

Immunotherapy in the Context of Hematopoietic Stem Cell Transplantation: The Emerging Role of Natural Killer Cells and Mesenchymal Stromal Cells

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- Immunotherapy • Hematopoietic stem cell transplantation
- Natural killer cells • Mesenchymal stromal cells

Immunotherapy in the context of hematopoietic stem cell transplantation (HSCT) has been dominated for many years by T cell- and dendritic cell-based treatment modalities. During the last decade, insight into the biology of natural killer (NK) cells and mesenchymal stromal cells (MSC) has rapidly increased and resulted in NK- and MSC-based therapeutic strategies in clinical practice. This article reviews current knowledge of the biology and clinical aspects of NK cells and MSC.

PART 1: NK CELLS

Biology of NK Cells

NK cells were first identified in 1975 in mice as a distinct subpopulation of lymphocytes with the capacity of killing tumor cells without prior sensitization.¹ In humans, NK cells represent about 5% to 20% of peripheral blood lymphocytes and are defined as CD56+CD3- lymphocytes, which often have a granular morphology.² Extensive

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research has revealed that NK cells are a heterogeneous population of cells with various functions in cytokine production and cytotoxicity.³ In contrast to T-lymphocytes, NK cells do not recognize foreign antigens, but rather detect changes in self-molecules displayed at the surface of autologous cells. Based on initial observations that syngeneic tumor cells with a deficient expression of major histocompatibility complex (MHC) class I molecules are selectively rejected by NK cells, the concept of the “missing-self” recognition was introduced.^{4,5} This model provided an explanation why virally infected or malignant cells with decreased MHC class I expression are preferentially lysed by NK cells, whereas autologous cells with normal expression of MHC class I antigens are protected.

NK cells express various surface receptors with inhibitory and activatory functions that play a role in various diseases including cancer.⁶ The functional response of NK cells is determined by the activation of these receptors in combination with the signaling of various coreceptors and other surface structures on binding to their cognate ligands.⁷ **Fig. 1** presents a schematic overview of the inhibitory, activatory and coreceptors and the various NK receptor ligands. The inhibitory receptors possess different specificities for HLA class I molecules. The 2 main groups are the killer immunoglobulin (Ig)-like receptors (KIRs), which are receptors for human leukocyte antigen (HLA) class I ligands⁸ and the CD94-NKG2A/B, which recognize HLA-E.⁹ Although HLA-E shows little polymorphism,¹⁰ many inhibitory KIRs are specific for polymorphic domains of MHC class I.¹¹ KIR2DL1 (CD158a) recognizes HLA-C alleles with lysine in position 80 (C2 specificity, eg, Cw2,4,5,6; group 2), whereas KIR2DL2 and KIR2DL3 (CD158b1/b2) recognize HLA-C alleles with asparagine in position 80 (C1 specificity, eg, Cw1,3,7,8; group 1). KIR 3DL1 (CD158e1) is specific for HLA-B alleles bearing the Bw4 motif and KIR3DL2 (CD158k) recognizes HLA-A epitopes (A3, A11). HLA class I- and KIR-genes are encoded on distinct chromosomes 6p21.3 and 19q13.4, respectively, and inherited independently.¹² KIR genes are encoded by a set of 15 loci and 2 pseudogenes. These KIR genes are closely linked and inherited as a haplotype, and the variability of KIR gene content can be organized into KIR haplotype A and B.¹³ Haplotype group A contains a variable number of inhibitory receptor genes, whereas group B haplotype additionally contains several

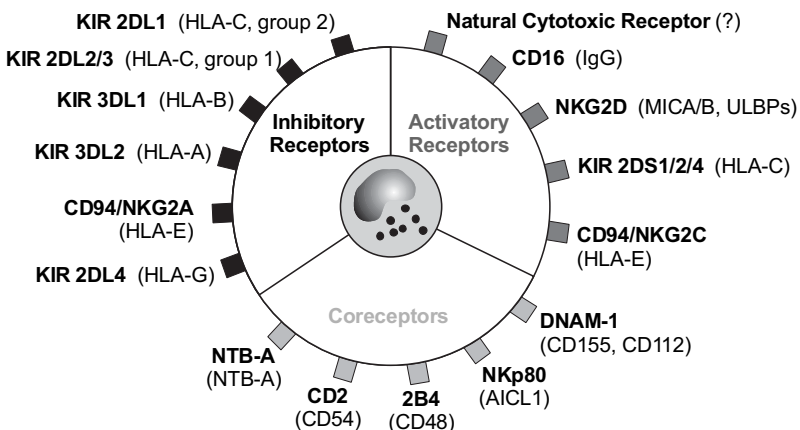


Fig. 1. The inhibitory, activatory, and coreceptors expressed on NK cells and their corresponding ligands in parentheses.

activating receptor genes. Killer activatory Ig-like receptors (KARs) are also triggered by HLA class I alleles,⁷ but additional activatory receptors exist, such as NKG2D, the leukocyte adhesion molecule DNAX accessory molecule (DNAM-1; CD226) and natural cytotoxicity receptors NKp46, NKp30, and NKp44, which recognize so far unknown ligands expressed on hematopoietic cells.^{7,8,14} The Fc-receptor CD16, binding the Fc portion of IgG, mediates the antibody-dependent cellular cytotoxicity of NK cells. NKG2D and DNAM-1 are receptors for stress-induced ligands, such as MHC class I polypeptide-related sequence A and B (MICA and MICB), UL16-binding proteins, and poliovirus receptor (CD155) and Nectin-2 (CD112), respectively.^{15–17} Other surface molecules contribute to the functional status of NK cells, among them the 2B4, NTB-A, and NKp80 coreceptors, CD18/CD11, CD2 adhesion molecules, and Toll-like receptors.^{14,18} Incubation of NK cells with various cytokines leads to their stimulation and expression of additional surface and intracellular molecules such as perforin, granzymes, Fas ligand, and tumor necrosis factor-related apoptosis-inducing ligand treatment (TRAIL), which enables them to kill a wide spectrum of tumor cells effectively via induction of necrosis or apoptosis.¹⁹

In summary, the output signal (ie, the functional status of NK cells) is regulated by various input signals and the large numbers of inhibitory, activatory, and modulating coreceptors allow cross talk of NK cells with other immune or tissue cells.

NK Cells and Cancer

Most evidence for a role of NK cells in tumor surveillance in humans comes from a clinical long-term follow-up study,²⁰ in which the inherent NK activity of 3625 individuals was measured longitudinally and the results compared with the incidence of cancer. Individuals who had a high spontaneous NK activity had a lower risk of developing cancer, whereas individuals with lower activity had a higher incidence of malignant diseases. It is thought that the main target of NK cell activity is within the hematopoietic system, as shown by the hybrid resistance model, in which NK cells reject allogeneic bone marrow but not skin or solid organs.²¹ In patients with leukemia, functional impairment of NK cells has prognostic significance and NK cell-mediated cytotoxicity against autologous blasts tested either *in vitro*^{22–24} or in a xenogeneic *in vivo* model²⁵ correlated with the duration of remission. In addition, leukemic blasts can shed MICA, a ligand of the activating NK receptor NKG2D, which can negatively affect the cytotoxicity of NK cells.²⁶ A decreased number and impaired function of NK cells have also been described in children with acute leukemia at the time of diagnosis and in relapse.²⁷ The low cytotoxicity of NK cells against autologous leukemic blasts could be restored *in vitro* after stimulation with interleukin 2 (IL-2).^{28,29} The role of NK cells in tumor surveillance of solid tumors is less clear. It has been shown that the infiltration of tumors with NK cells can be a positive prognostic marker in carcinomas.^{30,31} *In vitro* activation of NK cells obtained from children with malignant solid tumors with interferon α (IFN- α) and IL-2 also enhanced their cytotoxicity against solid tumor cell lines.³² Therefore, strategies to augment the antitumor activity of the NK cell system in the autologous or allogeneic setting could be beneficial in the treatment of pediatric patients with acute leukemia or solid tumors.

NK Cells in the Context of Allogeneic Stem Cell Transplantation

The concept of alloreactive NK cells

This concept is based on the observation that NK cells attack lymphohematopoietic target cells that express HLA class I molecules for which they do not express the corresponding inhibitory receptor as would be predicted based on the missing self model described earlier.³³ As depicted in **Fig. 2A**, NK cells are in a permanent

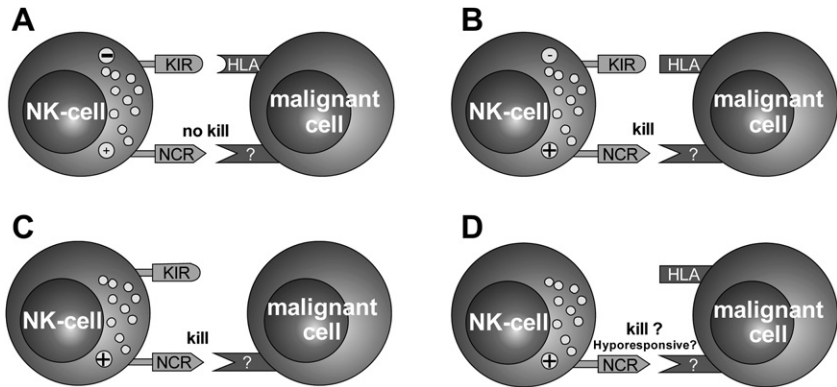


Fig. 2. Possible constellations of the expression of inhibitory receptors (KIR) on NK cells and the expression of KIR-binding or KIR-nonbinding HLA class I molecules on malignant cells (A–D), all of which influence the overall cytotoxic activity of NK cells (see text for details). NCR refers to the natural cytotoxicity receptors, for which ligands have not yet been identified.

activated status via binding of natural cytotoxic receptors (NCRs) to their yet unknown ligands expressed on normal or malignant hematopoietic cells. Only in the presence of a corresponding ligand for the inhibitory receptor, such as self HLA-Cw alleles, -Bw4 alleles and some HLA-A alleles, the cytotoxic function of NK cells is inhibited and the target cells are resistant to NK-mediated lysis. In contrast, if the target cell does not express the corresponding inhibitory ligand for the KIR, NK cells lyse their target cells because of the lack of inhibition. This situation is often encountered in HLA-mismatched but also in HLA-matched allogeneic stem cell transplantation because of the disparity of the donors' KIR repertoire and the HLA class I type of the recipients (Fig. 2B and discussed earlier). Therefore, the term alloreactive NK cells is used to describe this situation.³⁴ However, regardless of donor-recipient KIR-HLA matching status (discussed in greater detail later), additional clinical situations can be envisioned, which are shown in Fig. 2C and D: Tumor cells can have a reduced expression or complete lack of HLA class I molecules, which can be encountered in leukemic blasts³⁵ or certain tumors, such as neuroblastoma (Fig. 2C).³⁶ This constellation leads to killing of the tumor cells and the intensity of killing is dependent on the amount of residual HLA molecules expressed on the surface of the target cells.³⁵ Another situation seen clinically in the early phase of immunoreconstitution of NK cells after pediatric haploidentical transplantation is the absence of killer inhibitory or killer activatory receptors for HLA class I alleles (KIRs or KARs) on the reconstituting NK cells.³⁷ This situation either results in NK cell killing of their target independent of the expression of the amount, or the specificity of the HLA class I molecules on the tumor target cells, or a hyporesponsive status of NK cells via yet unknown mechanisms (Fig. 2D).³⁸

What predicts NK alloreactivity between donor and recipient?

Two models have been described to best predict NK alloreactivity in allogeneic transplantation to be used for donor selection: The KIR ligand mismatch model compares the HLA typing of donor and recipient (ligand-ligand model). This model assumes that all NK cells in an individual express at least 1 inhibitory receptor for a self HLA class I molecule to avoid autoimmunity. Based on this assumption, NK alloreactivity between

donor and recipient can be predicted when the donor is mismatched at inhibitory HLA class I antigens in the direction of graft-versus-host disease (GVHD).³⁹ In **Fig. 3**, the ligand-ligand model is shown in detail. In this example, the patient's HLA type is HLA-Cw1, Cw8 (group 1). If the donor is mismatched at the HLA-C locus in GVHD direction and is for example positive for HLA-Cw2 and Cw8 (groups 1 and 2), the donor's KIR repertoire can be predicted based on the assumption that each NK cell of this mismatched donor should express at least 1 inhibitory receptor for self HLA-Cw2 and Cw8 (KIR2DL1 and KIR2DL2/3, respectively). The donor's NK cells could express single KIRs for self HLA-Cw8. These NK cells would be inhibited by the patient's shared HLA-Cw8 allele (**Fig. 3A**) and thus not be alloreactive. Donor NK cells could also express both KIRs but would still be nonalloreactive because of inhibition by the patient's shared Cw8 allele (**Fig. 3B**). However, donor NK cells expressing single KIR2DL1 receptors cannot be inhibited by the patient's HLA-Cw1 nonshared antigen and the donor NK cells will exert an alloreactive antileukemic effect (**Fig. 3C**). In the case of HLA identity between donor and recipient, alloreactive NK cells

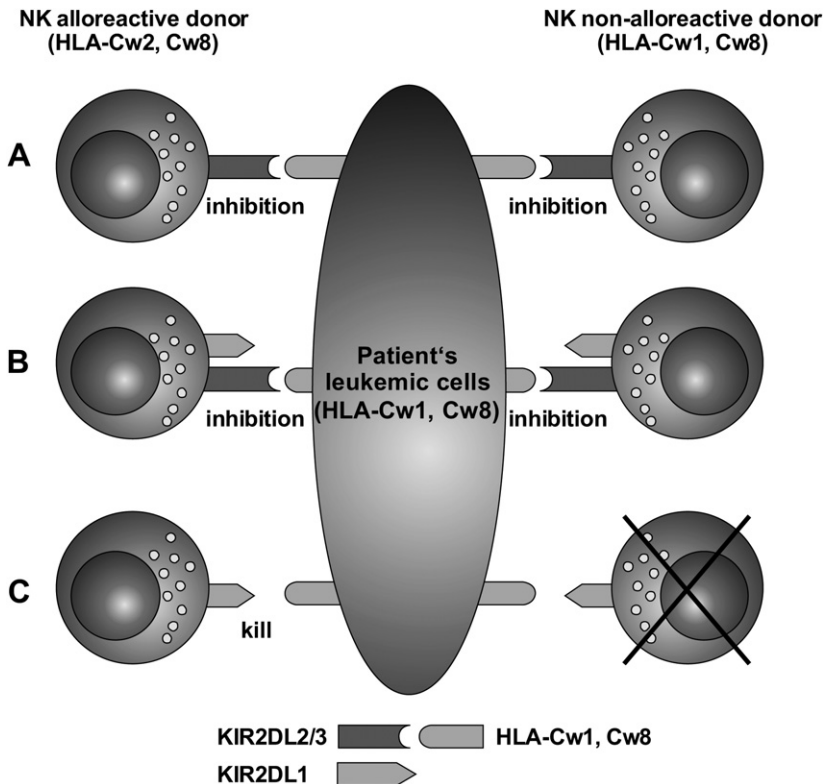


Fig. 3. The ligand-ligand model is based on the assumption that all NK cells have at least 1 KIR for self HLA class I alleles (A). In this example (patient's HLA type: HLA-Cw1, Cw8), a donor mismatched at the HLA-Cw allele in GVHD direction (donor HLA-Cw2, patient HLA-Cw1) has NK cells in his repertoire that express at least the inhibitory receptor KIR2DL1 (B) (receptor for self HLA-Cw2). These NK cells cannot be inhibited by the patient's HLA-Cw1 allele and the donor is NK alloreactive (depicted in C). Because all NK cells should have at least 1 KIR for self HLA class I alleles, NK cells expressing only KIR2DL1 would not be present in an HLA-matched donor's repertoire and the donor is nonalloreactive.

would not be encountered in the KIR repertoire of an HLA-matched donor. Using this model, impressive antileukemic effects based on NK alloreactivity have been reported, especially in adult patients with acute myeloid leukemia (AML).⁴⁰

An alternative model is the missing KIR ligand model (receptor-ligand model).⁴¹ In this model, the HLA type of the donor is not taken into account and only the donor's actual KIR repertoire (as opposed to predicted as in the ligand-ligand model) and the patient's HLA type are considered. The KIR expression on the surface of the donor's NK cells can be determined by flow cytometry or by genotyping.^{41,42} Compared with the ligand-ligand model, this model allowed a more accurate prediction of the risk of relapse in children with acute lymphoblastic leukemia after haploidentical stem cell transplantation and, as expected, patients transplanted from a receptor-ligand mismatched haploidentical donor had a lower risk of relapse compared with patients transplanted from a receptor-ligand-matched donor.^{41,43} Not all mismatches in these studies were predicted based on the ligand-ligand model. Further comparison of KIR genotyping and phenotyping for selection of the best NK-alloreactive donor showed that genotyping alone may not be sufficient (ie, gene expression does not always determine actual KIR expression) and a simplified algorithm for donor KIR typing has been suggested.⁴³ The receptor-ligand model has also been applied in patients with AML or myelodysplastic syndrome who received a transplant from HLA-identical siblings and patients lacking HLA ligands for the donors KIRs had significantly less risk of relapse.⁴⁴ Because all donor-recipient pairs in this study were HLA-identical siblings, the ligand-ligand model would not have predicted NK alloreactivity in any of the donor-recipient pairs. Therefore, the determination of donor KIR expression among HLA-identical donors may facilitate identification of donors expected to have potent antileukemic activity by alloreactive NK cells. In a large retrospective cohort of more than 2000 unrelated donor transplants,⁴⁵ the lack of KIR ligands significantly reduced the risk of relapse in patients with early-stage myeloid cells. However, another study of unrelated donors⁴⁶ failed to reveal any differences in leukemia recurrence among patients receiving grafts from KIR-ligand-matched or -mismatched donors. However, KIR-ligand incompatibility in the graft-versus-host direction, according to the ligand-ligand model, improved the outcome after umbilical cord blood transplantation for patients with acute leukemia.⁴⁷ The conflicting results in HLA-matched transplantation might be caused by the heterogeneity of the different transplant procedures (conditioning regimen, graft composition, posttransplant immunosuppression) and of the patients (disease, risk category).⁴⁸ It has been shown in this context that T cell alloreactivity dominates NK cell alloreactivity in minimally T cell-depleted HLA-nonidentical pediatric bone marrow transplantation.⁴⁹ More insights into the complex regulation of the cytotoxicity of NK cells via inhibitory and activatory receptors are necessary to further optimize the donor selection in HLA-matched or-mismatched transplantation.

CLINICAL EXPLOITATION OF NK CELLS AFTER TRANSPLANTATION

Hematological Malignancies

Especially in light of recent findings that AML CD34+CD38- stem cells are susceptible to allorecognition and lysis by single KIR-expressing NK cells,⁵⁰ it is foreseeable that NK cells will play an increasingly important role in the KIR-mismatch setting after allogeneic transplantation in the eradication of leukemic blasts. NK cells form the first wave of immunologic recovery after high-dose chemotherapy/radiotherapy and patients with higher numbers of NK cells at 30 days post transplant had a lower risk of relapse and better survival.⁵¹ Although the first NK cells recovering after

transplantation are CD94/NKG2A-positive and KIR-negative, patients acquire the donor's KIR repertoire after about 3 months post transplant.⁴¹ A biased early post-transplant NK cell reconstitution toward group C1-specific NK cells and the absence of group 2-specific NK cells was associated with a significantly reduced survival in the presence of C1 ligands.⁵² The absence of KIR expression on NK cells in the early phase after transplantation might render these cells cytotoxic against leukemic blasts independent of the patients' inhibitory KIR ligands.³⁸ Such NK cells might be initially hyporesponsive (for reasons that are unknown), but their cytotoxic function might be induced after stimulation with cytokines and the clinical application of IL-2 post transplant may be considered in patients receiving T cell-depleted grafts.⁵³

In addition to their antileukemic properties, it has been shown in mouse models that alloreactive NK cells also facilitate engraftment by the elimination of residual host hematopoiesis,⁵⁴ thus increasing the likelihood of donor engraftment following reduced intensity conditioning (RIC) regimens in allogeneic and especially in haploidentical transplantation.^{55,56} In addition, recent advances in T cell depletion technologies from mobilized peripheral stem cells allow the coinfusion of large numbers of NK cells.⁵⁷ This technology in combination with RIC regimens is associated with a lower transplant-related mortality (TRM) with the same rate of engraftment compared with the previous approach using CD34+ positively selected haploidentical stem cell grafts and fully ablative conditioning regimens.⁵⁵

T cell-depleted and NK cell-enriched grafts can be further activated *ex vivo* before infusion with cytokines, such as IL-15. This cytokine seems to be more efficient than IL-2 in expanding the NK cells because of promotion of survival and protection of NK cells from activation-induced cell death.⁵⁸ Overnight incubation of T cell-depleted mobilized grafts from haploidentical donors led to an increase of their cytotoxicity, and the infusion of such IL-15-activated NK cells was well tolerated in a small series of pediatric patients.⁵⁹ In the presence of suitable monoclonal antibodies, such as anti-CD19 or anti-CD20 targeting molecules expressed by the patient's leukemia, engagement of Fc-receptors expressed on NK cells overrides the KIR-mediated inhibition, thus mediating antibody-dependent cytotoxicity and could further increase the antileukemic activity of NK cells.^{60,61} Purified alloreactive haploidentical NK cells have been given several days after conditioning and transplantation of purified CD34+ stem cells from the same haploidentical donor.⁶² This approach resulted in a long-term remission in a patient with infant leukemia who relapsed after a standard myeloablative allogeneic transplantation and presented with refractory leukemia at time of second haploidentical transplantation. Approaches currently being investigated include posttransplant adoptive transfer of large numbers of *ex vivo* generated and genetically modified (to enhance function) donor-derived NK cells⁶³ and large-scale methods have been described that allow the expansion of donor-derived NK cells for clinical application.⁶⁴

Most of the clinical data obtained thus far have been collected in the transplant setting of sustained engraftment of full donor hematopoiesis. Another approach for the exploitation of the antitumor effect of alloreactive NK cells is in a nontransplant setting.⁶⁵ With this therapy, patients receive a moderate chemotherapy to induce lymphocyte expansion after infusion of alloreactive IL-2-activated NK cells from an allogeneic KIR-mismatched donor. Additional low-dose IL-2 is administered to the patients to induce and maintain *in vivo* proliferation of donor NK cells. Transient proliferation of donor NK cells for several weeks has been observed in patients and some impressive tumor responses have been reported. However, this approach does not result in a long-term engraftment of donor NK cells and further long-term follow-up studies are needed.

Solid Tumors

In addition to the studies in leukemia described earlier, the concept of alloreactive NK cells might also apply for the treatment of pediatric metastatic solid tumors.⁶⁶ It has been shown that KIR-ligand-mismatched alloreactive NK cells effectively lyse primary solid tumors⁶⁷ and tumor cell lines established from melanoma or renal cell carcinoma.⁶⁸ In addition, some pediatric tumors, such as neuroblastoma, express no or only low levels of HLA class I molecules on their surface and are therefore susceptible target cells for NK cells (as discussed earlier).³⁶ Tumor cell lines obtained from and Ewing sarcoma show a variable susceptibility to NK cell-mediated lysis.⁶⁹ Based on this concept and similar to the leukemia trials described earlier, clinical protocols using an RIC approach and haploidentical transplantation of CD3/19-depleted and NK cell-enriched stem cell grafts have been initiated in patients with advanced and refractory pediatric malignant solid tumors including neuroblastoma, rhabdomyosarcoma, and Ewing sarcoma.⁷⁰

Summary and Further Directions

New insights into the biology of NK cells have attracted significant interest of researchers and clinicians to exploit this lymphocyte subpopulation for the treatment of patients with malignancies. In the autologous setting, ex vivo induced NK cells have been used with some clinical responses. More convincing evidence exists in the allogeneic transplant setting, in which alloreactive NK cells have been shown to exert an antileukemic effect, especially after KIR-ligand mismatched haploidentical transplantation. Increasing insights into the biology of NK cells such as into their ontogeny, the development of the KIR repertoire (especially after transplantation), the cross talk of NK cells with other cells of the immune system, and the interaction of their multiple surface receptors with tumor cells will enable more effective use of this cell population in cancer treatment.

PART 2: MSC

Background to MSC

The bone marrow serves as a reservoir for different classes of stem cells. In addition to hematopoietic stem cells the bone marrow contains a population of marrow stromal or MSC. Stromal stem cells exhibit multilineage differentiation capacity, and are able to generate progenitors with restricted developmental potential, including fibroblasts, osteoblasts, adipocytes, and chondrocyte progenitors.⁷¹ Marrow stromal cells comprise a heterogeneous population of cells, including reticular endothelial cells, fibroblasts, adipocytes, and osteogenic precursor cells, which provide growth factors, cell to cell interactions, and matrix proteins that play a role in the regulation of hematopoiesis.⁷¹ Friedenstein and colleagues⁷² originally described a population of adherent cells from the bone marrow that were nonphagocytic and exhibited a fibroblastlike appearance. Following ectopic transplantation under the kidney capsule these cells (colony-forming unit fibroblastoids) gave rise to a broad spectrum of differentiated connective tissues, including bone, cartilage, adipose tissue, and myelosupportive stroma.^{72,73} Based on these observations it was proposed that these tissues were derived from a common precursor cell residing in the bone marrow, termed the stromal stem cell, the bone marrow stromal stem cell, the MSC, or the skeletal stem cell.⁷⁴ MSC secrete cytokines important for hematopoiesis and promote engraftment of hematopoietic stem cells in experimental animal models, especially when the dose of hematopoietic stem cells is limiting.⁷⁵⁻⁷⁷ In light of the controversy as to whether MSC at the single cell level truly fulfill the criteria of self-renewal and

multilineage differentiation capacity, it was recently proposed to use the term multipotent mesenchymal stromal cells (although not changing the acronym MSC) to describe fibroblastlike plastic adherent cells.⁷⁸

Sources of MSC

Although the bone marrow serves as the primary reservoir for MSC, their presence has been reported in various other tissues. These include periosteum and muscle connective tissue,^{79,80} fetal bone marrow, liver, and blood.⁸¹ MSC have been identified in cytokine (granulocyte-specific colony-stimulating factor [G-CSF]) mobilized peripheral blood by some investigators,⁸² although other studies have been unable to confirm their presence in peripheral blood.⁸³ Similarly, initial reports suggested that MSC could be isolated from umbilical cord blood.^{84,85} The low frequency of MSC in these sources may explain the initial contradictory findings of different researchers. However, MSC have been successfully isolated from human amniotic fluid.⁸⁶ The phenotype of the culture-expanded amniotic fluid-derived cells was similar to that reported for MSC derived from second-trimester fetal tissues and adult bone marrow. It has been reported that the *in vivo* functions of MSC depend on where they come from, irrespective of the expansion procedure used to obtain them. This finding may have consequences for their future clinical application.^{87–89}

Characterization of MSC

No unique phenotype has been identified that allows the reproducible isolation of MSC precursors with predictable developmental potential. The isolation and characterization of stromal cell function therefore still relies primarily on their ability to adhere to plastic and their expansion potential. The capacity of *ex vivo* expanded MSC to differentiate into multiple mesenchymal lineages, including bone, fat, and cartilage, is presently used as a functional criterion to define MSC.⁷¹

Similarly, no specific marker or combination of markers is available to identify MSC. Phenotypically, *ex vivo* expanded MSC express several nonspecific markers, but are devoid of hematopoietic and endothelial markers (Table 1).⁷¹

Expansion for Clinical Use

In recent years, new techniques have become available to isolate and grow mesenchymal progenitors and to manipulate their growth under defined *in vitro* culture

Table 1
Characteristic surface markers positively and negatively expressed by MSC

Marker	Expression
HLA-A,B,C	+
HLA-DR	–
CD31, PECAM	–
CD34	–
CD45	–
CD73	+
CD80	–
CD90, Thy-1	+
CD105, SH2	+
CD45	–
CD3	–

conditions. As a result MSC can be rapidly expanded (within 4–6 weeks) to numbers that are required for clinical application. Standard conditions for expansion of MSC include the presence of serum, in most instances fetal bovine serum (FBS), although platelet rich plasma is presently being evaluated as an alternative.^{88,90–92}

One of the risks related to *in vitro* expansion of MSC is of genetic instability and thus the risk of malignant transformation. This genetic instability has been reported during culture of MSC obtained from mice⁹³ but is believed to be caused by the inherent genetic instability of the species rather than the intrinsic nature of cultured MSC. When human MSC were subjected to prolonged culture conditions, standard karyotypic and genome-based analyses did not demonstrate the acquisition of chromosomal abnormalities, suggesting they remain genetically stable in culture and thus unlikely to induce malignant transformation.⁹⁴ However, the addition of exogenous growth factors to the culture medium can induce genetic transformation of expanded human MSC.⁹³

In most healthy donors, isolated and expanded MSC display a progressive decrease in proliferative capacity until reaching senescence and cannot be expanded in the long term. Most phase I/II published studies have used bone marrow-derived FBS-expanded MSC, without the addition of exogenous growth factors. It is therefore important to keep in mind that alternatively expanded MSC require extensive clinical testing to establish their efficacy and safety and to determine whether or not they can substitute MSC expanded in FBS.

Immune Modulatory Properties of MSC

Although it is widely accepted that MSC give rise to cells that form the structural network in support and maintenance of normal hematopoiesis, current opinion regarding MSC and their immunosuppressive function is derived from *in vitro* experiments of expanded MSC. Therefore it is still not known whether they play an important physiologic role in the regulation of immune homeostasis. Although much research has been undertaken to determine the immunomodulatory activities of MSC, the exact mechanisms on how they exert their influence have yet to be fully elucidated.

Experimental models suggest that expanded MSC have potent immunomodulatory effects, primarily through the inhibition of effector functions, thus offering a promising option for treating immune-mediated disorders such as acute GVHD.^{95–101} MSC are poor antigen-presenting cells and do not express MHC class II or costimulatory molecules. They were once considered nonimmunogenic but murine studies indicate that MSC can in an immunocompetent host elicit an immune response.¹⁰⁰ Thus, in the clinical setting, especially in those individuals who are not immunosuppressed, MSC should be considered as hypoimmune cells. Human MSC alter the cytokine secretion profile of dendritic cells (DCs), naive and effector T cells (T helper 1 [T_H1] and T helper 2 [T_H2]), and NK cells to induce a more antiinflammatory or tolerant phenotype.^{95–99}

T cell interactions

Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli⁹⁶ and inhibit the response of naive and memory antigen-specific T cells to their cognate peptide.⁹⁶ Accordingly, expanded MSC do not stimulate T cell proliferation in mixed lymphocyte reactions and are able to down-regulate alloreactive T cell responses when added to mixed lymphocyte cultures.^{96,97} As this inhibition is not dependent on MHC expression, allogeneic and autologous MSC can induce this effect. MSC do not induce T cell apoptosis but rather promote survival of T cells in a quiescent phase.^{102,103} IL-2 stimulation can partially overcome this MSC-mediated effect.¹⁰⁴ MSC-induced inhibition of T cell proliferation

is associated with a decreased IFN- γ and an increased IL-4 production, reflecting the induction of an antiinflammatory rather than a proinflammatory state.⁹⁸ MSC have also been reported to down-regulate MHC-restricted killing by CD8+ cytotoxic T lymphocytes (CTLs),¹⁰⁵ albeit they themselves are not CTL targets.¹⁰⁶ MSC can influence the generation of T regulatory cells that subsequently suppress activation of the immune system. This process is mediated by the release of IL-10 by plasmacytoid DCs (pDCs) and the release of sHLA-G5 isoform.^{95,98}

B cell interactions

Interactions of MSC with B cells are controversial with conflicting reports, which may reflect the variations in experimental procedures.⁹⁵ Most reports suggest that in vivo B cell proliferation and differentiation and expression of cytokines are inhibited by MSC.^{107,108} Conversely, in vitro data suggest that MSC support proliferation, differentiation, and survival of B cells from healthy individuals and from children with systemic lupus erythematosus.^{109,110} As T cells orchestrate B cell function, whatever the ultimate effects are of MSC directly on B cell function, B cells are more likely to be influenced indirectly by MSC-inhibiting T cells.⁹⁵

DC interactions

In vivo experiments have demonstrated that MSC inhibit the maturation of monocytes into dendritic DCs, and skew mature DCs to an immature DC state.^{111,112} Incubation of MSC with mature DCs reduces the latter's expression of class II and costimulatory molecules, inhibits tumor necrosis factor α (TNF- α) production, and impairs antigen presentation.^{111,113} MSC impair the stimulatory effect of mature DCs on resting NK cells and alter antigen presentation to T cells, preventing proliferation and clonal expansion.

The immune environment may alter the function of MSC. It has been hypothesized that early in the immune response when levels of IFN- γ are low, MSC may behave as nonprofessional antigen-presenting cells. With increasing levels of IFN- γ , MSC are then influenced to switch to their immune-suppressive function.¹¹⁴

NK cell interactions

MSC down-regulate the expression of Nkp30 and NK group 2, member D (NKG2D) activating receptors involved in NK activation and killing of target cells.⁹⁹ Resting NK cells stimulated with either IL-2 or IL-15 in the presence of MSC show markedly reduced proliferation and IFN- γ production, although activated NK cells are more resistant to the effects of MSC.¹¹⁵

The lack in MSC of MHC class I expression and the expression of NK-recognized ligands make them a natural target for activated NK cell killing. Cytokine-activated NK cells kill MSC via interaction of NKG2D expressed by NK cells with its ligands UL16-binding protein 3 (ULBP3) or class I polypeptide-related sequence A (MICA) expressed by MSC, and of NK cell-associated DNAM-1 with MSC-associated poliovirus receptor (CD155) or nectin-2 (CD112). This effect can be partially overcome by incubating MSC with IFN- γ , which up-regulates MHC class I expression.^{95,99}

It can be postulated that a microenvironment rich in IFN- γ would protect MSC from being attacked and destroyed by NK cells. However, the clinical relevance of these findings has not yet been demonstrated.

In summary, MSC have demonstrated broad immunosuppressive potential in vitro. So far the mode of action in vivo remains unclear.

Potential Mechanisms Involved in MSC-mediated Immunosuppression

Cell to cell contact and soluble factors are believed to be required for the induction of MSC-mediated immunosuppression.⁹⁵ Primary contact between MSC and the target cell is initiated by adhesion molecules.¹⁰⁷ Most studies demonstrate that soluble factors are involved, as the separation of MSC and peripheral blood mononuclear cells (PBMCs) by a trans-well permeable membrane does not prevent the inhibition of proliferation of PBMCs.^{96,116}

MSC release several soluble molecules either constitutively or following cross talk with other cells.⁹⁵ These include the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO)¹⁰¹ and nitric oxide. Release of IFN- γ by target cells induces the release of IDO by MSC, which in turn depletes tryptophan, an essential amino acid for lymphocyte proliferation.^{117,118} IDO is necessary to inhibit proliferation of T_{H1} cells and together with prostaglandin E₂ (PGE₂) inhibits NK cell function.^{95,118}

IFN- γ can, in a murine model with the addition of proinflammatory cytokines, stimulate chemokine production by MSC, resulting in T cell attraction and increased inducible nitric-oxide synthase (i-NOS). T cells are inhibited by the subsequent production of nitrous oxide.¹¹⁹

Other soluble factors that have been reported to be released by MSC include transforming growth factor β 1 (TGF β 1), heme oxygenase 1, hepatocyte growth factor (HGF), and IL-6.^{95,96,98,107,120,121} Cytokines produced by target cells can increase the release of some of these MSC-derived soluble factors.⁹⁵ sHLA-G5 released by MSC suppresses T cell proliferation and CD8+ T cell- and NK cell-mediated cytotoxicity.^{107,121} Conversely, MSC through the release of sHLA-G5 initiate the up-regulation of CD4+CD25+ Fox P3+ cells (T regulatory immune phenotype),^{97,107,121} albeit the depletion of regulatory T cells has no effect on the inhibition of T proliferation by MSC.⁹⁸

The complexity and mechanisms whereby MSC interact with cells, of the adaptive and innate immune system, are summarized in **Fig. 4**.

Despite most of the available understanding of MSC interactions being derived from in vitro experiments, the design of any future clinical studies needs to consider these findings.

Animal Studies

Almeida-Porada and colleagues¹¹⁶ observed that cotransplantation of human stromal cells into preimmune fetal sheep resulted in an enhancement of long-term engraftment of human cells in the bone marrow and higher levels of donor cells in the circulation during gestation and after birth. Infusion of bone-marrow-derived osteoblasts promoted the engraftment of allogeneic hematopoietic stem cells in mice.¹²² Other studies in NOD/SCID mice indicate that cotransplantation of MSC and cord blood enhances engraftment of human hematopoietic cells in the bone marrow of NOD/SCID mice.¹²³ Coinfusion of MSC derived from fetal lung and CD34⁺ cells derived from umbilical cord blood promoted the engraftment of myeloid and B-lymphoid cells in the marrow of recipient mice, showing that the engraftment-promoting effect of MSC was not lineage specific.⁷⁶ It was also found that enhancement of engraftment might be independent of the homing of MSC to the marrow and might be mediated by the release of cytokines that promote either the homing or proliferation of hematopoietic stem cells. Bartholomew and colleagues¹²² demonstrated that MSC infusions can suppress lymphocyte proliferation and prolong skin grafts in a nonhuman primate.

Infusion of allogeneic MSC ameliorated lethal GVHD in mice receiving haploidentical hematopoietic stem cell transplants, but only when MSC were administered early and

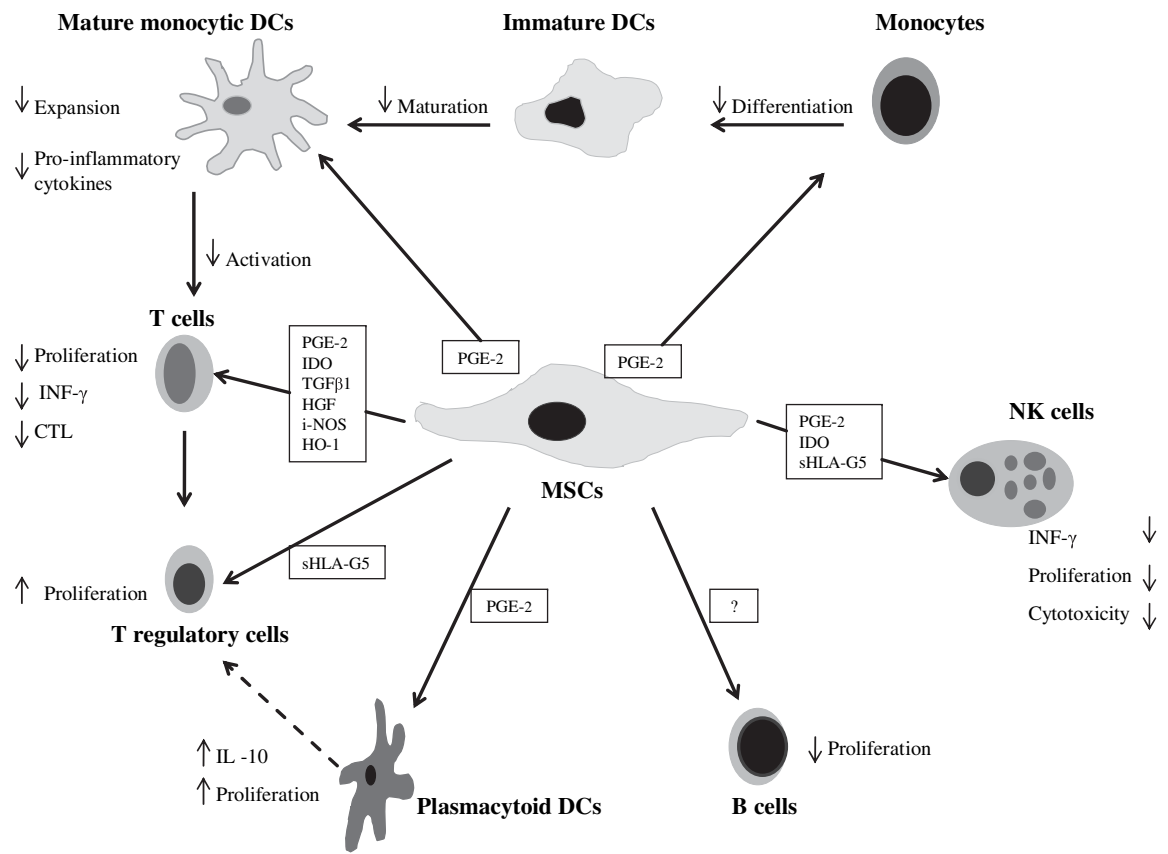


Fig. 4. Possible mechanisms of MSC interactions and cells of the immune system.

repeatedly after transplantation.¹²³ Effects of MSC have been reported in animal models of autoimmunity, such as amelioration of experimental autoimmune encephalitis in a murine model, raising the possibility of MSC use in autoimmune diseases.¹²⁴ However, in a collagen-induced model of rheumatoid arthritis, after infusion of MSC no observed improvement was evident.¹²⁵

The final destination of infused MSC in clinical studies is unknown. It has been difficult to demonstrate engraftment of donor-derived MSC following transplantation. In some studies, gene-marked culture-expanded MSC were infused along with unmodified bone marrow cells and evidence based on the polymerase chain reaction of the presence of marked MSC was found in the marrow several weeks after transplantation.^{116,126,127} How long MSC survive after injection and to what extent they are able to target tissues are unknown.¹²⁸ In nonhuman primates intravenously administered MSC distribute over a wide range of tissues.¹²⁹ Preferential homing to sites of injury has been shown.¹²⁸ MSC, like cells of the immune system, can extravasate from blood vessels as a result of expression of adhesion molecules on their surface. MSC display coordinated rolling and adhesion to endothelial cells, dependent on P-selectin and vascular cell adhesion molecule-1.¹³⁰ Several chemokines bind to cognate receptors expressed on the cell surface of MSC¹³¹ and facilitate their migration and extravasation.¹³²

Also MSC may become trapped in the vasculature, which limits their tissue distribution.¹³³ In future clinical studies, trafficking of infused MSC needs to be addressed, and consideration given to MSC that remain undifferentiated compared with those that differentiate.¹³³ Tracking infused MSC will require a suitable labeling method that can be safely administered to patients, without affecting the function of the MSC.

Clinical Results

Animal models may not predict the clinical situation, as the immune modulatory mechanisms between species (eg, murine and human MSC) may differ. Thus far, clinical application of ex vivo expanded MSC therapy in the HSCT setting has exploited their potential immune modulatory properties and their ability to support hematopoietic stem cell proliferation.

Adult HSCT studies

Initial phase I studies involving bone-marrow-derived MSC showed that MSC could be successfully collected, culture expanded ex vivo for 4 to 7 weeks and administered to patients with hematological malignancies in complete remission. The infusions contained up to 50×10^6 MSC and were well tolerated without adverse reactions.⁸²

In a subsequent phase I/II clinical trial in patients with breast cancer, autologous and expanded MSC were coinfused with autologous peripheral blood progenitor cells.¹³⁴ Clonogenic MSC could be detected in venous blood up to 1 hour after infusion in most patients. No toxicities were observed related to the infusion of MSC and hematopoietic reconstitution (defined as time to neutrophil and platelet recovery) was rapid, suggesting some effect of MSC infusion on hematopoietic reconstitution. In another multicenter phase I/II study, allogeneic donor bone-marrow-derived MSC were coinfused with allogeneic hematopoietic stem cells in patients with hematological malignancies undergoing matched-sibling stem cell transplantation.¹³⁵ Preliminary data suggest that there was no immediate toxicity following infusion of MSC and that there was more rapid engraftment and a low incidence of acute GVHD in comparison with historical controls.

Pediatric HSCT studies

Correction of inborn errors MSC express high levels of arylsulfatase A and α -L-iduronidase.¹³⁶ The deficiency of these enzymes is associated with specific inborn errors of metabolism. Arylsulfatase A deficiency is the cause of metachromatic leukodystrophy, and α -L-iduronidase deficiency is the cause of Hurler disease, disorders that may be cured by allogeneic HSCT.^{137,138} Expanded MSC were administered to patients with metachromatic leukodystrophy and Hurler syndrome, who had previously undergone HSCT but had residual symptoms of their disease.¹³⁹ Four of 5 patients with metachromatic leukodystrophy showed improvement in nerve conduction velocity, but no clinical effects were demonstrated in patients with Hurler disease.

MSC have been used to treat bone disease, namely osteogenesis imperfecta.¹⁴⁰ Five children with osteogenesis imperfecta undergoing HSCT had donor osteoblast engraftment, with new bone formation, an increase in total bone mineral content, and an increase in growth velocity and noticeable reduced fracture frequencies.¹⁴¹ MSC identified by specific gene markers were given to 6 children undergoing HSCT for severe osteogenesis imperfecta.¹⁴² Engraftment of MSC was identified and this was associated with acceleration of growth velocity.

Overcoming graft failure MSC had not been shown to overcome graft rejection, although, given the physiologic role of MSC in hematopoietic stem cell support, it was hypothesized that they could be a valuable adjunct in the haploidentical peripheral blood stem cell transplantation (PBSCT) setting, where graft failure had been reported as a major concern despite large doses of purified CD34+ stem cells being administered. If MSC were to overcome graft failure then there was a considerable opportunity to improve on the historical results of transplantation from HLA-disparate donors.

The feasibility and safety of administering haploidentical bone-marrow-derived expanded MSC to children undergoing haploidentical PBSCT was recently shown.¹⁴³ Thirteen patients received haploidentical bone-marrow-derived ex vivo expanded MSC at a dose of 1 to 2×10^6 /kg recipient weight. In addition they received haploidentical G-CSF mobilized CD34+ selected peripheral blood stem cells, 4 hours after the infusion of MSC, with a target dose of 20×10^6 /kg CD34+. The outcome in feasibility, toxicity, stable engraftment, rates of infection, acute GVHD, and relapse were compared with 52 nonselected historical controls, who received haploidentical PBSCs alone. Epidemiologically there was no difference between the study patients and the historical controls other than the administration of MSC. There was a trend to a statistically significant improvement in sustained engraftment ($P = .06$) without increasing the rate of infections, relapse, or transplant-related toxicities in children receiving MSC compared with controls. Engraftment of infused MSC was less than 1% in serial bone marrow samples up to 1 year post MSC infusion. Despite these encouraging results, the study was limited in that children were assigned to the study in a nonrandomized fashion and compared with historical controls. Since the initial publication, 32 children have been cotransplanted with haploidentical PBSCTs and MSC with no documented graft failure. These results and the fact that posttransplant immune suppression is not required because of the intense T cell depletion suggest that this method may provide a suitable platform for additional cellular therapies.

Treatment of steroid-resistant GVHD Exploiting the immunosuppressive effects of MSC, Le Blanc and colleagues¹⁴⁴ reported the first successful treatment of severe steroid/treatment-resistant grade IV acute GVHD of the gut and liver with haploidentical third-party bone-marrow-derived MSC in a 9-year-old boy.

Subsequently, a phase I/II multicenter trial of 55 patients (30 adults and 25 children) treated with MSC infusions for severe grade II to IV steroid refractory acute GVHD was conducted.^{145,146} Patients varied in the time to MSC infusion and the type of previous immunosuppression administered before receiving MSC. Most patients (60%) had received second-line treatments, 25% third-line treatments, and 10% greater than third-line treatments, in addition to steroids, without documented clinical or pathologic response. MSC were expanded according to a common protocol. A median dose of bone-marrow-derived ex vivo expanded MSC of 1.4 (range 0.4 – 9) $\times 10^6$ cells/kg was administered. Twenty-seven patients received 1 infusion, 22 patients received 2 infusions, and 6 patients received 3 to 5 infusions. In light of the rapid progression of symptoms necessitating prompt treatment, MSC were mainly derived from third-party HLA-mismatched donors ($n = 69$), the rest being obtained from either HLA-identical sibling donors ($n = 5$) or haploidentical donors ($n = 18$).

No toxicities were documented associated with the infusion of MSC, even in the most seriously ill patients treated. Thirty patients (55%) showed a complete response and 8 showed improvement, resulting in a 69% overall response rate. The response rate was higher in children (80%) than in adults (60%; $P = .28$). Although not significant, the better response of children contributed to the improved overall survival. Patients with a complete response had a lower TRM 1 year after MSC infusion compared with partial or nonresponders (37% vs 72%; $P = .002$) and a higher overall survival 2 years after HSCT (52% vs 16%; $P = .018$). Nearly half the patients survived ($n = 21$) and 8 patients developed chronic GVHD. The study was hampered because patients were heterogeneous not only in timing of MSC infusions but also because of the variable immune-suppressive regimens administered before MSC. Although compared with historical controls and recent literature the survival rates seem favorable, the efficacy of MSC in the treatment of acute GVHD remains to be confirmed; a European randomized prospective study is under way.

A recent press release by Osiris showed that in a randomized study of third-party MSC used for steroid refractory GVHD MSC proved no better than alternative treatments except in children and severe gastrointestinal GVHD. Publication of this trial is awaited.

In summary, MSC seem to be a promising cellular therapy in children undergoing HSCT to overcome GVHD and graft dysfunction. However, the long-term safety and efficacy have not been proven. Multicenter collaborative studies are needed to determine how and when MSC can be administered most effectively, and determining their in vivo functional capacities. Future studies will also need to address such issues as trafficking of MSC to target tissues and monitoring potential long-term toxicities. These studies may include an increased risk of relapse of underlying malignancies and increased infection risks because of the broad immune-suppressive activities of MSC, together with the risk of malignant transformation of infused expanded MSC.

REFERENCES

1. Kiessling R, Klein E, Pross H, et al. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol* 1975;5:117–21.
2. Lanier LL, Phillips JH, Hackett J Jr, et al. Natural killer cells: definition of a cell type rather than a function. *J Immunother* 1986;137:2735–9.
3. Caligiuri MA. Human natural killer cells. *Blood* 2008;112:461–9.

4. Ljunggren HG, Karre K. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. *J Environ Monit* 1985; 162:1745–59.
5. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today* 1990;11:237–44.
6. Boyton RJ, Altmann DM. Natural killer cells, killer immunoglobulin-like receptors and human leucocyte antigen class I in disease. *Clin Exp Immunol* 2007;149: 1–8.
7. Moretta L, Bottino C, Pende D, et al. Different checkpoints in human NK-cell activation. *Trends Immunol* 2004;25:670–6.
8. Bottino C, Moretta L, Pende D, et al. Learning how to discriminate between friends and enemies, a lesson from Natural Killer cells. *Mol Immunol* 2004;41: 569–75.
9. Braud VM, Allan DS, O'Callaghan CA, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 1998;391:795–9.
10. Kaiser BK, Barahmand-Pour F, Paulsene W, et al. Interactions between NKG2x immunoreceptors and HLA-E ligands display overlapping affinities and thermodynamics. *J Immunother* 2005;174:2878–84.
11. Moretta A, Bottino C, Vitale M, et al. Receptors for HLA class-I molecules in human natural killer cells. *Annu Rev Immunol* 1996;14:619–48.
12. Yokoyama WM, Plougastel BF. Immune functions encoded by the natural killer gene complex. *Nat Rev Immunol* 2003;3:304–16.
13. Uhrberg M, Valiante NM, Shum BP, et al. Human diversity in killer cell inhibitory receptor genes. *Immunity* 1997;7:753–63.
14. Trinchieri G. Biology of natural killer cells. *Adv Immunol* 1989;47:187–376.
15. Pende D, Rivera P, Marcenaro S, et al. Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. *Cancer Res* 2002;62:6178–86.
16. Cerwenka A, Lanier LL. NKG2D ligands: unconventional MHC class I-like molecules exploited by viruses and cancer. *Tissue Antigens* 2003;61:335–43.
17. Pende D, Bottino C, Castriconi R, et al. PVR (CD155) and Nectin-2 (CD112) as ligands of the human DNAM-1 (CD226) activating receptor: involvement in tumor cell lysis. *Mol Immunol* 2005;42:463–9.
18. Sivori S, Carlomagno S, Moretta L, et al. Comparison of different CpG oligodeoxynucleotide classes for their capability to stimulate human NK cells. *Eur J Immunol* 2006;36:961–7.
19. Zamai L, Ponti C, Mirandola P, et al. NK cells and cancer. *J Immunother* 2007; 178:4011–6.
20. Imai K, Matsuyama S, Miyake S, et al. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* 2000;356:1795–9.
21. Cudkovicz G, Bennett M. Peculiar immunobiology of bone marrow allografts. II. Rejection of parental grafts by resistant F 1 hybrid mice. *J Environ Monit* 1971; 134:1513–28.
22. Lowdell MW, Ray N, Craston R, et al. The in vitro detection of anti-leukaemia-specific cytotoxicity after autologous bone marrow transplantation for acute leukaemia. *Bone Marrow Transplant* 1997;19:891–7.
23. Lowdell MW, Craston R, Samuel D, et al. Evidence that continued remission in patients treated for acute leukaemia is dependent upon autologous natural killer cells. *Br J Haematol* 2002;117:821–7.

24. Tajima F, Kawatani T, Endo A, et al. Natural killer cell activity and cytokine production as prognostic factors in adult acute leukemia. *Leukemia* 1996;10: 478–82.
25. Siegler U, Kalberer CP, Nowbakht P, et al. Activated natural killer cells from patients with acute myeloid leukemia are cytotoxic against autologous leukemic blasts in NOD/SCID mice. *Leukemia* 2005;19:2215–22.
26. Salih HR, Antropius H, Gieseke F, et al. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. *Blood* 2003; 102:1389–96.
27. Afify ZA, bdel-Mageed A, Findley HW, et al. Cytotoxicity of purified Leu 19+ cells from the peripheral blood of children with acute lymphocytic leukemia. *Cancer* 1990;66:469–73.
28. Mageed AA, Findley HW Jr, Franco C, et al. Natural killer cells in children with acute leukemia. The effect of interleukin-2. *Cancer* 1987;60:2913–8.
29. Findley HW Jr, Abdel MA, Nasr SA, et al. Recombinant interleukin-2 activates peripheral blood lymphocytes from children with acute leukemia to kill autologous leukemic cells. *Cancer* 1988;62:1928–31.
30. Coca S, Perez-Piqueras J, Martinez D, et al. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer* 1997;79: 2320–8.
31. Ishigami S, Natsugoe S, Tokuda K, et al. Prognostic value of intratumoral natural killer cells in gastric carcinoma. *Cancer* 2000;88:577–83.
32. Alvarado CS, Findley HW, Chan WC, et al. Natural killer cells in children with malignant solid tumors. Effect of recombinant interferon-alpha and interleukin-2 on natural killer cell function against tumor cell lines. *Cancer* 1989; 63:83–9.
33. Velardi A. Role of KIRs and KIR ligands in hematopoietic transplantation. *Curr Opin Immunol* 2008;20:581–7.
34. Velardi A, Ruggeri L, Mancusi A, et al. Natural killer cell allorecognition of missing self in allogeneic hematopoietic transplantation: a tool for immunotherapy of leukemia. *Curr Opin Immunol* 2009;21:525–30.
35. Pfeiffer M, Schumm M, Feuchtinger T, et al. Intensity of HLA class I expression and KIR-mismatch determine NK-cell mediated lysis of leukaemic blasts from children with acute lymphatic leukaemia. *Br J Haematol* 2007;138:97–100.
36. Prigione I, Corrias MV, Aioldi I, et al. Immunogenicity of human neuroblastoma. *Ann N Y Acad Sci* 2004;1028:69–80.
37. Handgretinger R, Lang P, Schumm M, et al. Immunological aspects of haploidentical stem cell transplantation in children. *Ann N Y Acad Sci* 2001;938: 340–57 [discussion: 357–8].
38. Feuchtinger T, Pfeiffer M, Pfaffle A, et al. Cytolytic activity of NK cell clones against acute childhood precursor-B-cell leukaemia is influenced by HLA class I expression on blasts and the differential KIR phenotype of NK clones. *Bone Marrow Transplant* 2009;43:875–81.
39. Ruggeri L, Capanni M, Casucci M, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood* 1999;94: 333–9.
40. Ruggeri L, Mancusi A, Capanni M, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood* 2007;110:433–40.
41. Leung W, Iyengar R, Turner V, et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunother* 2004;172:644–50.

42. Chen X, Knowles J, Barfield RC, et al. A novel approach for quantification of KIR expression in healthy donors and pediatric recipients of hematopoietic SCTs. *Bone Marrow Transplant* 2009;43:525–32.
43. Leung W, Iyengar R, Triplett B, et al. Comparison of killer Ig-like receptor genotyping and phenotyping for selection of allogeneic blood stem cell donors. *J Immunother* 2005;174:6540–5.
44. Hsu KC, Keever-Taylor CA, Wilton A, et al. Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. *Blood* 2005;105:4878–84.
45. Miller JS, Cooley S, Parham P, et al. Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. *Blood* 2007;109:5058–61.
46. Farag SS, Bacigalupo A, Eapen M, et al. The effect of KIR ligand incompatibility on the outcome of unrelated donor transplantation: a report from the center for international blood and marrow transplant research, the European blood and marrow transplant registry, and the Dutch registry. *Biol Blood Marrow Transplant* 2006;12:876–84.
47. Willemze R, Rodrigues CA, Labopin M, et al. KIR-ligand incompatibility in the graft-versus-host direction improves outcomes after umbilical cord blood transplantation for acute leukemia. *Leukemia* 2009;23:492–500.
48. Verheyden S, Demanet C. NK cell receptors and their ligands in leukemia. *Leukemia* 2008;22:249–57.
49. Lowe EJ, Turner V, Handgretinger R, et al. T-cell alloreactivity dominates natural killer cell alloreactivity in minimally T-cell-depleted HLA-non-identical paediatric bone marrow transplantation. *Br J Haematol* 2003;123:323–6.
50. Langenkamp U, Siegler U, Jorger S, et al. Human acute myeloid leukemia CD34+CD38- stem cells are susceptible to allorecognition and lysis by single KIR-expressing natural killer cells. *Haematologica* 2009;94:1590–4.
51. Savani BN, Rezvani K, Mielke S, et al. Factors associated with early molecular remission after T cell-depleted allogeneic stem cell transplantation for chronic myelogenous leukemia. *Blood* 2006;107:1688–95.
52. Fischer JC, Ottinger H, Ferencik S, et al. Relevance of C1 and C2 epitopes for hemopoietic stem cell transplantation: role for sequential acquisition of HLA-C-specific inhibitory killer Ig-like receptor. *J Immunother* 2007;178:3918–23.
53. Teltschik F, Lang P, Pfeiffer H, et al. Long-term IL2 therapy after transplantation of T-cell depleted stem cells from alternative donors in children [abstract]. *Bone Marrow Transplant* 2007;39(Suppl 1):S185.
54. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 2002;295:2097–100.
55. Handgretinger R, Chen X, Pfeiffer M, et al. Feasibility and outcome of reduced-intensity conditioning in haploidentical transplantation. *Ann N Y Acad Sci* 2007;1106:279–89.
56. Bethge WA, Faul C, Bornhauser M, et al. Haploidentical allogeneic hematopoietic cell transplantation in adults using CD3/CD19 depletion and reduced intensity conditioning: an update. *Blood Cells Mol Dis* 2008;40:13–9.
57. Barfield RC, Otto M, Houston J, et al. A one-step large-scale method for T- and B-cell depletion of mobilized PBSC for allogeneic transplantation. *Cytherapy* 2004;6:1–6.
58. Rodella L, Zamai L, Rezzani R, et al. Interleukin 2 and interleukin 15 differentially predispose natural killer cells to apoptosis mediated by endothelial and tumour cells. *Br J Haematol* 2001;115:442–50.

59. Pfeiffer M, Lang P, Schumm M, et al. IL-15 activated CD3/10 depleted grafts for haploidentical transplantation in children: strongly increased NK activity in vitro and excellent tolerability in vivo [abstract]. *Bone Marrow Transplant* 2008;41: S51.
60. Lang P, Barbin K, Feuchtinger T, et al. Chimeric CD19 antibody mediates cytotoxic activity against leukemic blasts with effector cells from pediatric patients who received T-cell-depleted allografts. *Blood* 2004;103:3982–5.
61. Pfeiffer M, Stanojevic S, Feuchtinger T, et al. Rituximab mediates in vitro antileukemic activity in pediatric patients after allogeneic transplantation. *Bone Marrow Transplant* 2005;36:91–7.
62. Triplett B, Handgretinger R, Pui CH, et al. KIR-incompatible hematopoietic-cell transplantation for poor prognosis infant acute lymphoblastic leukemia. *Blood* 2006;107:1238–9.
63. Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood* 2005;106:376–83.
64. Fujisaki H, Kakuda H, Shimasaki N, et al. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. *Cancer Res* 2009;69:4010–7.
65. Miller JS, Soignier Y, Panoskaltis-Mortari A, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood* 2005;105:3051–7.
66. Perez-Martinez A, Leung W, Munoz E, et al. KIR-HLA receptor-ligand mismatch associated with a graft-versus-tumor effect in haploidentical stem cell transplantation for pediatric metastatic solid tumors. *Pediatr Blood Cancer* 2009;53:120–4.
67. Re F, Staudacher C, Zamai L, et al. Killer cell Ig-like receptors ligand-mismatched, alloreactive natural killer cells lyse primary solid tumors. *Cancer* 2006;107:640–8.
68. Igarashi T, Wynberg J, Srinivasan R, et al. Enhanced cytotoxicity of allogeneic NK cells with killer immunoglobulin-like receptor ligand incompatibility against melanoma and renal cell carcinoma cells. *Blood* 2004;104:170–7.
69. Verhoeven DH, de Hooge AS, Mooiman EC, et al. NK cells recognize and lyse Ewing sarcoma cells through NKG2D and DNAM-1 receptor dependent pathways. *Mol Immunol* 2008;45:3917–25.
70. Lang P, Pfeiffer M, Muller I, et al. Haploidentical stem cell transplantation in patients with pediatric solid tumors: preliminary results of a pilot study and analysis of graft versus tumor effects. *Klin Padiatr* 2006;218:321–6.
71. Fibbe WE, Lazarus HM. Mesenchymal stem cells and hematopoietic stem cell transplantation. In: Atkinson K, Champlin R, Brenner MA, et al, editors. *Clinical bone marrow and blood stem cell transplantation*. 3rd edition. Cambridge: Cambridge University Press; 2004. p. 67–78.
72. Friedenstein AJ, Deriglasova UF, Kulagina NN, et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. *Exp Hematol* 1974;2:83–92.
73. Owen M. Marrow stromal stem cells. *J Cell Sci Suppl* 1988;10:63–76.
74. Prockop DJ. Marrow stromal cells as stem cells for non-hematopoietic tissues. *Science* 1997;276:71–4.
75. Majumdar MK, Thiede MA, Mosca JD, et al. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. *J Cell Physiol* 1998;176:57–66.
76. Almeida-Porada G, Flake AW, Glimp HA, et al. Cotransplantation of stroma results in enhancement of engraftment and early expression of donor hematopoietic stem cells in utero. *Exp Hematol* 1999;27:1569–75.

77. In't Anker PS, Noort WA, Kruisselbrink AB, et al. Non expanded primary lung and bone marrow-derived mesenchymal cells promote the engraftment of umbilical cord blood-derived CD34 (+) cells in NOD/SCID mice. *Exp Hematol* 2003;31:881–9.
78. Horwitz EM, Le Blanc K, Dominici M, et al. Clarification of the nomenclature for MSC: the International Society for Cellular Therapy position statement. *Cytherapy* 2005;7:393–5.
79. Nathanson MA. Bone matrix-directed chondrogenesis of muscle in vitro. *Clin Orthop* 1985;200:142–58.
80. Nakahara H, Dennis JE, Bruder SP, et al. In vitro differentiation of bone and hypertrophic cartilage from periosteal-derived cells. *Exp Cell Res* 1991;195:492–503.
81. Campagnoli C, Roberts IA, Kumar S, et al. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood* 2001;98:2396–402.
82. Fernandez M, Simon V, Herrera G, et al. Detection of stromal cells in peripheral blood progenitor cell collections from breast cancer patients. *Bone Marrow Transplant* 1997;20:265–71.
83. Lazarus HM, Haynesworth SE, Gerson SL, et al. 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;16:557–64.
84. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol* 2000;109:235–42.
85. Bieback K, Kluter H. Mesenchymal stromal cells from umbilical cord blood. *Curr Stem Cell Res Ther* 2007;2:310–23.
86. In't Anker PS, Scherjon SA, Kleijburg-Van Der Keur C, et al. Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. *Blood* 2003;102:1548–9.
87. Bernardo ME, Emons JA, Karperien M, et al. Human mesenchymal stem cells derived from bone marrow display a better chondrogenic differentiation compared with other sources. *Connect Tissue Res* 2007;48:132–40.
88. Bernardo ME, Avanzini MA, Perotti C, et al. Optimization of in vitro expansion of human multipotent mesenchymal stromal cells for cell-therapy approaches: further insights in the search for a fetal calf serum substitute. *J Cell Physiol* 2007;211:121–30.
89. Avanzini MA, Bernardo ME, Cometa AM, et al. Generation of mesenchymal stromal cells in the presence of platelet lysate: a phenotypical and functional comparison between umbilical cord blood- and bone marrow-derived progenitors. *Hematologica* 2009;94:1649–60.
90. Vogel JP, Szalay K, Geiger F, et al. Platelet-rich plasma improves expansion of human mesenchymal stem cells and retains differentiation capacity and in vivo bone formation in calcium phosphate ceramics. *Platelets* 2006;17:462–9.
91. Doucet C, Ernou I, Zhang Y, et al. Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. *J Cell Physiol* 2005;205:228–36.
92. Schallmoser K, Bartmann C, Rohde E, et al. Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. *Transfusion* 2007;47:1436–46.
93. Tolar J, Nauta AJ, Osborn MJ, et al. Sarcoma derived from cultured mesenchymal stem cells. *Stem Cells* 2007;25:371–9.

94. Bernardo ME, Zaffaroni N, Novara F, 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;67:9142–9.
95. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol* 2008;8:726–36.
96. Di Nicola M, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;99:3838–43.
97. Krampera M, Glennie S, Dyson J, et al. Bone marrow mesenchymal stem cells inhibit the response of naïve and memory antigen-specific T-cells to their cognate peptide. *Blood* 2004;101:3722–9.
98. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;105:1815–22.
99. Sotiropoulou PA, Perz SA, Gritzapis AD, et al. Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells* 2006;24:74–85.
100. Nauta AJ, Westerhuis G, Kruisselbrink AB, et al. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. *Blood* 2006;108:2114–20.
101. Meisel R, Ziber A, Laryea M, et al. Human bone marrow stromal cells inhibit allogeneic responses by indoleamine 2,3 dioxygenase mediated tryptophan degradation. *Blood* 2004;103:4619–21.
102. Glennie S, Soeiro I, Dyson PJ, et al. Bone marrow mesenchymal stem cells induce division arrest energy of activated T cells. *Blood* 2005;105:2821–7.
103. Benvenuto F, Ferrari S, Gerdoni E, et al. Human mesenchymal stem cells promote survival of T cells in a quiescent state. *Stem Cells* 2007;25:1753–60.
104. Rasmusson I, Ringden O, Sundberg B, et al. Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms. *Exp Cell Res* 2005;305:33–41.
105. Rasmusson I, Ringden O, Sundberg B, et al. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. *Transplantation* 2003;76:1208–13.
106. Morandi F, Raffaghello L, Bianchi G, et al. Immunogenicity of human mesenchymal stem cells in HLA-class-I-restricted T-cell responses against viral or tumor-associated antigens. *Stem Cells* 2008;26:1275–87.
107. Augello A, Tasso R, Negrini SM, et al. Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. *Eur J Immunol* 2005;35:1482–6.
108. Corcione A, Benvenuto F, Ferretti E, et al. Human mesenchymal stem cells modulate B-cell functions. *Blood* 2006;107:367–72.
109. Traggiai E, Volpi S, Schena F, 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;26:562–9.
110. Rasmusson I, Le Blanc K, Sundberg B, et al. Mesenchymal stem cells stimulate antibody secretion in human B cells. *Scand J Immunol* 2007;65:336–43.
111. Jiang XX, Zhang Y, Liu B, et al. Human mesenchymal stem cells inhibit differentiation and function of monocyte derived dendritic cells. *Blood* 2005;105:4120–6.
112. Nauta AJ, Kruisselbrink AB, Lurvink E, et al. Mesenchymal stem cells inhibit generation and function of both CD34⁺-derived and monocyte-derived dendritic cells. *J Immunol* 2006;177:2080–7.

113. Li YP, Paczesny S, Lauret E, et al. Human mesenchymal stem cells license adult CD34⁺ hemopoietic progenitor cells to differentiate into regulatory dendritic cells through activation of the Notch pathway. *J Immunol* 2008;180:1598–608.
114. Stagg J, Pommey S, Eliopoulos N, et al. Interferon- γ -stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. *Blood* 2006;107:2570–7.
115. Spaggiari GM, Capobianco A, Becchetti S, et al. 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;107:1484–90.
116. Almeida-Porada G, Porada CD, Tran N, et al. Co-transplantation of human stromal cell progenitors into pre-immune fetal sheep results in early appearance of human donor cells in circulation and boosts cell levels in bone marrow at later time points after transplantation. *Blood* 2000;95:3620–7.
117. Krampera M, Cosmi L, Angeli R, et al. Role for interferon- γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006;24:386–98.
118. Ryan JM, Barry F, Murphy JM, et al. Interferon- γ does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. *Clin Exp Immunol* 2007;149:353–63.
119. Ren G, Zhang L, Zhao X, et al. Mesenchymal stem-cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008;2:141–50.
120. Kogler G, Kögler G, Radke TF, et al. Cytokine production and hematopoiesis supporting activity of cord blood derived unrestricted somatic stem cells. *Exp Hematol* 2005;33:573–83.
121. Selmani Z, Naji A, Zidi I, 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⁺CD25^{high}FOXP3⁺ regulatory T cells. *Stem Cells* 2008;26:212–22.
122. Bartholomew A, Sturgeon C, Siatskas M, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* 2002;30:42–8.
123. Yanez R, Lamana ML, Garcia-Castro J, et al. Adipose tissue-derived mesenchymal stem cells (AD-MSC) have in vivo immunosuppressive properties applicable for the control of graft-versus-host disease (GVHD). *Stem Cells* 2006;24:2582–91.
124. Zhang J, Li Y, Chen J, et al. Human bone marrow stromal cell treatment improves neurological functional recovery in EAE mice. *Exp Neurol* 2005;195:16–26.
125. Djouad F, Fritz V, Apparailly F, et al. Reversal of the immunosuppressive properties of mesenchymal stem cells by tumor necrosis factor alpha in collagen-induced arthritis. *Arthritis Rheum* 2005;52:1595–603.
126. Devine SM, Bartholomew AM, Mahmud N, et al. Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. *Exp Hematol* 2001;29:244–55.
127. Noort WA, Kruisselbrink AB, In't Anker PS, et al. Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. *Exp Hematol* 2002;30:870–8.

128. Ringden O, Uzunel M, Rasmusson I, et al. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* 2006;81:1390–7.
129. Devine SM, Cobbs C, Jennings M, et al. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into non-human primates. *Blood* 2003;101:2999–3001.
130. Ruster B, Göttig S, Ludwig RJ, et al. Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood* 2006;108:3938–44.
131. Sordi V, Malosio ML, Marchesi F, et al. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood* 2005;106:419–27.
132. Son BR, Marquez-Curtis LA, Kucia M, 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;24:1254–64.
133. Karp JM, Teo GSL. Mesenchymal stem cell homing: the devil is in the details. *Review Cell Stem Cell* 2009;4:206–316.
134. Koc ON, Gerson SL, Cooper BW, et al. Rapid hematopoietic recovery after co-infusion 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;18:307–16.
135. Lazarus HM, Curtin P, Devine S, et al. Co-transplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. *Biol Blood Marrow Transplant* 2005;11:389–98.
136. Müller I, Kustermann-Kuhn B, Holzwarth C, et al. In vitro analysis of multipotent mesenchymal stromal cells as potential cellular therapeutics in neurometabolic diseases in pediatric patients. *Exp Hematol* 2006;34:1413–9.
137. Koc ON, Peters C, Aubourg P, et al. Bone marrow-derived mesenchymal stem cells remain host-derived despite successful hematopoietic engraftment after allogeneic transplantation in patients with lysosomal and peroxisomal storage diseases. *Exp Hematol* 1999;27:1675–81.
138. Krivit W, Shapiro EG, Lockman LA, et al. Bone marrow transplantation: treatment for globoid cell leukodystrophy, metachromatic leukodystrophy, adrenoleukodystrophy and Hurler syndrome. In: Moser HW, Vinken PJ, Bruyn GW, editors. *Handbook of clinical neurology*. Amsterdam: Elsevier Science; 1996. p. 87–106.
139. Koc ON, Day J, Nieder M, et al. Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH). *Bone Marrow Transplant* 2002;30:215–22.
140. Sillence DO, Rimo DL, Danks DM. Clinical variability in osteogenesis imperfecta-variable expressivity or genetic heterogeneity. *Birth Defects Orig Artic Ser* 1979;15:113–29.
141. Horwitz EM, Prockop DJ, Gordon PL, et al. Clinical responses to bone marrow transplantation in children with severe osteogenesis imperfecta. *Blood* 2001;97:1227–31.
142. Horwitz EM, Gordon PL, Koo WK, et al. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. *Proc Natl Acad Sci U S A* 2002;99:8932–7.

143. Ball LM, Bernardo ME, Roelofs H, et al. Co-transplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haplo-identical hematopoietic stem cell transplantation. *Blood* 2007;110:2765–7.
144. Le Blanc K, Rasmusson I, Sundberg B, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004;363:1439–41.
145. Le Blanc K, Frassoni F, Ball LM, et al. Use of bone marrow derived expanded mesenchymal stem cells in the treatment of steroid refractory acute GvHD. *Lancet* 2008;371:1579–86.
146. Available at: <http://investor.osiris.com/releases.cfm>.