Peripheral Progenitor Cell Graft in the Rat: A Technique of Graft Processing

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Summary

The aim of this study was to establish a procedure for blood progenitor cell graft processing in rats.

As a first step the mobilization protocol was optimized. The second step was dedicated to define the optimal source for subsequent graft manufacturing: either peripheral blood or spleen. The third step was designed to establish a protocol for purification of stem cells.

The best mobilization results in terms of white blood cell count, granulocyte colony forming units (CFU-G) and CD90 positive progenitor cells were obtained after pre-treatment of the donors for 5 days with recombinant human granulocyte colony stimulating factor (100 µg/kg) in combination with murine stem cell factor (33 µg/kg).

Splenectomy prior to mobilization increased the yield of stem cells from peripheral blood. The numbers of CD90-positive progenitor cells recovered from the spleen of one rat after stem cell mobilization were sufficient to generate one stem cell graft.

Grafts containing 1 x 10^6 progenitor cells – and thus sufficient for transplantation - were obtained after T-cell depletion and positive selection of CD90 positive cells. The grafts were characterized and showed a purity exceeding 70%, a T-cell depletion of 3.6 $\log_{10}$ and a 3-fold increase in CFU-G compared to the yield post mobilization.

Introduction

The long-term success of organ transplantation is hampered by severe side effects from the immunosuppressive therapy. Diabetes and hypertension, but ultimately also kidney failure are potential sequelae. Induction of stable donor-specific tolerance appears an appealing concept to overcome this problem.

The main, and meanwhile well-accepted strategy (*Ildstad et al., 1984*), to induce donor-specific tolerance is the generation of macrochimerism by way of bone marrow transplantation after myeloablative treatment of the recipient. Engraftment and recovery of the recipient is followed by organ transplantation from the same donor as the bone marrow; the organ graft is tolerated without any further immunosuppression (*Kadry et al., 2003; Kim et al., 2006*). Living organ donation opens the door for induction of donor specific tolerance, since the potential donor of the organ could also donate hematopoietic stem cells.

Successful induction of tolerance before organ transplantation depends on two prerequisites. First, the stem cell graft has to be purified to deplete alloreactive T-cells. Alloreactive, cytotoxic T-cells infused with a stem cell graft can cause severe, even fatal acute graft versus host disease. Prophylactic immunosuppressive treatment after stem cell trans-

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plantation can only be avoided if a highly effective removal of T-cells is achieved by effective selection of stem cells from the graft. Secondly, the toxicity of the immunoablative conditioning regimen has to be reduced in patients with end-stage organ failure. Currently, conditioning prior to transplantation of purified peripheral blood stem cells is based on aggressive myeloablative regimens employing total body irradiation or busulfan (*Urbano-Ispizua et al., 2002*).

In the clinical setting, granulocyte colony stimulating factor (G-CSF) induces mobilization of bone marrow derived stem cells into the peripheral blood. Peripheral blood stem cells obtained by leukapheresis are a superior stem cell source for graft manipulation procedures compared to bone marrow. Contamination with non-hematopoietic cells and cellular debris is lower in peripheral blood stem cells resulting in higher quality purification results. Clinical scale techniques to purify peripheral blood stem cells using immunomagnetic cell selection (MACS) of CD34-positive stem cells have been developed and established in clinical trials (*Beelen et al., 2000*). Graft processing by efficient enrichment of stem cells using a combination of positive and negative cell selection leads to a very efficient depletion of contaminating T-cells; compared to T-cell depletion alone purity is about 1 $\log_{10}$ higher. This approach was successfully used to reduce alloreactivity resulting in graft-versus-host disease (GVHD). Peripheral blood stem cell grafts from siblings but also from HLA-haploidentical family donors were successfully used in transplantions in leukemia patients without any prophylactic immunosuppression post-transplant (*Elmaagacli et al., 2003; Handgretinger et al., 2001*).

However, as leukapheresis is not applicable in small rodents, peripheral blood stem cells could not be introduced into experimental stem cell transplantation and tolerance induction experiments in the past. Therefore, the aim of this study was to establish a protocol for the production of a highly purified peripheral blood stem cell graft in the rat. A blood stem cell graft in combination with subsequent kidney but also liver and partial liver transplantation in the rat could be an extremely useful animal model for research on tolerance induction after organ transplantation.

Materials and Methods

Experimental design

The most efficient mobilization regimen was assessed by comparing different protocols in respect to the absolute and differential white blood count, relative frequency and absolute numbers of CD90 positive cells and number of granulocyte colony forming units (CFU-G). The compartment, peripheral blood or spleen, suited best for obtaining the peripheral progenitor cells, was identified by assessing the yield and purity isolated from either source. Splenectomy prior to mobilization was performed to confirm the relevance of this compartment. Finally a protocol for graft processing leading to a high purity and yield of positively selected stem cells was established.

Animals

Adult Lewis rats weighing 250-350g were obtained from Charles River (Charles River, Wiga GmbH, Sulzfeld, Germany). The animals were housed under standard animal care conditions (12h light/dark cycle at 22°C) and were fed with rat chow *ad libitum*. All procedures were carried out according to the German Animal Welfare Legislation.

Splenectomy before mobilization of stem cells

Inhalation anesthesia was performed using isoflurane at a concentration of 1.5% - 3% and an oxygen flow of 0.5 l/min (Sigma Delta Vaporizer, UNO, The Netherlands). After opening the abdomen with a transversal incision a splenectomy was performed.

Mobilization

Filgrastim (Amgen GmbH, Munich, Germany), a recombinant human G-CSF, was administered subcutaneously doses ranging from 10 µg/kg to a max-

imum of 250 µg/kg for 5 up to 8 days to establish the optimal dosing schedule.
Recombinant murine stem cell factor (R&D Systems Inc., Minneapolis, MN) was added and administered subcutaneously at a dose of 33 µg/kg together with G-CSF for 5 days.

Staining (NASDCL)
Liver and spleen tissue samples were fixed in 4.5% buffered formalin for at least 24h. Paraffin embedding was performed using standard techniques. Sections (4µm) were cut and a NASDCL staining was performed (*Leder, 1970*). In NASDCL stained sections, both granulocytes and precursor cells show a red staining of the cytoplasm. Precursor cells are characterized by the large cell size, a large round nucleus and the bright red cytoplasm. They can be differentiated from segmented granulocytes (which also show a red cytoplasm) morphologically, because of the smaller size and their segmented nuclei.
To count the number of precursor cells in the spleen and the liver, digital images were taken at x 200 magnification (low power field) under a light microscope (Leica DMLB, Germany) using a digital camera (Coolpix 995, Nikon, Japan). 10 pictures containing portal tract and periportal areas were captured for each liver section. Precursor cells were counted manually.

White blood cell count
White blood cells (WBC) were counted with an automated cell counter (ACT-8, Beckman Coulter GmbH, Krefeld, Germany). Differential white blood count was performed on Giemsa-stained smears at a magnification of x 1000.

Flow cytometry
The hematopoietic cells in peripheral blood, bone marrow and the splenic isolate were analyzed by flow cytometry (Epics XL, Beckman Coulter GmbH, Krefeld, Germany). 1 x 10^6 cells were stained with antibodies against CD45 (clone OX-1), CD3 (clone G4.18), the αβ-T-cell receptor (clone R73), Pan-T-cells (clone OX-52), CD4 (clone OX-38), CD8a (clone OX-8), CD25 (clone OX-39), CD45R (clone HIS24), CD161a (clone 10/78) and CD90 (clone OX-7) (BD Biosciences Inc., PharMingen, Heidelberg, Germany). Cells were also incubated with FITC-mouse IgG1 and PE-mouse IgG1 as an isotype control to show specificity. Cell viability after processing was confirmed by staining with 7-AAD Viability Dye (Beckman Coulter GmbH, Krefeld; Germany).

CFU assay
The granulocyte colony forming unit assay already established as a routine procedure for human bone marrow transplantation in our hospital was modified in respect to the cytokines used. Recombinant human G-CSF and recombinant murine stem cell factor were incorporated in the procedure.
Methylcellulose was dissolved in boiling sterile distilled water in a concentration of 4.2% and diluted 1:1 with Iscove's Modified Dulbecco's Medium (Gibco, Life Technologies, Paisley, UK). For plating, 30% fetal calf serum (Cytogen, Berlin, Germany) and mercaptoethanol (Merck KGaA, Darmstadt, Germany) 1:300.000 was added. Recombinant murine stem cell factor (100 ng) and Filgrastim (100 ng for peripheral blood or 10 ng for spleen cells) was added per 35 mm petri dish. Each dish was plated with 1 x 10^5 cells in 1 ml or 3 x 10^4 purified stem cells in 1 ml methylcellulose preparation respectively and incubated at 37°C in a humidified incubator with an atmosphere of 5% CO2. After 14 days of culture, colony-forming units-granulocyte (CFU-G) were scored under an inverted microscope (Leitz, Germany) using a magnification of x 25.

Harvesting of peripheral stem cells
Rats subjected to the mobilization protocol and control animals were sacrificed in general anesthesia. Peripheral blood and spleen were used as sources for graft processing.

Graft processing

Graft processing was performed under sterile conditions in a class II laminar air flow bench. The spleen cells were obtained by mincing the spleen into small pieces and passing the fragments through a BD Falcon™ 40 μm cell strainer (BD Biosciences Inc., Discovery Labware, Heidelberg, Germany). Mononuclear cells from either peripheral blood or spleen were separated by density gradient centrifugation with Lymphoprep® (Greiner Bio-One GmbH, Essen, Germany) at 1000 g for 10 minutes. For magnetic cell sorting the autoMACS device (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) was used according to the instruction manual in a two-step procedure.

In the first step, T-cells were depleted. Different general T-cell antibodies were tried with equal results; T cells were labeled either indirectly with the biotinylated antibody anti-CD3 (clone G4.18; BD Biosciences Inc., Heidelberg, Germany) and anti-Biotin Microbeads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) or directly with anti-Pan-T-cell Microbeads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). Indirect labeling with an antibody for the αβ-T-cell receptor (clone R73; BD Biosciences Inc., Heidelberg, Germany) was similarly effective, but leaves γσ-T-cells in the graft, a T-cell subset that possibly improves tolerance induction.

The second step included a positive selection of CD90 positive cells by indirect labeling with phycoerythrin (PE)-conjugated CD90 antibodies (BD Biosciences Inc., PharMingen, Heidelberg, Germany) and anti-PE Microbeads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). Purity and recovery were determined by flow cytometry using the other two T-cell-antibody clones than the one used for T-cell depletion; the CD90 positive cells were already stained with the PE-conjugated antibody used for sorting.

Results

Optimal mobilization protocol

For determination of the optimal mobilization protocol, G-CSF was used in doses between10 μg/kg and 250 μg/kg administered to the rat for 5 to 8 days prior to blood sampling and splenectomy. At a dose of 10 μg/kg G-CSF did not show an effect. Neither dose escalation above 100 μg/kg G-CSFdaily, nor a prolonged stimulation beyond 5 days improved mobilization of bone marrow derived stem cells further. Adding recombinant murine stem cell factor at a dose of 33 μg/kg led to an effective mobilization of stem cells into the periphery. Baseline white blood cell count was doubled and recovery of CD90-positive cells was increased 6-fold (see Figure 1). Treatment with G-CSF in combination with recombinant murine stem cell factor led to a median number of 17 colonies per 10^5 WBC plated in the granulocyte CFU-assay (see Figure 2) compared to a median of 6 colonies per 10^5 WBC in rats subjected to mobilization with Filgrastim only and nil to single colonies per 10^5 WBC in the control group.

Homing of mobilized progenitor cells to the spleen

Hematopoietic stem cells were mobilized in Lewis rats with G-CSF at a dose of 100 μg/kg body weight daily for 5 consecutive days. Splenectomy was performed in half of the animals before administration of Filgrastim.

The additional splenectomy did not only induce a substantial increase in the absolute number of circulating WBC compared to the mobilization only protocol (see Figure 3A), but did also lead to a relative enrichment of CD90 positive cells (see Figure 3B).

The WBC count increased from a maximum of 12/nl to 25/nl in animals subjected to mobilization only. After mobilization and splenectomy WBC count reached 48/nl. The relative frequency of CD90 positive cells was similar in naïve animals and those subjected to mobilization only, ranging between 5 and 15%, whereas it was almost doubled in the splenectomy group with a relative frequency ranging between 17 and 26%. These observations suggest a preferred mobilization of progenitor cells into the spleen.

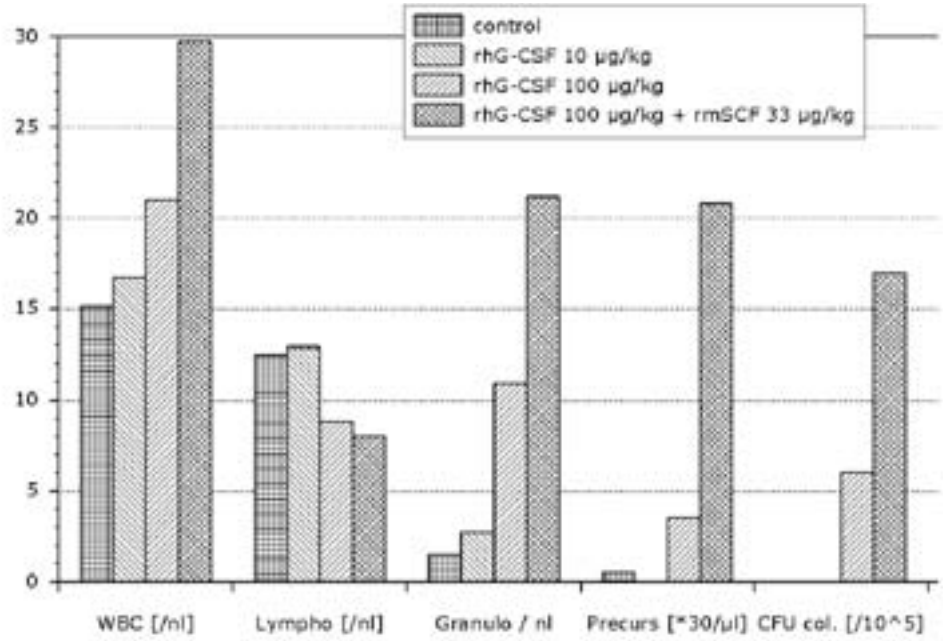

Figure 1. Mobilization of peripheral blood stem cells using 3 different protocols with daily cytokine application and sampling after 5 days. [WBC = white blood-cell count, Lympho = lymphocyte count, Granulo = neutrophil count, Precurs = hematopoietic progenitor cell count; rhG-CSF = recombinant human G-CSF, rmSCF = recombinant murine stem cell factor]

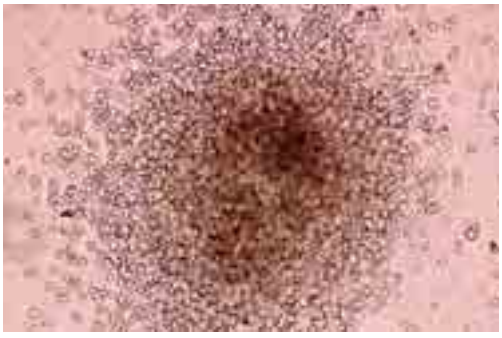

Figure 2. Colony forming unit.

Following mobilization, hematopoietic precursor cells did not only migrate to the spleen, but were also detected in the liver. Upon staining with NAS-DCL, large cells round nuclei and bright red staining of the cytoplasm were detected mainly in portal tracts, but also in liver parenchyma, suggesting the migration of progenitor cells into the liver (see figure 4).

Graft processing

About 20% of the CD90 positive cells constitute the stem cell compartment (*McCarthy et al., 1987a*). At least 4 x 10^6 stem cells per kg, and thus 20 x 10^6 CD90 positive cells per kg body weight of the recipient are needed for a sufficient graft (*Reddy, 2005*). Using rats with 250 gram body weight as recipients, at least 5 x 10^6 CD90 positive cells per graft are needed. Assuming a recovery of 30% after a 2-step selection procedure, a minimum of 15 x 10^6 CD90 positive cells is necessary prior to graft processing.

After gradient centrifugation using Ficoll (Amersham Pharmacia, USA), the splenic cell population contained a median of 6.5% CD90 positive cells corresponding to a median number of 15 x 10^6

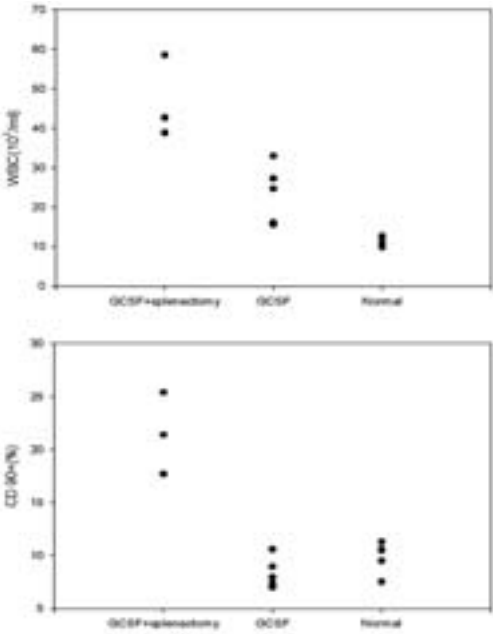

Figure 3. Effect of G-CSF treatment: A, Peripheral blood white blood cell count (WBC) with highest increase in splenectomized rats and B, percentage of CD90 positive cells showing an increased stem cell mobilization to the peripheral blood in asplenic animals.

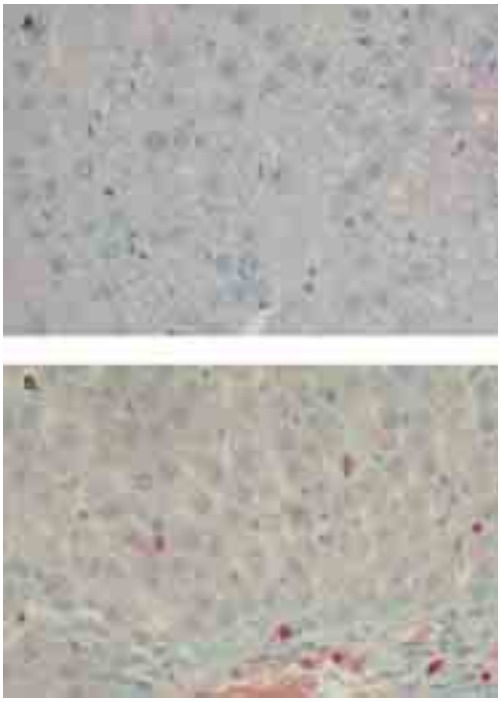

Figure 4. Stem cell clusters mobilized to the liver (NASDCL staining). A, untreated animal; B, G-CSF treated rat.

CD90 positive cells (see figure 5A). Immunomagnetic sorting using the two-step procedure described above resulted in a marked depletion of T-cells (see Figure 5B). As expected, graft processing using immunomagnetic selection caused losses of 30% to 50% of progenitor cells per step, therefore about 30% of progenitor cells could be recovered in the two step procedure. The T-cell depletion was further improved by nearly ten-fold using a second step of positive selection (CD90). The T-cell depletion efficacy reached 3.6 ± 0.5 $\log_{10}$ with a purity of CD90 positive cells of 76% ± 12% as shown in figure 5. The recovery of CD90 positive cells reached 34% ± 7%. The number of G-CFU colonies per 10^5 plated splenic white cells increased from 101 ± 33 post mobilization to 301 ± 5 after the selection procedure. The processing resulted in grafts containing 10.4 ± 0.5 x 10^6 CD90 positive cells and 3.7 ± 2.5 x 10^5 T-cells.

Excellent viability of the selected progenitor cell rich population was demonstrated using 7-AAD staining (data not shown).

Discussion

Induction of tolerance is the holy grail of organ transplantation *(Bluestone et al., 2000, Helderman et al., 2000; Norris, 2001*). One of the successful strategies to induce classical donor-specific transplantation tolerance is via the induction of mixed chimerism using bone marrow as source of donor stem cells (*Delis et al., 2004*).

Differences in immunologic properties of grafts prepared from peripheral stem cells compared with those processed from bone marrow have been

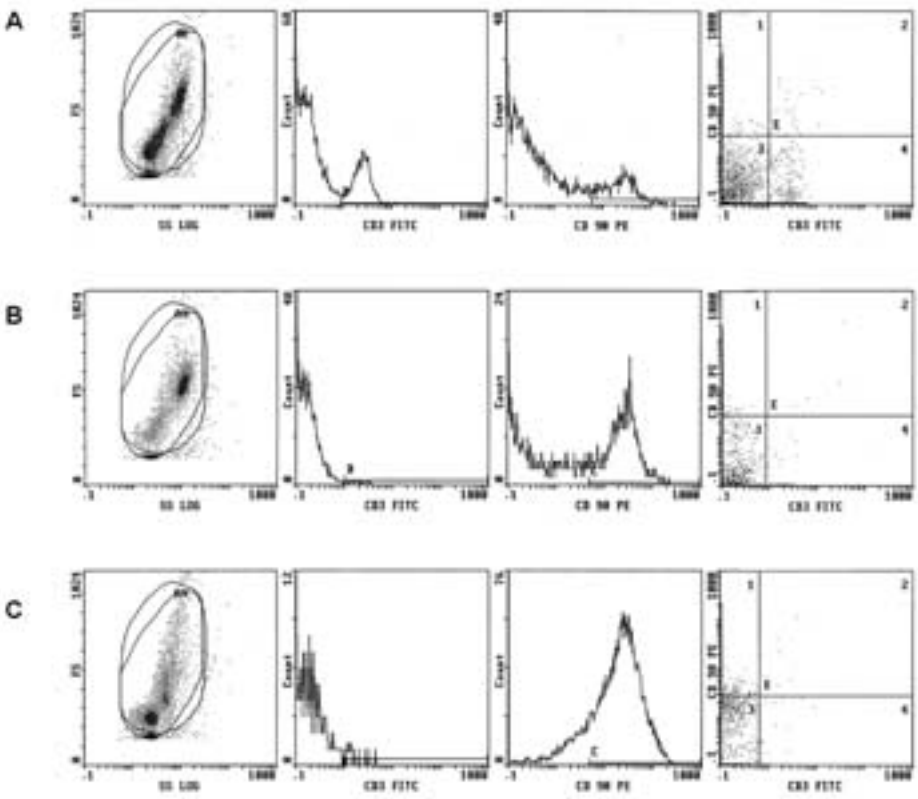

Figure 5. Results of stem cell purification by MACS: A, mononuclear cell population before processing, B, after T-cell depletion using the αβ-T-cell-receptor antibody clone R73; and C, after selection of CD90 positive cells.

described. Peripheral stem cell grafts have a higher absolute T-cell content, but T-helper cell populations shift to the TH_2-type after G-CSF treatment (*Stull, 2002*). There is an association between the presence of TH_2 phenotypic clones and a tolerant state (*Bromberg et al., 2001*). Furthermore clinical scale processing of hematopoietic grafts by MACS could be well established for peripheral blood, but this procedure is as suitable for bone marrow with its much higher content of contaminating non-hematopoietic cells, fat and bone particles.

Hematopoietic stem cells can be mobilized into the peripheral blood in rodents by the combined application of recombinant human G-CSF and recombinant murine stem cell factor (*Dunbar et al., 1996b*). The efficiency of different combinations of cytokines on Louvain rat blast cell cultures has been investigated by Lucas et al. (*Lucas et al., 1999b)*. Ex-vivo, the combination of stem cell factor with G-CSF showed the highest number of colonies in comparison to the combination of stem cell factor with other cytokines, an observation, which could be confirmed in-vivo in our experiments. Furthermore, the omission of stem cell factor resulted in apoptosis of the progenitor cell population their experiments.

Filgrastim, the recombinant human G-CSF, is used successfully for stem cell mobilization in humans and is readily available. The homology of the human G-CSF to rat colony stimulating factor 3 is

77% for *Rattus norvegicus* as calculated using BLAST 2.0 (The National Center for Biotechnology Information, NIH, USA). Injecting Filgrastim at a dose of 100 µg/kg for 5 consecutive days subcutaneously was sufficient to mobilize stem cells in the Lewis rat. Further dose escalation did not improve the results. Stem cell mobilization could be enhanced greatly by the combination of Filgrastim with murine stem cell factor at a dose of 33 µg/kg as published for mice (*Dunbar et al., 1996a*).

It is known from mouse experiments that bone-marrow derived mobilized stem cells migrate preferentially to the spleen (*Molineux et al., 1990*). This is the reason for splenectomy prior to cytokine application when stem cell mobilization into the peripheral blood is required in mice (*Lord et al., 1995*). We could demonstrate the same effect in the rat with preferential migration of most mobilized progenitor cells to the spleen, but also to the liver.

The spleen was chosen as source of mobilized progenitor cells in the rat as it was expected and later demonstrated, that one spleen was sufficient for processing one progenitor cell graft. The number of peripheral progenitor cells migrating to the spleen allowed for the production of grafts containing at least 4 x 10^6 hematopoietic progenitor cells per kg body weight of the recipient.

Highly specific progenitor cell antigens such as CD34 or CD133 in humans have not been defined in the rat yet. CD90 (Thy-1) is expressed on hematopoietic progenitors in high epitope density and reacts with the antibody clone OX-7. CD90 is not exclusively expressed on hematopoietic stem cells, but these can be found by flow cytometry in the upper 20% of the OX-7 positive cells (*Lucas et al., 1999a, McCarthy et al., 1987b*). However, CD90 is also expressed on subpopulations of T-cells (*Hosseinzadeh et al., 1993*), which could contribute to the development of acute graft versus host disease. Therefore, T-cells have to be depleted efficiently in a first step.

Multiple antibodies against T-cell antigens are available for the rat, as are CD3 (clone G4.18), CD6 (clone OX-52) or the αβ-T-cell receptor (clone R73). Taking advantage of the different properties of T-cell antigens defined by the respective antibody allows processing of grafts tailored to the specific needs. Depletion of T-cells using the anti-αβ-T-cell receptor antibody results in a graft containing the αβ-T-cell receptor negative T-cell subpopulation. This T-cell subpopulation has been shown to reduce the risk of acute graft-versus-host-disease (*Neipp et al., 1999*) and might contribute to the induction donor-specific transplantation tolerance. Immunomagnetic T-cell depletion and subsequent selection of CD90 positive cells resulted reproducibly in high quality grafts. These were characterized by an excellent viability tested in two different assays (7-AAD-staining and flow cytometric analysis, G-CFU assay), a sufficient number of CD90-positive progenitor cells and a nearly four $\log_{10}$ T-cell depletion.

In conclusion, we defined a procedure to produce highly purified progenitor grafts in the rat. This is the first step prior to experiments regarding tolerance induction via stem cell transplantation and subsequent challenge with a donor specific organ graft.

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