaque (Axis-Shield, Oslo, Norway) density gradient centrifugation (specific gravity, 1.077 g/ml). T cells were further purified using two rounds of a complement-mediated depletion of all non-T cells with lympho-KWIK-T reagent (One Lambda, Los Angeles, CA, USA) as previously described.[166] Briefly, the reagent was added to the mononuclear cells (0.8 ml per 20 - 30 x 10$^6$ PBMCs) and the mixture was incubated for 1 h at 800 rpm at 37°C. Cells were centrifuged at 1845 g for two minutes and washed once. A mixture of complement-fixing anti-NK cell monoclonal antibody (anti-CD16) of the IgM subclass (BD Biosciences, Erembodegem, Belgium) was then added to the cells. The cells were incubated for 30 minutes at 4°C and again washed once. Afterwards, incubation with lympho-KWIK-T reagent was repeated. Cells were again centrifuged at 1845 g for two minutes, washed once, and resuspended in complete culture medium consisting of RPMI 1640 supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Lonza) and 10% autologous heat-inactivated plasma. The purified T cell populations contained at least 95% CD3$^+$CD56$^-$ cells as determined by flow cytometry. In some experiments, CD8$^+$ CTLs were negatively selected from T cell fractions using the human CD8$^+$ T cell isolation kit (Miltenyi Biotec, Leiden, The Netherlands) according to the manufacturer's instructions. Purity of enriched CD3$^+$CD8$^+$ T cell fractions ranged from 87% to 97%.

To obtain alloantigen-specific T cells, freshly isolated T cells or purified CD8$^+$ CTL fractions (both 1 x 10$^6$ cells) were cocultured with either allogeneic irradiated (40 Gy) EBV-transformed B cells or allogeneic irradiated (30 Gy) PBMCs for 7 days in complete culture medium in flat-bottomed 24-well plates (Greiner Bio-One) at a stimulator:responder (S:R) ratio ranging from 1:2 to 1:20. For polyclonal activation of T cells, culture plates were coated with immobilized anti-CD3 mAb (5 µg/ml UCHT-1; obtained from hybridoma cultures, ATCC) in 300 µl phosphate-buffered saline (PBS) for 4 h at 37°C and washed three times. Together with the T cells, 1 µg/ml soluble anti-CD28 (Sanquin, Amsterdam, The Netherlands) was added to the culture plates.[208] After four days of culture, cells were harvested, washed and resuspended in complete medium to test CTL activity.

*Cytotoxicity assays*

The viability of (IFN-γ-pretreated) MultiStem in the presence of activated T cells was tested with the standard 4 h $^{51}$Cr-release method. Therefore, MultiStem cells were labeled with 100 µCi $^{51}$Cr (Perkin Elmer, Zaventem, Belgium) per 10$^6$ cells for 90 minutes and seeded at 5 x 10$^3$ cells/well in V-bottomed 96-well plates (Greiner Bio-One). As a control target cell, EBV$^+$ B cells from the corresponding MultiStem donor, which were also used to prime the naive T cells, were labeled with 200 µCi $^{51}$Cr per 10$^6$ cells for 60 minutes. The lytic potential of alloantigen-specific effector CTLs was tested by coculturing cells at effector:target (E:T) ratio 10:1 in a total volume of 200 µl complete medium. Spontaneous $^{51}$Cr-release of the target cells was verified, and saponin (Merck, Darmstadt, Germany) was added to measure the maximal $^{51}$Cr-release. Release of $^{51}$Cr from the target cells into the supernatant was measured by a Topcount gamma counter (Packard Instrument Company, Meriden, CT, USA). Results are expressed as percentage $^{51}$Cr-release, calculated as [(experimental release – spontaneous release) / (maximal release – spontaneous release)] x 100.

CTL activity was also checked regardless of antigen (Ag) specificity of the CTLs with an anti-CD3-redirected cytotoxicity system, as previously described.[208] Briefly, P815 cells (obtained from ATCC) were used as labeled target cells (100 µCi $^{51}$Cr per 10$^6$ cells for 90 minutes) in a standard $^{51}$Cr-release assay in the presence of an anti-CD3 mAb, being 2 µg/ml OKT3 [orthoclone OKT3 (muromonab-CD3), Janssen-Cilag, Berchem, Belgium] or 10 µg/ml UCHT1. The P815 cell line is an NK-resistant DBA/2-derived murine mastocytoma cell line which expresses mouse FcγRII and FcγRIII. By bridging the effector CTL to the target cell FcγR with the anti-CD3 mAb, this cytotoxicity system permits detection of CTL activity regardless of antigen specificity of the CTLs. Results are expressed as anti-CD3-dependent specific $^{51}$Cr-release (% SR), calculated as the difference between total release (in the presence of anti-CD3) and background release (in the absence of anti-CD3) of the target cell. The following formula was used: [(total release – experimental background release) / (maximal release – spontaneous release)] x 100.

*Immune regulation by MultiStem*

To analyze the immunogenicity of MultiStem, we stimulated T cells with either allogeneic irradiated (30 Gy) MultiStem or with allogeneic irradiated (30 Gy) PBMCs from the same donor for 7 days at S:R ratio 1:2. Afterwards, CTL response was measured.

To test the influence of MultiStem as a modulating cell population during either the activation phase or during the lytic effector phase of T cells, MultiStem cells were added to these cocultures respectively at the beginning of the 7-day stimulation period or at the start of the 4 h $^{51}$Cr-release assay at a suppressor:responder ratio ranging from 1:1 to 1:100. Afterwards, the proliferative and CTL responses were analyzed by 16 h [$^3$H]thymidine incorporation [1µCi/well; Perkin Elmer; measured on a liquid scintillation counter (Tri-Carb$^®$ 2100TR Liquid Scintillation Counter, PerkinElmer)] and by standard $^{51}$Cr-release assay, respectively. Proliferation results were analyzed as mean counts per minute (cpm) of quadruplicate wells, and were expressed as percentage response related to the control response in the absence of MultiStem. Alternatively, as a negative control, human umbilical vein endothelial cells (HUVECs; Lonza) were used as modulating cells in the same ratios.

In some experiments, 100 U/ml exogenous human recombinant (r)IL-2 (TECIN; Hoffmann-La Roche, Nutley, NJ, USA) was added to the cocultures of immune cells and stem cells. To test the effect of MultiStem on the T cell priming for memory response, T cells were stimulated in a primary MLC with allogeneic EBV$^+$ B cells in the presence or absence of MultiStem cells. After a 3-day resting period, these T cells were restimulated in the absence of MultiStem with the same alloantigens in a secondary MLC for 4 days.

The involvement of soluble factors in the immune regulation by MultiStem was evaluated by using 24-well plate Thincert$^{TM}$ inserts (Greiner Bio-One) with a semi-permeable polyethylene membrane (pore size 0.4 µm) to separate MultiStem cells from the MLC during the T cell activation phase (range 1:2 to 1:10 MultiStem:T cells). The MLC was performed in the bottom chamber of the transwell system, while MultiStem cells were placed in the upper chamber. After the T cell stimulation period, the inserts were removed and the T cells were tested for their cytotoxic activity. To address the specific role of indoleamine 2,3-dioxygenase (IDO) or prostaglandin E$_2$ (PGE$_2$) as immune suppressive mediators, respectively 200 µM/ml of the blocking molecules 1-methyl-trypthophan (1-MT; Sigma-Aldrich) or 2 µg/ml indomethacin (Cayman Chemical Company, Ann Arbor, MI, USA) were added to the coculture system.

The involvement in MultiStem-mediated immune modulation of the contact-dependent mechanisms programmed death ligand-1/2 (PD-L1/2) / programmed death-1 (PD-1) signaling pathway and apoptosis-inducing FasL/FasR interaction was investigated by adding their respective neutralizing mAbs: 5 µg/ml anti-PD-1, 2 µg/ml anti-PD-L1, 2 µg/ml anti-PD-L2 (all from eBioscience, San Diego, CA, USA) and 5 µg/ml anti-FasL (BD Pharmingen, Erembodegem, Belgium). The production of galectin-1 (Gal-1) by MultiStem cells was

analyzed by fluorescence microscopy by labeling the cells with anti-human Gal-1 mAb (1 µg/ml; Peprotech, London, UK), and by Western Blot (0.2 µg/ml). Secretion of soluble Gal-1 was verified by means of ELISA on cell supernatant.[209]

*Flow cytometry*

MultiStem cells, EBV⁺ B cells or (CD8⁺ cytotoxic) T cells (1 x $10^5$ per sample) were suspended in 100 µL PBS supplemented with 10% autologous heat-inactivated human plasma to block nonspecific staining. Cells were subsequently surface stained with fluorescence-conjugated specific mAbs anti-PD-1 (BD Pharmingen), anti-PD-L1 (BioLegend, San Diego, CA, USA), anti-PD-L2 (eBioscience), anti-FasL (BD Pharmingen), anti-CD45 (BD Biosciences), anti-CD3 (eBioscience), anti-CD8 (BD Pharmingen), anti-CD25 (BioLegend), anti-CD127 (BD Biosciences), anti-CD69 (BioLegend), anti-AnnexinV (BD Pharmingen), anti-CD54, anti-CD58, anti-CD80, anti-CD86, anti-HLA-ABC and anti-HLA-DR (all from BD Biosciences). In some experiments, the viability dyes propidium iodide (PI; BD Biosciences) or Zombie Yellow (BioLegend) were used. For intracellular perforin staining, cells were first fixed and permeabilized with Fix&Perm® cell permeabilization kit (ADG Bioresearch, Vienna, Austria) and afterwards stained with anti-perforin (BD Pharmingen). Fluoresence compensation was set to correct for spectral overlap by using UltraComp eBeads (eBioscience) as compensation controls and fluorescence minus one (FMO) staining controls were included. Acquisition was done using a FACSort or BD LSRFortessa™ (both BD Biosciences) and analysis was performed with respectively CellQuest Pro (BD Biosciences) or FlowJo software (Tree Star, Inc., Ashland, OR, USA).

*Statistical analysis*

Statistics were calculated with Prism software 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was calculated with (un)paired *t* tests for comparisons between two groups. Values of $p < 0.05$ were considered significant.

# 4. Results

## 4.1 Human MultiStem cells are nonstimulatory for allogeneic T cells *in vitro*

In previous work, we already demonstrated that hMAPCs did not induce alloreactive T cell proliferation nor $T_h1/T_h2$ cytokine production when cocultured *in vitro* with allogeneic T cells.[166] To assess whether MultiStem could possibly induce cytotoxic effector function in T cells, purified responder CD3$^+$ T cells were stimulated with irradiated allogeneic MultiStem on the one hand, and irradiated allogeneic PBMCs of the MultiStem donor on the other hand as a control APC population. Standard $^{51}$Cr-release assay revealed that allogeneic PBMC-stimulated T cells efficiently killed $^{51}$Cr-labeled P815 target cells in the presence of an anti-CD3 mAb (mean ± SEM % $^{51}$Cr-release: 56.75 ± 4.63 %; $n = 5$; **Fig. 1A**). In contrast, MultiStem induced only a minimal anti-CD3-redirected cytotoxic response when cocultured for one week with allogeneic T cells (21.32 ± 4.91 %; $n = 5$). In the alloantigen-specific cytotoxicity assay, EBV$^+$ target B cells were not lysed when T cells were prestimulated with allogeneic MultiStem (1.39 ± 1.11 %; $n = 3$; **Fig. 1B**) compared to prestimulation with allogeneic PBMCs (43.89 ± 4.34 %; $n = 3$). The MultiStem cells or PBMCs were from the same donor as the EBV$^+$ target B cells used during the cytotoxicity assay. These results suggest the lack of immunogenicity of MultiStem cells in the *in vitro* setting.
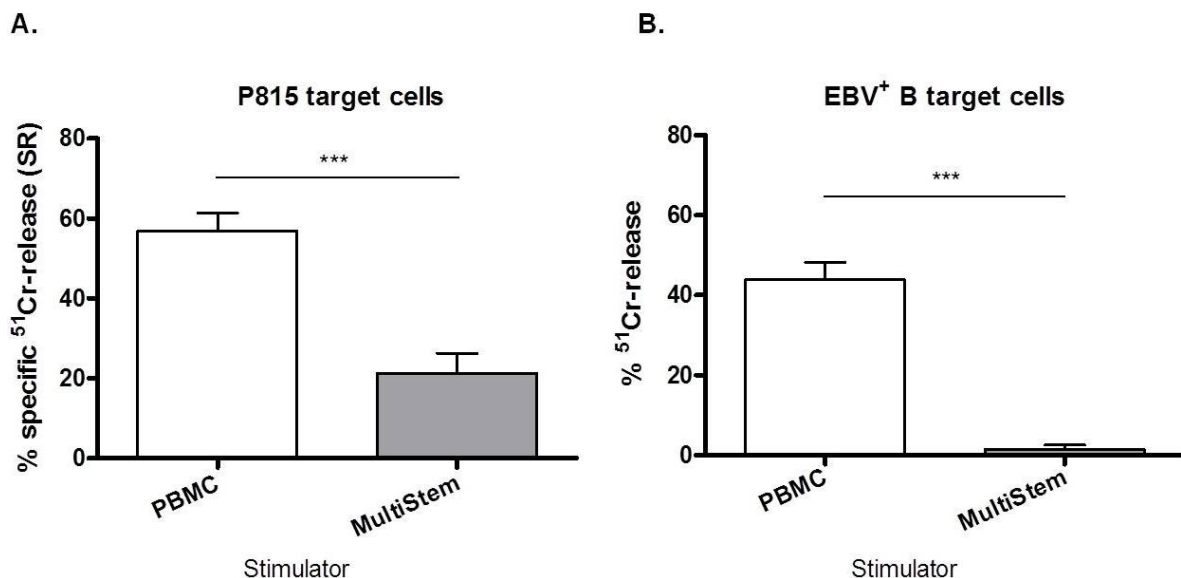


**Figure 1. MultiStem (MS) does not induce cytotoxic activity in T cells.**

Freshly isolated responder CD3$^+$ T cells were stimulated with either allogeneic irradiated (30 Gy) PBMCs or allogeneic irradiated (30 Gy) MultiStem (PBMCs and MS from the same donor) at S:R ratio of 1:2 for 7 days. Coculture was followed by an assessment of (A) anti-CD3-redirected cytotoxic activity against murine P815 mastocytoma target cells or (B) alloantigen-specific cytotoxic activity

against EBV$^+$ B cells at E:T ratio 10:1 in a standard $^{51}$Cr-release assay. Data are expressed as (A) mean ± SEM anti-CD3-dependent specific $^{51}$Cr-release (% SR) of five independent experiments with four different T cell donors and three different PBMC/MultiStem donors and (B) mean ± SEM percentage $^{51}$Cr-release of three independent experiments with two different T cell donors and two different PBMC/MultiStem/B cell donors. Statistical significance was calculated with the unpaired $t$ test. ***$p$ < 0.001.

## 4.2 MultiStem is insensitive to alloantigen-specific CTL-mediated lysis

To address the interaction between activated CD8$^+$ CTLs and allogeneic MultiStem, we first investigated the susceptibility of the stem cell population to CTL-mediated killing. To achieve this, we stimulated purified CD3$^+$CD8$^+$ T cells with allogeneic irradiated EBV$^+$ B cells for 7 days. This was followed by an assessment of CTL activity against murine P815 cells in the presence of an anti-CD3 mAb ($n = 6$), against alloantigen-specific MultiStem ($n = 6$) and against EBV$^+$ B cells ($n = 3$) as MHC-specific control target cells. As shown in **Figure 2**, activated T cells killed anti-CD3 coated P815 cells (36.30 ± 4.85 %), whereas MultiStem cells (from the same donor as the EBV$^+$ B cells used for stimulation) were insensitive to an alloantigen-specific CTL attack (2.54 ± 1.72 %). When EBV$^+$ B cells were used as target cells ($n = 3$), it was demonstrated that the T cell antigen-specific cytotoxic activity (13.50 ± 3.71 %) was considerably higher than the specific lysis of MultiStem. In some experiments ($n = 4$), MultiStem cells were pretreated with IFN-$\gamma$ (100 U/ml for 48 h) to increase MHC class I molecule expression.[166,206] However, this MHC class I upregulation did not result in a higher sensitivity to CTL-mediated lysis compared to untreated MultiStem cells, confirming the immune privileged status of these clinical-grade hMAPCs (data not shown).
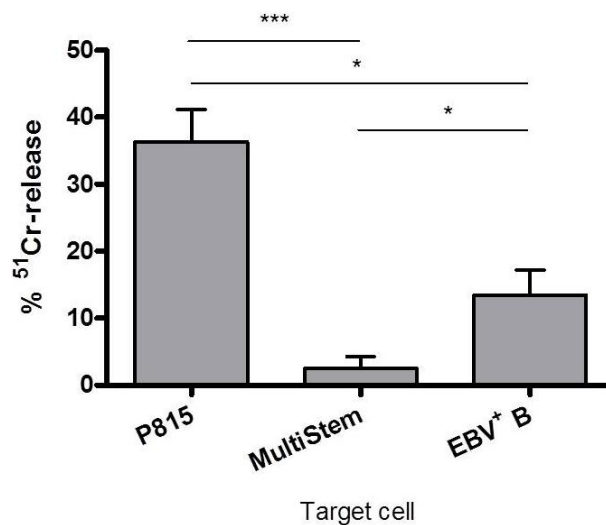


**Figure 2. Activated T cells do not lyse allogeneic MultiStem cells.**

Results of anti-CD3-redirected and alloantigen-specific cytotoxic activity of irr adiated (40 Gy) EBV$^+$ B cell-stimulated CD8$^+$ CTLs (S:R 1:10 for 7 days) against respectively anti-CD3 coated P815 target cells, MultiStem or EBV$^+$ B cells at E:T ratio 10:1. MultiStem cells and EBV$^+$ B target cells were from the same donor as the EBV$^+$ B cells used for CTL activation. Target cell killing is expressed as mean ± SEM percentage $^{51}$Cr-release of $n$ independent experiments (P815 targets: $n = 6$; MultiStem targets: $n = 6$; EBV$^+$ B targets: $n = 3$). Four different CTL donors and three different MultiStem/B cell donors were used. Statistical significance was calculated with the paired $t$ test. $*p < 0.05$, $***p < 0.001$.

## 4.3 MultiStem cells impair proliferation, perforin expression and cytotoxic function of CD8$^+$ T cells

In a next set of experiments, we studied whether MultiStem – besides impairing the alloantigen-, mitogen- and recall antigen-induced T cell proliferation [166] – could also interfere with the clonal expansion of antigen-specific CD8$^+$ T cells or with the generation of CTL function. Therefore, freshly isolated CD3$^+$CD8$^+$ T cells were primed with allogeneic EBV$^+$ B cells for 7 days. Proliferation was measured with thymidine incorporation. Lysis of P815 cells and EBV$^+$ target B cells was analyzed by means of $^{51}$Cr-release assay. Irradiated third-party MultiStem cells were added to the MLC either at the beginning of the 7-day EBV$^+$ B cell-induced activation phase or at the beginning of the 4 h cytolytic effector phase. In the presence of MultiStem, CTLs had a lower proliferative response (**Fig. 3A**). MultiStem-modulated CTLs demonstrated a slightly but significantly reduced lytic capacity in both cytotoxicity systems compared to CTLs that were not exposed to MultiStem during the activation phase (anti-CD3-redirected cytotoxicity: 36.86 ± 3.39 % *versus* 46.48 ± 2.55 %; $n = 32$; alloantigen-specific cytotoxicity: 13.47 ± 2.15 % *versus* 20.82 ± 1.74 %; $n = 9$; **Fig. 3B**). The presence of MultiStem for 4 h during the cytotoxic effector phase also significantly diminished the killing of both target cell populations (P815 targets: 33.44 ± 4.27 % *versus* 40.10 ± 3.50 %; $n = 18$; EBV$^+$ B targets: 12.50 ± 1.33 % *versus* 21.18 ± 2.92 %; $n = 15$; **Fig. 3C**). These data suggest that MultiStem impairs both the CD8$^+$ T cell proliferation and the induction of CTL activity, and the lytic T cell effector function itself.

**A.**



**B.**



**C.**
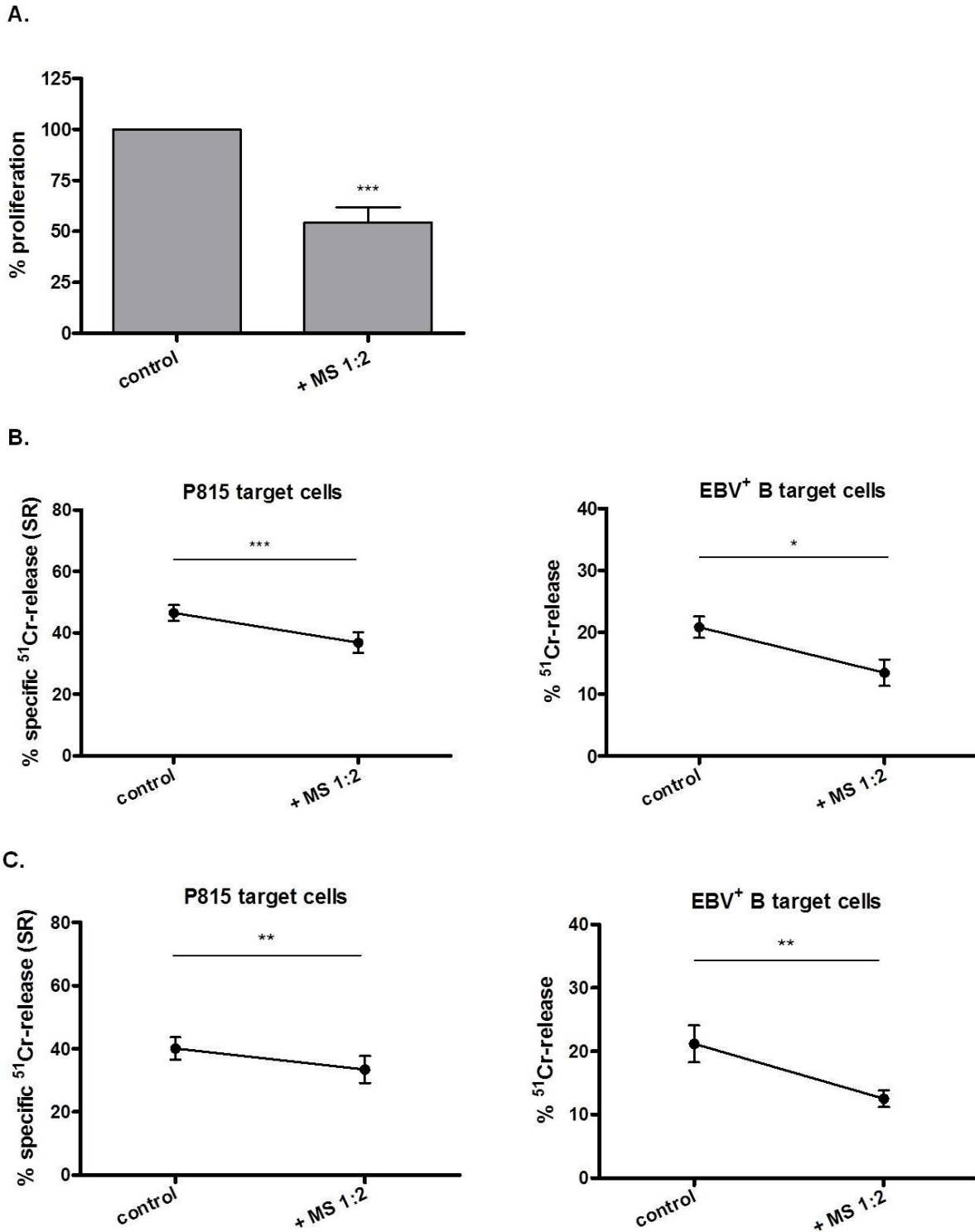


**Figure 3. MultiStem impairs the proliferation and (the induction of) the killer activity of CD8$^+$ T cells.**

(A) Purified CD8$^+$ CTLs were stimulated with irradiated (40 Gy) allogeneic EBV-transformed B cells (S:R 1:10) during 6 days in the absence (control) or presence of third-party irradiated (30 Gy) MultiStem cells at suppressor:responder ratio 1:2. The proliferative CTL response was measured on day 6 by [$^3$H]thymidine incorporation. Results are expressed as mean ± SEM percentage proliferation relative to the control culture in the absence of MultiStem [average cpm: 46801] of quadruplicates in five independent experiments with five different CTL donors and two different MultiStem donors. Statistical significance was calculated with the paired *t* test. ***$p < 0.001$.

(B) Results of anti-CD3-redirected cytotoxic activity of irradiated EBV^{+} B cell-stimulated CD8^{+} CTLs (S:R 1:10) against P815 target cells (E:T 10:1 ; *left panel*) or alloantigen-specific cytotoxic activity against EBV-transformed target B cells (E:T 10:1 ; *right panel*) in the absence (control) or presence of irradiated third-party MultiStem cells at MS:CTL ratio of 1:2 during the **7-day activation phase**. Data are expressed as mean ± SEM anti-CD3-dependent specific $^{51}$Cr-release (% SR) of 32 independent experiments with 19 different CTL donors and four different MultiStem donors (*left panel*) or percentage $^{51}$Cr-release of nine independent experiments with six different CTL donors and three different MultiStem donors (*right panel*). Statistical significance was calculated with the paired *t* test. *$p < 0.05$, ***$p < 0.001$.

(C) Results of anti-CD3-redirected cytotoxic activity of irradiated EBV^{+} B cell-stimulated CD8^{+} CTLs (S:R 1:10) against P815 target cells (E:T 10:1 ; *left panel*) or alloantigen-specific cytotoxic activity against EBV-transformed target B cells (E:T 10:1 ; *right panel*) in the absence (control) or presence of irradiated third-party MultiStem cells at MS:CTL ratio of 1:2 during the the **4 h cytotoxic effector phase**. Data are expressed as mean ± SEM anti-CD3-dependent specific $^{51}$Cr-release (% SR) of 18 independent experiments with ten different CTL donors and four different MultiStem donors (*left panel*) or percentage $^{51}$Cr-release of 15 independent experiments with eight different CTL donors and four different MultiStem donors (*right panel*). Statistical significance was tested with the paired *t* test. **$p < 0.01$.

To verify if MultiStem had a similar suppressive effect on the cytotoxicity induction of the total CD3^{+} T cell fraction, we performed a similar experiment with total T cells. In fact, MultiStem addition led to comparable inhibition of cytotoxicity induction of CD3^{+} T cells. This effect was dose-dependent (**Fig. 4**) and characteristic for MultiStem, as we compared the effect of addition of MultiStem cells as a modulating population with the addition of an unrelated third-party endothelial cell line (HUVECs). The latter did not impair T cell cytotoxicity induction (data not shown). Again, the suppressive effect was not enhanced by IFN-γ-pretreatment of MultiStem (data not shown).
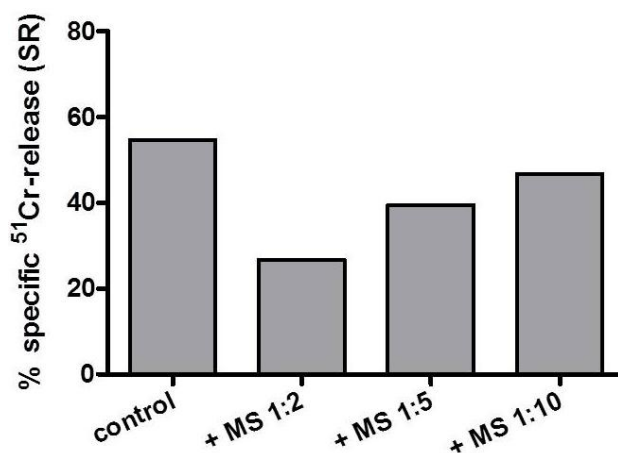


**Figure 4. The MultiStem-mediated cytotoxicity suppression is dose-dependent.**

Results of anti-CD3-redirected cytotoxic activity of irradiated EBV^{+} B cell-stimulated CD3^{+} T cells (S:R 1:20) against P815 target cells (E:T 10:1) in the absence (control) or presence (+ MS) of

irradiated third-party MultiStem cells at different suppressor:responder ratios during the 7-day activation phase. Data are expressed as mean specific $^{51}$Cr-release (% SR) of one representative experiment out of three.

Next, we studied the influence of MultiStem addition during the T cell stimulation phase on the perforin expression. Flow cytometric analysis revealed that the amount of perforin-positive CD8$^+$ T cells was reduced when MultiStem was present as a modulating cell population during the T cell activation phase (% CD8$^+$perforin$^+$ cells of stimulated CD3$^+$ lymphocytes: 3.24 % and 3.46 % in the presence of respectively MS donor 2 and 4 *versus* 7.07 % in the absence of MS; **Fig. 5**). MultiStem had variable effects on perforin expression in CD8$^-$ T cells. Collectively, these observations could be a plausible explanation for the reduced cytotoxic activity of stimulated T cells when cocultured with allogeneic MultiStem.
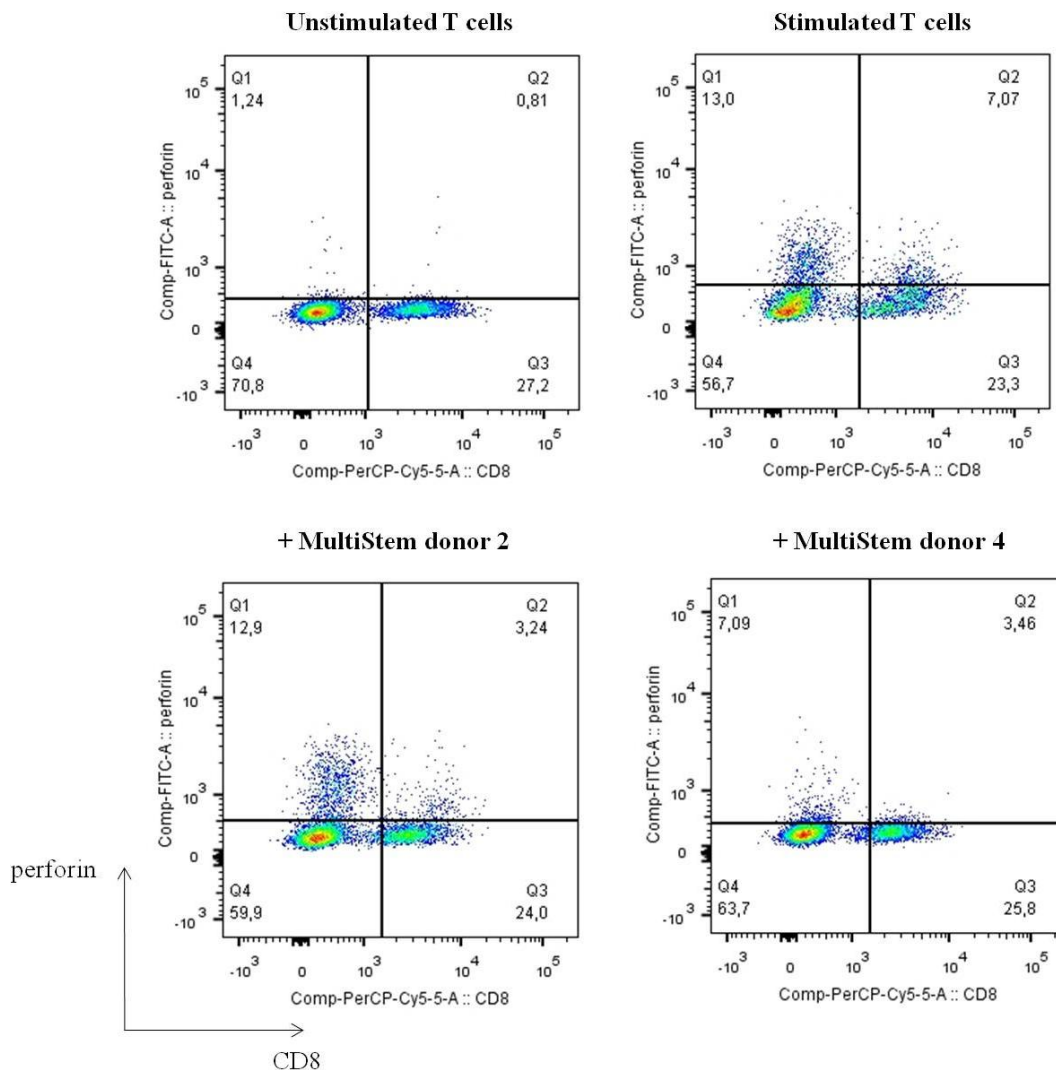


**Figure 5. MultiStem impairs the expression of perforin in CD8$^+$ T cells.**

Flow cytometric analysis of irradiated EBV$^+$ B cell-stimulated T cells (S:R 1:20) for intracellular perforin expression after a 7-day stimulation period in the absence (*upper panels*) or presence (*lower panels*) of irradiated third-party MultiStem cells (suppressor:responder 1:2). Results are expressed as % positive cells in the CD3$^+$ lymphocyte gate of one representative experiment with one T cell donor and two different MultiStem donors (donors 2 and 4).

We excluded the possibility that MultiStem could indirectly suppress the activation and functioning of T cells through interference with the allogeneic EBV$^+$ B cells. Flow cytometric analysis revealed no change in surface marker expression – and hence the alloantigen-presenting capacity – of the stimulator EBV$^+$ B cells after exposure to MultiStem (data not shown).

We confirmed this finding by exploring the suppressive effect of MultiStem on the cytolytic activity of T cells, when the latter were activated with polyclonal stimulation (anti-CD3/28). The presence of MultiStem during the activation phase resulted in a significantly reduced cytotoxic activity (**Fig. 6**). These data indicate that the inhibitory effect of MultiStem on CTL generation is at the level of the T cells.
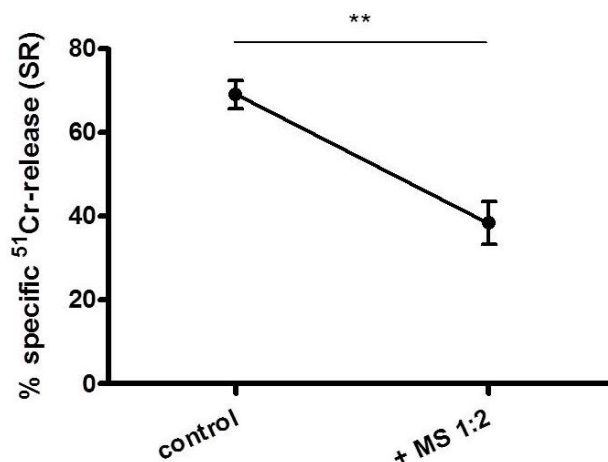


**Figure 6. MultiStem impairs cytotoxicity of polyclonal activated T cells.**

Responder CD3$^+$ T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 mAbs for 4 days in the absence (control) or presence of irradiated third-party MultiStem cells at suppressor:responder ratio 1:2. Coculture was followed by an assessment of anti-CD3-redirected cytotoxic activity of activated T cells against P815 target cells (E:T 10:1) in a standard $^{51}$Cr-release assay. Data are expressed as mean ± SEM anti-CD3-dependent specific $^{51}$Cr-release (% SR) of three independent experiments with two different T cell donors and two different MultiStem donors. Statistical significance was tested with the paired *t* test. **$p < 0.01$.

## 4.4 CTLs have an altered pattern of activation marker expression in the presence of MultiStem

To investigate the mechanism of the suppressive effect of MultiStem on the responder T cells, we first performed a set of experiments in which we verified whether addition of MultiStem resulted in T cell apoptosis, anergy or tolerance. As explained above, MultiStem blocked the induction of CD8$^+$ CTL activity in the MLC. Addition of exogenous rIL-2 only partially restored cytotoxic activity of the alloantigen-primed T cells (**Fig. 7A**). To further explain the lack of rescued CTL activity in spite of IL-2 addition, we analyzed the MultiStem-mediated induction of CTL apoptosis by means of AnnexinV/PI staining. In the presence of MultiStem, no induction of T cell apoptosis could be detected (data not shown). Furthermore, we also restimulated CTLs, suppressed during a primary MLC by third-party MultiStem, with the same alloantigens in a secondary MLC in the absence of MultiStem and then analyzed their cytotoxic function. This showed that these CTLs still displayed a secondary memory immune response similar to control T cells that had been stimulated in the absence of MultiStem (**Fig. 7B**). Taken together, these findings show that MultiStem cells do not induce apoptosis and do not render T cells anergic.
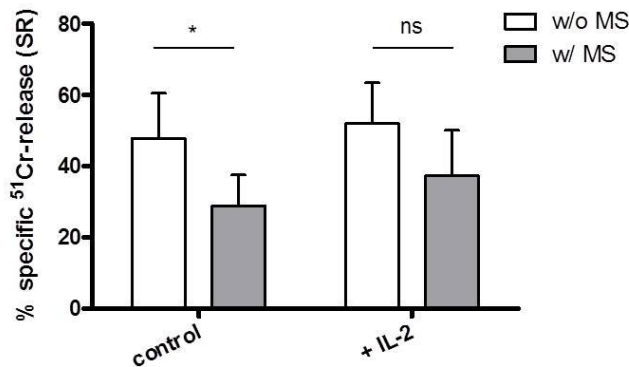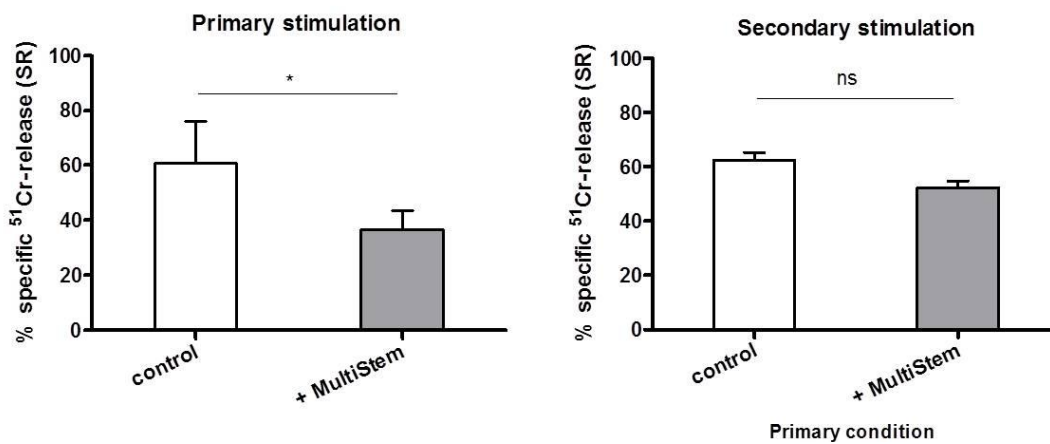
**Figure 7. Alloantigen-stimulated CD8$^+$ CTLs in the presence of MultiStem have a normal secondary cytotoxic response.**

(A) Purified CD8$^+$ T cells were primed with irradiated (40 Gy) allogeneic EBV$^+$ B cells (S:R 1:10) in the absence (w/o MS) or presence (w/ MS) of irradiated (30 Gy) allogeneic MultiStem cells (MultiStem:CTL ratio of 1:2), and with or without exogenous rIL-2 (100 U/ml) for 7 days. Data are expressed as mean ± SEM percentage anti-CD3-dependent specific $^{51}$Cr-release (% SR) of P815 target cells (E:T 10:1). Results are pooled from four independent experiments with three different CTL donors and two different MultiStem donors. Statistical significance was calculated with the paired $t$ test. *$p < 0.05$, ns = not significant.

(B) *Left panel* : Purified CTLs were stimulated for 7 days in a primary MLC with irradiated allogeneic EBV$^+$ B cells (S:R 1:10) in the absence (control) or presence of irradiated third-party MultiStem cells at suppressor:responder ratio 1:2. Afterwards anti-CD3-redirected cytotoxic activity against P815 target cells was tested with $^{51}$Cr-release (E:T 10:1). *Right panel* : After a 3-day resting period, T cells from the primary MLC cultured in the absence or presence of MultiStem, were restimulated during 4 days with the same irradiated alloantigens (S:R 1:10) in the absence of MultiStem, and thereafter anti-CD3-redirected cytotoxic activity against P815 target cells (E:T 10:1) was tested. Results are expressed as mean ± SEM anti-CD3-dependent specific $^{51}$Cr-release (% SR) of three independent experiments with two different CTL donors and two different MultiStem donors. Statistical significance was tested with the paired $t$ test. *$p < 0.05$, ns = not significant.

Secondly, we examined the influence of MultiStem on T cell activation marker expression. T cells were stimulated with EBV$^+$ B cells in the presence or absence of MultiStem cells. We observed an increased expression of the early activation marker CD69 on both CD8$^-$ and CD8$^+$ fractions, measured after 2 days of allogeneic T cell stimulation, and this expression was significantly higher in the presence of MultiStem (% CD8$^-$CD69$^+$ fraction of CD3$^+$ lymphocytes: 59.8 % and 53.0 % in the presence of respectively MS donor 2 and 4 *versus* 23.3 % in the absence of MS; % CD8$^+$CD69$^+$ cells: 26.3 % and 21.8 % *versus* 9.32 % ; **Table 2A** or **Fig. 8A**). Most of the CD69$^+$ cells were CD25-negative and CD127-positive. While the level of CD69 expression decreased again after 6 days, CD69 expression remained high in the MultiStem-modulated conditions, especially in the CD8$^-$ fraction (% CD8$^-$CD69$^+$ fraction of CD3$^+$ lymphocytes: 30.0 % and 46.1 % in the presence of respectively MS donor 2 and 4 *versus* 6.04 % in the absence of MS; % CD8$^+$CD69$^+$ cells: 9.13 % and 12.9 % *versus* 3.42 %). On the other hand, CD25 (IL-2Rα chain; **Table 2B** or **Fig. 8B**) upregulation was dramatically reduced after 6 days of coculture of T cells with MultiStem, compared to the control condition without MultiStem. This downregulation was most pronounced for CD8$^+$ cells (% CD8$^-$CD25$^+$ fraction of CD3$^+$ lymphocytes: 11.8 % and 15.3 % in the presence of respectively MS donor 2 and 4 *versus* 36.3 % in the absence of MS; % CD8$^+$CD25$^+$ cells: 1.69 % and 0.96 % *versus* 28.5 %). The remaining CD25$^+$ cells in the CD8$^-$ fraction of MultiStem-modulated T cells were CD69-positive and consisted of relatively more regulatory T cells (Tregs; CD4$^+$CD25$^+$CD127$^{dim}$), compared to the control condition (% CD25$^+$CD127$^{dim}$ cells in the CD8$^-$ gate: 38.2 % and 35.8 % in the presence of respectively MS donor 2 and 4 *versus* 17.3 % in the control condition). Finally, HLA-DR (**Table 2C** or **Fig. 8C**) upregulation obviously declined after 6 days in MultiStem-modulated T cells, compared to the control condition (% CD8$^-$HLA-DR$^+$ fraction of CD3$^+$ lymphocytes: 1.95 % and 0.84 % in the presence of respectively MS donor 2 and 4 *versus* 19.1 % in the absence of MS; % CD8$^+$HLA-DR$^+$ cells: 0.52 % and 0.13 % *versus* 18.9 %). In summary, these observations indicate an important effect of MultiStem on the activation and differentiation of T cells.

**Table 2: T cell activation marker expression**

**A.**

| Population | unstimulated | | stimulated | | + MS donor 2 | | + MS donor 4 | |
|---|---|---|---|---|---|---|---|---|
| | DAY 2 | DAY 6 | DAY 2 | DAY 6 | DAY 2 | DAY 6 | DAY 2 | DAY 6 |
| CD8^{-}CD69^{+} (%) | 2.43 | 1.32 | <u>23.3</u> | <u>6.04</u> | <u>59.8</u> | <u>30.0</u> | <u>53.0</u> | <u>46.1</u> |
| CD8^{-}CD69^{+}CD25^{-} (%) | NA | NA | 71.2 | NA | 74.8 | 63.1 | 67.9 | 63.5 |
| CD127 (MFI) | NA | NA | 1961 | NA | 2419 | 2568 | 2558 | 1649 |
| CD8^{+}CD69^{+} (%) | 1.29 | 0.68 | <u>9.32</u> | <u>3.42</u> | <u>26.3</u> | <u>9.13</u> | <u>21.8</u> | <u>12.9</u> |
| CD8^{+}CD69^{+}CD25^{-} (%) | NA | NA | 94.4 | NA | 96.7 | 78.5 | 95.7 | 91.5 |
| CD127 (MFI) | NA | NA | 2198 | NA | 2438 | 1957 | 2373 | 1456 |

**B.**

| Population | unstimulated | | stimulated | | + MS donor 2 | | + MS donor 4 | |
|---|---|---|---|---|---|---|---|---|
| | DAY 2 | DAY 6 | DAY 2 | DAY 6 | DAY 2 | DAY 6 | DAY 2 | DAY 6 |
| CD8^{-}CD25^{+} (%) | 20.4 | 5.94 | 16.1 | <u>36.3</u> | 16.0 | <u>11.8</u> | 18.7 | <u>15.3</u> |
| CD8^{-}CD25^{+}CD127^{dim} (%) | 18.9 | 60.8 | 37.7 | <u>17.3</u> | 31.4 | <u>38.2</u> | 31.1 | <u>35.8</u> |
| CD69 (MFI) | 226 | 402 | 500 | 1104 | 1426 | 1964 | 1119 | 2160 |
| CD8^{+}CD25^{+} (%) | 1.22 | 0.20 | 0.94 | <u>28.5</u> | 0.87 | <u>1.69</u> | 1.12 | <u>0.96</u> |
| CD8^{+}CD25^{+}CD127^{dim} (%) | NA | NA | NA | 0.00 | NA | NA | NA | NA |
| CD69 (MFI) | NA | NA | NA | NA | NA | NA | NA | NA |

**C.**

| Population | unstimulated | | stimulated | | + MS donor 2 | | + MS donor 4 | |
|---|---|---|---|---|---|---|---|---|
| | DAY 2 | DAY 6 | DAY 2 | DAY 6 | DAY 2 | DAY 6 | DAY 2 | DAY 6 |
| CD8^{-}HLA-DR^{+} (%) | 1.74 | 1.56 | 7.63 | <u>19.1</u> | 4.21 | <u>1.95</u> | 2.61 | <u>0.84</u> |
| CD8^{+}HLA-DR^{+} (%) | 0.69 | 0.21 | 3.40 | <u>18.9</u> | 1.72 | <u>0.52</u> | 0.98 | <u>0.13</u> |

Flow cytometric analysis of CD3^{+} T cells for expression of T cell activation markers (A) CD69, (B) CD25 and (C) HLA-DR on day 2 and day 6 of a 6-day stimulation period with irradiated (40 Gy) allogeneic EBV^{+} B cells (S:R 1:20) in the absence [(un)stimulated T cells] or presence (+ MultiStem donor 2/4) of irradiated (30 Gy) third-party MultiStem cells (suppressor:responder 1:1). Data are presented as % of cells within the CD3^{+} lymphocyte gate or within the parent population, or as median

fluorescence intensity (MFI) of the specific marker expressed on cells of the parent population. One representative experiment with one T cell donor and two different MultiStem donors (donors 2 and 4) is shown. Highlighted values are explained in the preceding text. NA = not applicable.
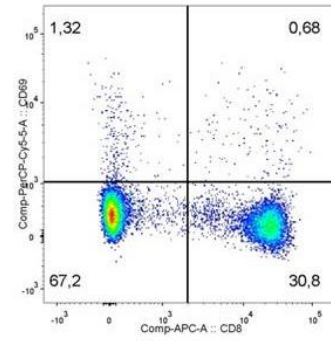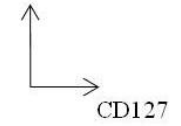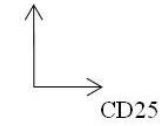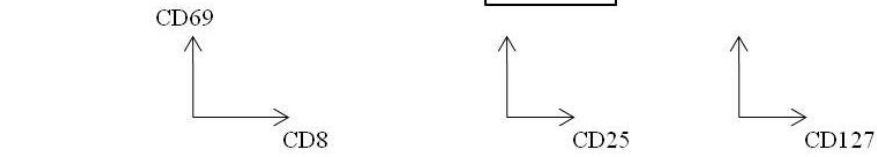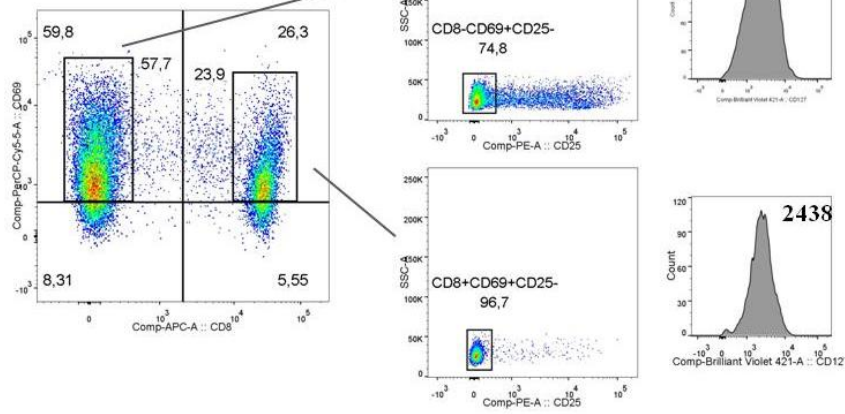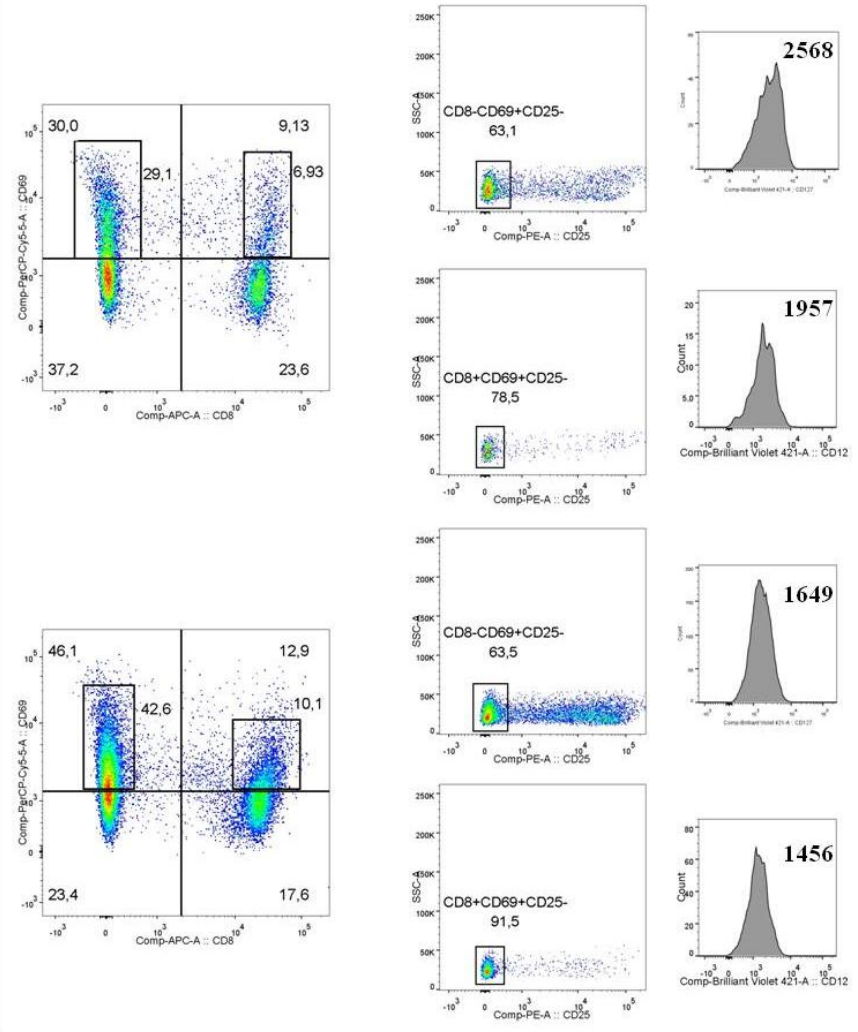
**C.**
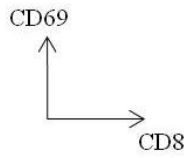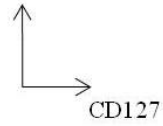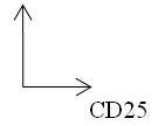
DAY 2

DAY 6
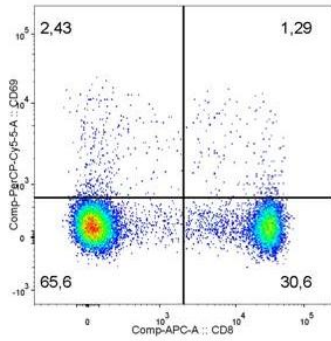
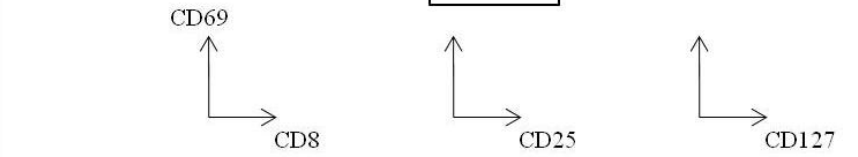**Unstimulated T cells**



**Stimulated T cells**
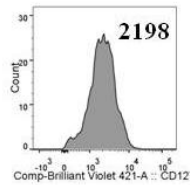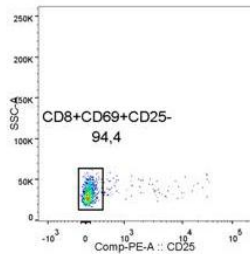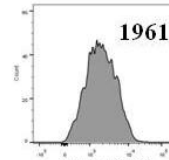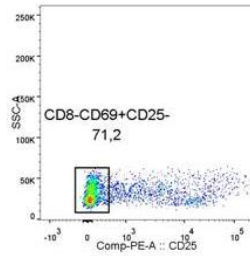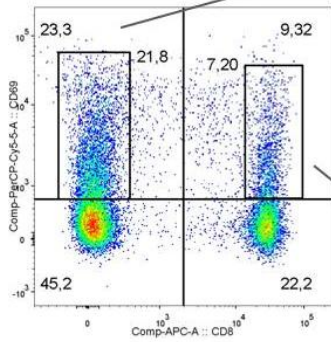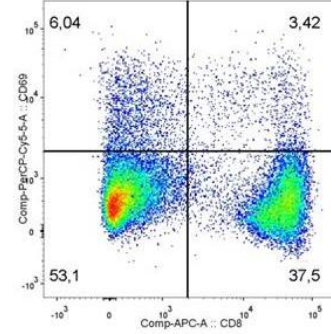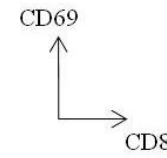
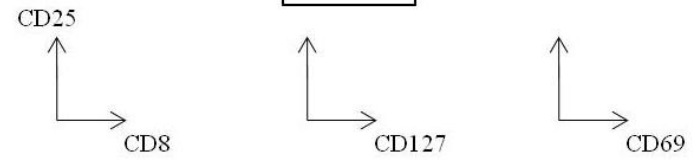## C. (continued)

DAY 2

DAY 6

+ **MultiStem donor 2**
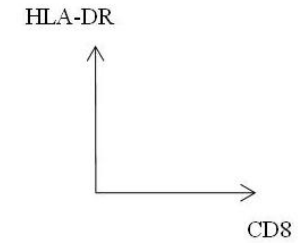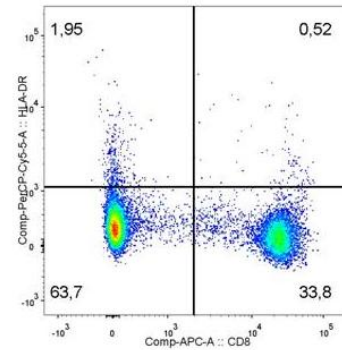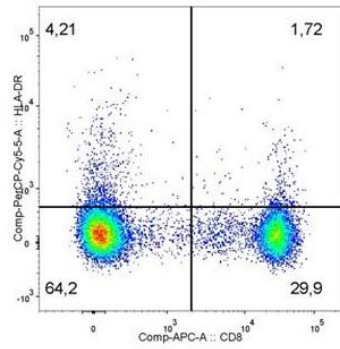


+ **MultiStem donor 4**
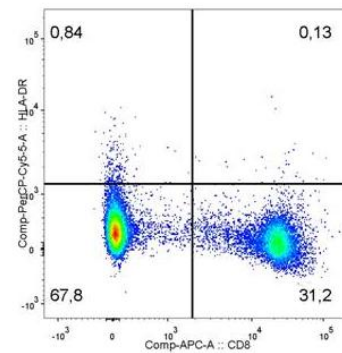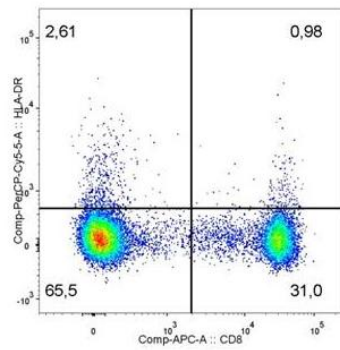
**Figure 8. MultiStem modulates T cell activation marker expression.**

Flow cytometric analysis of CD3$^+$ T cells for (A) early (CD69) and (B) – (C) late (CD25 and HLA-DR) T cell activation marker expression after a 6-day stimulation period with irradiated allogeneic EBV$^+$ B cells (S:R 1:20) in the absence [(un)stimulated T cells] or presence (+ MultiStem donor 2/4) of irradiated third-party MultiStem cells (suppressor:responder 1:1). One representative experiment with one T cell donor and two different MultiStem donors (donors 2 and 4) is shown.

(A) **CD69** expression after two days (*left panel*) or six days (*right panel*) of stimulation. Results are expressed as % positive cells in the CD3$^+$ lymphocyte gate. CD8$^-$CD69$^+$ and CD8$^+$CD69$^+$ subpopulations are selected (*left*) and tested for their expression of CD25 (*middle*). CD25$^-$ subpopulations are gated and tested for the presence of CD127 (*right*). Histograms represent CD127 expression of corresponding subpopulations with MFI values.

(B) **CD25** expression after two days (*left panel*) or six days (*right panel*) of stimulation. Results are expressed as % positive cells in the CD3$^+$ lymphocyte gate. CD8$^-$CD25$^+$ and CD8$^+$CD25$^+$ subpopulations are selected (*left*) and tested for their expression of CD127 (*middle*). CD127$^{dim}$ and CD127$^{high}$ subpopulations are gated and tested for the presence of CD69 (*right*). Histograms represent CD69 expression of corresponding subpopulations with MFI values.

(C) **HLA-DR** expression after two days (*left panel*) or six days (*right panel*) of stimulation. Results are expressed as % positive cells in the CD3$^+$ lymphocyte gate.

## 4.5 MultiStem cells mediate T cell cytotoxicity suppression through contact-dependent mechanisms
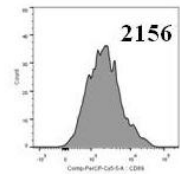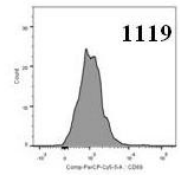
To further elaborate the exact mechanism of the MultiStem-mediated T cell cytotoxicity reduction, transwell inserts were used to separate the stem cells from the immune cells during the activation phase. As shown in **Figure 9**, an immune suppressive effect of MultiStem was found only in the condition in which both cell populations were in close proximity. Moreover, neither blocking IDO activity nor inhibiting PGE$_2$ synthesis was able to restore cytotoxicity, indicating that these two molecules, although they are important in the context of MSC-based modulation of T cell proliferation, play no role in MultiStem-mediated T cell cytotoxicity suppression (data not shown). These findings indicate that suppression of the cytotoxic capacity of T cells occurs mainly via contact-dependent mechanisms or via contact-induced soluble factors.
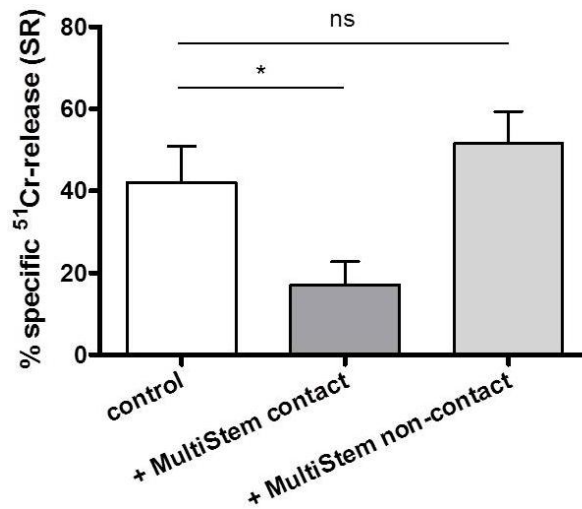
**Figure 9. MultiStem-mediated suppression of T cell cytotoxicity is contact-dependent.**

Freshly isolated CD3$^+$ T cells were primed with irradiated (40 Gy) allogeneic EBV$^+$ B cells (S:R 1:20) in the absence (control) or presence of irradiated (30 Gy) third-party MultiStem cells at MultiStem:T cell ratio of 1:2, either in direct contact (+ MS contact) or separated by means of a transwell system (+ MS non-contact). After 7 days, anti-CD3-redirected cytotoxicity was analyzed. Data are expressed as mean ± SEM percentage anti-CD3-dependent specific $^{51}$Cr-release (% SR) of P815 target cells (E:T 10:1). Results are pooled from six independent experiments, in which five different T cell donors and two different MultiStem donors were used. Statistical significance was calculated with the paired *t* test. *$p < 0.05$, ns = not significant.

To identify surface receptors or ligands that could be responsible for the contact-dependent immune modulating pathway, we analyzed MultiStem surface expression of the PD-L1/PD-L2/PD-1 molecules and of FasL by means of flow cytometry. These molecules were all expressed in variable amounts on the surface of MultiStem cells, with the highest expression of PD-L1 and PD-L2 ligands upon IFN-γ pretreatment (**Fig. 10A-B**). They were not identified as mediators of the MultiStem-related cytotoxicity suppression on the basis of functional blocking experiments with mAbs (data not shown). Given the fact that galectin-1 (Gal-1), a β-galactoside-binding immune suppressive protein, has recently been discovered as a ligand for CD69 on DCs [210], and that CD69 expression is in our hands prominently increased on T cells during coculture with MultiStem, we also studied the presence of Gal-1 on MultiStem cells. Fluorescence microscopy revealed Gal-1 expression on MultiStem (**Fig. 10C**), and this was confirmed by Western Blot analysis and ELISA on cell supernatant (data not shown).

**A.**



**B.**



| Marker | unstained | - IFN-γ | + IFN-γ |
|--------|-----------|---------|---------|
| PD-1   | $154 \pm 13$ | $1839 \pm 207$  | $1831 \pm 222$  |
| PD-L1  | $253 \pm 12$ | $3977 \pm 1565$ | $6183 \pm 3225$ |
| PD-L2  | $94 \pm 18$  | $4874 \pm 1767$ | $8987 \pm 4355$ |
| FasL   | $74 \pm 7$   | $704 \pm 124$   | $675 \pm 15$    |

**C.**
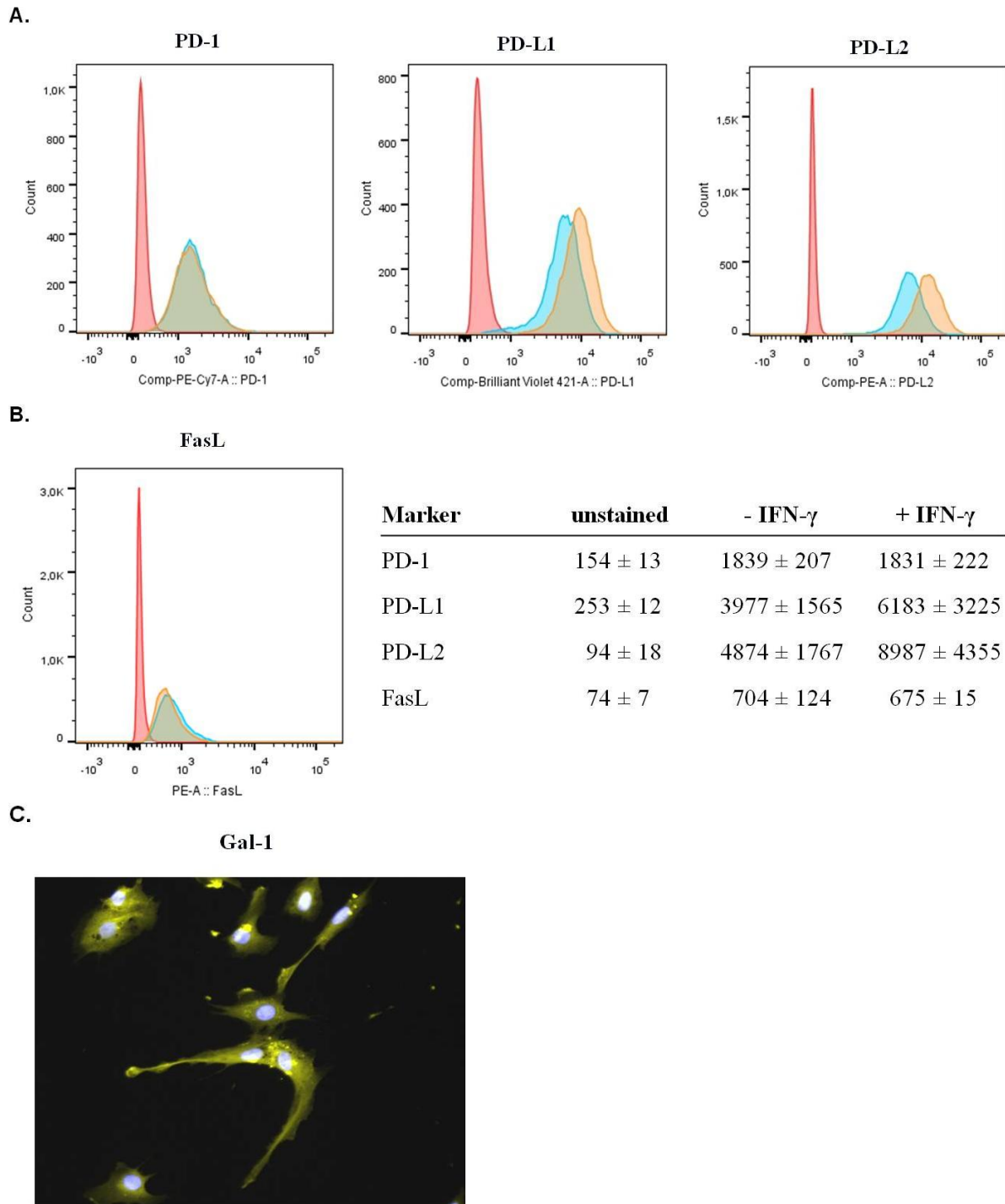


**Figure 10. MultiStem cells express PD-1/PD-L1/PD-L2, FasL and Gal-1.**

Flow cytometric analysis of MultiStem for the receptor (PD-1) and its ligands (PD-L1/2) of the PD-1 signaling pathway (A) and for the ligand (FasL) of the apoptosis-inducing FasL/FasR pathway (B). Histograms in red represent unstained cells, the blue and brown histograms represent respectively MultiStem before and after treatment with 100 U/ml IFN-γ for 48 h. One representative experiment (MultiStem donor 2) out of three is shown. Average mean fluorescence intensity (MFI) values ± SD of three experiments (MultiStem donors 2, 4 and 5) are shown in the table. (C) Fluorescent microscopic analysis of MultiStem (donor 4) for Gal-1 (yellow). Nuclear control staining with DAPI (4',6-diamidino-2-phenylindole; blue) is included.

## 5. Discussion

In the present study, we report for the first time on the effect of clinical-grade hMAPCs (MultiStem) on the cytotoxic function of T cells. MultiStem cells are immune privileged as they do not induce an alloreactive CTL response and as they are able to escape antigen-specific immune recognition by activated T cells. MultiStem decreases alloantigen-induced CD8$^+$ T cell proliferation and induction of CTL activity by significantly impairing the perforin expression after T cell priming, resulting in a diminished killer effect during the CTL effector phase. This modulatory effect is dose- and cell contact-dependent. In addition, MultiStem alters the T cell activation marker expression. When added to a total T cell population, MultiStem shifts activated CD8$^-$ T cells towards a regulatory phenotype. When added only during the cytotoxic effector phase, MultiStem cells are still capable of slightly inhibiting the lytic activity of activated T cells.

Our data on the immunogenicity and CTL susceptibility of MultiStem are similar with previously published reports on hMSCs. hMSCs do not induce T cell proliferation nor cytotoxic activity nor proinflammatory cytokine [IFN-γ and tumor necrosis factor (TNF)-α] production and induce little to no levels of activation markers (CD25, CD38 and CD69).[72,78,102,104] Regarding the CTL sensitivity of adult stem cells, hMSCs were neither susceptible to alloantigen-specific T cell-mediated lysis, even despite (upregulation of) MHC class I expression.[92,101,104] However, some studies show that MSCs are in fact sensitive to MHC-specific T cell-mediated lysis, which is even enhanced after IFN-γ pretreatment.[102,211,212] These contradictory results could be explained by the different experimental designs used and the origin of MSCs and their culture procedure. Based on the data presented here and our published findings on the mutual interaction between hMAPCs and NK cells [206], we can hypothesize in general that MultiStem cells escape recognition and lysis by the adaptive (MHC-specific CTLs) and innate (KIR-mismatched resting NK cells) immune system *in vitro*. Whether this will be the same for the *in vivo* setting, which eventually will depend on the *in situ* inflammatory microenvironment as we have seen in case of NK-cell mediated lysis, has to be further explored. Moreover, MultiStem might be processed by antigen-presenting DCs of the host in an *in vivo* setting. This indirect manner of alloantigen presentation to T cells should be investigated further in an *in vitro* setting.

CTL priming in the presence of MultiStem led to an impaired cytotoxic potential against target cells *in vitro*. Similar to other hMSC studies, they exert a dose-dependent inhibitory effect on the differentiation of CTL precursors into CTL effectors during the T cell priming

phase.[92,101,102] This suppressive effect was characteristic for MultiStem and not simply due to sterical hindrance or an increased cell density during the MLC, as demonstrated by the absence of any modulatory effect of third-party HUVECs. Moreover, the reduced T cell cytotoxicity was due to a direct effect of MultiStem on the responder T cells, as the stem cells had no influence on the surface phenotype and functionality of the alloantigen-presenting B cells. In contrast to most previous reports, addition of adult stem cells to antigen-primed effector CTLs at the time of the cytotoxic reaction also diminished target cell killing. Our results were corroborated in the MSC setting by Potian *et al.*, who neither observed any influence of BM-derived fibroblasts, confirming the absence of sterical cell hindrance.[213] However, the group of Rasmusson *et al.* saw no suppressive effect of MSCs added on a later time point during the T cell priming phase (day 3) or during the effector phase, suggesting that MSCs may predominantly inhibit the afferent phase of alloreactivity and prevent the development and proliferation of antigen-specific CTLs rather than their killer function.[92,101,103] Our results imply that MultiStem reduce the total cytotoxic response of CD8⁺ T cells, not only by inhibiting clonal expansion of the antigen-specific CD8⁺ T cell population, but also by directly and dose-dependently impairing the acquirement of cytotoxic capacity of the cells during the activation phase.

The mechanism for immune suppression by stem cells is not yet completely understood. No induction of tolerance, anergy or apoptosis could be demonstrated in our study, in accordance with previously published MSC studies.[72,75,81] Angoulvant *et al.* showed that exogenous IL-2 addition can partially restore target cell lysis after MSC-mediated suppression, while we observed only minimal improvement of cytotoxicity or proliferation in the presence of exogenous IL-2.[102,166] The inhibitory effect was reversed after removal of the stem cells, as we have shown in restimulation experiments and as described for MSCs in case of T cell proliferation suppression.[72,75,79,103] In our hands, third-party MultiStem addition led to a significant alteration in activation-associated surface marker expression of alloantigen-stimulated T cells, involving a significant reduction of CD25 and HLA-DR upregulation on day 6 of the stimulation phase. On the other hand, CD69 expression was strongly upregulated during the priming phase. The group of Le Blanc *et al.* showed MSC-mediated decreased expression of CD25 and CD38 on PHA-stimulated T cells on day 3, while other groups could not demonstrate any influence on CD25 and CD69 expression of allostimulated or PHA-stimulated T cells after 2 or 3 days respectively.[78,81,103] Our results could indicate a somehow dysregulated T cell activation in the presence of MultiStem, which might be associated with an impaired acquirement of cytotoxic properties. Besides the crucial effect on the survival and

expansion of CD4^{+} and CD8^{+} T cell subsets, IL-2 exerts a direct and independent enhancing effect on perforin and granzyme expression by CD8^{+} T cells, as demonstrated by Janas *et al.*[214] Accordingly, the observed MultiStem-mediated lack of CD25 (IL-2Rα chain) expression on CTLs and a consequently lower amount of high-affinity IL-2 receptors will lead to a disturbed autocrine IL-2 signaling cascade and lower perforin expression. The reduced CD25 upregulation on CTLs can also explain the lack of IL-2 effects to overcome the cytotoxicity suppression, while the priming of T cells remains unaffected.

Another crucial point is the fact that, when the total CD3^{+} T cell population instead of the CD8^{+} cells is used as responding population, MultiStem cells have an additional influence on the CD8^{-} (or CD4^{+}) fraction. MultiStem inhibits CD25 upregulation on both T cell subsets, but to a lower extent on CD8^{-} T cells. This remaining CD25^{+} fraction in the presence of MultiStem contains relatively more Tregs (CD127^{dim}) and has a higher expression of CD69, compared to the control condition of stimulated T cells. Therefore, we suggest that, in the presence of MultiStem, the balance of regulatory T cells *versus* effector T cells (Treg/Teff) is altered in favor of Tregs, which have higher levels of CD69 as well. A remarkable and sustained increase in CD69 expression on activated T lymphocytes was already shown for hMSCs, and accordingly, was seen in all CD4^{+} and CD8^{+} T cell subsets, including distinct regulatory subsets.[215] Recently, it has been shown that the previously known early activation marker CD69 also has an important regulatory role in the control of immune and inflammatory responses.[216,217] Saldanha-Araujo *et al.* have demonstrated that the late and sustained expression of CD69 (as an immunoregulatory molecule) could be controlled by the non-canonical NF-κB pathway, while its early expression (as an activation marker) is regulated by the canonical pathway.[215] However, we found also a CD8^{-}CD69^{+} population which was CD25^{-} and CD127^{+}. Several groups have demonstrated that a recently discovered subset of nontraditional regulatory T cells in tumor-bearing mice [218], in humans [219] and in patients with hepatocellular carcinoma are CD4^{+}CD25^{-}CD69^{+}.[220] Based on our findings and on the recent literature, we hypothesize that MultiStem addition is able to induce a similar suppressor T cell population, either by differentiation of CD8^{-}CD69^{+} T cells towards this regulatory phenotype or by an aberrant T cell activation (no upregulation of CD25 or HLA-DR) in the presence of MultiStem. The exact immune suppressive mechanism of this population is not yet revealed, but in the murine setting, these cells suppress T cell proliferation through membrane-bound TGF-β.[218] On the other hand, CD8^{+}CD25^{-}CD69^{+} T cells have been described in patients with chronic viral hepatitis [221] or rheumatoid arthritis.[222] Their exact function is yet unclear, but they are likely related to their CD4^{+} counterparts.[217] In

summary, MultiStem appears to exert a direct inhibitory effect on the expansion and functional activity of CD8$^+$ cytotoxic T cells, but they might as well have an indirect immune suppressive effect via CD4$^+$ T cells through shifting the Treg/Teff balance and through the induction of a distinct CD8$^-$CD69$^+$ suppressor population. Further research is required on this effect, and functional tests should be performed to demonstrate the regulatory activity of these populations.

Several candidate molecules [transforming growth factor (TGF)-β1, hepatocyte growth factor (HGF), PGE$_2$, IDO, etc.] have been proposed as the responsible soluble immunosuppressive factor produced by hMSCs, although data are contradictory probably because of variable experimental designs. In our hands, no role for soluble factors could be demonstrated in the MultiStem-mediated suppression of T cell cytotoxicity. Transwell experiments showed contact-dependency of MultiStem to exert its suppressive effects. Furthermore, blockage of neither IDO activity nor PGE$_2$ synthesis in the presence of MultiStem did restore cytotoxic T cell activity to its baseline levels. Although we reported for the first time on the expression of the ligands and receptor of the PD-1 pathway and of FasL on MultiStem cells, we were not able to identify one single responsible contact-dependent mechanism. Sotiropoulo *et al.* confirmed contact-dependency of hMSCs in case of suppression of NK cell cytotoxicity, while on the contrary proliferation and cytokine production suppression were mediated by soluble factors.[113] Krampera and colleagues observed that, in the murine system, MSCs inhibit naive and memory T cell responses, only when the cell populations are in close proximity to each other.[79] However, other MSC studies showed CTL cytotoxicity suppression by MSCs in transwell culture systems or via supernatant of cultured MSCs.[101,102] Our data show that MultiStem modulates cytotoxic function of T cells mainly through (for the moment not further specified) contact-dependent mechanism(s), while soluble factors are also involved in proliferation modulation.[166] This suggests that suppressive factor(s) of cytotoxicity are not constitutively secreted by MultiStem, but a dynamic cross-talk between MultiStem and immune cells is required.

Based on the MultiStem-mediated elevated levels of CD69 on T cells and the presence of its ligand Gal-1 [210] on MultiStem, Gal-1/CD69 binding could be an important mechanism of immune modulation by adult stem cells, independent of cytotoxicity suppression. De la Fuente *et al.* already proved inhibition of T$_h$17 differentiation and function in mice and humans upon CD69 recruitment.[210] Further research is mandatory.

According to our findings, we can conclude that MultiStem cells have broad immune suppressive properties, modulating both NK and T cell expansion and – as shown here – also cytotoxic functionality, validating their applicability in a range of immune-related diseases.

# Chapter 5 – General conclusions and future perspectives

The results presented in this manuscript provide further insights into the immunological effects of (clinical-grade) hMAPCs on NK cells and on CD8$^+$ T cells, by a number of *in vitro* studies (**Fig. 1**). Given the fact that cytotoxic immune effector cells play a vital role in immune homeostasis (e.g. host protection and GvHD/GvL balance) and are important in the pathogenesis of some autoimmune diseases, our data on the mutual interaction between this stem cell population and NK cells on the one hand and the stem cell influence on CTLs on the other hand, are highly relevant.

## 1. Immunogenicity

First, we have studied the immunogenicity of hMAPCs towards a functional NK and T cell response. We have shown that hMAPCs are not able to induce an antigen-specific – and only a minimal aspecific – cytotoxic response of naive T cells towards target cells. In line with previously published findings by our group [166], we can assume that hMAPCs are non-stimulatory for a T cell response, at least *in vitro*, including proliferation, $T_h1/T_h2$ cytokine secretion and cytotoxic activity. However, hMAPCs can be slightly immunogenic for alloreactive T cells since they induce a slight increase in activation marker expression [CD25, HLA-DR and inducible T cell costimulator (ICOS)].[166] Furthermore, we have demonstrated that allogeneic hMAPCs are insensitive to antigen-specific CTL-mediated lysis and to killing by resting KIR-mismatched NK cells, despite expression of some ligands (PVR and ULBP-2/5/6) for activating NK cell receptors (respectively DNAM-1 and NKG2D). On the other hand, when NK cells were preactivated with exogenous IL-2, efficient lysis of allogeneic hMAPCs was demonstrated. In contrast to hMSCs, hMAPCs express low levels of MHC class I molecules, which interact with inhibitory NK cell receptors and can be significantly upregulated upon IFN-γ-pretreatment.[56] Pretreatment of hMAPCs did not result in higher MHC-I-dependent allorecognition by CTLs and resulted in a diminished susceptibility to preactivated NK cell-mediated killing by shifting the balance towards MHC-I-associated inhibitory NK cell signalling. Taken together, these results indicate low immunogenicity of clinical-grade hMAPCs *in vitro*.

Upon administration *in vivo*, the survival and fate of hMAPCs will primarily be dictated by the inflammatory status and the cytokine balance in the local microenvironment. Thus, for example, while NK cells can lyse MAPCs in an inflammatory milieu in the presence of IL-2,

it is possible that the IFN-γ production by NK cells interacting with allogeneic MAPCs, in turn, will lead to protection of MAPCs from NK cell-mediated killing by upregulation of MHC I molecules. These findings underscore the complexity of the issue. With regard to MSCs, it has been shown that these cells are not intrinsically immunoprivileged. MSCs are also lysed by IL-2-activated NK cells *in vitro* [114], and immunocompetent MHC-mismatched mice were immune responsive to infusion of allogeneic MSCs, resulting in their rejection.[190] Most clinical reports on MSC therapy have confirmed low immunogenicity of MSCs *in vivo*. MSCs are capable of homing to inflamed tissues after systemic administration, regulate local inflammatory responses and promote endogenous tissue repair.[179] Nonetheless, some studies report on the short longevity of functional MSCs located in the host, implying a quick clearance of the stem cells and supporting their so-called 'touch-and-go' mechanism through secreting various trophic factors.[204]

## 2. Immune modulation

Secondly, we have assessed the immune modulatory capacities of (clinical-grade) hMAPCs on the cytotoxic functioning of NK and CTL effectors *in vitro*. We have proved that hMAPCs are able to suppress alloreactive CTL proliferation, upon stimulation with allogeneic APCs (PBMCs or EBV$^+$ B cells), and IL-2-induced expansion of allogeneic NK cells. In addition, we have shown a reduced cytotoxic function of resting NK cells and alloantigen- or anti-CD3/CD28-stimulated CTLs against antigen-(a)specific targets in the presence of allogeneic third-party hMAPCs during the lytic effector phase. hMAPCs also directly and dose-dependently impaired induction of CTL cytotoxicity during their priming phase by interfering with their perforin expression. On the other hand, in contrast to their inhibitory effect on NK cell proliferation, they had no influence on the intrinsic NK cell cytotoxic properties during the IL-2-mediated activation phase. Because of the cold target inhibition effect, we cannot conclude whether hMAPCs influence the killer function of IL-2-activated NK cells during their effector phase. Collectively, our observations imply that hMAPCs impair both proliferative and cytotoxic NK and T cell responses, resulting in a depressed immune response *in vitro*.

Whether this will be the same in the more complicated *in vivo* setting has to be evaluated. It has become clear that the local environment has a crucial effect on the immune suppressive capacity of MSCs, making it even more difficult to translate *in vitro* results to *in vivo* effects. For instance, MSC functionality can be influenced by the cytokine milieu. High

concentrations of IFN-γ enhance the inhibitory MSC activity, while low concentrations render MSCs proinflammatory (see below).[81] Furthermore, in the absence of IL-6, MSCs can switch macrophages to an anti-inflammatory M2 type.[223] The detection of microbial molecules can also polarize MSCs into a pro- or anti-inflammatory state: TLR3-mediated signalling gives rise to anti-inflammatory MSCs (MSC1), while TLR4-signalling leads to proinflammatory MSCs (MSC2).[224] Whether these factors can also act as a switch for hMAPCs has to be further examined. Considering the exact timing of stem cell addition, we could hypothesize that, based on results in this manuscript and on previous results [166,206], hMAPCs are somehow able to downregulate ongoing T cell responses in the *in vitro* setting, in contrast to NK cell responses. This hypothesis should be investigated more closely.

## 3. Immune modulatory mechanism

In our studies on the responsible mechanism and mediator(s) of the hMAPC-associated immunosuppressive effect, we have demonstrated that the mode of action is multifactorial, as is the case for hMSCs. Despite intensive research over the last decade, the exact mechanism behind the immune modulatory effect of hMSCs could not be fully elucidated so far. An overwhelming body of proof points out that both cell-to-cell contact-dependent pathways and a huge variety of (constitutively secreted or induced) soluble factors can play a major role in the suppression. Identification of these factors is apparently depending on the specific study design. In our hands, we observed that the mode of action depends on the immune effector response under study. In case of <u>proliferation</u> suppression, we could ascribe a partial role to IDO for hMAPC-mediated impairment of NK cell proliferation, in line with previous results obtained for T cell expansion inhibition.[166] In contrast, we could not identify a single responsible soluble factor in NK cell <u>cytotoxicity</u> suppression after testing some well-known mediators (IDO, $PGE_2$, IL-10 and TGF-β) of hMSC-mediated immune suppression. The same phenomenon was observed with T cells. Transwell experiments have shown contact-dependency of hMAPCs to exert their suppressive function on T cell cytotoxicity, while we know from previous work that hMAPCs separated from T cells by transwell inserts still partly reduce their proliferation.[166] As Sotiropoulo *et al.* described a similar phenomenon on NK cells [113], this difference in mode of action requires a more in-depth investigation. Until now, no single responsible contact-dependent mechanism could be identified to play a role in cytotoxicity suppression. Though, we described for the first time (clinical-grade) hMAPC expression of galectin-1 and ligands of the immune inhibitory PD-1 pathway and of the Fas-

mediated apoptosis pathway, all previously reported as responsible contact-dependent mechanisms in hMSC-mediated immune suppression.[97,98] Functional blocking studies however did not confirm the latter two pathways as candidates responsible for the suppressive activity. Based on the elevated expression of the immunoregulatory CD69 molecule on both CD8$^-$ and CD8$^+$ T cell subsets and on the fact that its recently discovered ligand, Gal-1 [210], is present on (clinical-grade) hMAPCs, we could suggest a possible role for this pathway in MAPC-mediated suppression. Although this pathway has been shown to be involved in impairment of T$_h$17 differentiation and function [210], this has to be examined further with regard to T cell cytotoxicity inhibition.

With regard to the suppressed T cell phenotype, we have observed that the presence of hMAPCs does not result in apoptosis or anergy and that T cells retain their memory response. However, hMAPCs change their pattern of activation marker expression, induced upon stimulation. The decrease in CD25 (IL-2Rα) upregulation seen in hMAPC-modulated CTLs might be indirectly responsible for the reduced perforin expression and for the lack of IL-2 effects to overcome cytotoxicity suppression.[214] Hypothetically, the clear and sustained increase of the immunoregulatory molecule CD69 on T cells after coculture with hMAPCs – together with the increase in Treg/Teff ratio – might reflect an alternative inhibitory mechanism. Besides directly targeting immune effector cells and their function, clinical-grade hMAPCs may also act indirectly by inducing a relatively higher abundance of other immunoregulatory cell populations with suppressive functions [217,220], as has been shown for M2 macrophages.[123] Alltogether, further investigation on this complex and pleiotropic mechanism of adult stem cell-mediated immune suppression is necessary for a better understanding.
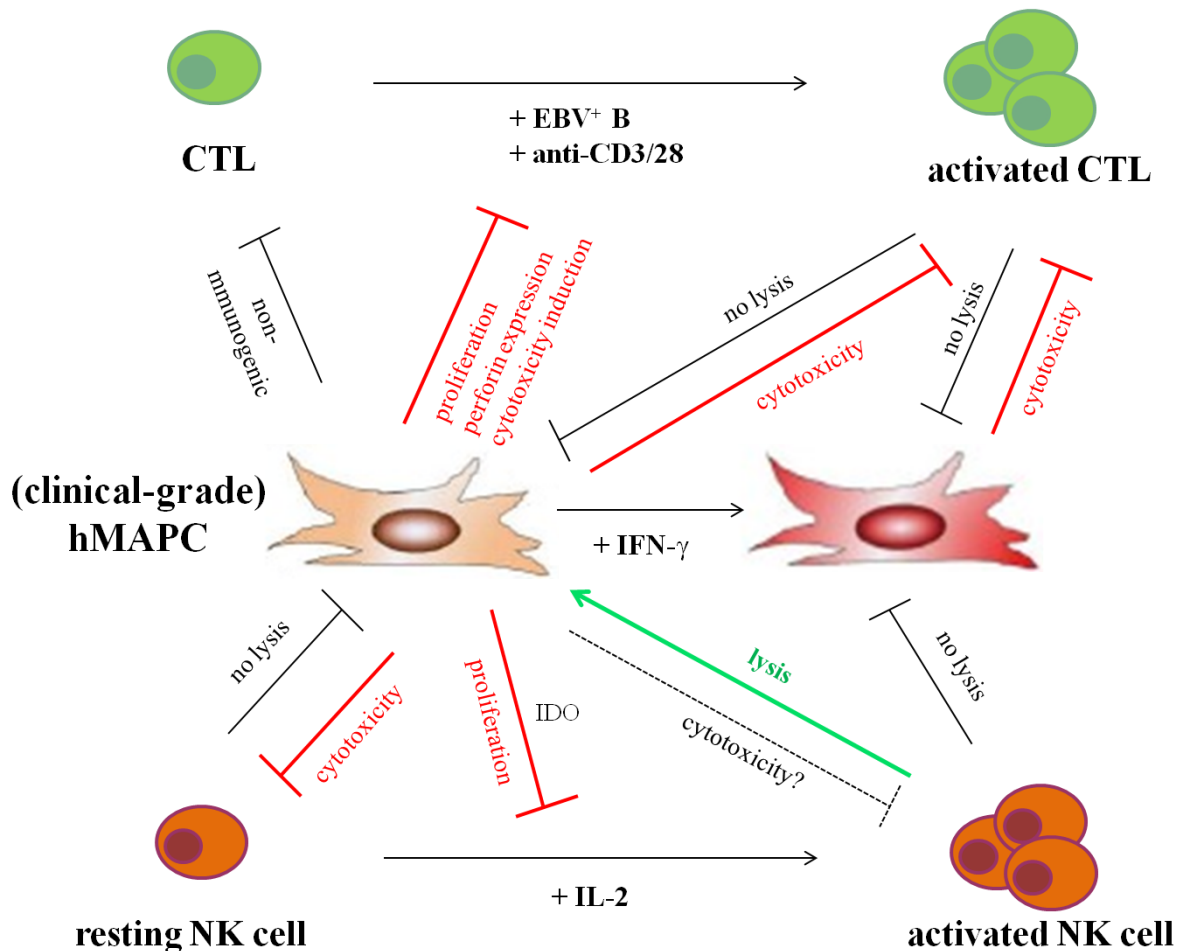
**Figure 1. Interplay between (clinical-grade) hMAPCs and NK cells or CTLs.**

Schematic representation of the influence of (clinical-grade) hMAPCs (± IFN-γ-pretreatment) on CTLs, and the interaction between hMAPCs and NK cells. hMAPCs do not induce alloreactive CTL function and are insensitive to lysis by antigen-specific CTLs, even after IFN-γ pretreatment of hMAPCs. hMAPCs are able to impair proliferation, perforin expression and cytotoxicity induction of CTLs during the T cell activation phase, next to suppressing the cytolytic function of activated CTLs during the effector phase. hMAPCs are not killed by allogeneic resting NK cells and are able to suppress the cytotoxic function of resting NK cells. IL-2-induced proliferation of NK cells is impaired in the presence of allogeneic hMAPCs in an IDO-dependent manner. IL-2-activated NK cells, on the other hand, are capable of killing allogeneic hMAPCs, unless latter cells are pretreated with IFN-γ. Because hMAPCs are targeted by IL-2-activated NK cells, it is unclear whether hMAPCs can influence the cytotoxic activity of activated NK cells.

## 4. Comparison immune modulation of hMAPCs *versus* hMSCs

Based on literature and previously published findings of our group [166], we can assume that, in spite of being two phenotypically and functionally distinct cell populations, the immunological behavior of hMAPCs *in vitro* is quite similar to that of hMSCs. In the few comparative experiments we performed, we could only observe a slight difference between hMAPCs and hMSCs regarding the modulation of cytokine-induced NK cell activation.

Compared to hMSCs, hMAPCs are able to suppress IL-2-driven NK cell proliferation to a higher extent.[206] Another remark is that the exposure of MSCs to proinflammatory cytokines (especially IFN-γ) enhances the immune suppressive capacity of MSCs, by inducing or upregulating inhibitory factors such as IDO and PD-L1.[81,225] IFN-γ-pretreated MSCs are considered to be highly immunosuppressive and to have enhanced migration and tissue regeneration properties [226], which validate the current strategy to license MSCs with IFN-γ prior to their *in vivo* administration. However, the concentration of IFN-γ appears to be crucial, since low levels stimulate the antigen-presenting function and result in MSC-mediated proinflammatory effects.[73] Based on our work and on previously published material of our group [166], we conclude that *ex vivo* IFN-γ pretreatment clearly alters the phenotype of hMAPCs with increased expression of MHC class I, ICAM-1, PD-L1/2 and kynurenine, but does not result in an increase of the immune regulatory hMAPC-mediated effect. The latter includes both immunogenic and immune suppressive properties with regard to T cell proliferation and cytotoxicity. The different outcomes of our experiments compared to the hMSC literature might be explained by the variability in experimental study design, including the kind of responder immune cell population (unfractionated PBMCs *versus* purified cell fractions), the stimulation (antigen- *versus* mitogen-induced), the dose and timing of stem cell addition or to a true functional difference between hMSCs and hMAPCs (e.g. origin, culture procedure). In a recent study by Mora-Lee *et al.*, murine intracranial injections of hMAPCs *versus* hMSCs after induction of stroke revealed that hMAPCs had stronger neuroprotective effects, more pronounced effects on the attenuation of inflammation, and more potency to promote endogenous tissue regeneration than hMSCs.[227] This observation was recently confirmed by Sindberg *et al.*, who have shown higher potency *in vitro* for hMAPCs.[43] This highlights the fact that there are differences in activity, which should be further examined.

## 5. Future perspectives and conclusions

Future studies need to further elaborate on the effects of hMAPCs on other innate and adaptive immune cell populations (e.g. DCs, macrophages, MDSCs, B cells, etc.). As outlined in the introduction, hMSCs have been demonstrated to interfere with DC differentiation, maturation and function.[119,120] Furthermore, hMSCs suppress B cell proliferation and differentiation.[107,108] In addition, it will be interesting to study the role of hMAPCs on macrophages. Busch *et al.* demonstrated in an *in vitro* rat model of spinal cord injury that rat MAPCs induce a shift in macrophages from a proinflammatory M1 state to an anti-

inflammatory M2 state.[124] Thus far, no data are available on the effects of hMAPCs on all these important immune and inflammatory cell types, which will again lead to a better understanding of the immunological behavior of hMAPCs.

Although the adoptive transfer of (clinical-grade) hMAPCs has already reached phase II testing in the clinical setting, some main concerns should not be neglected. As a first concern, the safety of the delivery of hMAPCs to the patients needs to be critically assessed. So far, several studies have been performed to evaluate the safety of the MultiStem product *in vivo*.[61,170,176] The results from these studies are consistent with our *in vitro* findings on the low immunogenicity of (clinical-grade) hMAPCs without any reported infusional toxicities or adverse reactions, confirming the immune privileged status of these cells. However, the risk of immune sensitization following a repeated dose regimen of allogeneic cells should be taken in consideration.

A second issue relates to the standardization and reproducibility of *ex vivo* generated stem cell cultures. MultiStem provides the advantage that cell expansion can be performed on an (automated) industrial scale in a well-validated and reproducible manner. Cells obtained from a single donor can be used to generate banks yielding a large amount of clinical doses, available at the time of need ('off-the-shelf') and usable without patient matching. Clinical trials can be performed with a single or only a few batches of cells, so that the results from these trials will not depend on the quality of the different isolations. Quality, product consistency and safety of the batches is ensured by an extensive characterization and a standard battery of biosafety tests (e.g. karyotyping and epigenetic analysis).[175] However, a disadvantage that is shared between adherent stem cell cultures is the use of FBS in culture media. Immune responses against serum components of MSC cultures have been detected [228], but no significant alloantibody production against MSCs has been described.[229] Nowadays, serum-free isolation, expansion and cryopreservation methods are being explored to circumvent the problem of batch-to-batch differences, the transmission of adventitious xenogeneic pathogens and to generate an optimized and functionally equivalent stem cell product. In addition, it remains crucial to validate a standardized immune assay to quantify the immunomodulatory potency of each batch prior to clinical use.

Another main concern about the *in vivo* use of clinical-grade hMAPCs is that they might render the host more vulnerable for infections by interfering with the normal protective

immune response against pathogens. For instance, we observed that hMAPCs might suppress the killing of viruses as they interfere with the cytotoxic capacities of (resting) NK cells and CTLs. Similarly, the effect of hMAPCs on the desired GvL effect after HSCT is being questioned. In case of hMSCs, Ning *et al.* reported on a randomized clinical trial in which patients with hematological malignancies received HSCs with or without hMSCs.[142] hMSC therapy had a beneficial effect on the occurrence of GvHD, but was associated with a higher relapse rate. In contrast, Baron *et al.* demonstrated that MSC coinfusion did not abrogate GvL effects in patients receiving allogeneic HLA-mismatched HSCT following nonmyeloablative conditioning.[131] This underscores the importance to further study the influence of hMAPCs on the GvL effect in allogeneic HSCT.

A last issue is the question as whether the immunosuppressive capacities of hMAPCs will be influenced by other immunosuppressive drugs (CsA, tacrolimus and MMF) and this has thus far not been studied for hMAPCs. As outlined in the introduction, Le Blanc *et al.* proved an *in vitro* synergistic effect of CsA on the hMSC-mediated immune suppression of T cell reactivity.[78] In contrast, Buron *et al.* observed that CsA, tacrolimus and rapamycin antagonized the inhibitory effects of hMSCs, whereas MMF promoted them.[191] Moreover, Eggenhofer *et al.* demonstrated in a rat model of heart transplantation that MSCs and MMF synergistically prevented the infiltration of antigen-presenting cells and T cells into the graft.[192] By contrast, calcineurin inhibitors have been shown to abrogate the immunosuppressive effect of rat MSC therapy.[193] These observations emphasize the need to study the appropriate drugs in combination with the adoptive stem cell-mediated immunotherapy.

In conclusion, we can state that the rationale for therapeutic use of (clinical-grade) hMAPCs for immunological disorders is legitimate. Notwithstanding the encouraging preliminary safety results, the number of studies and evaluated patients is still fairly limited. Therefore, further studies are needed to determine optimal cell dose, next to timing, frequency and route of administration in various clinical settings. Randomized MultiStem trials should be performed with large cohorts of patients subjected to a strict and long-term follow-up to exclude late complications, immunogenicity risk, ectopic tissue formation and unwanted adverse events. Scrutiny is still required and the immunological mechanism should be further elucidated *in vitro* and in various animal models to acquire new knowledge about the complex

cross-talk between stem cells and the immune system, and to pave the way for a continuation of this research.

# SUMMARY

Because of their long-term self-renewal ability and functional multi-lineage differentiation capacity, stem cells are highly attractive candidates for many applications. Both embryonic and adult stem cells have been explored as therapeutic strategies in the context of tissue regeneration. Over the years, it has become clear that adult stem cells also possess a wide range of immune and inflammation modulating properties. Many research groups have studied the immunological behavior of mesenchymal stem cells (MSCs) *in vitro* and their role as an adoptive immune regulatory cell population in several immune-related disorders. Nowadays, other adult adherent stem cell populations have been characterized and are being considered to exhibit more potency compared to MSCs. Recently, human multipotent adult progenitor cells (hMAPCs) have thoroughly been studied and have been shown to be clearly distinct from hMSCs. In contrast to hMSCs, hMAPCs can be expanded for a significantly longer time and – besides mesenchymal cell types – also differentiate into functional endothelium *in vitro* and *in vivo*. Clinical-grade production of MAPCs has been achieved and, currently, this proprietary large-scale expanded stem cell product (MultiStem®) is being evaluated as an allogeneic 'off-the-shelf' stem cell product in the clinic in the fields of regenerative medicine for cardiovascular (acute myocardial infarction; AMI) and neurological diseases (ischemic stroke) and as immunotherapy in immune-associated disorders [graft-*versus*-host disease (GvHD) after allogeneic hematopoietic stem cell transplantation (HSCT), solid organ transplantation and autoimmunity).

Even though administration of clinical-grade hMAPCs appears to be safe and well-tolerated, and leads to beneficial (preliminary) clinical results, data regarding their immunological behavior *in vitro* are scarce. A better understanding of the exact immune regulatory mechanism would be very valuable to optimize future clinical studies on the immunotherapeutic function of hMAPCs and of adult stem cells in general. In the presented manuscript, in continuation of a former project in this specific research line, we addressed the issue by investigating the reciprocal interaction between (clinical-grade) hMAPCs and immune cells of a functional human immune system *in vitro*. Given the fact that cytotoxic immune effector cells play a crucial role in immune homeostasis and in the pathogenesis of some autoimmune diseases, we analyzed the influence of (clinical-grade) hMAPCs on the phenotype and functionality of natural killer (NK) cells and CD8$^+$ cytotoxic T cells (CTLs).

The first specific objective of this research was to explore the **immunogenic and immune modulatory properties** of (clinical-grade) hMAPCs, with regard to functional cytotoxic responses of NK cells and T cells. More specific, it was questioned whether these stem cells of allogeneic origin could act as stimulators, as foreign target cells or as modulators of NK cells and CTLs. We showed that hMAPCs are not able to induce an antigen-specific – and only a minimal anti-CD3-redirected – CTL response *in vitro*. Furthermore, allogeneic hMAPCs were insensitive to antigen-specific CTL-mediated lysis and to killing by resting KIR-mismatched NK cells, despite expression of some ligands (PVR and ULBP-2/5/6) of activating NK cell receptors (respectively DNAM-1 and NKG2D). On the other hand, IL-2-preactivated NK cells efficiently lysed allogeneic hMAPCs. However, increased MHC class I expression after IFN-γ-pretreatment of hMAPCs did not result in higher MHC-I-dependent allorecognition by CTLs and reduced hMAPC susceptibility to preactivated NK cell-mediated killing, by shifting the balance towards MHC-I-associated inhibitory NK cell signalling. These results indicate low immunogenicity of (clinical-grade) hMAPCs *in vitro*, and suggest that survival of hMAPCs *in vivo* will primarily be dictated by the inflammatory status and the cytokine balance in the local microenvironment.

Secondly, we proved that hMAPCs exert strong immune suppressive effects, as they are able to suppress alloreactive CTL proliferation and IL-2-induced expansion of allogeneic NK cells. The suppressive effect on NK cell proliferation is dose- and IDO-dependent, and is more pronounced for hMAPCs than for hMSCs from the same donor. In addition, we observed a reduced cytotoxic function of resting NK cells and activated CTLs (in an antigen-specific and anti-CD3-redirected cytotoxicity system) in the presence of allogeneic third-party hMAPCs during the lytic effector phase. hMAPCs also directly and dose-dependently impaired induction of CTL cytotoxicity during their priming phase (alloantigen-induced or polyclonal activation) by interfering with their perforin expression. On the other hand, in contrast to their inhibitory effect on NK cell proliferation, hMAPCs had no influence on the intrinsic cytotoxic properties of NK cells during the IL-2-mediated activation phase. Because of competition between hMAPCs and K562 cells as target cells for IL-2-activated NK cell-mediated killing, we could not conclude if hMAPCs influence the killer function of IL-2-activated NK cells during their effector phase. Taken together, our observations imply that (clinical-grade) hMAPCs impair both proliferative and cytotoxic NK and T cell responses, resulting in a lower immune response *in vitro*.

The second specific goal of this thesis was to further elaborate the **immune modulatory mechanism** of (clinical-grade) hMAPCs and to identify responsible immune regulatory factors and pathways. We demonstrated that the mode of action is multifactorial and depends on the studied immune effector response. In case of <u>proliferation</u> suppression, we could ascribe a partial role to IDO for hMAPC-mediated impairment of NK cell proliferation, in line with previously obtained results for T cell expansion inhibition. In contrast, we could not identify a single responsible soluble factor (IDO, PGE$_2$, IL-10 and TGF-β) in NK cell <u>cytotoxicity</u> suppression. Transwell experiments showed contact-dependency of hMAPCs to exert their suppressive function on T cell cytotoxicity. Until now, as is the case for NK cells, we did not detect a single responsible contact-dependent mechanism in hMAPC-mediated T cell cytotoxicity suppression. Though, we described for the first time hMAPC expression of galectin-1 and ligands of the immune inhibitory PD-1 pathway (PD-L1/2) and of the Fas-mediated apoptosis pathway (FasL). Functional blocking studies however did not confirm the latter two pathways as suppressive candidates in CTL function inhibition by hMAPCs. With regard to the suppressed T cell phenotype, we observed that the presence of hMAPCs does not result in T cell apoptosis or anergy and that T cells retain their memory response during a secondary immune reaction after removal of hMAPCs. However, hMAPCs largely disturb the activation marker expression of T cells. In hMAPC-modulated T cells, CD69 expression was clearly and persistently increased, in both CD8$^-$ and CD8$^+$ T cell populations. Moreover, HLA-DR and CD25 (IL-2Rα) upregulation were significantly decreased in the presence of hMAPCs. hMAPCs downregulated CD25 expression to a lower extent on CD8$^-$ T cells and shifted those cells possibly towards a regulatory phenotype.

In conclusion, we have provided evidence that (clinical-grade) hMAPCs are low immunogenic and have potent immunosuppressive effects *in vitro* on NK cells and CTLs. In addition, we have contributed to a better understanding of the complex immunomodulatory mechanism. Based on these findings, we can state that the rationale for therapeutic use of hMAPCs or its clinical-grade counterpart MultiStem as an off-the-shelf adoptive cell population in the context of immune-related disorders is legitimate. These data may represent an extensive contribution to the current knowledge and, in combination with the results of future phase II/III trials using MultiStem, will lead to an intriguing continuation of stem cell-based research for immunotherapy.

# SAMENVATTING

Stamcellen zijn veelbelovende kandidaten voor vele toepassingen, omwille van hun capaciteit om zichzelf gedurende lange tijd te regenereren en om uit te groeien tot verscheidene functionele celtypes. Zowel embryonale als volwassen stamcellen zijn reeds onderzocht als therapie voor weefselherstel. De laatste jaren is het duidelijk geworden dat adulte stamcellen ook een brede waaier aan immuun- en ontstekingsmodulerende eigenschappen bezitten. Vele onderzoeksgroepen hebben reeds het immunologisch gedrag van mesenchymale stamcellen (MSCs) *in vitro* onderzocht, en hun rol als immuunregulerende celpopulatie in verscheidene immuungemedieerde aandoeningen getest. Tegenwoordig zijn er andere adulte stamcelpopulaties beschreven die beschouwd worden meer potentieel te vertonen in vergelijking met MSCs. Humane multipotente adulte progenitorcellen (hMAPCs) zijn recentelijk grondig bestudeerd en zijn volgens onderzoek duidelijk verschillend van humane (h)MSCs. hMAPCs kunnen in tegenstelling tot hMSCs significant langer geëxpandeerd worden en kunnen, behalve tot mesenchymale celtypes, ook differentiëren tot functionele endotheelcellen *in vitro* en *in vivo*. Grootschalige klinische productie van MAPCs is bewerkstelligd en momenteel wordt dit gepatenteerde stamcelproduct (MultiStem®) geëvalueerd als een allogeen 'off-the-shelf' stamcelproduct in de klinische wereld op gebied van regeneratieve geneeskunde voor cardiovasculaire aandoeningen (acuut myocardinfarct; AMI) en voor neurologische ziektes (cerebrovasculair accident), en als immuuntherapie in immuungeassocieerde aandoeningen ['graft-*versus*-host disease' (GvHD) na allogene transplantatie van hematopoietische stamcellen, orgaantransplantatie en auto-immuunziekten]. Hoewel toediening van 'clinical-grade' hMAPCs veilig en goed verdraagbaar blijkt, en tot (voorlopige) voordelige klinische resultaten leidt, zijn gegevens omtrent hun immunologisch gedrag *in vitro* eerder schaars. Het zou zeer waardevol zijn om het precieze immuunregulerende mechanisme beter te leren kennen, met het oog om toekomstige klinische studies over de immuuntherapeutische functie van hMAPCs en van adulte stamcellen in het algemeen te optimaliseren. In het gepresenteerde onderzoek wordt dit probleem, in voortzetting van een voormalig project in dit specifieke onderzoeksveld, behandeld door de wederzijdse interactie tussen 'clinical-grade' hMAPCs en immuuncellen van een functioneel humaan immuunsysteem *in vitro* te onderzoeken. Omwille van het feit dat cytotoxische immunologische effectorcellen een cruciale rol spelen in de homeostase van het immuunsysteem en in de pathogenese van enkele auto-immuunziekten, hebben we de invloed

van 'clinical-grade' hMAPCs op het phenotype en de functionaliteit van natural killer (NK) cellen en CD8-positieve cytotoxische T lymphocyten (CTLs) geanalyseerd.

De eerste specifieke doelstelling van dit onderzoek was het nagaan van de **immunogene en immuunmodulerende eigenschappen** van 'clinical-grade' hMAPCs met betrekking tot functioneel cytotoxische responsen van NK cellen en T cellen. Meer concreet is er onderzocht of deze allogene stamcellen zich kunnen gedragen als stimulerende cellen, als vreemde doelwitcellen of als modulerende cellen voor NK cellen en CTLs. We hebben aangetoond dat hMAPCs niet in staat zijn om een antigeenspecifieke – en enkel een minimaal anti-CD3 afhankelijke – CTL respons *in vitro* te induceren. Bovendien werden allogene hMAPCs niet gedood door antigeenspecifieke CTLs en evenmin door rustende vreemde NK cellen, ondanks expressie van enkele liganden (PVR en ULBP-2/5/6) voor activerende NK celreceptoren (respectievelijk DNAM-1 en NKG2D). Anderzijds werden allogene hMAPCs efficiënt gelyseerd door IL-2-gepreactiveerde NK cellen. Een verhoogde MHC klasse I expressie van hMAPCs na pre-incubatie met IFN-γ resulteerde echter niet in een hogere MHC-I afhankelijke lyse door CTLs en reduceerde de gevoeligheid van hMAPCs voor lyse door gepreactiveerde NK cellen, door de balans te doen overhellen richting MHC-I geassocieerde inhiberende NK celsignalisatie. Deze resultaten wijzen op de lage immunogeniciteit van 'clinical-grade' hMAPCs *in vitro*, en suggereren dat het voortbestaan van hMAPCs *in vivo* vooral afhankelijk zal zijn van de inflammatiestatus en de cytokinebalans in de lokale micro-omgeving.

Ten tweede hebben we bewezen dat hMAPCs sterke immuunsuppressieve effecten uitoefenen, aangezien zij in staat zijn alloreactieve CTL proliferatie en IL-2-geïnduceerde expansie van allogene NK cellen te onderdrukken. Het inhiberend effect op de NK celproliferatie is dosis- en IDO-afhankelijk, en is sterker voor hMAPCs dan voor hMSCs van dezelfde donor. Daarnaast hebben we, in de aanwezigheid van allogene 'third-party' hMAPCs tijdens de lytische effectorfase, een gereduceerde cytotoxische functie van rustende NK cellen en geactiveerde CTLs (in een antigeenspecifiek en anti-CD3-gemedieerd cytotoxiciteitssysteem) geobserveerd. hMAPCs verstoren ook op rechtstreekse en dosisafhankelijke wijze de inductie van CTL cytotoxiciteit tijdens de activatiefase (alloantigeengeïnduceerde of polyclonale activatie), door te interfereren met de expressie van perforine. Anderzijds, en in tegenstelling tot hun inhiberend effect op NK celproliferatie, hadden hMAPCs geen invloed op de intrinsieke cytotoxische eigenschappen van NK cellen tijdens de IL-2-gemedieerde activatiefase. Omwille van competitie tussen hMAPCs en K562

cellen als doelwitcellen voor lyse door IL-2-geactiveerde NK cellen, konden we niet besluiten of hMAPCs de lytische functie van IL-2-geactiveerde NK cellen beïnvloeden tijdens hun effectorfase. Samenvattend impliceren onze observaties dat 'clinical-grade' hMAPCs zowel de proliferatie- als de cytotoxiciteitsrespons van NK cellen en T cellen verstoren, wat uiteindelijk leidt tot een verminderde immuunreactie *in vitro*.

Het tweede specifieke doel van deze thesis was om het **immuunmodulerende mechanisme** van 'clinical-grade' hMAPCs verder uit te werken en verantwoordelijke immuunregulerende factoren en pathways te identificeren. We hebben aangetoond dat het werkingsmechanisme multifactorieel is en afhankelijk is van de bestudeerde immuunrespons. Ingeval van suppressie van proliferatie konden we een gedeeltelijke rol toeschrijven aan IDO voor hMAPC-gemedieerde inhibitie van NK celproliferatie, in lijn met vorige resultaten met betrekking tot inhibitie van T celproliferatie. Daarentegen konden we geen verantwoordelijke solubele factor (IDO, $PGE_2$, IL-10 en TGF-$\beta$) voor suppressie van NK celcytotoxiciteit identificeren. Experimenten met transwell systemen toonden de contactafhankelijkheid van hMAPCs aan om hun suppressieve functie op T cel cytotoxiciteit uit te oefenen. Totnogtoe hebben we geen enkel verantwoordelijk contactafhankelijk mechanisme gevonden voor hMAPC-gemedieerde suppressie van T celcytotoxiciteit, zoals het geval is voor NK cellen. We hebben echter wel voor de eerste keer beschreven dat hMAPCs galectine-1 en liganden voor de immuuninhiberende PD-1 pathway (PD-L1/2) en de Fas-gemedieerde apoptosepathway (FasL) tot expressie brengen. Studies waarin we de functie van deze moleculen hebben geblokkeerd, hebben echter geen bevestiging gebracht dat deze laatste twee pathways als suppressieve kandidaten hun rol spelen in de inhibitie van CTL functie door hMAPCs. Met betrekking tot het onderdrukte T celphenotype hebben we gezien dat de aanwezigheid van hMAPCs niet resulteert in T celapoptose of T celanergie, en dat T cellen hun geheugenrespons niet verliezen tijdens een secundaire immuunreactie na het verwijderen van de hMAPCs. Niettemin verstoren hMAPCs de expressie van T cel activatiemerkers grondig. In hMAPC gemoduleerde T cellen was de expressie van CD69 duidelijk en blijvend verhoogd, in zowel CD8-negatieve als -positieve T celpopulaties. Daarenboven was de stijging van HLA-DR en CD25 (IL-2R$\alpha$) significant afgenomen in de aanwezigheid van hMAPCs. hMAPCs verminderden de expressie van CD25 minder sterk bij CD8-negatieve T cellen en brachten mogelijks een verschuiving teweeg richting CD8-negatieve cellen met een regulerend phenotype.

We kunnen besluiten dat we het bewijs hebben geleverd dat 'clinical-grade' hMAPCs een lage immunogene capaciteit en sterke immuunsuppressieve effecten op NK cellen en CTLs *in vitro* hebben. Daarnaast hebben we bijgedragen tot een beter begrip van het gecompliceerde immuunmodulerend mechanisme. Gebaseerd op deze bevindingen kunnen we stellen dat de rationale voor het therapeutisch gebruik van hMAPCs of de 'clinical-grade' variant MultiStem als een off-the-shelf celpopulatie in de context van immuungerelateerde aandoeningen gerechtvaardigd is. Deze data kunnen mogelijks een reële bijdrage leveren voor de hedendaagse kennis en, samen met de resultaten van toekomstige fase II/III studies met MultiStem, leiden tot een intrigerende voortzetting van stamcelgerelateerd onderzoek voor immuuntherapie.

# REFERENCE LIST

1. Bongso A, Lee EH. Stem cells: their definition, classification and sources. In: Bongso A, Lee EH, editors. Stem cells: From Bench to Bedside. Toh Tuck: World Scientific Publishing; 2005. p. 1-13.
2. Weissman IL. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science. 2000 Feb 25;287(5457):1442-1446.
3. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A. 1981 Dec;78(12):7634-7638.
4. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998 Nov 6;282(5391):1145-1147.
5. Hayes M, Curley G, Ansari B, Laffey JG. Clinical review: Stem cell therapies for acute lung injury/acute respiratory distress syndrome - hope or hype? Crit Care. 2012;16(2):205.
6. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981 Jul 9;292(5819):154-156.
7. Zeng X, Rao MS. Human embryonic stem cells: long term stability, absence of senescence and a potential cell source for neural replacement. Neuroscience. 2007 Apr 14;145(4):1348-1358.
8. Bradley A, Evans M, Kaufman MH, Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature. 1984 May 17-23;309(5965):255-256.
9. Smith AG. Embryo-derived stem cells: of mice and men. Annu Rev Cell Dev Biol. 2001;17:435-462.
10. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol. 2000 Apr;18(4):399-404.
11. Adewumi O, Aflatoonian B, Ahrlund-Richter L, Amit M, Andrews PW, Beighton G, et al. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol. 2007 Jul;25(7):803-816.
12. Lensch MW, Schlaeger TM, Zon LI, Daley GQ. Teratoma formation assays with human embryonic stem cells: a rationale for one type of human-animal chimera. Cell Stem Cell. 2007 Sep 13;1(3):253-258.
13. Hayflick L. The Limited in Vitro Lifetime of Human Diploid Cell Strains. Exp Cell Res. 1965 Mar;37:614-636.
14. Morrison SJ, Uchida N, Weissman IL. The biology of hematopoietic stem cells. Annu Rev Cell Dev Biol. 1995;11:35-71.
15. Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. Cell. 2000 Jan 7;100(1):157-168.
16. Lewis JP, Trobaugh FE, Jr. Haematopoietic Stem Cells. Nature. 1964 Nov 7;204:589-590.
17. Weissman IL, Shizuru JA. The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. Blood. 2008 Nov 1;112(9):3543-3553.
18. Eckfeldt CE, Mendenhall EM, Verfaillie CM. The molecular repertoire of the 'almighty' stem cell. Nat Rev Mol Cell Biol. 2005 Sep;6(9):726-737.
19. Gage FH. Mammalian neural stem cells. Science. 2000 Feb 25;287(5457):1433-1438.
20. Alvarez-Buylla A, Garcia-Verdugo JM. Neurogenesis in adult subventricular zone. J Neurosci. 2002 Feb 1;22(3):629-634.
21. Blanpain C, Fuchs E. Epidermal homeostasis: a balancing act of stem cells in the skin. Nat Rev Mol Cell Biol. 2009 Mar;10(3):207-217.
22. van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol. 2009;71:241-260.
23. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol. 1974;2(2):83-92.
24. Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991 Sep;9(5):641-650.
25. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. 1997 Apr 4;276(5309):71-74.
26. Caplan AI. The mesengenic process. Clin Plast Surg. 1994 Jul;21(3):429-435.
27. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002 Dec;13(12):4279-4295.
28. Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, et al. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells. 2004;22(7):1330-1337.

29.     Fukumoto T, Sperling JW, Sanyal A, Fitzsimmons JS, Reinholz GG, Conover CA, et al. Combined effects of insulin-like growth factor-1 and transforming growth factor-beta1 on periosteal mesenchymal cells during chondrogenesis in vitro. Osteoarthritis Cartilage. 2003 Jan;11(1):55-64.

30.     Pierdomenico L, Bonsi L, Calvitti M, Rondelli D, Arpinati M, Chirumbolo G, et al. Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation. 2005 Sep 27;80(6):836-842.

31.     De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum. 2001 Aug;44(8):1928-1942.

32.     Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood. 2001 Oct 15;98(8):2396-2402.

33.     da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci. 2006 Jun 1;119(Pt 11):2204-2213.

34.     Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999 Apr 2;284(5411):143-147.

35.     Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-317.

36.     Sudres M, Norol F, Trenado A, Gregoire S, Charlotte F, Levacher B, et al. Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. J Immunol. 2006 Jun 15;176(12):7761-7767.

37.     Zhou YF, Bosch-Marce M, Okuyama H, Krishnamachary B, Kimura H, Zhang L, et al. Spontaneous transformation of cultured mouse bone marrow-derived stromal cells. Cancer Res. 2006 Nov 15;66(22):10849-10854.

38.     Meisel R, Brockers S, Heseler K, Degistirici O, Bulle H, Woite C, et al. Human but not murine multipotent mesenchymal stromal cells exhibit broad-spectrum antimicrobial effector function mediated by indoleamine 2,3-dioxygenase. Leukemia. 2011 Apr;25(4):648-654.

39.     Bianco P, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell. 2008 Apr 10;2(4):313-319.

40.     Prockop DJ. Repair of tissues by adult stem/progenitor cells (MSCs): controversies, myths, and changing paradigms. Mol Ther. 2009 Jun;17(6):939-946.

41.     Tolar J, Le Blanc K, Keating A, Blazar BR. Concise review: hitting the right spot with mesenchymal stromal cells. Stem Cells. 2010 Aug;28(8):1446-1455.

42.     Haniffa MA, Collin MP, Buckley CD, Dazzi F. Mesenchymal stem cells: the fibroblasts' new clothes? Haematologica. 2009 Feb;94(2):258-263.

43.     Sindberg GM, Lindborg BA, Wang Q, Clarkson C, Graham M, Donahue R, et al. Comparisons of phenotype and immunomodulatory capacity among rhesus bone-marrow-derived mesenchymal stem/stromal cells, multipotent adult progenitor cells, and dermal fibroblasts. J Med Primatol. 2014 Aug;43(4):231-241.

44.     Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell. 2007 Oct 19;131(2):324-336.

45.     Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell. 2008 Sep 11;3(3):301-313.

46.     da Silva Meirelles L, Caplan AI, Nardi NB. In search of the in vivo identity of mesenchymal stem cells. Stem Cells. 2008 Sep;26(9):2287-2299.

47.     Covas DT, Panepucci RA, Fontes AM, Silva WA, Jr., Orellana MD, Freitas MC, et al. Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. Exp Hematol. 2008 May;36(5):642-654.

48.     Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002 Jul 4;418(6893):41-49.

49.     Geraerts M, Verfaillie CM. Adult stem and progenitor cells. Adv Biochem Eng Biotechnol. 2009;114:1-21.

50.     Ulloa-Montoya F, Kidder BL, Pauwelyn KA, Chase LG, Luttun A, Crabbe A, et al. Comparative transcriptome analysis of embryonic and adult stem cells with extended and limited differentiation capacity. Genome Biol. 2007;8(8):R163.

51.     Subramanian K, Geraerts M, Pauwelyn KA, Park Y, Owens DJ, Muijtjens M, et al. Isolation procedure and characterization of multipotent adult progenitor cells from rat bone marrow. Methods Mol Biol. 2010;636:55-78.

52.     Sohni A, Verfaillie CM. Multipotent adult progenitor cells. Best Pract Res Clin Haematol. 2011 Mar;24(1):3-11.

53.     Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest. 2002 Feb;109(3):337-346.

54.     Schwartz RE, Reyes M, Koodie L, Jiang Y, Blackstad M, Lund T, et al. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest. 2002 May;109(10):1291-1302.

55.     Aranguren XL, Luttun A, Clavel C, Moreno C, Abizanda G, Barajas MA, et al. In vitro and in vivo arterial differentiation of human multipotent adult progenitor cells. Blood. 2007 Mar 15;109(6):2634-2642.

56.     Roobrouck VD, Clavel C, Jacobs SA, Ulloa-Montoya F, Crippa S, Sohni A, et al. Differentiation potential of human postnatal mesenchymal stem cells, mesoangioblasts, and multipotent adult progenitor cells reflected in their transcriptome and partially influenced by the culture conditions. Stem Cells. 2011 May;29(5):871-882.

57.     Roobrouck VD, Vanuytsel K, Verfaillie CM. Concise review: culture mediated changes in fate and/or potency of stem cells. Stem Cells. 2011 Apr;29(4):583-589.

58.     Jacobs SA, Roobrouck VD, Verfaillie CM, Van Gool SW. Immunological characteristics of human mesenchymal stem cells and multipotent adult progenitor cells. Immunol Cell Biol. 2013 Jan;91(1):32-39.

59.     Boozer S, Lehman N, Lakshmipathy U, Love B, Raber A, Maitra A, et al. Global Characterization and Genomic Stability of Human MultiStem, A Multipotent Adult Progenitor Cell. J Stem Cells. 2009;4(1):17-28.

60.     Van Bokkelen G. Company profile: Athersys. Regen Med. 2011 Jan;6(1):39-43.

61.     Penn MS, Ellis S, Gandhi S, Greenbaum A, Hodes Z, Mendelsohn FO, et al. Adventitial delivery of an allogeneic bone marrow-derived adherent stem cell in acute myocardial infarction: phase I clinical study. Circ Res. 2012 Jan 20;110(2):304-311.

62.     Lehman N, Cutrone R, Raber A, Perry R, Van't Hof W, Deans R, et al. Development of a surrogate angiogenic potency assay for clinical-grade stem cell production. Cytotherapy. 2012 Sep;14(8):994-1004.

63.     Burrows GG, Van't Hof W, Newell LF, Reddy A, Wilmarth PA, David LL, et al. Dissection of the human multipotent adult progenitor cell secretome by proteomic analysis. Stem Cells Transl Med. 2013 Oct;2(10):745-757.

64.     Noel D, Djouad F, Jorgense C. Regenerative medicine through mesenchymal stem cells for bone and cartilage repair. Curr Opin Investig Drugs. 2002 Jul;3(7):1000-1004.

65.     Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, et al. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med. 1999 Mar;5(3):309-313.

66.     Au P, Tam J, Fukumura D, Jain RK. Bone marrow-derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature. Blood. 2008 May 1;111(9):4551-4558.

67.     Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006 Aug 1;98(5):1076-1084.

68.     Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol. 2002 Jan;30(1):42-48.

69.     Bensidhoum M, Chapel A, Francois S, Demarquay C, Mazurier C, Fouillard L, et al. Homing of in vitro expanded Stro-1- or Stro-1+ human mesenchymal stem cells into the NOD/SCID mouse and their role in supporting human CD34 cell engraftment. Blood. 2004 May 1;103(9):3313-3319.

70.     Chou SH, Lin SZ, Day CH, Kuo WW, Shen CY, Hsieh DJ, et al. Mesenchymal stem cell insights: prospects in hematological transplantation. Cell Transplant. 2013;22(4):711-721.

71.     Bernardo ME, Cometa AM, Locatelli F. Mesenchymal stromal cells: a novel and effective strategy for facilitating engraftment and accelerating hematopoietic recovery after transplantation? Bone Marrow Transplant. 2012 Mar;47(3):323-329.

72.     Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, et al. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci. 2005;12(1):47-57.

73.     Stagg J, Pommey S, Eliopoulos N, Galipeau J. Interferon-gamma-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. Blood. 2006 Mar 15;107(6):2570-2577.

74. Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation. 2003 Feb 15;75(3):389-397.

75. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002 May 15;99(10):3838-3843.

76. Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol. 2003 Jan;57(1):11-20.

77. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005 Feb 15;105(4):1815-1822.

78. Le Blanc K, Rasmusson I, Gotherstrom C, Seidel C, Sundberg B, Sundin M, et al. Mesenchymal stem cells inhibit the expression of CD25 (interleukin-2 receptor) and CD38 on phytohaemagglutinin-activated lymphocytes. Scand J Immunol. 2004 Sep;60(3):307-315.

79. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood. 2003 May 1;101(9):3722-3729.

80. Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. Blood. 2005 Apr 1;105(7):2821-2827.

81. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells. 2006 Feb;24(2):386-398.

82. Benvenuto F, Ferrari S, Gerdoni E, Gualandi F, Frassoni F, Pistoia V, et al. Human mesenchymal stem cells promote survival of T cells in a quiescent state. Stem Cells. 2007 Jul;25(7):1753-1760.

83. Sivanathan KN, Gronthos S, Rojas-Canales D, Thierry B, Coates PT. Interferon-gamma modification of mesenchymal stem cells: implications of autologous and allogeneic mesenchymal stem cell therapy in allotransplantation. Stem Cell Rev. 2014 Jun;10(3):351-375.

84. Plumas J, Chaperot L, Richard MJ, Molens JP, Bensa JC, Favrot MC. Mesenchymal stem cells induce apoptosis of activated T cells. Leukemia. 2005 Sep;19(9):1597-1604.

85. Rasmusson I, Ringden O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms. Exp Cell Res. 2005 Apr 15;305(1):33-41.

86. Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood. 2004 Jun 15;103(12):4619-4621.

87. Mellor A. Indoleamine 2,3 dioxygenase and regulation of T cell immunity. Biochem Biophys Res Commun. 2005 Dec 9;338(1):20-24.

88. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell. 2008 Feb 7;2(2):141-150.

89. Chabannes D, Hill M, Merieau E, Rossignol J, Brion R, Soulillou JP, et al. A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells. Blood. 2007 Nov 15;110(10):3691-3694.

90. Selmani Z, Naji A, Gaiffe E, Obert L, Tiberghien P, Rouas-Freiss N, et al. HLA-G is a crucial immunosuppressive molecule secreted by adult human mesenchymal stem cells. Transplantation. 2009 May 15;87(9 Suppl):S62-66.

91. Lee RH, Pulin AA, Seo MJ, Kota DJ, Ylostalo J, Larson BL, et al. Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. Cell Stem Cell. 2009 Jul 2;5(1):54-63.

92. Maccario R, Podesta M, Moretta A, Cometa A, Comoli P, Montagna D, et al. Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. Haematologica. 2005 Apr;90(4):516-525.

93. Zhao ZG, Xu W, Sun L, You Y, Li F, Li QB, et al. Immunomodulatory function of regulatory dendritic cells induced by mesenchymal stem cells. Immunol Invest. 2012;41(2):183-198.

94. Cho DI, Kim MR, Jeong HY, Jeong HC, Jeong MH, Yoon SH, et al. Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. Exp Mol Med. 2014;46:e70.

95. Yen BL, Yen ML, Hsu PJ, Liu KJ, Wang CJ, Bai CH, et al. Multipotent human mesenchymal stromal cells mediate expansion of myeloid-derived suppressor cells via hepatocyte growth factor/c-met and STAT3. Stem Cell Reports. 2013;1(2):139-151.

96. Sioud M, Mobergslien A, Boudabous A, Floisand Y. Mesenchymal stem cell-mediated T cell suppression occurs through secreted galectins. Int J Oncol. 2011 Feb;38(2):385-390.

97. Augello A, Tasso R, Negrini SM, Amateis A, Indiveri F, Cancedda R, et al. Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. Eur J Immunol. 2005 May;35(5):1482-1490.

98. Akiyama K, Chen C, Wang D, Xu X, Qu C, Yamaza T, et al. Mesenchymal-stem-cell-induced immunoregulation involves FAS-ligand-/FAS-mediated T cell apoptosis. Cell Stem Cell. 2012 May 4;10(5):544-555.

99. Opitz CA, Litzenburger UM, Lutz C, Lanz TV, Tritschler I, Koppel A, et al. Toll-like receptor engagement enhances the immunosuppressive properties of human bone marrow-derived mesenchymal stem cells by inducing indoleamine-2,3-dioxygenase-1 via interferon-beta and protein kinase R. Stem Cells. 2009 Apr;27(4):909-919.

100. Ren G, Zhao X, Zhang L, Zhang J, L'Huillier A, Ling W, et al. Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. J Immunol. 2010 Mar 1;184(5):2321-2328.

101. Rasmusson I, Ringden O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. Transplantation. 2003 Oct 27;76(8):1208-1213.

102. Angoulvant D, Clerc A, Benchalal S, Galambrun C, Farre A, Bertrand Y, et al. Human mesenchymal stem cells suppress induction of cytotoxic response to alloantigens. Biorheology. 2004;41(3-4):469-476.

103. Ramasamy R, Tong CK, Seow HF, Vidyadaran S, Dazzi F. The immunosuppressive effects of human bone marrow-derived mesenchymal stem cells target T cell proliferation but not its effector function. Cell Immunol. 2008 Feb;251(2):131-136.

104. Rasmusson I, Uhlin M, Le Blanc K, Levitsky V. Mesenchymal stem cells fail to trigger effector functions of cytotoxic T lymphocytes. J Leukoc Biol. 2007 Oct;82(4):887-893.

105. Luz-Crawford P, Kurte M, Bravo-Alegria J, Contreras R, Nova-Lamperti E, Tejedor G, et al. Mesenchymal stem cells generate a CD4+CD25+Foxp3+ regulatory T cell population during the differentiation process of Th1 and Th17 cells. Stem Cell Res Ther. 2013;4(3):65.

106. Beyth S, Borovsky Z, Mevorach D, Liebergall M, Gazit Z, Aslan H, et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. Blood. 2005 Mar 1;105(5):2214-2219.

107. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, et al. Human mesenchymal stem cells modulate B-cell functions. Blood. 2006 Jan 1;107(1):367-372.

108. Tabera S, Perez-Simon JA, Diez-Campelo M, Sanchez-Abarca LI, Blanco B, Lopez A, et al. The effect of mesenchymal stem cells on the viability, proliferation and differentiation of B-lymphocytes. Haematologica. 2008 Sep;93(9):1301-1309.

109. Rasmusson I, Le Blanc K, Sundberg B, Ringden O. Mesenchymal stem cells stimulate antibody secretion in human B cells. Scand J Immunol. 2007 Apr;65(4):336-343.

110. Traggiai E, Volpi S, Schena F, Gattorno M, Ferlito F, Moretta L, et al. Bone marrow-derived mesenchymal stem cells induce both polyclonal expansion and differentiation of B cells isolated from healthy donors and systemic lupus erythematosus patients. Stem Cells. 2008 Feb;26(2):562-569.

111. Glenn JD, Whartenby KA. Mesenchymal stem cells: Emerging mechanisms of immunomodulation and therapy. World J Stem Cells. 2014 Nov 26;6(5):526-539.

112. Moretta A, Bottino C, Mingari MC, Biassoni R, Moretta L. What is a natural killer cell? Nat Immunol. 2002 Jan;3(1):6-8.

113. Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells. 2006 Jan;24(1):74-85.

114. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood. 2006 Feb 15;107(4):1484-1490.

115. Poggi A, Prevosto C, Massaro AM, Negrini S, Urbani S, Pierri I, et al. Interaction between human NK cells and bone marrow stromal cells induces NK cell triggering: role of NKp30 and NKG2D receptors. J Immunol. 2005 Nov 15;175(10):6352-6360.

116. Spaggiari GM, Capobianco A, Abdelrazik H, Becchetti F, Mingari MC, Moretta L. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood. 2008 Feb 1;111(3):1327-1333.

117. Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, et al. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. Stem Cells. 2008 Jan;26(1):212-222.

118. Nauta AJ, Kruisselbrink AB, Lurvink E, Willemze R, Fibbe WE. Mesenchymal stem cells inhibit generation and function of both CD34+-derived and monocyte-derived dendritic cells. J Immunol. 2006 Aug 15;177(4):2080-2087.

119. Spaggiari GM, Abdelrazik H, Becchetti F, Moretta L. MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived prostaglandin E2. Blood. 2009 Jun 25;113(26):6576-6583.

120. Jiang XX, Zhang Y, Liu B, Zhang SX, Wu Y, Yu XD, et al. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. Blood. 2005 May 15;105(10):4120-4126.

121. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. J Pathol. 2013 Jan;229(2):176-185.

122. Eggenhofer E, Hoogduijn MJ. Mesenchymal stem cell-educated macrophages. Transplant Res. 2012;1(1):12.

123. Francois M, Romieu-Mourez R, Li M, Galipeau J. Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. Mol Ther. 2012 Jan;20(1):187-195.

124. Busch SA, Hamilton JA, Horn KP, Cuascut FX, Cutrone R, Lehman N, et al. Multipotent adult progenitor cells prevent macrophage-mediated axonal dieback and promote regrowth after spinal cord injury. J Neurosci. 2011 Jan 19;31(3):944-953.

125. Koc ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI, et al. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol. 2000 Jan;18(2):307-316.

126. Lazarus HM, Haynesworth SE, Gerson SL, Rosenthal NS, Caplan AI. Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. Bone Marrow Transplant. 1995 Oct;16(4):557-564.

127. Maitra B, Szekely E, Gjini K, Laughlin MJ, Dennis J, Haynesworth SE, et al. Human mesenchymal stem cells support unrelated donor hematopoietic stem cells and suppress T-cell activation. Bone Marrow Transplant. 2004 Mar;33(6):597-604.

128. Almeida-Porada G, Flake AW, Glimp HA, Zanjani ED. Cotransplantation of stroma results in enhancement of engraftment and early expression of donor hematopoietic stem cells in utero. Exp Hematol. 1999 Oct;27(10):1569-1575.

129. Lee ST, Jang JH, Cheong JW, Kim JS, Maemg HY, Hahn JS, et al. Treatment of high-risk acute myelogenous leukaemia by myeloablative chemoradiotherapy followed by co-infusion of T cell-depleted haematopoietic stem cells and culture-expanded marrow mesenchymal stem cells from a related donor with one fully mismatched human leucocyte antigen haplotype. Br J Haematol. 2002 Sep;118(4):1128-1131.

130. Le Blanc K, Samuelsson H, Gustafsson B, Remberger M, Sundberg B, Arvidson J, et al. Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. Leukemia. 2007 Aug;21(8):1733-1738.

131. Baron F, Lechanteur C, Willems E, Bruck F, Baudoux E, Seidel L, et al. Cotransplantation of mesenchymal stem cells might prevent death from graft-versus-host disease (GVHD) without abrogating graft-versus-tumor effects after HLA-mismatched allogeneic transplantation following nonmyeloablative conditioning. Biol Blood Marrow Transplant. 2010 Jun;16(6):838-847.

132. Poloni A, Leoni P, Buscemi L, Balducci F, Pasquini R, Masia MC, et al. Engraftment capacity of mesenchymal cells following hematopoietic stem cell transplantation in patients receiving reduced-intensity conditioning regimen. Leukemia. 2006 Feb;20(2):329-335.

133. Ball LM, Bernardo ME, Roelofs H, Lankester A, Cometa A, Egeler RM, et al. Cotransplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. Blood. 2007 Oct 1;110(7):2764-2767.

134. Fouillard L, Bensidhoum M, Bories D, Bonte H, Lopez M, Moseley AM, et al. Engraftment of allogeneic mesenchymal stem cells in the bone marrow of a patient with severe idiopathic aplastic anemia improves stroma. Leukemia. 2003 Feb;17(2):474-476.

135. Fouillard L, Chapel A, Bories D, Bouchet S, Costa JM, Rouard H, et al. Infusion of allogeneic-related HLA mismatched mesenchymal stem cells for the treatment of incomplete engraftment following autologous haematopoietic stem cell transplantation. Leukemia. 2007 Mar;21(3):568-570.

136. Macmillan ML, Blazar BR, DeFor TE, Wagner JE. Transplantation of ex-vivo culture-expanded parental haploidentical mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood: results of a phase I-II clinical trial. Bone Marrow Transplant. 2009 Mar;43(6):447-454.

137. Meuleman N, Tondreau T, Ahmad I, Kwan J, Crokaert F, Delforge A, et al. Infusion of mesenchymal stromal cells can aid hematopoietic recovery following allogeneic hematopoietic stem cell myeloablative transplant: a pilot study. Stem Cells Dev. 2009 Nov;18(9):1247-1252.

138. Baron F, Storb R. Mesenchymal stromal cells: a new tool against graft-versus-host disease? Biol Blood Marrow Transplant. 2012 Jun;18(6):822-840.

139. Yanez R, Lamana ML, Garcia-Castro J, Colmenero I, Ramirez M, Bueren JA. Adipose tissue-derived mesenchymal stem cells have in vivo immunosuppressive properties applicable for the control of the graft-versus-host disease. Stem Cells. 2006 Nov;24(11):2582-2591.

140. Polchert D, Sobinsky J, Douglas G, Kidd M, Moadsiri A, Reina E, et al. IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. Eur J Immunol. 2008 Jun;38(6):1745-1755.

141. Tisato V, Naresh K, Girdlestone J, Navarrete C, Dazzi F. Mesenchymal stem cells of cord blood origin are effective at preventing but not treating graft-versus-host disease. Leukemia. 2007 Sep;21(9):1992-1999.

142. Ning H, Yang F, Jiang M, Hu L, Feng K, Zhang J, et al. The correlation between cotransplantation of mesenchymal stem cells and higher recurrence rate in hematologic malignancy patients: outcome of a pilot clinical study. Leukemia. 2008 Mar;22(3):593-599.

143. Liu K, Chen Y, Zeng Y, Xu L, Liu D, Chen H, et al. Coinfusion of mesenchymal stromal cells facilitates platelet recovery without increasing leukemia recurrence in haploidentical hematopoietic stem cell transplantation: a randomized, controlled clinical study. Stem Cells Dev. 2011 Oct;20(10):1679-1685.

144. Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet. 2004 May 1;363(9419):1439-1441.

145. Ringden O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lonnies H, et al. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. Transplantation. 2006 May 27;81(10):1390-1397.

146. Muller I, Kordowich S, Holzwarth C, Isensee G, Lang P, Neunhoeffer F, et al. Application of multipotent mesenchymal stromal cells in pediatric patients following allogeneic stem cell transplantation. Blood Cells Mol Dis. 2008 Jan-Feb;40(1):25-32.

147. von Bonin M, Stolzel F, Goedecke A, Richter K, Wuschek N, Holig K, et al. Treatment of refractory acute GVHD with third-party MSC expanded in platelet lysate-containing medium. Bone Marrow Transplant. 2009 Feb;43(3):245-251.

148. Fang B, Song Y, Lin Q, Zhang Y, Cao Y, Zhao RC, et al. Human adipose tissue-derived mesenchymal stromal cells as salvage therapy for treatment of severe refractory acute graft-vs.-host disease in two children. Pediatr Transplant. 2007 Nov;11(7):814-817.

149. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet. 2008 May 10;371(9624):1579-1586.

150. Ball LM, Bernardo ME, Roelofs H, van Tol MJ, Contoli B, Zwaginga JJ, et al. Multiple infusions of mesenchymal stromal cells induce sustained remission in children with steroid-refractory, grade III-IV acute graft-versus-host disease. Br J Haematol. 2013 Nov;163(4):501-509.

151. Ringden O, Le Blanc K. Mesenchymal stem cells for treatment of acute and chronic graft-versus-host disease, tissue toxicity and hemorrhages. Best Pract Res Clin Haematol. 2011 Mar;24(1):65-72.

152. Kebriaei P, Isola L, Bahceci E, Holland K, Rowley S, McGuirk J, et al. Adult human mesenchymal stem cells added to corticosteroid therapy for the treatment of acute graft-versus-host disease. Biol Blood Marrow Transplant. 2009 Jul;15(7):804-811.

153. Prasad VK, Lucas KG, Kleiner GI, Talano JA, Jacobsohn D, Broadwater G, et al. Efficacy and safety of ex vivo cultured adult human mesenchymal stem cells (Prochymal) in pediatric patients with severe refractory acute graft-versus-host disease in a compassionate use study. Biol Blood Marrow Transplant. 2011 Apr;17(4):534-541.

154. Zhao K, Lou R, Huang F, Peng Y, Jiang Z, Huang K, et al. Immunomodulation Effects of Mesenchymal Stromal Cells on Acute Graft-versus-Host Disease after Hematopoietic Stem Cell Transplantation. Biol Blood Marrow Transplant. 2015 Jan;21(1):97-104.

155.    von Bahr L, Sundberg B, Lonnies L, Sander B, Karbach H, Hagglund H, et al. Long-term complications, immunologic effects, and role of passage for outcome in mesenchymal stromal cell therapy. Biol Blood Marrow Transplant. 2012 Apr;18(4):557-564.

156.    Zhou H, Guo M, Bian C, Sun Z, Yang Z, Zeng Y, et al. Efficacy of bone marrow-derived mesenchymal stem cells in the treatment of sclerodermatous chronic graft-versus-host disease: clinical report. Biol Blood Marrow Transplant. 2010 Mar;16(3):403-412.

157.    Weng JY, Du X, Geng SX, Peng YW, Wang Z, Lu ZS, et al. Mesenchymal stem cell as salvage treatment for refractory chronic GVHD. Bone Marrow Transplant. 2010 Dec;45(12):1732-1740.

158.    Hare JM, Traverse JH, Henry TD, Dib N, Strumpf RK, Schulman SP, et al. A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. J Am Coll Cardiol. 2009 Dec 8;54(24):2277-2286.

159.    Uccelli A, Laroni A, Freedman MS. Mesenchymal stem cells for the treatment of multiple sclerosis and other neurological diseases. Lancet Neurol. 2011 Jul;10(7):649-656.

160.    Ciccocioppo R, Bernardo ME, Sgarella A, Maccario R, Avanzini MA, Ubezio C, et al. Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease. Gut. 2011 Jun;60(6):788-798.

161.    Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, et al. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood. 2005 Sep 1;106(5):1755-1761.

162.    Lee RH, Seo MJ, Reger RL, Spees JL, Pulin AA, Olson SD, et al. Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. Proc Natl Acad Sci U S A. 2006 Nov 14;103(46):17438-17443.

163.    Deng W, Han Q, Liao L, You S, Deng H, Zhao RC. Effects of allogeneic bone marrow-derived mesenchymal stem cells on T and B lymphocytes from BXSB mice. DNA Cell Biol. 2005 Jul;24(7):458-463.

164.    Duijvestein M, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, et al. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. Gut. 2010 Dec;59(12):1662-1669.

165.    Karussis D, Karageorgiou C, Vaknin-Dembinsky A, Gowda-Kurkalli B, Gomori JM, Kassis I, et al. Safety and immunological effects of mesenchymal stem cell transplantation in patients with multiple sclerosis and amyotrophic lateral sclerosis. Arch Neurol. 2010 Oct;67(10):1187-1194.

166.    Jacobs SA, Pinxteren J, Roobrouck VD, Luyckx A, van't Hof W, Deans R, et al. Human multipotent adult progenitor cells are nonimmunogenic and exert potent immunomodulatory effects on alloreactive T-cell responses. Cell Transplant. 2013;22(10):1915-1928.

167.    Tolar J, O'Shaughnessy M J, Panoskaltsis-Mortari A, McElmurry RT, Bell S, Riddle M, et al. Host factors that impact the biodistribution and persistence of multipotent adult progenitor cells. Blood. 2006 May 15;107(10):4182-4188.

168.    Luyckx A, De Somer L, Rutgeerts O, Waer M, Verfaillie CM, Van Gool S, et al. Mouse MAPC-mediated immunomodulation: Cell-line dependent variation. Exp Hematol. 2010 Jan;38(1):1-2.

169.    Highfill SL, Kelly RM, O'Shaughnessy MJ, Zhou Q, Xia L, Panoskaltsis-Mortari A, et al. Multipotent adult progenitor cells can suppress graft-versus-host disease via prostaglandin E2 synthesis and only if localized to sites of allopriming. Blood. 2009 Jul 16;114(3):693-701.

170.    Kovacsovics-Bankowski M, Mauch K, Raber A, Streeter PR, Deans RJ, Maziarz RT, et al. Pre-clinical safety testing supporting clinical use of allogeneic multipotent adult progenitor cells. Cytotherapy. 2008;10(7):730-742.

171.    Kovacsovics-Bankowski M, Streeter PR, Mauch KA, Frey MR, Raber A, van't Hof W, et al. Clinical scale expanded adult pluripotent stem cells prevent graft-versus-host disease. Cell Immunol. 2009;255(1-2):55-60.

172.    Aranguren XL, McCue JD, Hendrickx B, Zhu XH, Du F, Chen E, et al. Multipotent adult progenitor cells sustain function of ischemic limbs in mice. J Clin Invest. 2008 Feb;118(2):505-514.

173.    Van't Hof W, Mal N, Huang Y, Zhang M, Popovic Z, Forudi F, et al. Direct delivery of syngeneic and allogeneic large-scale expanded multipotent adult progenitor cells improves cardiac function after myocardial infarct. Cytotherapy. 2007;9(5):477-487.

174.    Walker PA, Shah SK, Jimenez F, Gerber MH, Xue H, Cutrone R, et al. Intravenous multipotent adult progenitor cell therapy for traumatic brain injury: preserving the blood brain barrier via an interaction with splenocytes. Exp Neurol. 2010 Oct;225(2):341-352.

175.    Vaes B, Van't Hof W, Deans R, Pinxteren J. Application of MultiStem((R)) Allogeneic Cells for Immunomodulatory Therapy: Clinical Progress and Pre-Clinical Challenges in Prophylaxis for Graft Versus Host Disease. Front Immunol. 2012;3:345.

176. Maziarz RT, Devos T, Bachier CR, Goldstein SC, Leis JF, Devine SM, et al. Single and Multiple Dose MultiStem(R) (Multi-Potent Adult Progenitor Cell) Therapy Prophylaxis of Acute GVHD in Myeloablative Allogeneic Hematopoietic Cell Transplantation: A Phase I Trial. Biol Blood Marrow Transplant. 2014 Dec 30.

177. Hess DC, Sila CA, Furlan AJ, Wechsler LR, Switzer JA, Mays RW. A double-blind placebo-controlled clinical evaluation of MultiStem for the treatment of ischemic stroke. Int J Stroke. 2014 Apr;9(3):381-386.

178. Obermajer N, Popp FC, Johnson CL, Benseler V, Dahlke MH. Rationale and prospects of mesenchymal stem cell therapy for liver transplantation. Curr Opin Organ Transplant. 2014 Feb;19(1):60-64.

179. Devine SM, Cobbs C, Jennings M, Bartholomew A, Hoffman R. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood. 2003 Apr 15;101(8):2999-3001.

180. Gao J, Dennis JE, Muzic RF, Lundberg M, Caplan AI. The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. Cells Tissues Organs. 2001;169(1):12-20.

181. Liechty KW, MacKenzie TC, Shaaban AF, Radu A, Moseley AM, Deans R, et al. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. Nat Med. 2000 Nov;6(11):1282-1286.

182. Iso Y, Spees JL, Serrano C, Bakondi B, Pochampally R, Song YH, et al. Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. Biochem Biophys Res Commun. 2007 Mar 16;354(3):700-706.

183. Ruster B, Gottig S, Ludwig RJ, Bistrian R, Muller S, Seifried E, et al. Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. Blood. 2006 Dec 1;108(12):3938-3944.

184. Son BR, Marquez-Curtis LA, Kucia M, Wysoczynski M, Turner AR, Ratajczak J, et al. Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. Stem Cells. 2006 May;24(5):1254-1264.

185. Yagi H, Soto-Gutierrez A, Parekkadan B, Kitagawa Y, Tompkins RG, Kobayashi N, et al. Mesenchymal stem cells: Mechanisms of immunomodulation and homing. Cell Transplant. 2010;19(6):667-679.

186. Lalu MM, McIntyre L, Pugliese C, Fergusson D, Winston BW, Marshall JC, et al. Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. PLoS One. 2012;7(10):e47559.

187. Moermans C, Lechanteur C, Baudoux E, Giet O, Henket M, Seidel L, et al. Impact of cotransplantation of mesenchymal stem cells on lung function after unrelated allogeneic hematopoietic stem cell transplantation following non-myeloablative conditioning. Transplantation. 2014 Aug 15;98(3):348-353.

188. Troeger A, Meisel R, Moritz T, Dilloo D. Immunotherapy in allogeneic hematopoietic stem cell transplantation--not just a case for effector cells. Bone Marrow Transplant. 2005 Mar;35 Suppl 1:S59-64.

189. Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J, et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood. 2003 Nov 15;102(10):3837-3844.

190. Nauta AJ, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. Blood. 2006 Sep 15;108(6):2114-2120.

191. Buron F, Perrin H, Malcus C, Hequet O, Thaunat O, Kholopp-Sarda MN, et al. Human mesenchymal stem cells and immunosuppressive drug interactions in allogeneic responses: an in vitro study using human cells. Transplant Proc. 2009 Oct;41(8):3347-3352.

192. Eggenhofer E, Steinmann JF, Renner P, Slowik P, Piso P, Geissler EK, et al. Mesenchymal stem cells together with mycophenolate mofetil inhibit antigen presenting cell and T cell infiltration into allogeneic heart grafts. Transpl Immunol. 2011 Apr 15;24(3):157-163.

193. Inoue S, Popp FC, Koehl GE, Piso P, Schlitt HJ, Geissler EK, et al. Immunomodulatory effects of mesenchymal stem cells in a rat organ transplant model. Transplantation. 2006 Jun 15;81(11):1589-1595.

194. Casiraghi F, Remuzzi G, Abbate M, Perico N. Multipotent mesenchymal stromal cell therapy and risk of malignancies. Stem Cell Rev. 2013 Feb;9(1):65-79.

195. Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, et al. Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. Cancer Res. 2007 Oct 1;67(19):9142-9149.

196. Rubio D, Garcia-Castro J, Martin MC, de la Fuente R, Cigudosa JC, Lloyd AC, et al. Spontaneous human adult stem cell transformation. Cancer Res. 2005 Apr 15;65(8):3035-3039.

197. Uccelli A, Mancardi G, Chiesa S. Is there a role for mesenchymal stem cells in autoimmune diseases? Autoimmunity. 2008 Dec;41(8):592-595.

198. Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, et al. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy. 2005;7(5):393-395.

199. Moretta L, Locatelli F, Pende D, Marcenaro E, Mingari MC, Moretta A. Killer Ig-like receptor-mediated control of natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation. Blood. 2011 Jan 20;117(3):764-771.

200. Moretta L, Bottino C, Pende D, Mingari MC, Biassoni R, Moretta A. Human natural killer cells: their origin, receptors and function. Eur J Immunol. 2002 May;32(5):1205-1211.

201. Moretta L, Mingari MC, Pende D, Bottino C, Biassoni R, Moretta A. The molecular basis of natural killer (NK) cell recognition and function. J Clin Immunol. 1996 Sep;16(5):243-253.

202. De Vleeschouwer S, Spencer Lopes I, Ceuppens JL, Van Gool SW. Persistent IL-10 production is required for glioma growth suppressive activity by Th1-directed effector cells after stimulation with tumor lysate-loaded dendritic cells. J Neurooncol. 2007 Sep;84(2):131-140.

203. Gotherstrom C, Lundqvist A, Duprez IR, Childs R, Berg L, le Blanc K. Fetal and adult multipotent mesenchymal stromal cells are killed by different pathways. Cytotherapy. 2011 Mar;13(3):269-278.

204. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. Nat Rev Immunol. 2008 Sep;8(9):726-736.

205. Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. Nat Rev Immunol. 2002 Jun;2(6):401-409.

206. Jacobs SA, Plessers J, Pinxteren J, Roobrouck VD, Verfaillie CM, Van Gool SW. Mutual interaction between human multipotent adult progenitor cells and NK cells. Cell Transplant. 2014;23(9):1099-1110.

207. Antwiler GD, Nguyen KT, McNiece IK. Bioreactor Design and Implementation. In: Parekkadan B, Yarmush ML, editors. Stem Cell Bioengineering. Boston: Artech House; 2009. p. 49-62.

208. Van Gool SW, de Boer M, Ceuppens JL. CD28 ligation by monoclonal antibodies or B7/BB1 provides an accessory signal for the cyclosporin A-resistant generation of cytotoxic T cell activity. J Immunol. 1993 Apr 15;150(8 Pt 1):3254-3263.

209. Verschuere T, Van Woensel M, Fieuws S, Lefranc F, Mathieu V, Kiss R, et al. Altered galectin-1 serum levels in patients diagnosed with high-grade glioma. J Neurooncol. 2013 Oct;115(1):9-17.

210. de la Fuente H, Cruz-Adalia A, Martinez Del Hoyo G, Cibrian-Vera D, Bonay P, Perez-Hernandez D, et al. The leukocyte activation receptor CD69 controls T cell differentiation through its interaction with galectin-1. Mol Cell Biol. 2014 Jul;34(13):2479-2487.

211. Crop MJ, Korevaar SS, de Kuiper R, JN IJ, van Besouw NM, Baan CC, et al. Human mesenchymal stem cells are susceptible to lysis by CD8(+) T cells and NK cells. Cell Transplant. 2011;20(10):1547-1559.

212. Roemeling-van Rhijn M, Reinders ME, Franquesa M, Engela AU, Korevaar SS, Roelofs H, et al. Human Allogeneic Bone Marrow and Adipose Tissue Derived Mesenchymal Stromal Cells Induce CD8+ Cytotoxic T Cell Reactivity. J Stem Cell Res Ther. 2013 Dec 12;3(Suppl 6):004.

213. Potian JA, Aviv H, Ponzio NM, Harrison JS, Rameshwar P. Veto-like activity of mesenchymal stem cells: functional discrimination between cellular responses to alloantigens and recall antigens. J Immunol. 2003 Oct 1;171(7):3426-3434.

214. Janas ML, Groves P, Kienzle N, Kelso A. IL-2 regulates perforin and granzyme gene expression in CD8+ T cells independently of its effects on survival and proliferation. J Immunol. 2005 Dec 15;175(12):8003-8010.

215. Saldanha-Araujo F, Haddad R, Farias KC, Souza Ade P, Palma PV, Araujo AG, et al. Mesenchymal stem cells promote the sustained expression of CD69 on activated T lymphocytes: roles of canonical and non-canonical NF-kappaB signalling. J Cell Mol Med. 2012 Jun;16(6):1232-1244.

216. Sancho D, Gomez M, Sanchez-Madrid F. CD69 is an immunoregulatory molecule induced following activation. Trends Immunol. 2005 Mar;26(3):136-140.

217. Gonzalez-Amaro R, Cortes JR, Sanchez-Madrid F, Martin P. Is CD69 an effective brake to control inflammatory diseases? Trends Mol Med. 2013 Oct;19(10):625-632.

218. Han Y, Guo Q, Zhang M, Chen Z, Cao X. CD69+ CD4+ CD25- T cells, a new subset of regulatory T cells, suppress T cell proliferation through membrane-bound TGF-beta 1. J Immunol. 2009 Jan 1;182(1):111-120.

219. Gandhi R, Farez MF, Wang Y, Kozoriz D, Quintana FJ, Weiner HL. Cutting edge: human latency-associated peptide+ T cells: a novel regulatory T cell subset. J Immunol. 2010 May 1;184(9):4620-4624.

220. Zhu J, Feng A, Sun J, Jiang Z, Zhang G, Wang K, et al. Increased CD4(+) CD69(+) CD25(-) T cells in patients with hepatocellular carcinoma are associated with tumor progression. J Gastroenterol Hepatol. 2011 Oct;26(10):1519-1526.

221. Garcia-Monzon C, Moreno-Otero R, Pajares JM, Garcia-Sanchez A, Lopez-Botet M, de Landazuri MO, et al. Expression of a novel activation antigen on intrahepatic CD8+ T lymphocytes in viral chronic active hepatitis. Gastroenterology. 1990 Apr;98(4):1029-1035.

222. Afeltra A, Galeazzi M, Ferri GM, Amoroso A, De Pita O, Porzio F, et al. Expression of CD69 antigen on synovial fluid T cells in patients with rheumatoid arthritis and other chronic synovitis. Ann Rheum Dis. 1993 Jun;52(6):457-460.

223. Bernardo ME, Fibbe WE. Mesenchymal stromal cells: sensors and switchers of inflammation. Cell Stem Cell. 2013 Oct 3;13(4):392-402.

224. Waterman RS, Tomchuck SL, Henkle SL, Betancourt AM. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. PLoS One. 2010;5(4):e10088.

225. Sheng H, Wang Y, Jin Y, Zhang Q, Zhang Y, Wang L, et al. A critical role of IFNgamma in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1. Cell Res. 2008 Aug;18(8):846-857.

226. Duijvestein M, Wildenberg ME, Welling MM, Hennink S, Molendijk I, van Zuylen VL, et al. Pretreatment with interferon-gamma enhances the therapeutic activity of mesenchymal stromal cells in animal models of colitis. Stem Cells. 2011 Oct;29(10):1549-1558.

227. Mora-Lee S, Sirerol-Piquer MS, Gutierrez-Perez M, Gomez-Pinedo U, Roobrouck VD, Lopez T, et al. Therapeutic effects of hMAPC and hMSC transplantation after stroke in mice. PLoS One. 2012;7(8):e43683.

228. Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, Lynch PJ, et al. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Mol Ther. 2004 May;9(5):747-756.

229. Sundin M, Ringden O, Sundberg B, Nava S, Gotherstrom C, Le Blanc K. No alloantibodies against mesenchymal stromal cells, but presence of anti-fetal calf serum antibodies, after transplantation in allogeneic hematopoietic stem cell recipients. Haematologica. 2007 Sep;92(9):1208-1215.

# CURRICULUM VITAE

Jeroen Plessers was born in Neerpelt (Belgium) on April 8th, 1987. He graduated from secondary school in 2005 at the Wico Campus Mater Dei in Overpelt with a major in science and mathematics. In 2005 he started his studies in Biomedical Sciences at the KU Leuven. During this study he performed internships at the Stem Cell Institute Leuven (SCIL) and in the laboratories of Clinical and Experimental Endocrinology and Clinical and Epidemiological Virology. In 2009 he started his master thesis under supervision of prof. Dr. Peter Carmeliet at the Laboratory of Angiogenesis and Neurovascular Link (Vesalius Research Centre). In June 2010 he graduated *magna cum laude* as Master in Biomedical Sciences, with a major in Biomedical Research and a minor in Management and Communication in the Biomedical Sciences. Subsequently, he started his PhD in the Laboratory of Pediatric Immunology under the supervision of prof. Dr. Stefaan Van Gool and prof. Dr. Jan Ceuppens.

# BIBLIOGRAPHY

## List of publications

Jacobs SA\*, **Plessers J\***, Pinxteren J, Roobrouck VD, Verfaillie CM, Van Gool SW. Mutual interaction between human multipotent adult progenitor cells and NK cells. Cell Transplant. 2014;23(9):1099-1110.

*\* equal contribution*

**Plessers J**, Roobrouck VD, Pinxteren J, Verfaillie CM, Van Gool SW. Influence of clinical-grade human multipotent adult progenitor cells on cytotoxic T lymphocytes.

*Manuscript in preparation*

## Communications at scientific meetings

**9th HGG-IMMUNO meeting, Leuven, Belgium, October 2011**
<u>Oral presentation</u>: Stem cell-based immunomodulation
**Plessers J**, Jacobs SA, Pinxteren J, Roobrouck VD, Verfaillie CM, Van Gool SW.

**6th Symposium for Tumor Immunology in Childhood, Rostock, Germany, February 2012**
<u>Oral presentation</u>: Mutual interaction between human multipotent adult progenitor cells and NK cells
**Plessers J**, Jacobs SA, Pinxteren J, Roobrouck VD, Verfaillie CM, Van Gool SW.

**EHA-ESH Scientific Workshop on Anti-inflammatory and Immune modulatory properties of MSCs, Mandelieu, France, April 2012**
<u>Poster presentation</u>: Mutual interaction between human multipotent adult progenitor cells and NK cells
**Plessers J**, Jacobs SA, Pinxteren J, Roobrouck VD, Verfaillie CM, Van Gool SW.

**EAB-meeting Stem Cell Institute Leuven (SCIL), Leuven, Belgium, May 2012**

Poster presentation: Mutual interaction between human multipotent adult progenitor cells and NK cells

**Plessers J**, Jacobs SA, Pinxteren J, Roobrouck VD, Verfaillie CM, Van Gool SW.


**7th Symposium for Tumor Immunology in Childhood, Rostock, Germany, February 2013**

Oral presentation: Stem cell-based immunomodulation

**Plessers J**, Jacobs SA, Pinxteren J, Roobrouck VD, Verfaillie CM, Van Gool SW.


**2nd Summer school of Immunology, Leuven, Belgium, September 2014**

Oral presentation: Stem cell-based immunomodulation

**Plessers J**, Roobrouck VD, Pinxteren J, Verfaillie CM, Van Gool SW.


**9th Symposium for Tumor Immunology and Brain Tumor Research in Pediatrics, Rostock, Germany, February 2015**

Oral presentation: Influence of clinical-grade human multipotent adult progenitor cells on cytotoxic T lymphocytes

**Plessers J**, Roobrouck VD, Pinxteren J, Verfaillie CM, Van Gool SW.