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D I S S E R T A T I O N

Use of mobilized peripheral blood stem cells (mPBSC) for the induction of mixed chimerism and tolerance

ausgeführt zum Zwecke der Erlangung des akademischen Grades eines Doktors der Naturwissenschaften unter der Leitung von

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CONTENT OF THE THESIS

This dissertation was performed in Transplant Laboratory of Vienna General Hospital, Medical University of Vienna in team of Prof. MD. Thomas Wekerle under project "Tolerance through hematopoietic cell transplantation with costimulation blockade".
Most of the work in this dissertation was done by me including; preparation of experiments, mice splenectomy, preparation, isolation and transplantation of mPBSC and BMC, in vitro MACS T cell depletion of mPBSC, analysis and interpretation of data, Flow Cytometry, MLR assays, serum cytokine measurement, experimental follow up, analyses and interpretation of data.

Due to the enormous quantity of work, especially at the start day (d0) of experiment, which require a team work, some technical help was provided from the colleagues of Transplant Laboratory; Peter Blaha MD, Sinda Biegenzahn MD and Ines Pree Ms.Sc. helped in some experiments by BMC isolation and transplantation as well by procedure of skin grafting. Patrich Nierlich MD additionally helped in two of 13 allogeneic experiments by mice splenectomy and monitoring of skin graft test. Elahi Fariborz Ph.D. experimentally performed **C**olony-**F**orming **U**nit assay, and Edgar Selzer MD as a specialist of radiotherapy and radiobiology was involved in the process of mice irradiation. Franz Winkler technician of Transplant Laboratory often assisted in mice bleeding and flow cytometry staining.

Prof. MD. Thomas Wekerle was the scientific supervisor of this work.

SUMMARY

The number of allotransplantations performed from the grafts obtained from the living donor has dramatically increased in the past few decades. Living (un)related donor transplantation offers advantage of a scheduled procedure in which potential donor manipulation (exp. mPBSC) is feasible before organ harvest. However, due to the acute rejection episodes, chronic graft dysfunction and the toxicity of long-term immunosuppression, outcome of living (un)related donor transplantation is still far from being optimal. A possible solution to this problem would be an induction of specific tolerance through the state of chimerism (representation of donor cells in the host of more than 1% and less 100 %, induced after transplantation of donor hematopoietic cells), free of general immunosuppression. Such irradiation free protocol employing costimulatory blocking antibodies (anti-CD40L and CTLA4Ig) and a high dose of allogeneic fully MHC mismatched BMC [10x more than standard dose ($20x10^6$ BMC)] has been recently developed in a experimental murine model [1]. The major obstacle for clinical application of this protocol is that such a high number of bone marrow cells (BMC) is clinically unobtainable. At the present, highest number of heamatopoietic stem cells (HSC) can currently be obtained through the collection of mPBSC from a living donor by the process called leukapharesis. Thereof, the aim of my study was to explore the possibility to substitute the BMC with mPBSC and to eventually develop an irradiation-free murine model employing mPBSC, which could possibly serve as the base for the future clinical application.

The first part of my study revealed the comparison of the engraftment properties of BMC vs. mPBSC in the murine syngeneic model. The major conclusion of this part is that unseparated mPBSC can successfully engraft, inducing long-term chimerism at significantly lower level than the one induced with the same number of BMC. I found that even the transplantation of similar amounts of c-kit$^+$ (CD-117$^+$) cells led to the lower levels of established chimerism in the recipients of mPBSC. Nevertheless, under non-myeloablative conditions murine progenitor cells contained in either mPBSC or BMC have similar engraftment characteristics, inducing comparable multi-lineage

macrochimerism. These findings strongly support evaluation of allogeneic mPBSC aiming to develop clinically more relevant mixed chimerism protocols.

In the second part of my work, I tried to substitute the BMC with mPBSC in a murine allogenic non-myeloablative model. Surprisingly, even the transplantation of high dose of mPBSC combined with co.bl. (costimulatory blockade) and non-myeloablative doses of total body irradiation (TBI) failed to induce mixed chimerism in any of transplanted mice. This effect was even more surprising knowing that the same amount of BMC transplanted with co.bl and even without TBI, induced chimerism in 60% of transplanted mice [1]. mPBSC recipients showed typically higher donor reactivity in mixed lymphocyte reaction assays (MLR) compared with the recipients of BMC. In an effort to overcome immunological rejection of mPBSC, I found that only intensified conditioning which involves both donor specific transfusion (DST) and treatment with immunosuppressive drugs (IS), induces mixed chimerism and tolerance. However it must be noted that this conditioning also induce unwished side effect in terms of the graft versus-host disease (GVHD). In order to clarify the immunological rejection mechanism of mPBSC, I performed several different experiments in which $60x10^6$ mPBSC were transplanted together with standard bone marrow transplantation (BMT) protocol ($20x10^6$ BMC, 3Gy TBI plus co. bl.). With this BMT protocol it is usually possible to routinely induce mixed chimerism and tolerance in majority of transplanted mice. This co-transplantation of BMC and mPBSC led to the prompt rejection of transplanted cells. Moreover, I found that the later transplantation of mPBSC (d+94) in previously mentioned model abolishes the abrogation of chimerism, thus translating the chimeric recipients into the full chimeras. These findings are in accordance with previously published data (Sykes et al, Blood 2004) [2] which show that late donor lymphocyte infusion (d35) after HSCT, induce full chimerism. Further, using the same experimental model and either by the in vitro T cell depletion of mPBSC or by additional injection of recipient mice with in vivo Tc depleting mAb (αCD4 and αCD8 at d0), I clearly demonstrated that the T cells contained in mPBSC are the cell subpopulation responsible for immunological rejection. Consequently, due to those additional conditionings, transplanted mice developed stable long-term chimerism and tolerance.

Similarly as in costimulatory model, transplantation of mPBSC in murine model without co.bl. (employing the αCD4 and αCD8 depleting mAb at d-5 and d-1) showed that contrarily to BMC recipients, none of the mice transplanted with mPBSC developed long-term chimerism. This result strongly confirmed hypothesis that costimulatory blockade doesn't differently influence the outcome of mPBSCT in comparison with BMT.

These findings of mPBSC behaviour in recipients of co.bl., warrant consideration in the development of (pre-) clinical tolerance protocols employing mPBSC.

ZUSAMMENFASSUNG

Die Zahl der allogenen Organtransplantate mit Transplantaten von Lebendspendern ist in den letzten Jahrzehnten dramatisch angestiegen. Transplantate von lebenden (nicht)verwandten Spendern bieten die Möglichkeit einer vorgeplanten Behandlung, welche die Manipulation des Organs vor der Entnahme ermöglich. Trotzdem ist das Ergebnis von Lebendspender-Transplantaten wegen einer Vielzahl von Komplikationen, wie akute Abstoßung, Fehlfunktion des Transplantats und der Toxizität einer Langzeit-Immunsuppression, längst nicht optimal. Eine mögliche Lösung für dieses Problem wäre die Induktion einer spezifischen Toleranz durch Chimärismus (das Vorhandensein einer Population > 1% und < 100% von Spenderzellen im Empfänger durch die Transplantation von hämatopoetischen Spenderzellen) ohne allgemeine Immunsupression. Im experimentellen Mausmodell wurde kürzlich ein Protokoll entwickelt, welches ohne Bestrahlung dafür mit Einsatz von Kostimulations-Blockern (anti-CD40L und CTLA4Ig Antikörper) und einer hohen Dosis von allogenen Knochenmarkszellen (10x mehr als die Standarddosis von $20x10^6$ Zellen) Toleranz induziert [1]. Das größte Hindernis für die klinische Anwendbarkeit stellt dabei die hohe Anzahl an Knochenmarkszellen dar. Derzeit kann durch einen Prozess namens Leukapharese die größtmögliche Zahl an hämatopoetischen Stammzellen gewonnen werden. Hierbei werden periphere Blutstammzellen des Lebendspenders aufgereinigt. Das Ziel dieser Studie war deshalb zu untersuchen ob die Möglichkeit besteht Knochenmarkszellen durch periphere Blutstammzellen zu ersetzen und ein bestrahlungsfreies Transplantationsprotokoll im Mausmodell zu entwickeln. Ein solches Protokoll könnte in der Zukunft als Basis für klinische Anwendungen dienen.

Der erste Teil meiner Studie beschäftigt sich mit dem Vergleich Möglichkeiten der Verpflanzung von Knochenmarkszellen und peripheren Blutstammzellen im syngenen Mausmodell. Die Hauptaussage die man aus diesem Teil des Projekts ableiten kann ist dass unseparierte periphere Blutstammzellen zwar erfolgreich verpflanzt werden können und Langzeitchimärismus induzieren, jedoch signifikant niedrigere Chimärismus-Spiegel als mit der gleichen Zahl an Knochenmarkszellen erreicht werden. Selbst die Transplantation von gleichen Zahlen an aufgereinigten Zellen die den Stammzellmarker

c-kit exprimieren, führte zu niedrigeren Chimärismus-Spiegeln in Empfängern von peripheren Blustammzellen. Trotzdem zeigten unter nicht-myeloablativen Bedingungen die murinen Vorläufer-Zellen in sowohl peripherem blut als auch Knochenmark die gleichen Verpflanzungs-Charakteristika und induzierten vergleichbaren Makrochimärismus. Diese Resultate untermauern die Bedeutung peripherer Blutstammzellen mit dem Ziel der Entwicklung klinisch relvanter Protokolle im gemischten Chimärismus Modell.

Im zweiten Teil meiner Arbeit versuchte ich, in einem allogenen Mausmodell bei nicht-myeloablativer Bestrahlung, Knochenmarkzellen durch periphere Blutstammzellen zu ersetzen. Überraschenderweise konnten nicht einmal hohe Dosen an peripheren Blutstammzellen, in Kombination mit Kostimulationsblockade und nicht-myeloablativer Bestrahlung, gemischten Chimärismus in den behandelten Mäusen induzieren. Dieses Resultat war deshalb so unerwartet, weil bekannt ist, dass die gleiche Menge an Knochenmarkzellen ausreicht, um mit Kostimulationsblockade aber ohne Bestrahlung, Chimärismusraten von 60% zu erzielen. Empfänger von peripheren Bluttammzellen zeigten typischerweise eine höhere Spenderreaktivität in Lymphozyten-proliferations-Experimenten verglichen mit Empfängern von Knochenmark. Mit dem Bestreben, die immunologische Abstossung der Blustammzellen zu umgehen, fanden wir heraus, dass nur zusätzliche Maßnahmen, die sowohl Spenderspezifische Infusionen als auch Immunsuppression beinhalten, gemischten Chimärismus und somit Toleranz erzielen. Dabei muss man beachten, dass diese Behandlung auch unerwünschte Nebenwirkungen im Sinne von GVHD. Um den Mechanismus der immunologischen Abstoßung der Blutstammzellen zu klären, führten wir einige unterschiedliche Experimente durch, in denen $60x10^6$ Periphere Blutstammzellen in Kombination mit dem Standard-Knochenmark-Transplantationsprotokoll verabreicht wurden. Mit diesem Protokoll ist es normalerweise routinemäßig möglich, gemischten Chimärismus und Toleranz im Grossteil der Mäuse zu erzielen. Diese Kotransplantation von Knochenmark und Blutstammzellen führt zu einer sofortigen Abstoßung der transplantierten Zellen. Weiters haben wir herausgefunden, dass eine spätere Transplantation der Blustammzellen (Tag 94) im eben beschriebenen Modell Chimärismus ermöglicht und bei den gemischten Chimären Empfängern vollen Chimärismus erzielt. Diese Resultate stimmen mit bereits

früher publizierten Daten überein (Sykes et al, Blood 2004), welche zeigen, dass späte Spender-Lymphozyten Infusion (Tag 35) nach Stammzelltransplantation, vollen Chimärismus induziert. Mit dem gleichen experimentellen Modell wurde durch T-Zell Depletion des Spenderblutes bzw. zusätzliche Gabe von T-Zell depletionierenden Antikörpern (antiCD4 und antiCD8 am Tag 0) in die Empfängermaus, gezeigt, dass die im Spenderblut vorhandenen T-Zellen für die immunologische Abstoßung verantwortlich sind. Aufgrund dieser zusätzlichen Maßnahmen konnten die transplantierten Mäuse stabilen Langzeitchimärismus und damit Toleranz entwickeln. Ähnlich dem Kostimulations-Modell, zeigt Transplantation von peripheren Blustammzellen im Mausmodell ohne Kostimulations Blockade (zusätzlich Gabe von antiCD4 und antiCD8 Antikörper am Tag -5 und -1) dass im Gegensatz zu Knochmarkempfängern keine dieser mit peripheren Blutstammzellen transplantierten Mäusen Langzeitchimärismus entwickelte. Diese Resultate bestätigten die Hypothese, dass Kostimulationsblockade keinen wesentlichen Einfluss auf Unterschiede im Ergebnis von Transplantation von peripheren Blutstammzellen im Vergleich zu Knochenmarkzellen hat.

Diese Resultate über die Wirkung und Verhaltensweise von peripheren Blutstammzellen in Empfängern von Kostimulationsblockade müssen bei der Entwicklung von (vor)klinischen Toleranz-Modellen , in denen Blutstammzellen verwendet werden, berücksichtigt werden.

1

INTRODUCTION AND SCOPE OF THIS THESIS

1 INTRODUCTION AND SCOPE OF THE THESIS

1.1. INTRODUCTION

1.1.1. Allotransplantation

There are different types of organ transplantations; *Allogenic transplantation* or *Allotransplantation* (Greek allos: other) means transplantation (organ or cells) between genetically disparate individuals of the same species (human to human or mouse to mouse), *Syngenic transplantation* where transplants are transferred between genetically identical individuals and *Autologous transplantation* where transplants are transferred from one site to another within the same organism. Clinically, due to the limited supply of donor organs, potential of the Xenotransplantation, transplantation between different species (e.g., from pigs to human) has been in the focus of scientific interest for a while. Although the xenotransplantation has recently achieved some fascinating results [3], mostly because of the remaining immunological (immunological disparity between donor and recipient, rejection cell- or antibody-mediated), but also because of the safety (transmission of diseases such as due to some known or unknown bacteria, viruses and prions), and ethical concerns and problems, this type of transplantation still require future exploration and development, and will not be discussed further in this thesis.

From its early beginnings, when in 1902 in Vienna, Ullmann reported the heterotopic placement of kidneys in the necks of dogs and goats, thru the successful transplantation between identical twins in late 1954 in Boston performed by Murray, till today, the transplantations medicine has due to the continued progress, significantly improved the survival of allografts during the first years post-transplant. Unfortunately, still the rate of the long-term morbidity and mortality remains to be substantial problem. The lost of transplanted organs due to the chronic rejection and toxicity of immunosuppressive therapy which is life-long required, are the major limitating issues of this outcome [4]. For possible improvement of the long-term outcome after the allotransplantation the induction of robust donor-specific tolerance would be crucial issue, what is clinically still till nowadays concerned to be a dream or a vision.

1.1.2. Immunosupression

That allogeneic transplant would survive, some degree of immunsuppression is required. Usually immunosuppressive treatments are characterized with unspecific immunosupression of responses to all antigens (not only to those of allograft), what can dramatically increase the risk of infections in recipient. There is also a present risk of cancer, hypertension, and metabolic disease in patients under long-term immunosuppressive treatment. Generally used immunosuppressive treatments involve the use of corticosteroids (such as prednisone and dexamethasone) [5] which are strong anti-inflammatory agents exerting their effects at many levels of the immune response. Corticosteroids are often combined with mycophenolate mofetil (CellCept, Roche). Mycophenolate mofetil (MMF) is the 2-morpholinoethyl ester of mycophenolic acid (MPA), and immunosuppressive agent which is monophosphate dehydrogenase (IMPDH) inhibitor. After oral administration, MMF is rapidly adsorbed following oral administration and hydrolyzed to form MPA, which is the active metabolite and inhibiting IMPDH inhibits the de novo pathway of guanosine nucleotide synthesis without incorporation into DNA. Since T- and B- cells depend critically for their proliferation on de novo synthesis of purines (other cells can utilize salvage pathways), MPA has strong cytostatic effects on T and B cells. There are several other chemical compounds such as Cyclosporin A (CsA) [6], FK506 [7], and rapamycin [8] which are fungal metabolites with potent immunosuppressant characteristics. Calcineurin inhibitors CsA and FK506 are the most important immunosuppressive drugs used at the moment. They function similarly, by blocking the activation of genes encoding IL-2 and the high-affinity IL-2 receptor (IL-2R), which are essential for the T-cell activation. Although a major component of the immunosuppressive effects of FK506 and CsA is concerned to be due to the binding the calcium/calmodulin-dependent phosphatase (calcineurin), leading to inhibition of the activation and nuclear translocation of nuclear factor of activated T cells (NF-AT), it is apparent that FK506 inhibits steps distal to calcineurin activation in the T-cell activation cascade [9]. This important difference between FK506 and CsA may explain why CsA is ineffective in the treatment of allograft rejection [9;10]. Structurally rapamycin is a macrocyclic lactone which was extracted from a soil sample

containing *Streptomyces hygroscopicus* and was named so, because the soil sample was collected on Rapa Nui which is the domicile name for Easter Island. Rapamycin is a potent immunosuppressive agent [11;12], which blocks the proliferation and differentiation of activated T_H cells in the G_1 phase of the cell cycle. Mayor obstacle of previously mentioned immunosuppressant treatments are their unspecifity, meaning that such treatments produce generalized immunosuppression and thereby increase the patients risk for the infections. By the use of monoclonal antibodies (mAb) [13] directed against specific targets on T cells or certain cytokines, more specific immunosupression can be achieved. Injecting the monoclonal antibodies to the allograft recipients directed against certain receptors on T cells (IL2R), can prolong survival of allograft. Anti-IL2R-mAb are directed against α chain (CD25 or Tac: T-cell activation) of the IL2R. IL2R is up-regulated upon the activation on T cells through binding of the allo-antigen to the T-cell receptor. Such mAb used clinically is Daclizumab (Zenapax®), which is humanized mAb with estimated elimination half-life of 20 days in renal transplant patients [14]. Additional advantage of anti-IL2R mAbs is that their use allows substantial reduction of more toxic drugs. Another widely used mAb are directed against CD52. Such mAb is CAMPATH-1 which causes profound T-cell depletion through a process of complement-dependent lysis [15]. There are several types of the CAMPATH-1 antibodies which differs under their immunoglobulin subtype and origin, but their advantage under the other T-cell depletion antibodies are that they can fix both human and rabbit complements, making them extremely useful for both *in vivo* (injected into the patients) and *in vitro* [used by bone marrow transplantation (BMT) or peripheral blood stem cells transplantation (PBSCT)] application [16].

1.1.3. Transplantation tolerance

The precise definition of transplant tolerance can be regarded as the lack of a destructive immune response toward the graft in the absence of ongoing immunosuppressive therapy [17;18] what is clinically manifested by normal graft function in the absence of acute and chronic rejection [19]. More than thirty years ago it was found that human renal allografts in sensitized recipients may undergo strong rejection within minutes or hours of

revascularization [20-23]. This rejection is called hyperacute and today is almost never seen in clinical transplantation. It occurs only a few minutes after the transplantation due to the presence of antibodies [6;24;25] in the host against the tissue to be transplanted, because of the previous transplants or blood transfusions. Besides hyperacute rejection there are also acute [26;27] which begins about 10 days after transplantation (driven by cell-mediated and humoral immunological response) and chronic [28] which develops months or years after acute rejection have subsided.

1.1.4. T- cell tolerance

Induction of T-cell tolerance is concerned to be the solution for prevention of allograft rejection. There are three generally accepted mechanisms of T-cell tolerance: deletion, anergy and suppression (or immunoregulation). Depending on the place where they occur, in thymus (the central organ of T cell development) or outside, they are called *peripheral* or *central* tolerance mechanisms. The main **peripheral tolerance mechanisms** involve *anergy*, *suppression (or immunoregulation*), and *extrathymic deletion*. **Central tolerance mechanisms** involve the *intrathymic anergy* and major central mechanism of tolerance *intrathymic clonal deletion*.

1.1.4.1. *Peripheral tolerance*

As mentioned before peripheral tolerance mechanism involves *anergy*, *suppression* and *extrathymic deletion. Anergy* indicates that a cell is unresponsive to what would normally be appropriate activating stimuli, meaning that reactive T cells are functionally inactivated. To be fully activated T cells require a second ("costimulatory") signal in addition to the signal thru the T-cell receptor which recognizes the antigen in the context of MHC [29-31]. By blocking this second costimulatory signal, anergy could be induced [32]. Nevertheless, anergic T cell is still capable of responding to cytokines [33] (such is IL2, which can reverse this anergic state) if provided by other cells. To remain tolerant, *in vivo* anergized T cells require continual exposure to antigen. Molecular mechanisms underlying *suppression* or *immunoregulation* still remains poorly understood. This mechanism is driven by specialized T cells, which display regulatory and suppressor

function. A major subset of regulatory T cells (T_R) display characteristic $CD4^+CD25^+$ phenotype and act as natural inhibitors of T cell reactivity to autoantigens. "Natural" T_R arise in the thymus, are present at birth and persists through adult life. They constitutively express the α chain of the interleukin2R (CD25) and compromise a minor population of $CD4^+$ T cells (~10% in rodents and ~13% in humans) [34]. Some reports show also that regulatory activity is found in $CD4^+CD25^-$ T-cell population in transplantation models, although at a lower frequency where approximately 10 times more $CD4+CD25^-$ cells than $CD4^+CD25^+$ cells are required to prevent graft rejection [35;36]. Nevertheless, recently it has been shown that irrespective to CD25 expression, suppressor activity of $CD4^+$ cells correlates with expression of FOXP3 (a member of forkhead family of transcription factors) in $CD4^+$ cells [37]. Also some data demonstrates that FoxP3 levels regulate T cell function, and that FoxP3 itself is dynamically regulated during effector T cell differentiation [38]. Although the ideal marker for the regulatory cells has not been found yet, there are several candidate markers; CD45RB, cytotoxic T-lymphocyte antigen 4 (CTLA4; CD152), glucocorticoid-induced tumor-necrosis factor receptor family-related gene (GITR; TNFRSF18), CD122, CD103 and the previously mentioned transcription factor forkhead box P3 (FOXP3) [35]. However, regulatory activity is not limited only on $CD4^+$ T cells. $CD8^+$ [39;40], $CD8^+CD28^-$ [41] and T-cell receptor $(TCR)^+CD4^-CD8^-$ (double negative) cells, as well as natural killer cells (NKT cells) [42;43], have also been shown to have regulatory activities in different situations after transplantation [35]. T_R`s derived from the CD8+ cells have been also described like IL-10 producing $CD8^+$ T_R cells (which seems to be induced by both mature or immature plasmacytoid dendritic cells, and immature myeloid dendritic cells) [44]. Both $CD4^+CD25^+$ and CD8+CD25+ human thymocytes express Foxp3 and GITR mRNA, as well as surface CCR8 and TNFR2 and cytoplasmic CTLA-4 proteins, which are common features of mature Treg cells [45]. Following activation they do not proliferate or produce cytokines, but express surface CTLA-4 and TGF-beta1 [46]. $CD8^+CD28^+$ T suppressor (T_S) cells are generated after stimulation of T cells with allogeneic, xenogeneic or antigen-pulsed autologous APCs [47]. T_S cells are HLA class I/peptide specific with a restricted TCR repertoire, produce no cytokines and are negative for CD28, CD40L and perforin. When APCs come in the contact with these cells, they start to upregulate two inhibitory receptors named ILT3 and

ILT4 [48] and transmit a negative signal to $CD4^+$ T cells, which recognize HLA class II/peptide complexes on the tolerogenic APCs, and become anergic $CD4^+CD25^+$ T cells which than inhibit the activation and differation of the Th1 cells by inducing upregulation of ILT3 and ILT4 on APC which in turn become tolerogenic. $CD4^+CD25^+$ T cells generated under costimulations blockade has been shown to be capable of suppressing rejection initiated by a donor-alloantigen-specific CD8+ TCR-transgenic cells, demonstrating the potency of $CD4^+CD25^+$ T cells.

To maintain the peripheral tolerance, except the anergy and suppression the evidence of *extrathimic deletion* (antigen-specific deletion of T cells in the periphery) are reported as well [49-52]. Extrathimic deletion was noticed also after bone marrow transplantation (BMT) with costimulatory blockade [53;54]. This event appears in the early period after BMT and costimulatory blockade and allows the engraftment of fully MHC-mismatched, allogeneic pluripotent stem cells, which induce central tolerance among T cells that subsequently develop in the thymus [53]. This mechanism could provide effective way for specific elimination (or reduction) of pre-existing donor reactive mature T-cells in a host, without global T-cell destruction. Recently, another peripheral mechanism of deletion has been reported as well. The mechanism of "neglected death" allows that self-reactive $CD8^+$ T cells activated by hepatocytes die by neglect due to the poor production of IL-2 and insufficient expression of survival gene bcl-x_L before they become harmful to body [55].

1.1.4.2. *Central tolerance*

Although it has been shown that T cells can go under anergy [56] or suppression and then be released to periphery, it has also been shown that this cells can again become fully reactive under certain circumstances. *Intrathymic clonal deletion* is concerned as a central mechanism of tolerance. Term "deletion" means that during the maturation in the thymus, T cell expressing T cell receptors (TCR) specific for self-antigens (high-avidity self-reactive thymocytes) are physically destroyed [57-59]. This process is also known as a negative selection [60-62]. Pre-thymocytes are precursors of the T cells (T lymphocytes); they mature in the bone marrow and fetal liver, where the rearrangement of T-cell receptors (TCR) and the change in genetic information required for gamma chains also

occurs. These precursor cells are characterized by the presence of terminal deoxynucleotidyl transferase (TdT) enzyme [63]. After entering in the thymus, these cells differentiate into early thymocytes which carry CD2 and CD7 antigens (first stage of T-cell differentiation). Here in the thymus, transcription of the T-cell receptor's gamma chain and beta chain rearrangement occurs. In this phase cells are so called "double negative" since they do not contain CD4 or CD8 antigen. In the second stage thymocytes contain CD1 antigen but also CD4 and CD8 surface antigen making them "double positive". TCR expression on the cell surface occurs in this phase in conjunction with the alpha and beta chains formation. In this stage, CD3 antigens is being expressed at the cell surface as well. In the third phase of thymocytes maturation (now actual T cells), CD1 antigen is lost and the cells divide in two "single positive" populations that express either CD4 (characteristic for the T-helper T_H cells) or the CD8 antigen [characteristic for cytotoxic T cell population (T_C, CTL)]. More than 99% of T cells express TCRα/β and minority has TCRγ/δ. The process of maturation in thymus ensures that cells released into circulation function in conjunction with the MHC genes of the body's immune system, but do not identify endogenous substances as foreign material. So entering thymus, thymocytes come in contact with thymic epithelial cells leading them into two selection processes. *Positive selection* of thymocytes (expressing receptors capable of binding self-MHC molecules) results in MHC restriction. If the thymocytes are unable to bind to MHC molecule or this bound is too strong they undergo the *programmed cell death* or *apoptosis*, event which leaves these cells without positive, life-saving signal which terminate the process of programmed cell death. In difference to deletional mechanism of "death by neglect" by which T cells activated by hepatocytes die prematurely before they could become harmful to the body [55], process of *negative selection*, eliminates thymocytes bearing high affinity receptors for self-MHC molecules alone or self-antigen presented by self-MHC what results in self-tolerance. T cells which recognize autoantigens presented from dendritic cells, do not receive a life saving signal and go to apoptosis [64]. Without this negative selection, such "autoimmune" T cells could destroy the organism [65]. Since this self reactive T cells are everlasting destroyed, <u>this mechanism is considered as a most effective mechanism of tolerance induction</u>, leading to robust, not easily abrogated form of tolerance. Due to this effect, this form of tolerance is clinically

desirable. Without physical destruction of the donor reactive T cells by clonal deletion, potential risk of repeated activation of anergized or suppressed self-reactive T cells is simply to huge that only peripheral mechanisms of tolerance would be concern as an attractive base for further clinical setting.

1.1.5. Strategies for T-cell tolerance induction

1.1.5.1. *Tolerance through macrochimerism*

The induction of the robust donor-specific tolerance, can be routinely achieved in rodent studies by the induction of mixed chimerism, where the tolerance is maintained thru the inthrathymic clonal deletion [66-69]. Term *chimerism* is used to characterize the presence of the foreign (donor) cells in an individual [29]. It can occur spontaneously after organ transplantation leading to the state of the *microchimerism* (existence of donor hematopoietic cells with donor representation of less than 1%). Higher representation of donor hematopoietic cells in the host is considered as a *macrochimerism*. It can be a *full chimerism* (with a 100% representation of donor cells in host), or a mixed chimerism (actively induced after transplantation of donor hematopoietic cells with specific, non-myeloablative, host conditioning with representation of donor cells in the host of more than 1% and less 100%). This approach of tolerance induction thru the induction of macrochimerism has several advantages [4]; it relies in a large part of clonal deletion as a mechanism of tolerance what is a robust form of tolerance, it has been developed in large animal models, it can be readily measured, there are evidence that machrochimerism can lead to tolerance in humans and also there are first clinical studies that rely on machrocimerism have been initiated successfully. Major limitation of tolerance induction thru macrochimerism induced by transplantation of BM or PBSC is that it requires the conditioning of the recipient in a target to overcome MHC barriers, what is known to be associated with morbidity and death [4]. This conditioning regiments are either use of irradiation or cytotoxic drugs in a target to promote engraftment of transplanted hematopoietic stem cells (HSC) and use of T cell-depletion antibodies for global destruction of host T cells to avoid rejection of donor cells leaving the recipient temporarily immunocompromised until newly developed T cell repertoire is not

established. First macrochimerism protocols for the induction of tolerance included lethal total-body irradiation (TBI) of recipient before BMT [70].

1.1.5.2. *Less toxic protocols for the induction of tolerance thru macrochimerism*

There have been several regimens developed for the induction of tolerance thru macrochimerism which included milder non-myeloablative conditioning with non-specific T cell depletion [71-74]. Since even syngeneic bone marrow do not engraft without cytoreductive host treatment if given in standard dose [75], it seemed that avoidance of myelosuppression would be impossible until it was found that it can be overcame in murine transplantation models by the injection of very high numbers of bone marrow cells [1;76]. To avoid TBI or use of cytotoxic drugs in allogeneic settings with similarly high doses of MHC-mismatched bone marrow together with T cell depletion, moderate dose of thymic irradiation was required to induce machrochimerism. Complete avoidance of non-specific T-cell depletion and myelosuppresion was not possible until the costimulations blocking reagents were introduced as a part of BMT protocols.

1.1.5.3. *Costimulation-blocking reagents*

Monoclonal antibody therapy can be used except for depletion of the recipient from a certain broad or specific (like against CD3 or CD4) [77] cell population, as well to block costimulatory signals required for the T_H-cell activation. This costimulatory signal is mediated by the antigen presenting cells (APCs) B7.1/B7.2 and CD40 molecules interacting with the T cells CTLA-4, CD28 and CD154. If this signal is missing, T cells become anergic. Signaling via CTLA-4 (expressed at lower levels than CD28 and only on activated T cells, but binding B7 with a 20-fold higher affinity) induces inhibitory signals in T-cells, whereas signaling via CD28 (expressed on both resting and activated T cells binding the B7 with moderate affinity) induces activating signals. The signaling via CD40-CD154 (CD40 expressed on APCs, and CD154 or CD40Ligand on T cell) activates antigen presenting cells and may also activate T-cells (Figure 1).

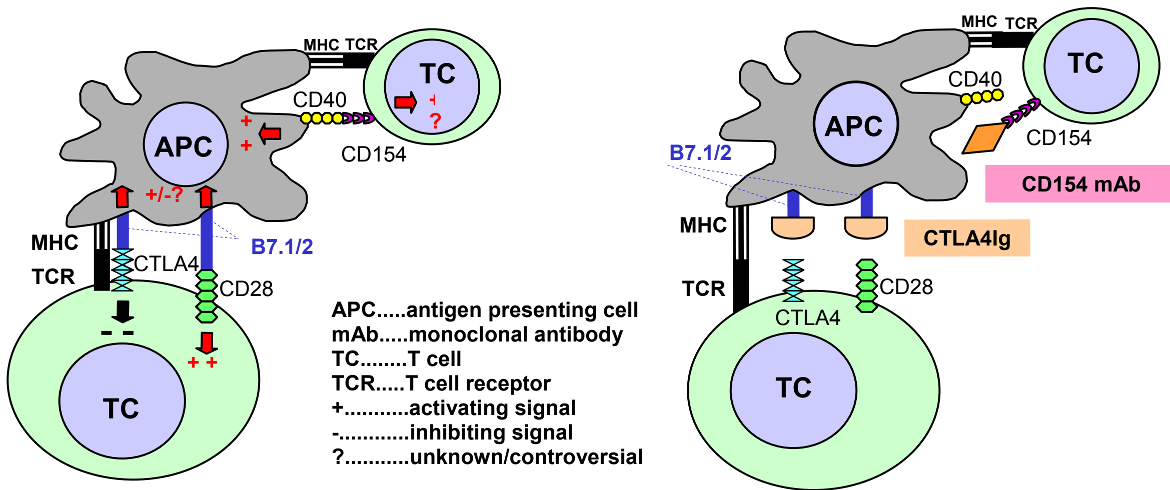


Figure 1. Model illustrating the CD28 and CD40 costimulation pathways, on the left side during normal interactions between T-cell and APC, and on the right side blockade of costimulatory signal (interaction between TCR-MHC is not compromised) by anti-CD154mAb and CTLA4Ig (adapted from *Wekerle et al., Current Opinion in Immunology 2002*).

There are two groups of commonly used costimulations signals blocking reagents; first are directed against the CD80/86(B7.1/B7.2) or CD28 aimed at interfering with the CD28/CD80/CD86 pathway or CTLA4Ig mAbs (soluble fusion protein consisting of the extracellular domains of CTLA4 and the constant region of the IgG1 heavy chain), and second are mAbs directed against CD40/CD-154 pathway or anti-CD154 mAbs (also known as MR-1). These costimulatory pathways blocking reagents have demonstrated strong immunosuppressive properties [78;79;80]. It has been proved that anti-CD154 or CTLA4Ig induce regulation and anergy in several experimental models without BMT [81;82;83] . Treatment with anti-CD154 alone has been showed to induce immunregulation [84;85]. On the other hand, CTLA4Ig induces linked suppression in vitro [86]. Nevertheless, it has been reported as well that this costimulatory blocking reagents are less effective in some mice strains (C57BL/6) [87]. These blocking antibodies induce robust form of tolerance only when they are combined with BMT. The use of antibodies alone do not form tolerance in rodents which could pass the stringent tolerance test (permanent acceptance of skin grafts over MHC barrier in euthimic recipients) [88;89] and also do not

induce tolerance in monkeys [79]. CTLA4Ig also blocks signaling through the inhibitory receptor CD152, binding its only ligands CD80 and CD86. Delayed administration of CTLA4Ig [90] which is beneficial in some models, probably allows upregulation and engagement of CD152 in the first days after antigen contact but still blocks the CD28 early enough to prevent full activation of the T cells [81].

1.1.5.4. *Transplantation tolerance through the mixed chimerism induced after BMT with costimulation blockade*

The experimental transplantation protocols [53] employing the BMT with costimulation blockade [CTLA4g + anti-CD 154 mAb (CD40L)] specifically eliminate donor-reactive T cells without destroying a general T cell repertoire, thus inducing macrochimerism and tolerance with substantially less toxicity [1;4;29;54;60;76;91-93] and without GVHD (Figure 2).

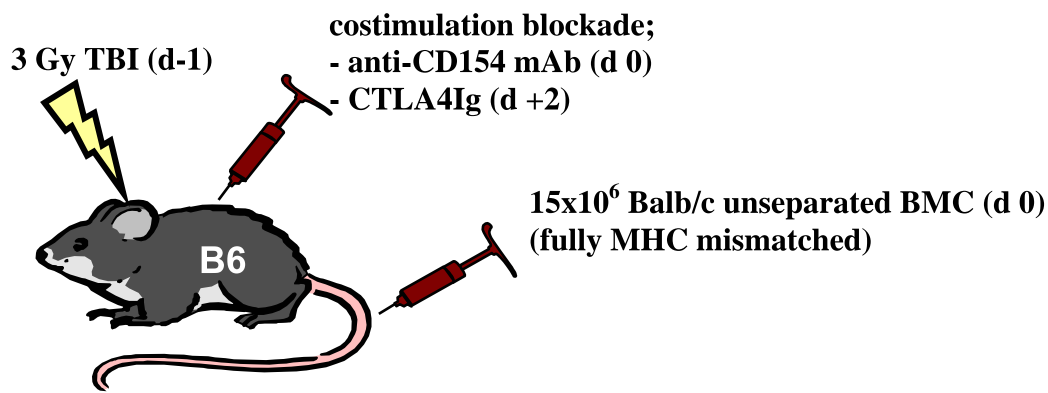


Figure 2. Protocol for the induction of stable multillineage chimerism and tolerance without development of GVHD. (Adapted from *Wekerle , The Journal of Heart and Lung Transplantation 2001*.)

By this protocol non-depleted pre-existing donor-reactive T cells are strongly eliminated during the first weeks after BMT. Interestingly, tolerance in this model occurs before deletion is fully complete, pointing the role of non-deletional mechanisms during the early phase of tolerance induction (immediately after BMT) [94]. The major mechanism of tolerance induced by this protocol seemed to be the extrathimic clonal deletion, allowing the development of long-lasting mixed chimerism, which than maintain the tolerance thru the mechanism of intrathimic clonal deletion [54]. Interestingly, tolerance can be found even before peripheral deletion is complete, and elimination of recipient CD4+ cells at

the time of BMT prevents tolerance induction [53]. Recent study performed in our laboratory [95] showed that $CD4^+$ cell mediated regulation is critical for the induction of robust tolerance early after BMT and costimulatory blockade but appears to have no critical role in the maintenance of tolerance once when peripheral deletion of donor-reactive T cells is complete. Unfortunately, non-myeloablative dose of TBI remains an essential part of this treatment, since the engraftment barrier exists even when the syngeneic bone marrow cells (BMC) are transplanted. When combined with this protocol, calcineurin inhibitors (cyclosporine A [CyA] or tacrolimus [FK]) inhibit development of long-term chimerism and abrogate tolerance induction. Nevertheless, used dose of 3 Gy TBI can be reduced to the dose of 1Gy by the additional use of compatible immunosuppressive drugs (methylprednisolone+ mycophenolate mofetil+ rapamycin) [96]. Recently published protocol [1] (Figure 3) demonstrates that the need for cytoreduction (irradiation, cytotoxic drugs or T-cell depletion antibodies) can be avoided by the transplantation of extremely high doses of bone marrow cells (13-times more than conventional BMC dose). Long lasting mixed chimerism and donor-specific skin graft tolerance were accomplished in the 60% of the experimental mice, without clinical signs of GVHD in a murine model where recipient strain (C57BL/6) is reported to be one of the most difficult for achieving graft prolongation with costimulatory blockade [97].

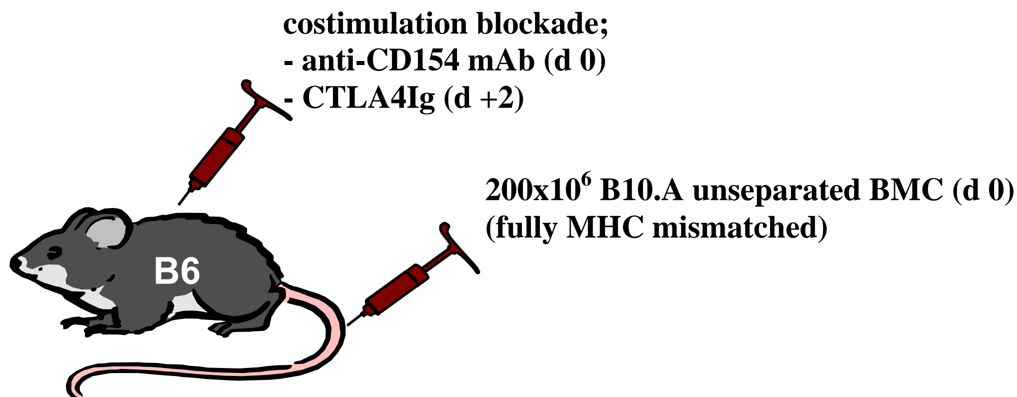


Figure 3. Immunological engraftment barrier can be overcame either by irradiation or by transplantation of extremely high dose of bone marrow cells. (Adapted from Wekerle, The Journal of Heart and Lung Transplantation 2001.)

Although this model proofs that completely non-myelosuppresive BMT regimens are possible, major obstacle for clinical application of this protocol, is the limited number of BMC available from the living donor.

1.1.6. Mobilized peripheral blood stem cells

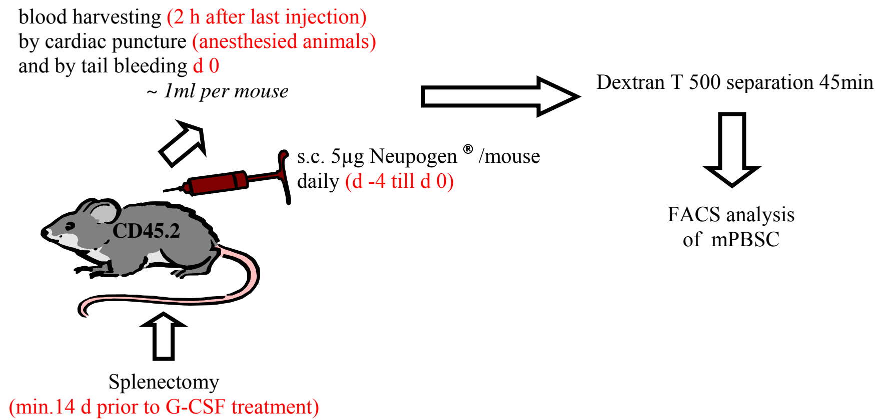
Theoretically, the major obstacle concerning the lack of available haematopoietic stem cells (HSC) required for previously mentioned treatment could be solved by the collection of mPBSC from peripheral blood. Clinically, the collection of mobilized peripheral blood stem cells (mPBSC) thru the process called leukapheresis from a living donor, offers the highest numbers of HSC (5-10 more than BMC) [98;99] and is frequently used as the source of cells for HSCT [100]. Donor patients are treated for several days with certain cytokines, what leads to the strong increase in number of HCS in peripheral blood from where they can be harvested by leukapheresis. Currently, clinically the most commonly used cytokine for this purpose is granulocyte colony-stimulating factor (G-CSF) [101]. G-CSF is seemingly safe, even though some side effects were described [102;103]. This process is generally concerned to be “less painful” for the donor patients without need for general anesthesia like is the case in BMC harvest [104]. Although it was shown that in the murine experimental model G-CSF has worse mobilization properties when compared with flt3 ligand [105], some uncertainty regarding this comparison still remains, because C57BL/6 strain used in this study has also been described as a low responder to G-CSF [106;107].

BM and mPBSC are used for similar indications, but they differ substantially with respect to several major characteristics. One important difference regards cell composition, with human mPBSC containing more $CD3^+$ and $CD56^+$ cells (about twice as many on per cell basis than marrow) [99;108]. Also, mPBSC have significantly different immunological characteristics. Further advantages of the PBSC transplantation compared with BMT are faster engraftment properties of PBSC [101;98;109;110] and less post-transplant infections in recipients due to the faster granulocyte recovery and a higher lymphocyte counts [111;112]. However, they show an increased incidence of severe hemolyis episodes in cases of

"minor" ABO incompatibilities [113;114]. Till recently, it was considered that despite containing much higher numbers of donor T cells in the inoculum, allogeneic mPBSC transplantation seemed not to be associated with a significantly increased incidence of acute GVHD [108-110]. Moreover, G-CSF treatment was shown to polarize donor T cells towards a Th2-type immune response, which might contribute to the lower than expected incidence of acute GVHD after mPBSC transplantation [115-117]. This immune deviation is mediated by increased numbers of Th2-inducing DC after G-CSF mobilization [118] what evidently does not effect DC function in the blood [119]. Recent studies also suggest that G-CSF administration suppresses T cell proliferation and cytokine response to allogeneic stimulation by indirectly modulating monocyte function [120]. Nevertheless, considerable uncertainty persists whether the incidence of chronic GVHD after mPBSC transplantation is increased. Some studies suggest a similar risk compared with BMT [108;110] or similar with higher number of successive treatments needed to control chronic GVHD after PBSCT [121]. Others in opposite indicate an increased incidence [122;123], and in particular increased risk of chronic GVHD compared with BMT [124]. In a recent large retrospective analysis, the one-year probability for the development of chronic GVHD after MHC-identical sibling mPBSC transplantation was significantly increased (65% versus 53% for BMT) [109]. Finally, according to Meta-Analysis of sixteen pooled studies, both acute and chronic GVHD were more common after PBSCT than BMT. Like shown, this increased risk could be associated with increased amount of transplanted CD34+ cells [125] namely with magnitude of the transfused T-cell load [126].

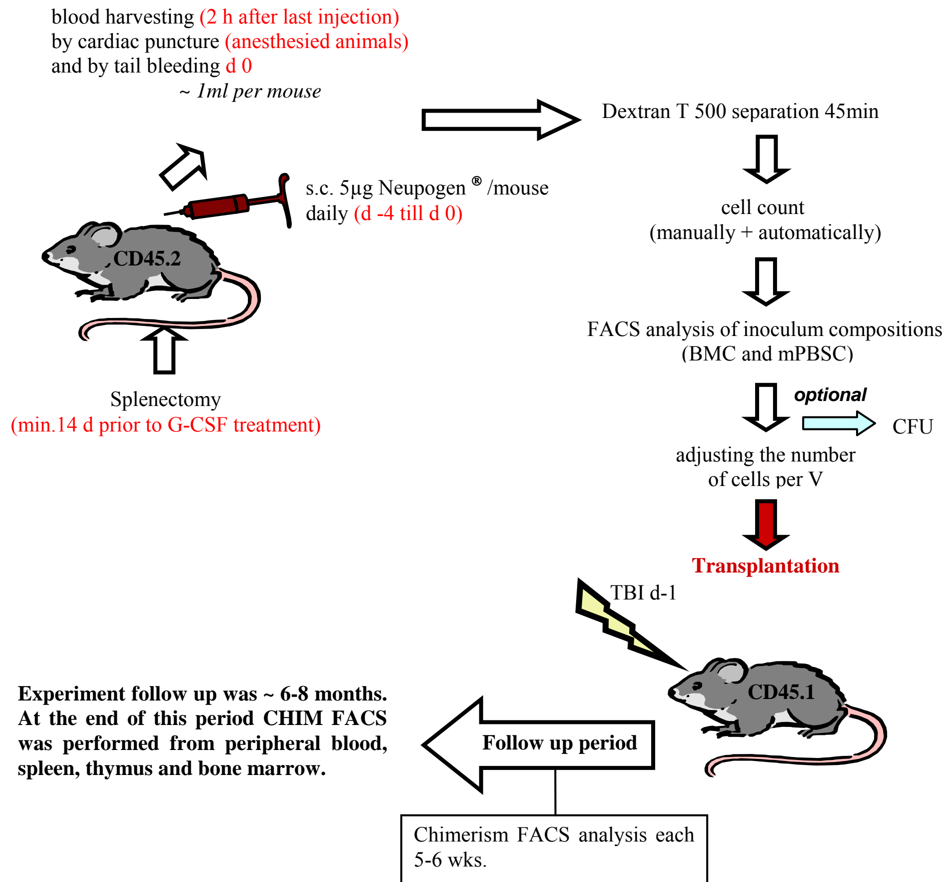
There have been several studies performed regarding mPBSC reconstitutions capabilities in lethally and sublethally irradiated mice in allogeneic and congenic systems [105]. Induction of chimerism and tolerance by the use of PBSC has already been shown in large animal models [127;128]. Usually, murine tolerance models require less intense protocols than large animal models. Although mPBSC are an attractive candidate for the tolerance induction through costimulation blockade and mixed chimerism (due to the large number of hematopoietic cells obtainable from single donor), till nowadays, it hasn't been explored whether this cells can be used for this purpose in the non-myeloablative or non-cytoreductive murine protocols.

1.1.7. Preinvestigations



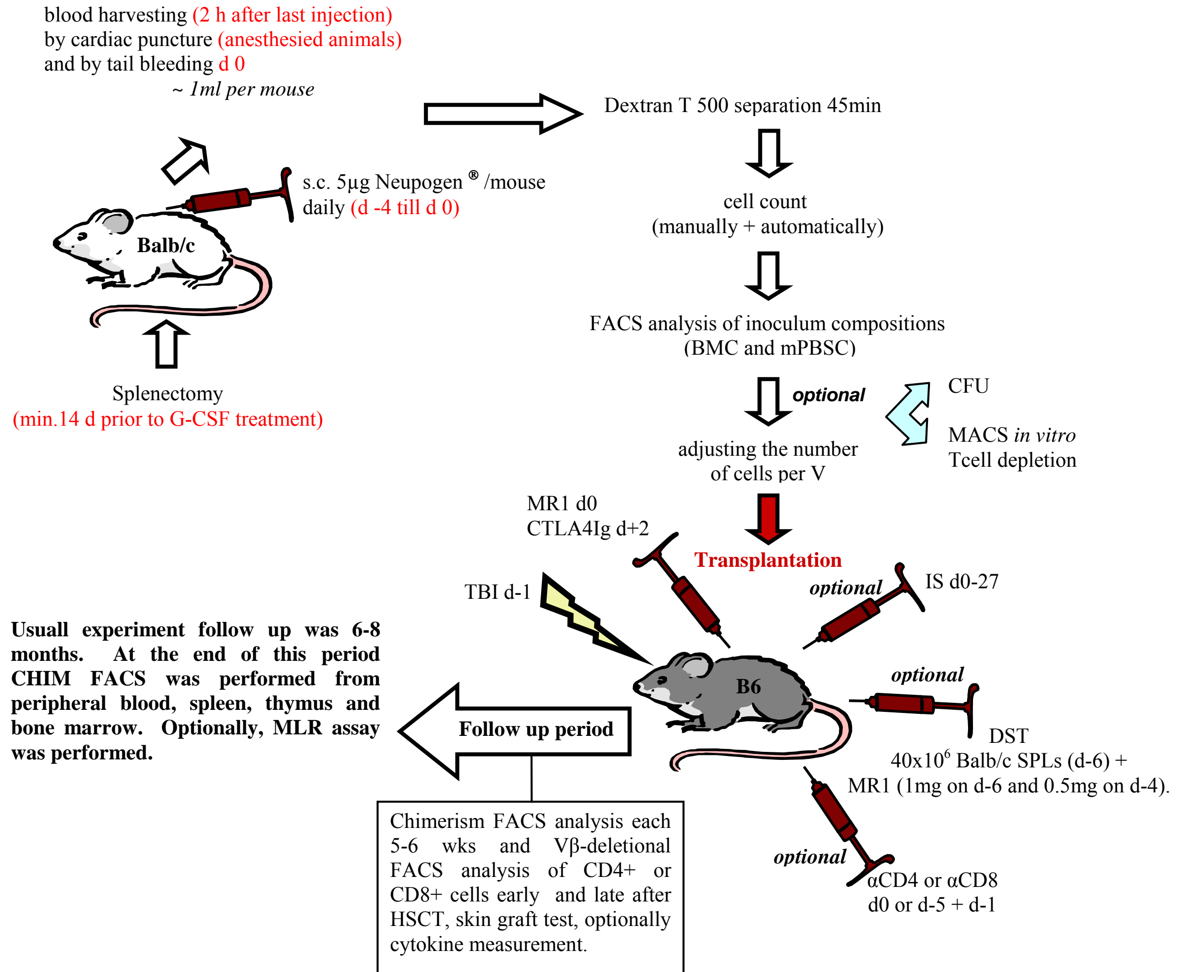
- Learning and practicing splenectomy (SPX) and other surgical techniques
- Determination of the best bleeding technique
- Determination of the best mobilizing cytokine and mobilization procedure
  (5 days/daily, subcutaneous injections)
- Determination of the best leukocyte separation technique
  (by dextran T500, 45 min at 37ºC)
- Determination of mPBSC composition by FACS

1.1.8. General experimental scheme for syngeneic (CD45 congenic) murine model (CD45.1 *recipient* – CD45.2 *donor*)



1.1.9. General experimental scheme for allogeneic murine model

[B6 *recipient* -Balb/c *donor* (fully MHC mismatched)]



1.2. SCOPE OF THIS THESIS

Based on the current state of knowledge, the following points warrant further examinations.

- Can mPBSC successfully engraft in a murine syngeneic (CD45 congenic) model?
- If yes, would the level of induced mixed chimerism be different than that induced with BMC?
- Could BMC be simply substituted with mPBSC in a murine non-myeloablative allogeneic model with or without the use of costimulatory blockade (CTL4Ig + anti-CD154 mAb)?
- If not, would it be possible to further condition the transplantation protocol in order to induce mixed chimerism and tolerance using the mPBSC?
- Would it be possible to further determine the immunological differences between BMC and mPBSC?

2

Induction of Mixed Chimerism through Transplantation of CD45-congenic mobilized Peripheral Blood Stem Cells after non-myeloablative irradiation

ABBREVIATIONS USED IN THIS PAPER

BM, bone marrow; **BMC**, bone marrow cells; **BMT**, bone marrow transplantation; **CFU**, colony forming unit; **CFU-GM**, colony forming unit-granulocyte/monocyte progenitor; **FCM**, flow cytometry; **G-CSF**, granulocyte colony stimulating factor; **Gy**, Gray; **HSC**, hematopoietic stem cells; **HSCT**, hematopoietic stem cell transplantation; **mPBSC**, mobilized peripheral blood stem cells; **mPBSCT**, mobilized peripheral blood stem cell transplantation; **PB** peripheral blood; **SPL**, spleen; **TBI**, total body irradiation

ABSTRACT

Background: Clinical translation of the mixed chimerism approach for inducing transplantation tolerance would be facilitated if mobilized peripheral blood stem cells (mPBSC) could be used instead of bone marrow cells (BMC). Since the use of mPBSC for this purpose has not been investigated in non-myeloablative murine protocols, we explored the engraftment potential of mPBSC in a CD45-congenic model as a first step.

Materials and Methods: Following 2, 1.5 or 1 Gy total body irradiation (TBI), CD45.1 B6 hosts received unseparated G-CSF-moblized CD45.2 B6 PBSC, or unseparated CD45.2 B6 BMC. The same total cell numbers, or aliquots of mPBSC and BMC containing similar numbers of c-kit$^+$ cells, were transplanted, with or without a short course of rapamycin-based immunosuppression (IS).

Results: Transplantation of mPBSC induced long-term multilineage macrochimerism, but chimerism levels were significantly lower than among recipients of the same number of BMC. Transplanting aliquots containing similar numbers of c-kit$^+$ cells reduced the difference between mPBSC and BMC, but still lower levels of chimerism were observed in recipients of mPBSC. Chimerism levels more closely correlated with the number of transplanted progenitor cells as determined by CFU assays. IS did not affect chimerism levels, indicating that the donor CD45 isoform or other minor disparities do not pose a major barrier to engraftment.

Conclusion: Thus, under non-myeloablative conditions progenitor cells contained in mPBSC have similar engraftment capacity as those from BMC, allowing induction of lasting mixed chimerism with moderate cell numbers. On a cell-per-cell basis, unseparated BMC have some advantages which may be minimized if the number of

progenitor cells is equalized. These results are expected to facilitate the development of mPBSC-based allogeneic tolerance protocols.

INTRODUCTION

Induction of donor-specific tolerance is a major research goal of transplantation medicine. Mixed chimerism, established through the transplantation of donor hematopoietic stem cells (HSC), is an attractive tolerance strategy for clinical development [1]. A major obstacle in the clinical translation of this approach, however, is the required recipient conditioning.

While mild conditioning protocols using costimulation blockers have been developed for the induction of lasting allogeneic mixed chimerism employing relatively realistic doses of BMC (approx. $15x10^6$ BMC per mouse), they require non-myeloablative TBI or cytotoxic drug treatment [2-5]. Such conditioning requirements would still be regarded as too toxic by many for routine use in organ transplant recipients. We and others have shown that allogeneic BM engraftment can be achieved experimentally without any cytoreductive conditioning if extremely high doses of BMC are transplanted (approx. $200x10^6$ BMC per mouse) [6-9]. Although these models provide proof-of-principle that completely non-cytoreductive BMT regimens are possible, they are not clinically practicable because such high numbers of BMC cannot be routinely obtained from a single human donor.

In the clinic, the highest numbers of HSC are obtained through the collection of mPBSC from a living donor. Transplantation of G-CSF mobilized PBSC has an established role in the treatment of hematological diseases [10;11], with the cell yield of a mPBSC collection typically being 5 to 10-fold higher than from a conventional BM harvest from the iliac

crests. However, while BM and mPBSC are used for similar clinical indications, they differ substantially with respect to several major biological and immunological characteristics [12-16]. These differences are of concern as they could potentially influence the induction of mixed chimerism and tolerance in a significant manner. In particular, it is unknown whether costimulation blockade retains the same tolerance-inducing effect if allogeneic PBSC are transplanted instead of BMC. Before investigating allogeneic mPBSC in combination with costimulation blockade, however, we considered it necessary to establish the behavior of murine mPBSC after non-myeloablative conditioning in the absence of alloreactivity. For this purpose we transplanted congenic mPBSC after non-myeloablative TBI.

Engraftment properties of murine PBSC mobilized with G-CSF and/or other growth factors has previously been evaluated in lethally irradiated recipients, and their reconstituting capability has been established [17-24]. While mostly allogeneic models were used, congenic systems have been investigated as well, although after myeloablative conditioning [22;24]. In one study the graft-versus-leukemia effect of allogeneic G-CSF mobilized-PBSC was evaluated after 7 Gy of sublethal TBI, leading to high levels of chimerism (>75%) [25]. The therapeutic effect of transplantation of a mixture of allogeneic and syngeneic murine mPBSC was demonstrated in a myeloablative SLE-like autoimmune disease model (BXSB) [19]. To the best of our knowledge, however, murine mPBSC have not been investigated in detail after low-dose (1-3 Gy) non-myeloablative TBI. Thus, their engraftment properties under these conditions remain unknown.

Hence, while mPBSC are an attractive candidate for tolerance induction through costimulation blockade and mixed chimerism due to the large number of hematopoietic cells obtainable from a single donor, non-myeloablative or non-cytoreductive murine protocols employing mPBSC have not been explored for this purpose. As an initial step toward the development of mPBSC-based mixed chimerism protocols for tolerance induction, we show here that CD45-congenic mPBSC transplanted after non-myeloablative TBI led to lasting multi-lineage mixed chimerism. Consistent with the frequency of progenitor cells, transplantation of *unseparated* mPBSC resulted in significantly lower levels of chimerism than the same number of *unseparated* BMC, but engraftment efficiency per number of progenitor cells transplanted was comparable between mPBSC and BMC. These results should facilitate the development of allogeneic mPBSC-based tolerance protocols.

MATERIALS AND METHODS

Animals. Female B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (CD45.1, hosts) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and congenic female C57BL/6NCrl (B6:H-2^{b}) mice (CD45.2, donors) were purchased from the Charles River Laboratories (Sulzfeld, Germany). Animals were kept under specific pathogen-free conditions and were used between 6 and 10 weeks of age. All experiments were approved by the local review board of the University of Vienna, and were performed in accordance with national and international guidelines of laboratory animal care.

BMT and mobilized peripheral blood stem cell transplantation (mPBSCT). CD45.1 hosts received non-myeloablative TBI (2, 1.5 or 1 Gy, as indicated) one day (d-1) before either mPBSCT or BMT (d0). To avoid pooling of mPBSC in spleen, donor CD45.2 mice were splenectomized at least 14 days prior to the mobilization procedure, as described by Weissman and colleagues [22]. Thereafter, 5µg of human G-CSF (approx. 250 µg/kg) (filgrastim, Neupogen®, Amgen Europe BV Breda, Netherlands) were injected subcutaneously (s.c.) for 5 consecutive days. Two hours after the last injection, mice were maximally bled (using tail bleeding and heart puncture), and the heparinizied blood was pooled and diluted with PBS (1:1). Subsequently the same volume of 2% dextran T500 solution was added (to give a final concentration of 1% dextran). Red blood cells were separated by sedimentation for 45 minutes at 37ºC, before the supernatant fraction containing the mobilized leukocytes (mPBSC) was collected. BM was harvested from tibiae, femura and humeri, as described in detail previously [26]. Both BMC and mPBSC were filtered through a 70 µm filter. BMC were counted manually (by trypan blue

exclusion of dead cells) and mPBSC were both counted manually and automatically. Cells were diluted with cold BM media [500ml Medium 199 (Sigma, Vienna, Austria), supplemented with 5 ml HEPES buffer (ICN, Biomedica, Vienna, Austria), 5 mg DNAse (Sigma) and 2 mg Gentamycin (Sigma)] and were injected in a volume of 1 ml into a tail vein of recipient mice (d0). BMC and mPBSC were used without further manipulation (without enrichment or depletion).

Flow-cytometric analysis (FCM). Two-color FCM was used to distinguish donor and host cells of particular lineages, by staining with fluorescein isothiocyanate (FITC)-conjugated antibodies against CD4, CD8, B220, MAC-1, NK1.1 and biotin-conjugated CD45.2 (developed with phycoerythrin-avidin). Mice were considered chimeric if they showed at least 2 % of donor cells within the myeloid lineage and at least one lymphoid lineage. To analyze cell composition, BMC and mPBSC were stained with FITC-conjugated antibodies against CD4, CD8, B220, MAC-1, CD117 (c-kit), CD49b, TCR-β and biotin-conjugated CD45.2 (developed with phycoerythrin-avidin). The percentage of subpopulations among CD45.2^{+} live cells was calculated. Irrelevant isotype controls were included, and propidium iodide staining was used to exclude dead cells.

CFU-assay. BMC and mPBSC were suspended at a cell concentration of $1x10^{6}$ per ml in BM medium. $1x10^{5}$ of either BMC or mPBSC (in 100µl) were cultivated in duplicates inside Petri plates (Falcon Nr. 3003) with one ml of cultivating medium prepared by mixing of 75µl of recombinant mouse IL-3 (25ng/ml - R&D Systems, Minneapolis, MN, USA) and 2.3ml of MyeloCult^{TM} H5100 medium (StemCell Technologies, Vanccouver,

BC, Canada). CFU-GM were counted by an experienced hematologist after 14 days of incubation.

Chimerism per transplanted progenitor cells. The total number of progenitor cells transplanted per mouse was calculated by multiplying the number of CFU colonies grown from $1x10^5$ plated cells [288 for BMC and 64 for mPBSC, see page 14] by 100 for BMC (as $10x10^6$ cells were transplanted), and by 300 for mPBSC (as $30x10^6$ cells were transplanted). Accordingly, 28,800 progenitors were transplanted per mouse in the BMC group and 19,200 in the mPBSC group. The level of chimerism per $1x10^4$ transplanted progenitor cells was then obtained by multiplying the measured percentage of donor chimerism (as determined by FCM) by $1x10^4$ divided by the total number of transplanted progenitors (chimerism per $1x10^4$ transplanted progenitor cells = % measured chimerism x [$1x10^4$ /total number of transplanted progenitors]; thus % measured chimerism was multiplied by a factor of 0.35 for BMC [$1x10^4$ / 28,800] and 0.52 for mPBSC [$1x10^4$ / 19,200]).

Immunosuppression. In the indicated groups, mice were injected with immunosuppressive drugs daily from day 0 to 27. Drugs were used at following doses: rapamycin: 0.2 mg/kg/d; methylprednisolone (MP): 10 mg/kg/d; mycophenolate mofetil (MMF): 20mg/kg/d. Drugs were diluted and administered as described previously [26]. Rapamycin was kindly provided by Wyeth-Ayerst (Princton, NJ, USA), and MMF by Roche (Vienna, Austria). MP was purchased from Aventis (Vienna, Austria).

Statistics. A two-tailed Student's T test was used for comparing chimerism levels between groups. A P value less than 0.05 was considered to be statistically significant.

RESULTS

Unseparated mPBSC successfully engraft after non-myeloablative conditioning, although with reduced efficiency compared to BMC.

Since only BMC have been investigated so far for the purpose of tolerance induction through non-myeloablative hematopoietic stem cell transplantation (HSCT) and mixed chimerism, and since little is known about engraftment properties of murine mPBSC under non-myeloablative conditions, the aim of this study was to investigate the capability of ‘syngeneic’ (i.e. congenic) mPBSC for the induction of mixed chimerism after non-myeloablative TBI.

In order to compare the engraftment of mPBSC and BMC in the absence of alloreactivity, a CD45-congenic model was chosen. CD45.1 recipients received 2, 1.5 or 1 Gy TBI one day prior to transplantation of $20x10^6$ unseparated mPBSC or BMC, respectively (6 mice per group).

All mice receiving either $20x10^6$ mPBSC or BMC after 2, 1 or 1.5 Gy of TBI developed long-term multi-lineage macrochimerism (chimerism rates: mPBSC 6/6; 6/6; 6/6 and BMC 6/6; 6/6; 6/6) (Figure 1). Chimerism was stable for 29 weeks in all groups. Levels of chimerism obtained both with mPBSC and BMC correlated with the dose of TBI. The transplantation of mPBSC led to significantly lower levels of hematopoietic chimerism compared to the transplantation of the same number of BMC with all tested TBI doses. This difference was, for instance, at the end of follow up (shown as BMC vs. mPBSC chimerism, after 2 Gy TBI): 75% vs. 31% $CD4^+$ ($p<0.0005$), 52% vs. 16% $CD8^+$

($p<0.0005$), 86% vs. 44% B cell ($p<0.0005$), 75% vs. 35% myeloid ($p<0.0005$), and 63% vs. 17% NK ($p<0.0005$) cell chimerism (Figure 1). Similarly, substantial and significant differences were also observed in the groups treated with 1.5 and 1 Gy TBI.

We also determined chimerism levels in BM and spleen (SPL) of randomly selected mice (two per group, 1 Gy and 2 Gy TBI), at the end of follow-up (32 weeks post-transplantation) (Table 1). mPBSC recipients showed substantially lower levels of chimerism (e.g. in BM: 78% vs. 42% myeloid [$p<0.05$] or 79% vs. 40% B cell [$p<0.005$] chimerism and in SPL: 65% vs. 29% $CD4^+$ [$p<0.005$], 59% vs. 27% $CD8^+$ [$p<0.05$] or 86% vs. 47% B cell [$p<0.05$], using 2 Gy TBI).

The composition of BMC and mPBSC was analyzed by FCM, revealing that, as expected, mPBSC contain higher percentages of $CD4^+$ and $CD8^+$ cells (17% vs. 2% for $CD4^+$ and 12% vs. 2% for $CD8^+$ cells, Table 2), and B cells (39% vs. 28%), and lower percentages of MAC-1^+ cells (32% vs. 65%) than BMC. Of note, a higher percentage of c-kit^+ (CD117) cells was found among BMC than among mPBSC (11.1% vs. 3.7%).

Hence, CD45-congenic mPBSC successfully engraft and induce stable long-term multilineage macrochimerism after non-myeloablative TBI. Furthermore, the same number of unseparated mPBSC resulted in significantly lower chimerism levels in all tested lineages compared to BMC (with all 3 doses of TBI used). The lower engraftment of unseparated mPBSC was associated with a lower percentage of c-kit^+ cells. These

results suggest a reduced per-cell potential of unseparated murine mPBSC to induce mixed chimerism under non-myeloablative conditions.

The chimerism achieved with mPBSC and BMC correlates with the number of transplanted progenitor cells.

The results described above suggest that the difference in chimerism levels might be due to the higher percentage of c-kit$^+$ cells among BMC implying a higher number of progenitors [27]. We therefore next transplanted aliquots of mPBSC and BMC containing similar numbers of c-kit$^+$ cells. BMC contained approximately 3 times as many c-kit$^+$ cells as mPBSC (11.1% vs. 3.7%, Table 2). According to this ratio, we transplanted 30×10^6 unseparated mPBSC and 10×10^6 unseparated BMC (with 1.5 Gy TBI).

While the transplantation of unseparated mPBSC or BMC containing a similar number of c-kit$^+$ cells again led to lower levels of chimerism among the recipients of mPBSC than among recipients of BMC, the differences were overall substantially smaller and the difference reached statistical significance only in some lineages and only at certain time points (Figure 2). For instance, chimerism levels at 26 weeks post-HSCT were (shown as BMC vs. mPBSC): 36% vs. 17% CD4$^+$ ($p<0.05$), 26% vs. 11% CD8$^+$ ($p<0.05$), 42% vs. 31% B cell (p=n.s.), 29% vs. 20% myeloid (p=n.s.), and 17% vs. 11% NK cell chimerism (p=n.s.) (Figure 2, panels A+B). Furthermore, CFU assays of donor BMC and mPBSC performed in this experiment showed that 4.5 times as many colonies grew from BMC than from mPBSC (mean of two plates: 288 vs. 64 CFU-GM/1×10^5 plated cells for BMC

vs. mPBSC), suggesting that the actual difference in the frequencies of progenitors might be larger than estimated by the percentages of c-kit$^+$ cells.

We hypothesized that the determined percentages of c-kit$^+$ cells underestimate the true difference in progenitor content. Thus we calculated whether chimerism levels correlate more closely with the number of transplanted progenitor cells as estimated by CFU assay. Since we transplanted only three times as many mPBSC as BMC (following the percentages of c-kit$^+$ cells), but 4.5 times as many colonies grew from BMC than from mPBSC, there might have been still 50% more progenitor cells transplanted in the BMC group than in the mPBSC group (28,800 progenitor cells contained in $10x10^6$ BMC vs. 19,200 progenitor cells in $30x10^6$mPBSC). Consistent with this assessment, we found that in BMC recipients chimerism levels were 49% higher among CD4$^+$ cells, 40% higher among CD8$^+$ cells and 57% higher among B cells, for instance (in SPL, 29 weeks post HSCT; results from the two best chimeras from each group, Table 3). The difference in chimerism levels among various lineages in blood over time was gernerally of a similar magnitude.

We thus also calculated levels of measured chimerism in relation to the estimated number of progenitor cells transplanted (according to CFU results, for details of the calculation please see the Methods section) (Figure 3). Levels of donor chimerism *per $1x10^4$ transplanted progenitors* determined in this way were very similar for recipients of BMC and mPBSC (Figure 3). Higher levels of T cell chimerism detected among the mPBSC

recipients during the first few weeks after HSCT were likely due to the higher percentage of $CD4^+$ and $CD8^+$ cells contained in mPBSC.

Thus the lower levels of multilineage chimerism among recipients of unseparated mPBSC compared with recipients of BMC correlate with a lower number of progenitor cells contained in transplanted unseparated mPBSC. On a per cell basis, progenitor cells from mPBSC and BMC seem to have very similar engraftment potential under non-myeloablative conditions, leading to similar levels of long-lasting multi-linear macrochimerism.

Short-course immunosuppression does not significantly improve engraftment.

While CD45-congenic strain combinations are frequently thought of as essentially syngeneic systems that allow the tracking of donor cells, it has been recognized that CD45 isoforms can elicit immune responses [28]. Furthermore, it cannot be ruled out that minor transplantation antigen differences between the recipient and donor strains develop over time due to spontaneous mutations during breeding. Thus, we explored the possibility that the lower levels of mPBSC chimerism were due to an increased alloresponse compared to the alloresponse elicited by BMC. We thus treated recipients with a combination of immunosuppressive drugs (rapamycin, mycophenolate mofetil and methylprednisolone; from day 0 to 27), which has been shown to effectively increase engraftment of allogeneic BMC in combination with costimulation blockade [26;29]. As shown in Figure 2, transient immunosuppression did not significantly affect chimerism rates or levels after either BMT or mPBSCT. After mPBSCT, chimerism developed in

4/4 mice treated with immunosuppression vs. 5/6 mice without, and after BMC in 5/5 mice with immunosuppression and 5/5 without (using 1.5 Gy of TBI). Chimerism levels were similar between the groups with and without immunosuppression for the length of observation of 26 weeks. At the end of follow-up the mean percentage of donor chimerism in peripheral blood of BMC recipients was (with IS vs. without IS): 36% vs. 36% $CD4^+$, 26% vs. 26% $CD8^+$, 42% vs. 50% B cell, 29% vs. 35% myeloid and 17% vs. 26% for NK cell chimerism; of mPBSC recipients: 17% vs. 20% $CD4^+$, 11% vs. 13% $CD8^+$, 31% vs. 32% B cell, 20% vs. 19% myeloid and 11% vs. 11% NK.

As additional use of immunosuppression did not significantly improve chimerism, the reduced engraftment of mPBSC is most likely not due to an alloresponse against CD45 or other putative antigenic strain differences.

DISCUSSION

While tolerance induction through mixed chimerism has been investigated for a long time, its translation into routine clinical practice of organ transplantation has not been achieved so far [1]. A recent pilot trial simultaneously transplanting kidney and bone marrow grafts from the same donor to HLA-identical recipients suffering from end-stage kidney failure and from multiple myeloma, provides proof-of-principle that tolerance can indeed be achieved with this approach [30]. At the same time, application of this protocol to patients without concomitant malignancy might be problematic. Substantially milder BMT protocols are needed to facilitate widespread use of this tolerance strategy. The clinically unattainable number of required donor BMC is one reason why the mildest regimens developed in murine studies could not be translated to the clinic so far [6-9;31]. The use of mPBSC would allow transplantation of substantially higher numbers of hematopoietic cells from a single donor, and could therefore potentially permit the application of milder regimens. We thus began to investigate whether mPBSC could be substituted for BMC in costiumulation blockade-based mixed chimerism protocols designed for the induction of transplantation tolerance.

As a first step towards developing murine mPBSC-based tolerance regimens, we investigated the engraftment of unseparated murine mPBSC in the absence of alloreactivity. To our knowledge, the transplantation of murine mPBSC after low-dose non-myeloablative TBI has not been investigated in detail so far, as most previous studies used lethal conditioning of the recipient [17-24]. The results show that sufficient numbers of HSC are contained in $20x10^6$ mPBSC to allow successful engraftment and lasting

reconstitution under non-myeloablative conditions, as evidenced by multi-lineage macrochimerism remaining stable for more than 6 months. As expected, chimerism levels correlated with the dose of irradiation. Notably, at all tested TBI doses, chimerism induced with mPBSC was lower among all lineages than chimerism achieved with the same number of BMC. This difference correlated with a lower percentage of c-kit$^+$ cells and CFU generating cells contained in mPBSC. Our data suggest that murine BMC contain approximately three to four times as many progenitors as mPBSC, which is consistent with data of Glass et al. [20], showing approximately four times as many CFU-GM in BMC than mPBSC of DBA/2 mice. Likewise, the percentage of CD34$^+$ cells is higher in human BMC than in human mPBSC [32]. Thus, under non-myeloablative conditions murine progenitor cells contained in either mPBSC or BMC have similar engraftment characteristics, leading to comparable multi-lineage macrochimerism.

These results serve as a rough indicator for the maximum possible chimerism levels that could ideally be expected after transplantation of a given number of unseparated murine allogeneic mPBSC if alloreactivity is completely overcome. According to our data, ~4.5 times as many murine mPBSC than BMC would have to be transplanted to achieve similar chimerism. In the clinic, the cell yield of a mPBSC collection is typically 5 to 10-fold higher than from a conventional BM harvest. Thus, while substantially higher chimerism levels can be expected from transplanting a mPBSC harvest, these results raise doubt whether the advantage of harvesting mPBSC is sufficiently big to make mPBSC a viable solution for obtaining enough donor hematopoietic cells clinically to induce

chimerism without any cytoreduction [6;29]. This question is currently being addressed in our laboratory.

One caveat, however, that needs to be considered when trying to extrapolate these results from a syngeneic model to tolerance protocols, pertains to the role of donor T cells in promoting engraftment in the allogeneic setting. Thus, mPBSC, which are enriched for T cells, theoretically might lead to better engraftment when transplanted across an allogeneic barrier.

The CD45.1 isoform may induce a weak T cell-mediated [28] immune response which is strong enough to reduce BM engraftment [33]. This immunogenicity is of concern especially when minimal recipient conditioning leaves the host immune system largely intact. Even though significant immunogenicity was not found in the (reverse) strain combination which we used (CD45.2 into CD45.1) [34;35], we nevertheless wanted to rule out that mPBSC, due to the different cell composition, elicit a stronger immune response against CD45.2, or any other minor antigenic difference between the donor and recipient strain, than BMC. We thus treated recipients with a combination of immunosuppressive drugs that promotes chimerism in allogeneic models [26;29]. Immunosuppression did not detectably increase engraftment, thus indicating that alloreactivity is not responsible for the lower chimerism achieved with mPBSC in our studies.

mPBSC have already been used in a limited number of large animal models for inducing chimerism and tolerance [36-38]. In a haploidentical swine model, for instance,

transplantation of megadoses of mPBSC after thymic irradiation and recipient T cell depletion led to long-term chimerism and tolerance. While substantial levels of chimerism were achieved, it remains unclear how the engraftment potential of swine mPBSC compares to BMC since no direct comparison is available within the same model. For numerous reasons, large animal models of tolerance generally are less successful and require more intense protocols than murine models. These regimens were usually developed in mice by employing BMC, but not mPBSC [39]. It has not been convincingly shown, however, that allogeneic mPBSC behave similarly enough to allow substitution without requiring modifications of the protocols. Given the numerous known immunological differences between BMC and mPBSC [12-16], it appears likely that their properties regarding the ability to induce tolerance might differ. This is of particular concern when costimulation blockers are used in combination with HSCT, as their mechanisms of action depend critically on the details of a specific experimental protocol. Based on the results of the congenic models presented herein, studies are currently under way in our laboratory to develop costimulation-based allogeneic murine PBSC protocols.

From the present studies we conclude that in the absence of alloreactivity transplantation of unseparated murine mPBSC effectively leads to stable long-term multi-lineage chimerism. However, due to the lower frequency of progenitor cells mPBSC induce lower chimerism levels than the same number of unseparated BMC. A typical mPBSC harvest in the clinic, however, yields 5-10 times as many hematopoietic cells as bone marrow [40;41], more than offsetting this difference and allowing the transplantation of more HSC. Thus, as our data show that very similar chimerism levels can be expected per

transplanted progenitor, they support the evaluation of allogeneic mPBSC for the purpose of developing clinically more relevant mixed chimerism models.

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REFERENCES

1. Wekerle T, Sykes M: Mixed chimerism and transplantation tolerance. Annu Rev Med 52: 353-370, 2001

2. Wekerle T, Sayegh MH, Hill J, Zhao Y, Chandraker A, Swenson KG, Zhao G, Sykes M: Extrathymic T cell deletion and allogeneic stem cell engraftment induced with costimulatory blockade is followed by central T cell tolerance. J Exp Med 187: 2037-2044, 1998

3. Adams AB, Durham MM, Kean L, Shirasugi N, Ha J, Williams MA, Rees PA, Cheung MC, Mittelstaedt S, Bingaman AW, Archer DR, Pearson TC, Waller EK, Larsen CP: Costimulation blockade, busulfan, and bone marrow promote titratable macrochimerism, induce transplantation tolerance, and correct genetic hemoglobinopathies with minimal myelosuppression. J Immunol 167: 1103-1111, 2001

4. Taylor PA, Lees CJ, Waldmann H, Noelle RJ, Blazar BR: Requirements for the promotion of allogeneic engraftment by anti-CD154 (anti-CD40L) monoclonal antibody under nonmyeloablative conditions. Blood 98: 467-474, 2001

5. Quesenberry PJ, Zhong S, Wang H, Stewart M: Allogeneic chimerism with low-dose irradiation, antigen presensitization, and costimulator blockade in H-2 mismatched mice. Blood 97: 557-564, 2001

6. Wekerle T, Kurtz J, Ito H, Ronquillo JV, Dong V, Zhao G, Shaffer J, Sayegh MH, Sykes M: Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. Nature Med 6: 464-469, 2000

7. Seung E, Mordes JP, Rossini AA, Greiner DL: Hematopoietic chimerism and central tolerance created by peripheral-tolerance induction without myeloablative conditioning. J Clin Invest 112: 795-808, 2003

8. Durham MM, Bingaman AW, Adams AB, Ha J, Waitze S-Y, Pearson TC, Larsen CP: Administration of anti-CD40 ligand and donor bone marrow leads to hematopoietic chimerism and donor-specific tolerance without cytoreductive conditioning. J Immunol 165: 1-4, 2000

9. Lambert JF, Colvin GA, Zhong S, Wang H, D'Hondt L, Abedi M, Frimberger AE, Stewart FM, Quesenberry PJ: H2-mismatched transplantation with repetitive cell infusions and CD40 ligand antibody infusions without myeloablation. Br J Haematol 119: 155-63, 2002

10. Dreger P, Schmitz N: Allogeneic transplantation of blood stem cells: coming of age? Ann Hematol 80: 127-36, 2001

11. Korbling M, Anderlini P: Peripheral blood stem cell versus bone marrow allotransplantation: does the source of hematopoietic stem cells matter? Blood 98: 2900-2908, 2001

12. Arpinati M, Green CL, Heimfeld S, Heuser JE, Anasetti C: Granulocyte-colony stimulating factor mobilizes T helper 2-inducing dendritic cells. Blood 95: 2484-90, 2000

13. Cutler C, Antin JH: Peripheral blood stem cells for allogeneic transplantation: A Review. Stem Cells 19: 108-117, 2001

14. Gyger M, Stuart RK, Perreault C.: Immunobiology of allogeneic peripheral blood mononuclear cells mobilized with granulocyte-colony stimulating factor. Bone Marrow Transplant 26: 1-16, 2000

15. Pan L, Delmonte J, Jalonen CK, Ferrara JLM: Pretreatment of donor mice with granulocytes colony-stimulating factor polarizes donor T lymphocytes toward type-2 cytokine production and reduces severity of experimental graft-vs-host disease. Blood 86: 4422-4429, 1996

16. Sloand EM, Kim S, Maciejewski JP, Van Rhee F, Chaudhuri A, Barrett J, Young

NS: Pharmacologic doses of granulocyte colony-stimulating factor affect cytokine production by lymphocytes in vitro and in vivo. Blood 95: 2269-74, 2000

17. Neipp M, Zorina T, Domenick MA, Exner BG, Ildstad ST: Effect of FLT3 ligand and granulocyte colony-stimulating factor on expansion and mobilization of facilitating cells and hematopoietic stem cells in mice: kinetics and repopulating potential. Blood 92: 3177-3188, 1998

18. Yamamoto Y, Yasumizu R, Amou Y, Watanabe N, Nishio N, Toki J, Fukuhara S, Ikehara S: Characterization of peripheral blood stem cells in mice. Blood 88: 445-54, 1996

19. Yamamoto Y, Wang B, Fukuhara S, Ikehara S, Good RA: Mixed peripheral blood stem cell transplantation for autoimmune disease in BXSB/MpJ mice. Blood 100: 1886-1893, 2002

20. Glass B, Uharek L, Zeis M, Dreger P, Loffler H, Steinmann J, Schmitz N: Allogeneic peripheral blood progenitor cell transplantation in a murine model: evidence for an improved graft-versus-leukemia effect. Blood 90: 1694-700, 1997

21. Wagers AJ, Allsopp RC, Weissman IL: Changes in integrin expression are associated with altered homing properties of Lin-/loThy1.1loSca-1+c-kit+ hematopoietic stem cells following mobilization by cyclophosphamide/granulocyte colony-stimulating factor. Exp. Hematol 30: 176-185, 2002

22. Morrison SJ, Wright DE, Weissman IL: Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. Proc Natl Acad Sci USA 94: 1908-1913, 1997

23. Brasel K, McKenna HJ, Charrier K, Morrissey PJ, Williams DE, Lyman SD: Flt3 Ligand synergizes with granulocyte-macrophage colony-stimulating factor or granulocyte colony-stimulating factor to mobilize hematopoietic progenitor Cells Into the Peripheral Blood of Mice. Blood 90: 3781-3788, 1997

24. Szilvassy SJ, Meyerrose TE, Ragland PL, Grimes B: Differential homing and engraftment properties of hematopoietic progenitor cells from murine bone marrow, mobilized peripheral blood, and fetal liver. Blood 98: 2108-2115, 2001

25. Weiss L, Or R, Slavin S, Naparstek E, Reich S, Abdul-Hai A: Immunotherapy of murine leukemia following non-myeloablative conditionin with naive or G-CSF mobilized blood or bone marrow stem cells. Cancer Immunol Immunother 53: 358-362, 2004

26. Blaha P, Bigenzahn S, Koporc Z, Schmid M, Langer F, Selzer E, Bergmeister H, Wrba F, Kurtz J, Kiss C, Roth E, Muehlbacher F, Sykes M, Wekerle T: The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. Blood 101: 2886-2893, 2003

27. Okada S, Nakauchi H, Nagayoshi K, Nishikawa S, Nishikawa S, Miura Y, Suda T: Enrichment and characterization of murine hematopoietic stem cells that express c-kit molecule. Blood 78: 1706-1712, 1991

28. Xu H, Exner BG, Chilton PM, Schanie C, Ildstad ST: CD45 congenic bone marrow transplantation: Evidence for T cell-mediated immunity. Stem Cells 22: 1039-1048, 2004

29. Blaha P, Bigenzahn S, Koporc Z, Sykes M, Muehlbacher F, Wekerle T: Short-term immunosuppression facilitates induction of mixed chimerism and tolerance after BMT without cytoreductive conditioning. Transplantation 80:237-43, 2005

30. Bühler LH, Spitzer TR, Sykes M, Sachs DH, Delmonico FL, Tolkoff-Rubin N, Saidman SL, Sackstein R, McAffee S, Dey B, Colby C, Cosimi AB: Induction of kidney allograft tolerance after transient lymphohematopoietic chimerism in patients with multiple myeloma and end-stage renal disease. Transplantation 74: 1405-1409, 2002

31. Graca L, Le Moine A, Lin C-Y, Fairchild PJ, Cobbold SP, Waldmann H: Donor-specific transplantation tolerance: The paradoxical behavior of CD4+CD25+ T

cells. Proc. Natl. Acad. Sci. 101: 10122-10126, 2004

32. To L, Haylock D, Dowse T, Simmons P, Trimboli S, Ashman L, Juttner C: A comparative study of the phenotype and proliferative capacity of peripheral blood (PB) CD34+ cells mobilized by four different protocols and those of steady-phase PB and bone marrow CD34+ cells. Blood 84: 2930-2939, 1994

33. van Os R, Sheridan T, Robinson S, Drukteinis D, Ferrara J, Mauch P: Immunogenicity of Ly5 (CD45)-antigens hampers long-term engraftment following minimal conditioning in a murine bone marrow transplantation model. Stem Cells 19: 80-87, 2001

34. Sykes M, Chester CH, Sundt TM, Romick ML, Hoyles KA, Sachs DH: Effects of T cell depletion in radiation bone marrow chimeras: III. Characterization of allogeneic bone marrow cell populations that increase allogeneic chimerism independently of graft-vs-host disease in mixed marrow recipients. J.Immunol. 143: 3503-3511, 1989

35. Ito H, Takeuchi Y, Shaffer J, Sykes M: Local irradiation enhances congenic donor pluripotent hematopoietic stem cell engraftment similarly in irradiated and nonirradiated sites. Blood 103: 1949-1954, 2004

36. Fuchimoto Y, Huang CA, Shimizu A, Kitamura H, Colvin RB, Ferrara V, Murphy MC, Sykes M, White-Sharf ME, Neville DM, Sachs DH: Mixed chimerism without whole body irradiation in a large animal model. J Clin Invest 105: 1779-1789, 2000

37. Colby C, Chang Q, Fuchimoto Y, Ferrara V, Murphy M, Sackstein R, Spitzer TR, White-Sharf ME, Sachs DH: Cytokine-mobilized peripheral blood progenitor cells for allogeneic reconstitution of miniature swine. Transplantation 69: 135-140, 2000

38. Buhler L, Awwad M, Treter S, Chang Q, Basker M, Alwayn IP, Teranishi K, Ericsson T, Moran K, Harper D, Kurilla-Mahon B, Huang CA, Sackstein R, Sykes M, White-Scharf ME, Sachs DH, Down JD, Cooper DK: Pig hematopoietic cell chimerism in baboons conditioned with a nonmyeloablative regiment and CD154

blockade. Transplantation 73: 12-22, 2002

39. Sykes M, Szot GL, Swenson K, Pearson DA: Induction of high levels of allogeneic hematopoietic reconstitution and donor-specific tolerance without myelosuppressive conditioning. Nature Med. 3: 783-787, 1997

40. Bensinger WI, Martin PJ, Storer B, Clift R, Forman SJ, Negrin R, Kashyap A, Flowers ME, Lilleby K, Chauncey TR, Storb R, Appelbaum FR: Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. N Engl J Med 344: 175-81, 2001

41. Ringden O, Remberger M, Runde V, Bornhauser M, Blau IW, Basara N, Holig K, Beelen DW, Hagglund H, Basu O, Ehninger G, Fauser AA: Peripheral blood stem cell transplantation from unrelated donors: a comparison with marrow transplantation. Blood 94: 455-64, 1999

TABLES

Table 1. Chimerism levels in marrow and spleen correlate with chimerism levels in blood after BMT or mPBSCT.

	CD4	CD8	B220	MAC1	NK 1.1
I					
2 Gy TBI					
$20x10^6$ BMC					
Mean % of donor cells in BM	-	-	78.7	78.0	-
Mean % of donor cells in SPL	65.4	58.4	86.2	81.0	45.9
$20x10^6$ mPBSC					
Mean % of donor cells in BM	-	-	39.7	41.9	-
Mean % of donor cells in SPL	29.1	27.2	46.7	48.8	23.1
II					
1 Gy TBI					
$20x10^6$ BMC					
Mean % of donor cells in BM	-	-	41.5	43.6	-
Mean % of donor cells in SPL	28.6	26.2	48.1	39.3	22.5
$20x10^6$ mPBSC					
Mean % of donor cells in BM	-	-	12.5	11.7	-
Mean % of donor cells in SPL	6.3	5.0	12.9	10.5	5.4

Table 1. Chimerism was analyzed by FCM in bone marrow and spleen of recipients transplanted with $20x10^6$ BMC or mPBSC under 2 or 1 Gy of TBI (33 weeks post posttransplant). Chimerism levels in BM and SPL correlated with chimerism in peripheral blood. Two randomly selected mice per group were analyzed.

Table 2. FCM analysis of various lineage markers among BM and in mPBSC.

Cell marker	**CD4**	**CD8**	**B220**	**MAC1**	**CD117**	**CD49b**	**TCR-β**
% in **BM**	1.9	1.8	27.8	64.5	11.1	11.9	4.9
% in **mPBSC**	16.8	11.7	38.5	32.3	3.7	33.8	31.6

Table 2. mPBSC contain markedly more $CD4^+$ and $CD8^+$ cells. Almost three time as many c-kit^+ (CD117) cells are found in BMC than in mPBSC. One representative result of two similar experiments is shown.

Table 3. Chimerism levels in BM and spleen correlate with chimerism levels in blood after BMT or mPBSCT. Additional use of IS did not significantly improve chimerism.

	CD4	CD8	B220	MAC1	TCR-β
I					
1.5 Gy TBI					
10x10^6 BMC					
Mean % of donor cells in BM	-	-	37.8	43.9	-
Mean % of donor cells in SPL	47.2	36.5	54.3	-	-
Mean % of donor cells in THY	-	65.7	-	-	69.0
30x10^6 mPBSC					
Mean % of donor cells in BM	-	-	32.3	32.5	-
Mean % of donor cells in SPL	31.6	26.1	34.5	-	-
Mean % of donor cells in THY	-	57.4	-	-	61.1
II					
*1.5 Gy TBI, **IS***					
10x10^6 BMC					
Mean % of donor cells in BM	-	-	55.9	48.2	-
Mean % of donor cells in SPL	37.9	33.9	51.7	-	-
Mean % of donor cells in THY	-	53.7	-	-	57.4
30x10^6 mPBSC					
Mean % of donor cells in BM	-	-	36.9	23.8	-
Mean % of donor cells in SPL	26.1	24.8	45.4	-	-
Mean % of donor cells in THY	-	37.4	-	-	41.3

Table 3. Chimerism was determined by FCM in BM, spleen and thymus of recipients transplanted with 10x10^6 BMC or 30x10^6 mPBSC after 1.5 Gy of TBI, with [II] or without IS [I]) (29 weeks posttransplant). Similar differences in chimerism between

recipients of BMC vs mPBSC were found in these tissues as in blood. IS did not significantly influence chimerism. Two best chimeras were analyzed per group.

FIGURE LEGENDS

Figure 1. Transplantation of PBSC leads to significantly lower levels of long-term chimerism in comparison with BMC after non-myeloablative TBI. CD45.1 mice (n=6/group) received either 20x10^6 CD45.2 BMC (**A, B, C**) or 20x10^6 CD45.2 mPBSC (**D, E, F**) after various doses of TBI (2 Gy – **A, D**; 1.5 Gy – **B, E** or 1Gy – **C, F**). The percentages of donor-derived $CD4^+$ cells, $CD8^+$ cells, B cells, monocytes/granulocytes and NK cells among WBC were evaluated by FCM at different time points, and are shown as means. Levels of chimerism induced with mPBSC were significantly lower compared to those induced with BMC treated with same dose of TBI (**A vs. D; or B vs. E;** and **C vs. F**, $p<0.05$).

Figure 2. Transplantation of similar numbers of c-kit^+ mPBSC and c-kit^+ BMC still leads to differences in chimerism levels. CD45.1 mice (n=6/group) received either 10x10^6 CD45.2 BMC (**A, C**) or 30x10^6 CD45.2 mPBSC (**B, D**) with 1.5 Gy of TBI. In this way similar numbers of c-kit^+ cells were transplanted in both groups (according to the percentage of c-kit^+ cells as determined by FCM analysis among unseparated mPBSC and BMC). In order to evaluate if the lower chimerism induced in mPBSCT recipients is due to a remaining alloresponse against CD45.2, or other antigenic differences, some groups received immunosuppression (**C, D**) (rapa, MP, and MMF for four weeks) others remained untreated (**A, B**). Chimerism levels in various lineages as determined by FCM analysis over time are shown as means. *$p<0.05$ indicates a significant difference in chimerism between BMT and mPBSCT groups (**A vs. B** and **C vs. D**). IS treatment did

not significantly improve levels of chimerism (!p=n.s. in any lineage) either by BMT or mPBSCT (**A vs. C** or **B vs. D**). HSCT denotes hematopoietic stem cell transplantation.

Figure 3. Levels of chimerism induced with BMC and mPBSC recipients correlate with the number of transplanted progenitor cells as estimated from CFU assays. Chimerism per transplanted progenitor cell was calculated as described in detail in the Methods section. A significant difference in percentage of chimerism per $1x10^4$ transplanted progenitor cells, between BMC and mPBSC recipients (**A** vs. **B**) is noticeable only in the first few weeks after HSCT (**C**), probably due to the larger percentage of $CD4^+$ and $CD8^+$ cells contained in mPBSC. Data are from the same experiment for which results are also shown in Figure 2. HSCT denotes hematopoietic stem cell transplantation.

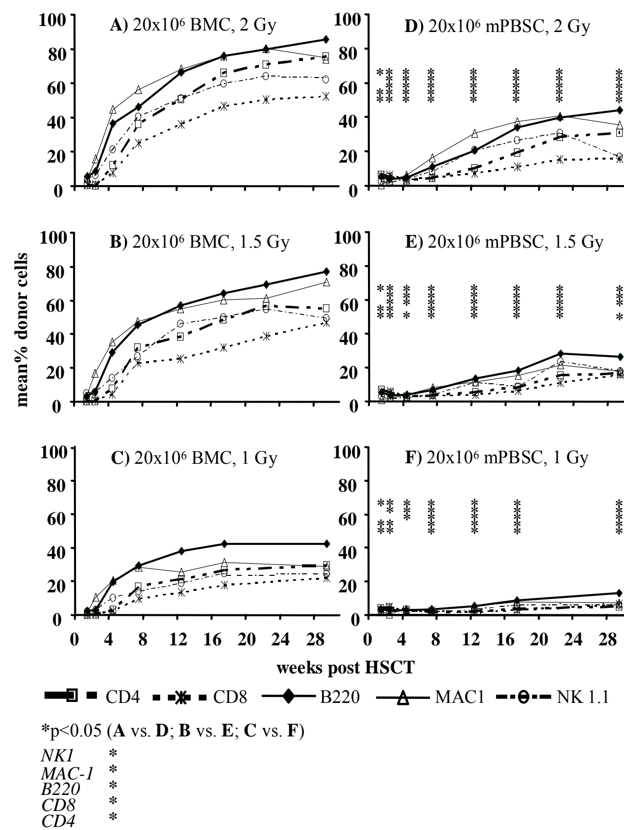

Figure 1

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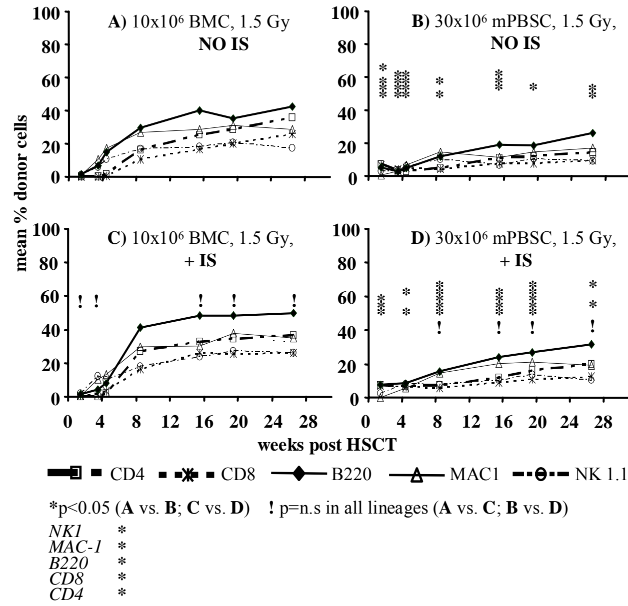

Figure 2

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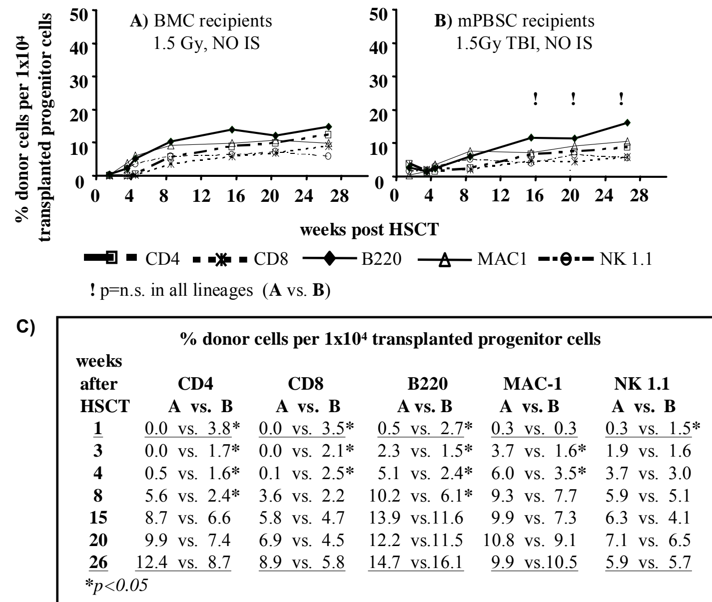

% donor cells per $1x10^4$ transplanted progenitor cells

weeks after HSCT	CD4 A vs. B	CD8 A vs. B	B220 A vs. B	MAC-1 A vs. B	NK 1.1 A vs. B
1	0.0 vs. 3.8*	0.0 vs. 3.5*	0.5 vs. 2.7*	0.3 vs. 0.3	0.3 vs. 1.5*
3	0.0 vs. 1.7*	0.0 vs. 2.1*	2.3 vs. 1.5*	3.7 vs. 1.6*	1.9 vs. 1.6
4	0.5 vs. 1.6*	0.1 vs. 2.5*	5.1 vs. 2.4*	6.0 vs. 3.5*	3.7 vs. 3.0
8	5.6 vs. 2.4*	3.6 vs. 2.2	10.2 vs. 6.1*	9.3 vs. 7.7	5.9 vs. 5.1
15	8.7 vs. 6.6	5.8 vs. 4.7	13.9 vs.11.6	9.9 vs. 7.3	6.3 vs. 4.1
20	9.9 vs. 7.4	6.9 vs. 4.5	12.2 vs.11.5	10.8 vs. 9.1	7.1 vs. 6.5
26	12.4 vs. 8.7	8.9 vs. 5.8	14.7 vs.16.1	9.9 vs.10.5	5.9 vs. 5.7

*$p<0.05$

Figure 3

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3

Mixed Chimerism and Tolerance through Transplantation of murine allogeneic mobilized Peripheral Blood Stem Cells

ABBREVIATIONS

BM, bone marrow

BMC, bone marrow cells

BMT, bone marrow transplantation

CFU, colony forming unit

CFU-GM, colony forming unit-granulocyte/monocyte progenitor

G-CSF, granulocyte colony stimulating factor

Gy, Gray

HSC, hematopoietic stem cells

HSCT, hematopoietic stem cell transplantation

PBSC, mobilized peripheral blood stem cells

PBSCT, mobilized peripheral blood stem cells transplantations

PB, peripheral blood

SPL, spleen

TBI, total body irradiation

ABSTRACT

Allogeneic bone marrow transplantation (BMT) under costimulation blockade allows induction of mixed chimerism and tolerance without global T cell depletion. The mildest such protocols without recipient cytoreduction, however, require clinically impracticable BM doses. The successful use of mobilized peripheral blood stem cells (PBSC) instead of BM in such regimens would provide a substantial advance, allowing transplantation of higher doses of hematopoietic donor cells. We thus transplanted fully allogeneic murine granulocyte colony-stimulating-factor (G-CSF) mobilized PBSC under costimulation blockade (anti-CD154 and CTLA4Ig). Unexpectedly, PBSC did not engraft, even when very high cell doses and non-myeloablative total body irradiation (TBI) were used. Paradoxically, T cells contained in the donor PBSC triggered rejection of the transplanted donor cells. Donor-specific transfusion and transient immunosuppression prevented PBSC-triggered rejection and mixed chimerism and tolerance were achieved, but graft-versus-host disease (GVHD) occurred. The combination of *in vivo* T cell depletion with costimulation blockade prevented rejection and GVHD. Thus, if allogeneic PBSC are transplanted instead of BMC costimulation blockade alone did not induce chimerism and tolerance without unacceptable GVHD-toxicity, and the addition of global T cell depletion was required for success.

INTRODUCTION

Induction of donor-specific tolerance in organ transplant recipients would substantially improve outcome by preventing graft loss due to acute and chronic rejection and by avoiding side effects of immunosuppressive drug therapy. The induction of mixed chimerism through transplantation of donor hematopoietic stem cells is a promising experimental strategy leading to robust tolerance [42]. Its clinical translation, however, has so far been prevented in large part by the toxicity of recipient conditioning

Mixed chimerism can be induced in rodent and large animal models by global destruction of the recipient T cell repertoire through the use of T cell depleting mAbs, in addition to non-myeloablative total body irradiation (TBI) [15;31;36]. Non-cytoreductive protocols entirely without irradiation or cytotoxic drugs were not achieved with this approach [11;40]. More recently the use of costimulation blockade (anti-CD40L with or without CTLA4Ig) allowed establishment of mixed chimerism and tolerance without global T cell depletion with either non-myeloablative conditioning [1;4;41], or entirely without cytoreduction [5;10;39]. Non-cytoreductive protocols, however, require transplanting amounts of bone marrow cells (BMC) substantially higher than clinically available. Since non-myeloblative conditioning is regarded as too toxic by many for routine use in organ transplant recipients, non-cytoreductive protocols using amounts of donor hematopoietic cells which are feasible in the clinical setting still need to be developed.

In the clinic, collection of G-CSF-mobilized peripheral blood stem cells (PBSC) allows the harvest of a many-fold higher number of hematopoietic cells (from a living donor).

Thus, the clinical development of the mixed chimerism approach for transplantation tolerance should be facilitated if PBSC could be used instead of BMC. While transplantation of mobilized allogeneic PBSC has an established clinical role in the treatment of hematological diseases [9;19], PBSC differ substantially from BMC with respect to several major biological and immunological characteristics [2;8;13;25;32]. To the best of our knowledge murine allogeneic mobilized-PBSC have not been investigated so far for the purpose of inducing transplantation tolerance. It is thus unknown whether the distinct properties of PBSC influence the induction of allogeneic mixed chimerism and tolerance, and, in particular, whether costimulation blockade is equally effective in established tolerance regimens when allogeneic PBSC are transplanted instead of BMC.

We have recently shown that transplantation of $20x10^6$ CD45-congenic PBSC leads to high levels of stable long-term multilineage chimerism (>6 months) after non-myeloablative TBI {Koporc, Wekerle et al. BBMT in press}. While roughly 3-4 times as many progenitor cells were found among unseparated BMC as among PBSC, progenitor cells contained in PBSC had similar engraftment capacity on a per-cell basis. These engraftment characteristics in the absence of an immunological barrier encouraged us to investigate transplantation of allogeneic PBSC for the purpose of tolerance induction.

Here we show that allogeneic PBSC behave markedly different from BMC in murine chimerism-based tolerance protocols. PBSC fail to engraft when transplanted even in high doses both in costimulation-based and T cell depletion-based non-myeloablative regimens that are successful when BMC are transplanted. Donor T cells contained in

PBSC trigger their own rejection. This rejection can be prevented by adding recipient conditioning with DST and immunosuppression to costimulation blockade and non-myeloablative TBI, however this is associated with severe GVHD. Adding *in vivo* T cell depletion to costimulation blockade avoids GVHD. Thus, if PBSC are used instead of BMC major modifications of mixed chimerism protocols are necessary.

MATERIALS AND METHODS

Animals. Female mice were purchased from the Charles River Laboratories (Sulzfeld, Germany), were kept under specific pathogen-free conditions and were used between 6 and 10 weeks of age. All experiments were approved by the local review board of the Medical University of Vienna, and were performed in accordance with national and international guidelines of laboratory animal care.

BMT and mobilized peripheral blood stem cell transplantation (PBSCT). Age-matched (6- to 12-week old) female C57BL/6 (B6: H-2^b) mice received TBI (1 – 3, or 10 Gy, as indicated) 1 day before the cell transplant (d-1). For harvesting murine PBSC a protocol described by Weissman et al. [24] was used with minor modifications. To avoid pooling of PBSC in spleen, donor Balb/c were splenectomized at least 14 days prior to the mobilization procedure [24]. Thereafter, 5µg of human G-CSF (approx. 250 µg/kg) (Neupogen®, Amgen Europe BV Breda, Netherlands) were injected subcutaneously (s.c.) for 5 consecutive days. Two hours after the last injection, mice were maximally bled (using tail bleeding and heart puncture), the heparinizied blood was pooled and diluted with PBS (1:1). Subsequently the same quantity of 2% dextran T500 solution was added (to give a final concentration of 1% dextran). Red blood cells (RBC) were separated by sedimentation for 45 minutes at 37ºC, before the supernatant fraction containing the mobilized leukocytes (PBSC) was collected and was used without further manipulation (enrichment or depletion), unless indicated otherwise. BM was harvested from tibiae, femurs and humeri, as described in detail previously [4]. Both BMC and PBSC were filtrated through a 70 µm filter. BMC were counted manually (by trypan blue exclusion

of dead cells) and PBSC both manually and automatically. Cells were diluted with cold BM media [500ml Medium 199 (Sigma, Vienna, Austria), supplemented with 5 ml HEPES buffer (ICN, Biomedica, Vienna, Austria), 5mg DNAse (Sigma) and 2 mg Gentamycin (Sigma)] and were injected in a volume of 1 ml into a tail vein of recipient mice (d0). In protocol J, $40x10^6$ splenocytes from Balb/c mice were injected on d-6, together with MR1 (1mg on d-6 and 0.5mg on d-4).

Costimulation blockade. Recipients were treated with a hamster anti-mouse-CD154 mAb (MR1; 1 mg injected intraperitoneally; d0) and with human CTLA4Ig (0.5 mg injected intraperitoneally; d+2). In two experiments of protocol J, higher doses of costimulation blockade were used (MR1: 1mg on d0, 0.5 mg on days 2, 4 and 6; CTLA4Ig: 0.5mg on days 2, 4, 6 and 8). MR1 was purchased from Bioexpress Inc. (New Hampshire, USA), hCTLA4Ig was generously provided by Bristol-Myers, Squibb Pharmaceuticals (Princeton, New Jersey),

***In vivo* and *in vitro* T cell depletion.** Where indicated, recipients were either injected at d -5 and -1 or only d 0 with a depleting anti-CD8 mAb (2.43, 1.4 mg) and a depleteing anti-CD4 mAb (GK1.5, 1.8 mg) or with PBSC depleted of T cell by anti-CD90 (Thy1.2) MACS separation (Miltenyi Biotec, Bergisch Gladbach, Germany). In vitro T cell depletion was typically 81-91% complete for CD4 cells, and 90-100% for CD8 cells.

***In vivo* cytokine release.** Serum concentration of cytokines was measured (d5) using the mouse Th1/Th2 10plex FlowCytomix system (Bender MedSystems, Vienna, Austria).

Secretion of IL-10, IL-6, IL-5, IL-2, IL-1a, IL-17, IL-4, GM-CSF, TNFα and IFNγ were determined by flow-cytometric analysis.

Flowcytometric analysis (FCM). Two-color FCM was used to distinguish donor and recipient cells of particular lineages, by staining with fluorescein isothiocyanate (FITC)-conjugated antibodies against CD4, CD8, B220, MAC1 and biotinylated H-$2D^d$ (34-2-12, developed with phycoerythrin streptavidin) and irrelevant isotype controls. To analyze cell composition, BMC and PBSC were stained with fluorescein isothiocyanate-conjugated antibodies against CD4, CD8, B220, MAC-1, CD117 (c-kit), CD49b, TCR-β, GR-1 and biotin-conjugated CD45.2 with irrelevant isotype controls (all antibodies from Pharmingen, San Diego, California). Propidium iodide staining was used to exclude dead cells. Mice were considered chimeric if they showed at least 2 % donor cells within the myeloid lineage plus at least one lymphoid lineage. Flow-cytometric analysis was performed as described in detail previously [4].

Skin grafting. Skin grafting was performed 3-10 weeks after cell transplantation. Full thickness tail skin from Balb/c mice (donor) and fully mismatched C3H mice (3rd party) was grafted on the lateral thoracic wall, secured with 4-0 sutures and adhesive bandages. Grafts were monitored at short intervals from day 7 onward. Grafts were considered to be rejected when less than 10% of the graft remained viable.

Immunosuppression. Mice were injected daily with immunosuppressive drugs in the indicated groups, (day 0 to day 20 or 27). Drugs were used at following doses:

rapamycin: 0.2 mg/kg/d; methylprednisolone (MP): 10 mg/kg/d; mycophenolate mofetil (MMF): 20mg/kg/d. Drugs were diluted and administered as described previously [4]. Rapamycin was kindly provided by Wyeth-Ayerst, New Jersey, USA, and MMF by Roche, Vienna Austria. MP was purchased from Aventis, Vienna, Austria.

Mixed lymphocyte reaction (MLR). MLRs were performed as descibed in more detail previously [4]. Briefly, $4x10^5$ responder splenocytes were incubated with $4x10^5$ irradiated (30 Gy) stimulator cells of either Balb/c (donor), C3H or SJL/JCrl (3rd party) and B6 (host) mice or only with medium. After 3 or 4 days, cells were pulsed with ^{3}H-thymidine and incubated for 18 hours. Stimulation indices (SI) were calculated by dividing the mean counts per minute (c.p.m.) from responses against host (B6), donor (Balb/c) or 3rd party (C3H or SJL/JCrl) by mean background c.p.m. (i.e., c.p.m. with no stimulator population).

GVHD observations. Mice were frequently screened for weight loss, diarrhea, hair loss, skin changes and hunched posture.

Statistics. A two-tailed, unpaired Student's T test was used for comparing percentages of chimerism, SI values between given groups and percentages of Vβ-positive populations between groups. The Chi-square test was used for comparing rates of chimeras, and rates of skin graft acceptance. Skin graft survival was calculated according to the Kaplan-Meier product limit method and compared between groups by using the log-rank test. A P value less than 0.05 was considered to be statistically significant.

RESULTS

PBSC behave differently from BMC in non-myeloablative chimerism protocols relying on costimulation blockade or on recipient T cell depletion

To investigate the use of PBSC for the induction of mixed chimerism and tolerance, we first transplanted escalating doses of fully mismatched G-CSF-moblized PBSC employing an established protocol successfully establishing chimerism and tolerance in a high proportion of recipients when BMC are transplanted (20x10^6 fully mismatched, unseparated Balb/c BMC, 2 or 3 Gy TBI, costimulation blockade with anti-CD154 plus CTLA4Ig [3;4;41]) (experimental protocols used in this paper are summarized in Table 1).

B6 mice receiving 20x10^6 Balb/c BMC developed long-term macrochimerism (2 Gy TBI: 6/6, 3 Gy: 10/10). In contrast, transplantation of the same, or substantially higher numbers of PBSC after 1 to 3 Gy TBI and costimulation blockade did not lead to long-term macrochimerism (<u>20x10^6:</u> 2 Gy TBI: 0/6, 3 Gy: 0/6); <u>75x10^6:</u> 1 Gy TBI: 0/6, 1.5 Gy: 0/6, 2 Gy: 0/6, 3 Gy: 0/4; <u>200x10^6:</u> 2/9 demonstrated chimerism at 2 weeks, but 0/9 developed long-term chimerism). Transplantation of 200x10^6 BMC under costimulation blockade can induce chimerism and tolerance without any TBI, or other cytoreduction [5;10;39]. Since it was shown that under certain circumstances irradiation negatively affects engraftment after BMT and costimulation blockade [5;33], we also transplanted 200x10^6 Balb/c PBSC without TBI. Again, chimerism was not induced (0/3) (Table 2).

To explore whether the different properties of PBSC compared to BMC occur only in relation to the effect of costimulation blockade, we transplanted PBSC using a protocol

relying on global *in vivo* recipient T cell depletion (3 Gy TBI, anti-CD4 plus anti-CD8 mAbs on days -5 and -1 [35]). B6 recipients treated with this regimen and 75x10^6 Balb/c PBSC developed only some early, but no long-term chimerism (0/5 at 7 weeks, Table 2), and did not become tolerant. In contrast, the majority of controls transplanted with 25x10^6 Balb/c BMC developed long-term chimerism (4/6 at week 7; 2/3 at week 26) and tolerance.

Thus, in contrast to BMC, allogeneic PBSC do not engraft with non-myeloablative conditioning protocols involving either only costimulation blockade, or only *in vivo* T cell depletion.

PBSC are more immunogenic triggering rejection of the transplanted donor cells

Two, not mutually exclusive, factors could be responsible for the failure of PBSC to induce chimerism: (non-immunologic) engraftment failure or rejection. To distinguish between these two possibilities, we transplanted T cell-depleted Balb/c PBSC (40x10^6) into lethally irradiated (10 Gy TBI) B6 recipients. Although animals shortly after the transplantation started to develop signs of GVHD (presumably due to incomplete T cell depletion), this protocol demonstrated that PBSC engrafted successfully, leading to full chimerism (5/5 at week 1, 3/3 at week 6; data not shown). Besides, we have recently shown that transplantation of 20x10^6 PBSC (harvested with the same technique used in the studies described herein) into CD45-congenic recipients after 1 Gy TBI led to substantial levels of stable long-term macrochimerism (approx. 13% MAC-1^+ chimerism at 29 weeks) (Koporc Biol Blood Marrow Transplant 2006 in press). From this we

conclude that 20x10^6 PBSC contain sufficient numbers of hematopoietic progenitors and stem cells to induce lasting macrochimerism in the absence of alloreactivity, and thus primary engraftment failure is unlikely to be the main factor preventing chimerism induction with allogeneic PBSC.

To determine whether the transplanted allogeneic PBSC were rejected, MLR assays were performed, which revealed donor-reactivity among the recipients of PBSC (with either costimulation blockade, or *in vivo* T cell depletion, protocols B and D), whereas BMC recipients typically demonstrated donor hyporesponsiveness (Table 2). Thus, allogeneic PBSC seem to be rejected after non-myeloablative TBI with costimulation blockade or *in vivo* T cell depletion.

To distinguish whether PBSC are solely less tolerogenic and are rejected because they fail to induce tolerance, or whether they actively trigger rejection, we co-transplanted BMC and PBSC. If PBSC are solely less tolerogenic, they will not negatively interfere with tolerance and chimerism induction achieved through the co-transplanted BMC. B6 received 3 Gy TBI and costimulation blockade and were injected with 20x10^6 Balb/c BMC together with 60x10^6 Balb/c PBSC (n=18; 4 separate experiments). In 3 of 4 experiments chimerism was undetectable in all co-transplanted mice as early as one week after transplantation (0/13) (Figure 1), and tolerance did not ensue (8/8 rejected donor skin [skin grafting was not performed in one experiment with 0/5 chimeric mice], <u>MLR SI against donor:</u> PBSC+BMC vs. BMC; 2.4 vs. 0.7 $p<0.001$, Table 3). In one experiment 5/5 co-transplanted mice developed long-term chimerism, but only 1/5

accepted donor skin long-term (MST=39d). Control groups included in each experiment, transplanted with $20x10^6$ BMC alone (without PBSC) developed long-term chimerism (19/22, p<0.001 compared to BMC+PBSC) and tolerance (14/19, p<0.001). To rule out that any traces of dextran injected with PBSC mediated rejection, $20x10^6$ BMC mixed with 2% dextran solution were transplanted, without negative effect. Thus, PBSC did not just fail to induce tolerance, but actively triggered rejection also of the transplanted donor BMC.

To determinate whether the detrimental effect of co-transplanted PBSC is dose-dependent, we transplanted $20x10^6$ BMC together with $2x10^6$, $5x10^6$, $10x10^6$ or $20x10^6$ PBSC (with 3 Gy TBI and costimulation blockade). Long-term chimerism was seen in 2/3 (p=n.s.), 2/5 (p=n.s.), 4/5 (p<0.05) and 3/4 (p=n.s.) recipients, respectively (12 weeks post-HSCT, compared to co-transplanting $60x10^6$ PBSC; donor skin was accepted >100 days in 0/3 mice co-transferred with $20x10^6$ PBSC, and 4/5 mice receiving BMC only [p<0.05]). Hence, the rejection-triggering effect of PBSC seems to be dose-dependent to some degree, but the full effect is observed with moderate, clinically relevant doses ($60x10^6$).

We also determined whether PBSC break tolerance when injected late after BMT into stable chimeras with healed-in donor skin grafts ($20x10^6$ BMC, 3 Gy TBI plus costimulation blockade; $60x10^6$ PBSC injected 94 days after BMT, protocl G). The PBSC transplantation late after BMT led to an increase in chimerism levels which persisted until the end of follow-up (19 weeks post PBSC infusion, Figure 2 I), and did not cause

rejection of donor grafts (Figure 3 B). Despite conversion to full chimerism, no signs of GVHD were noticed. These data suggest, that PBSC trigger rejection when administered early after conditioning, but do not have a detrimental effect when given at late time points, augmenting chimerism without causing GVHD.

Donor T cells contained in PBSC trigger rejection

Transplantation of PBSC has been reported to induce a Th2-shift in the cytokine response of recipients [2]. Some evidence suggests that costimulation blockade affects Th1 and Th2 responses differently [17;38], raising the possibility that a cytokine shift caused by PBSC negatively affects tolerance induction, at least in the costimulation-based mixed chimerism protocols. We therefore measured serum levels of prototypical Th1 and Th2 cytokines 5 days after HSCT in groups of mice transplanted with either $20x10^6$ BMC alone or $20x10^6$ BMC plus $60x10^6$ PBSC, (3 Gy TBI plus costimulation blockade). As shown in Figure 4A, cytokine levels varied considerably among individual mice within the same group, and no statistically significant differences between the two groups were evident. These results are only suggestive but make it less likely that a Th2 shift is the main cause of the failure of the PBSC protocols.

The cell composition of PBSC differs considerably from the one of BMC (Figure 4B). In particular, consistent with the published literature [2;28], PBSC contained markedly higher percentages of $CD4^+$ and $CD8^+$ cells [mean value 28.1% vs. 1.6% for $CD4^+$ ($p<0.0001$) and 10.8.% vs. 0.7% for $CD8^+$ cells ($p<0.0001$), pooled results of 13 experiments]. To assess whether it is the T cells contained in PBSC that trigger rejection, we co-

transplanted Balb/c PBSC that had been T cell-depleted *in vitro* by MACS separation, with $20x10^6$ BMC (3 Gy TBI and costimulation blockade). While chimerism was undetectable in all mice receiving BMC with un-depleted PBSC (Figure 2B) (MST for donor skin =7d, n=4), chimerism and tolerance were induced in 7/8 mice co-transplanted with T cell-depleted PBSC (MST>31d) (Table 3, Figure 2A). Despite T cell depletion, however, signs of GVHD developed from day 48 on, with only 4 mice (2/4 chimeric) alive on day 59. In a separate experiment, chimerism and tolerance were again observed after co-transplantation of T cell-depleted PBSC together with BMC (6/6 Figure 2 D, MST>142d for donor skin) (Table 3). No signs of GVHD were observed, possibly because depletion was more complete. Unexpectedly, however, long-term chimerism was seen after co-transplantation of un-depleted PBSC as well (5/5 mice Figure 2E, but 4/5 mice lost the donor graft [MST=39d]) (as mentioned above, this was the only of four experiments were such an outcome occurred, Figure 3C-pooled data). GVHD was observed in recipients co-transplanted with un-depleted PBSC, which was never seen in BMC only-recipients. Since the levels of chimerism were lower when un-depleted PBSC were transplanted compared to depleted PBSC (e.g. 66% vs. 34% B cell chimerism $p<0.05$ at week 22, Figure 2D+E) it is suggested that the transplanted donor cells were partially rejected in the group without T cell depletion. Furthermore, chimerism levels were higher in the group co-transplanted with T cell-depleted PBSC than in the BMC only group (e.g. 57% vs. 24% CD4 $p<0.05$, 66% vs. 41% B cell $p<0.05$ at week 22, Figure 2D+F), indicating that the PBSC have successfully engrafted and contributed to chimerism.

When *in vivo* T cell depletion was used in recipients co-transplanted with BMC and PBSC (3 Gy TBI, $20x10^6$ BMC plus $60x10^6$ PBSC, costimulation blockade, anti-CD4 + anti-CD8 [d0], protocol I) high levels of multilineage chimerism developed. Chimerism persisted for the length of follow-up (7/7; Figure 2G), and chimerims levels were significantly higher than in recipients of BMC alone [e.g. 53% vs. 23% CD4 $p<0.05$, 51% vs. 19% CD8 $p<0.05$ at week 8, Figure 2G+I (BMC alone till d +94)], indicating that the PBSC have successfully engrafted. All recipients accepted donor skin (MST>188d; Figure 3A), without signs of GVHD.

Thus, donor T cells contained in PBSC trigger rejection of donor cells, which can be prevented by T cell depletion (*in vitro* or *in vivo*).

Additional immunosuppression and DST allow induction of chimerism and tolerance after transplantation of PBSC with costimulation blockade

Both i*n vitro* T cell depletion [22;23;27], and *in vivo* recipient T cell depletion are problematic in the clinical setting. Thus, protocols without T cell depletion would be desirable [29]. We, and others, have recently shown that rapamycin improves engraftment after BMT [4;20;43] and DST reduces recipient donor-reactivity in several protocols [21;33;37]. Therefore, we investigated whether the rejection triggered by PBSC is prevented by the addition of transient immunosuppression and DST to the non-myeloablative costimulation-based protocol ($40x10^6$ Balb/c SPL d-6 plus MR1; 3 Gy TBI d-1; $200x10^6$ or $75x10^6$ undepleted Balb/c PBSC; costimulation blockade; rapamycin+mycophenolate mofetil+methylprednisolone d0-27). The majority of mice developed full chimerism

when transplanted with $200x10^6$ PBSC (18/19, pooled data from four separate experiments) and skin graft tolerance (15/18; MST>99d, third party grafts were promptly rejected) (Figure 5A+C). MLR assays revealed donor-specific hyporesponsiveness (SI against donor 0.6; 3rd party 3.3, data from one experiment). However, approximately 2 months post-HSCT chimeric PBSC recipients developed clinical signs of chronic GVHD including weight loss, skin changes, and hunched posture, without evident diarrhea.

When only $75x10^6$ PBSC (which is a clinically more relevant dose) were transplanted with the regimen including DST and immunosuppression, 4/5 recipients developed mixed chimerism (at week 3), with 3/4 long-term chimeric mice surviving more than 100 days after HSCT (Figure 5B). Chimeras (n=4) permanently accepted donor skin (MST>69d), while promptly rejecting 3rd party grafts (Figure 5C). Howerver, the chimeric animals developed GVHD (starting from day 62). When $75x10^6$ PBSC were transplanted with additional DST, but without immmunosuppression, 3/5 (p=n.s.) mice showed chimerism at 1 week post-HSCT), and 0/3 at 11 weeks (two chimeras died before week 11). No mouse treated with additional IS but without DST showed chimerism at 1 week post-HSCT (0/5, p<0.01). Taken together, these results suggest that DST is critical for allowing the induction of chimerism and tolerance, and that the best results might be achieved when both DST and immunosuppression are given Lasting chimerism and tolerance are thus achieved after transplantation of a clinically relevant dose of PBSC under costimulation blockade and non-myeloablative TBI,. However, severe GVHD uniformly develops in such chimeras.

DISCUSSION

Unless minimally toxic mixed chimerism regimens are developed, the translation of this tolerance strategy into routine clinical use remains unlikely. Thus we investigated whether the transplantation of PBSC, instead of BMC, would allow the development of such clinically feasible and acceptable mixed chimerism protocols. However, we not only found that the use of PBSC failed to allow minimization of recipient conditioning, but on the contrary that PBSC are less tolerogenic and trigger their own rejection requiring intensified conditioning.

We started our investigation by substituting allogeneic PBSC for BMC in well-established non-myeloablative mixed chimerism protocols. Surprisingly, PBSC uniformly failed to engraft in costimulation blockade-based and in T cell depletion-based models. Chimerism was not achieved even when $200x10^6$ donor cells were transplanted which is the10-fold dose of PBSC which led to long-term chimerism in a CD45-congenic model {Koporc, Wekerle 2006}. Donor-reactivity observed in MLR assays also suggested that lack of chimerism is not just due to engraftment failure. Co-transplantation of PBSC together with BMC provided direct evidence that PBSC not merely fail to engraft or fail to induce tolerance but that they actively trigger rejection. Thus allogeneic PBSC are not just less tolerogenic but they are more immunogenic.

Co-transplantation of the same dose of PBSC late after HSCT did not break tolerance, but led to conversion into full chimerism without inducing GVHD. This time-dependent effect of PBSC is reminiscent of a cyclophosphamide-based murine non-myeloablative

mixed chimerism model, in which donor lymphocyte infusions were shown to have different effects depending on the time of injection [26]. While infusion of donor T cells early after BMT paradoxically triggered T cell-mediated rejection of donor BM, late donor lymphocyte infusions (>35 days post-BMT) converted mixed to full donor chimerism without causing GVHD. In this model donor CD4 cells triggered rejection by residual recipient T cells [16].

It was unexpected that neither costimulation blockade alone nor *in vivo* T cell depletion alone was sufficient to prevent PBSC-triggered rejection. Only their combination proved to be effective. After *in vivo* T cell depletion alone (without costimulation blockade), T cells contained in the transplanted PBSC are possibly not quickly or completely enough depleted by the circulating mAbs, and thus trigger rejection mediated by remaining recipient T cells escaping the T cell depleting antibodies. Adding costimulation blockade to *in vivo* T cell depletion might help in tolerising remaining donor-reactivity of the host, thereby allowing engraftment despite incomplete T cell depletion.

We and others have previously shown that short-term rapamycin-based immunosuppression promotes engraftment of allogeneic BMC in non-myeloablative, costimulation blockade-based protocols [4;14;34]. DST enhances chimerism after treatment with anti-CD40L mainly by overcoming host CD8 reactivity [30;33]. The additional use of immunosuppression and DST prevented the PBSC-triggered rejection when costimulation blockade and non-myeloablative TBI were given. GVHD was slightly delayed but not prevented.

GVHD does not occur after BMT with costimulation blockade, even when very high doses of (undepleted) BMC are transplanted [10;39]. Thus costimulation blockade injected at the time of BMT is evidently capable of tolerizing injected donor T cells towards recipient antigens, thereby preventing GVHD. In sharp contrast, costimulation blockade (together with DST and transient immunosuppression) did not prevent GVHD after transplantation of PBSC. This might be a purely quantitative phenomenon, as some donor T cells escape tolerization and go on to cause GVHD. Alternatively, T cells contained in PBSC differ qualitatively in a way that makes them costimulation blockade-resistant. T cells in G-CSF mobilized PBSC are known to be skewed towards a Th2-phenotype [2]. The role of cytokines in the induction of graft acceptance through costimulation blockade is somewhat complex [38], and remains incompletely understood, but IFNγ, a prototypic Th1 cytokine, was shown to be critical for the graft-prolonging effect of anti-CD154 plus CTLA4Ig in skin and heart graft models [18].

PBSC have been used for the induction of allo-tolerance [7;11;15] and xeno-tolerance [6] in a limited number of large animal models. In a pig model in which haplo-identical PBSC were transplanted at a dose of 1-2x10^{10}/kg after profound *in vivo* T cell depletion with an immunotoxin-conjugated anti-CD3 mAb, best results in terms of stable long-term chimerism and allograft tolerance required irradiation to the thymic area of 10 Gy [11;12].

Hence, lasting chimerism and tolerance can be achieved after transplantation of a clinically relevant dose of PBSC under costimulation blockade and non-myeloablative TBI, if recipient conditioning is intensified by the addition of DST and short-course

immunosuppression. However, severe GVHD uniformly develops in such chimeras, whose occurrence would prohibit the use of any such protocol in the clinic for the purpose of tolerance induction in organ recipients. Thus, some form of T cell depletion (*in vivo* or *in vitro*) seems to be a critical part of protocols inducing chimerism and tolerance through the transplantation of PBSC. The distinct properties of PBSC need to be considered when pre-clinical large animal tolerance protocols are developed.

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REFERENCES

1. Adams AB, Durham MM, Kean L, Shirasugi N, Ha J, Williams MA, Rees PA, Cheung MC, Mittelstaedt S, Bingaman AW, Archer DR, Pearson TC, Waller EK, Larsen CP: Costimulation blockade, busulfan, and bone marrow promote titratable macrochimerism, induce transplantation tolerance, and correct genetic hemoglobinopathies with minimal myelosuppression. J Immunol 167: 1103-1111, 2001

2. Arpinati M, Green CL, Heimfeld S, Heuser JE, Anasetti C: Granulocyte-colony stimulating factor mobilizes T helper 2-inducing dendritic cells. Blood 95: 2484-90, 2000

3. Bigenzahn S, Blaha P, Koporc Z, Pree I, Selzer E, Bergmeister H, Wrba F, Heusser C, Wagner K, Muehlbacher F, Wekerle T: The role of non-deletional tolerance mechanisms in a murine model of mixed chimerism with costimulation blockade. Am J Transplant 5: 1237-1247, 2005

4. Blaha P, Bigenzahn S, Koporc Z, Schmid M, Langer F, Selzer E, Bergmeister H, Wrba F, Kurtz J, Kiss C, Roth E, Muehlbacher F, Sykes M, Wekerle T: The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. Blood 101: 2886-2893, 2003

5. Blaha P, Bigenzahn S, Koporc Z, Sykes M, Muehlbacher F, Wekerle T: Short-Term Immunosuppression Facilitates Induction of Mixed Chimerism and Tolerance after Bone Marrow Transplantation without Cytoreductive Conditioning.

Transplantation 80: 237-243, 2005

6. Buhler L, Awwad M, Treter S, Chang Q, Basker M, Alwayn IP, Teranishi K, Ericsson T, Moran K, Harper D, Kurilla-Mahon B, Huang CA, Sackstein R, Sykes M, White-Scharf ME, Sachs DH, Down JD, Cooper DK: Pig hematopoietic cell chimerism in baboons conditioned with a nonmyeloablative regiment and CD154 blockade. Transplantation 73: 12-22, 2002

7. Colby C, Chang Q, Fuchimoto Y, Ferrara V, Murphy M, Sackstein R, Spitzer TR, White-Sharf ME, Sachs DH: Cytokine-mobilized peripheral blood progenitor cells for allogeneic reconstitution of miniature swine. Transplantation 69: 135-140, 2000

8. Cutler C, Antin JH: Peripheral blood stem cells for allogeneic transplantation: A Review. Stem Cells 19: 108-117, 2001

9. Dreger P, Schmitz N: Allogeneic transplantation of blood stem cells: coming of age? Ann Hematol 80: 127-36, 2001

10. Durham MM, Bingaman AW, Adams AB, Ha J, Waitze S-Y, Pearson TC, Larsen CP: Administration of anti-CD40 ligand and donor bone marrow leads to hematopoietic chimerism and donor-specific tolerance without cytoreductive conditioning. J Immunol 165: 1-4, 2000

11. Fuchimoto Y, Huang CA, Shimizu A, Kitamura H, Colvin RB, Ferrara V, Murphy MC, Sykes M, White-Sharf ME, Neville DM, Sachs DH: Mixed chimerism without whole body irradiation in a large animal model. J Clin Invest 105: 1779-1789, 2000

12. Gleit ZL, Fuchimoto Y, Yamada K, Melendy E, Scheier-Dolberg R, Monajati L, Coburn RC, Neville DMJr, Sachs DH, Huang CA: Variable relationship between chimerism and tolerance after hematopoietic cell transplantation without myelosuppressive conditioning. Transplantation 74: 1535-1544, 2002

13. Gyger M, Stuart RK, Perreault C.: Immunobiology of allogeneic peripheral blood mononuclear cells mobilized with granulocyte-colony stimulating factor. Bone Marrow Transplant 26: 1-16, 2000

14. Heitger A, Blaha P, Bigenzahn S, Muehlbacher F, Wekerle T: The influence of immunosuppressive drugs on cell-induced graft tolerance. Curr Opin Organ Transplant 9: 307-313, 2004

15. Huang CA, Fuchimoto.Y., Scheier-Dolberg R, Murphy MC, Neville DMJr, Sachs DH: Stable mixed chimerism and tolerance using a nonmyeloablative preparative regimen in a large-animal model. J Clin Invest 105: 173-181, 2000

16. Kim Y-M, Mapara MY, Down JD, Johnson KW, Boisgerault F, Akiyama Y, Benichou G, Pelot M, Zhao G, Sykes M: Graft-versus-host-reactive donor CD4 cells can induce T cell-mediated rejection of the donor marrow in mixed allogeneic chimeras prepared with nonmyeloablative conditioning. Blood 103: 732-739, 2004

17. Kishimoto K, Dong VM, Issazadeh S, Fedoseyeva EV, Waaga AM, Yamada A, Sho M, Benichou G, Auchincloss jr. H, Grusby MJ, Khoury SJ, Sayegh MH: The role of CD154-CD40 versus CD28-B7 costimulatory pathways in regulating allogeneic Th1 and Th2 responses in vivo. J. Clin. Invest. 106: 63-72, 2000

18. Konieczny BT, Dai Z, Elwood ET, Saleem S, Linsley PS, Baddoura FK, Larsen CP, Pearson TC, Lakkis FG: IFN-γ is critical for long-term allograft survival induced by blocking the CD28 and CD40 ligand T cell costimulation pathways. J.Immunol. 160: 2059-2064, 1998

19. Korbling M, Anderlini P: Peripheral blood stem cell versus bone marrow allotransplantation: does the source of hematopoietic stem cells matter? Blood 98: 2900-2908, 2001

20. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB: Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. Nature Med. 5: 1298-1302, 1999

21. Markees T, Pearson T, Cuthbert A, Pearson ASL, Leif J, Phillips N, Mordes J, Greiner DL, Rossini A: Evaluation of Donor-Specific Transfusion Sources: Unique Failure of Bone Marrow Cells to Induce Prolonged Skin Allograft Survival with Anti-CD154 Monoclonal Antibody. Transplantation 78: 1601-8, 2004

22. Martin PJ, Hansen JA, Buckner CD, Sanders JE, Deeg HJ, Stewart P, Appelbaum FR, Clift R, Fefer A, Witherspoon RP, Kennedy MS, Sullivan KM, Flournoy N, Storb R, Thomas ED: Effects of in vitro depletion of T cells in HLA-identical allogeneic marrow grafts. Blood 66: 664-672, 1985

23. Martin PJ, Hansen JA, Torok-Storb B, Durnam D, Przepiorka D, O'Quigley J, Sanders J, Sullivan KM, Witherspoon RP, Deeg HJ, Appelbaum FR, Stewart P, Weiden P, Doney K, Buckner CD, Clift R, Storb R, Thomas ED: Graft failure in

patients receiving T cell-depleted HLA-identical allogeneic marrow transplants. Bone Marrow Transplant. 3: 445-456, 1988

24. Morrison SJ, Wright DE, Weissman IL: Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. Proc Natl Acad Sci USA 94: 1908-1913, 1997

25. Pan L, Delmonte J, Jalonen CK, Ferrara JLM: Pretreatment of donor mice with granulocytes colony-stimulating factor polarizes donor T lymphocytes toward type-2 cytokine production and reduces severity of experimental graft-vs-host disease. Blood 86: 4422-4429, 1996

26. Pelot MR, Pearson DA, Swenson K, Zhao G, Sachs J, Yang Y-G, Sykes M: Lymphohematopoietic graft-vs-host reactions can be induced without graft-vs-host disease in murine mixed chimeras established with a cyclophosphamide-based non-myeloablative conditioning regimen. Biol.Blood Marrow Transplant. 5: 133-143, 1999

27. Pirsch JD, Maki DG: Infectious complications in adults with bone marrow transplantation and T-cell depletion of donor marrow. Increased susceptibility to fungal infections. Ann Intern Med. 104: 619-31, 1986

28. Ringden O, Remberger M, Runde V, Bornhauser M, Blau IW, Basara N, Holig K, Beelen DW, Hagglund H, Basu O, Ehninger G, Fauser AA: Peripheral blood stem cell transplantation from unrelated donors: a comparison with marrow transplantation. Blood 94: 455-64, 1999

29. Seidl MG, Fritsch G, Matthes-Martin S, Latwitschka A, Lion T, Potschger U, Rosenmayr A, Fischer G, Gadner H, Peters C: In vitro an in vivo T-cell depletion with myeloablative or reduced-intensity conditioning in pediatric hematopoietic stem cell transplantation. Haematologica 90: 1405-14, 2005

30. Seung E, Mordes JP, Rossini AA, Greiner DL: Hematopoietic chimerism and central tolerance created by peripheral-tolerance induction without myeloablative conditioning. J Clin Invest 112: 795-808, 2003

31. Sharabi Y, Sachs DH: Mixed chimerism and permanent specific transplantation tolerance induced by a non-lethal preparative regimen. J Exp Med 169: 493-502, 1989

32. Sloand EM, Kim S, Maciejewski JP, Van Rhee F, Chaudhuri A, Barrett J, Young NS: Pharmacologic doses of granulocyte colony-stimulating factor affect cytokine production by lymphocytes in vitro and in vivo. Blood 95: 2269-74, 2000

33. Takeuchi Y, Ito H, Kurtz J, Wekerle T, Ho L, Sykes M: Earlier low-dose TBI or DST overcomes $CD8^+$ T-cell-mediated alloresistance to allogeneic marrow in recipients of anti-CD40L. Am J Transplant 4: 31-40, 2004

34. Taylor PA, Lees CJ, Wilson JM, Ehrhardt MJ, Campbell MT, Noelle RJ, Blazar BR: Combined effects of calcineurin inhibitors or sirolimus with anti-CD40L mAb on alloengraftment under nonmyeloablative conditions. Blood 100: 3400-3407, 2002

35. Tomita Y, Khan A, Sykes M: Mechanism by which additional monoclonal antibody injections overcome the requirement for thymic irradiation to achieve mixed chimerism in mice receiving bone marrow transplantation after conditioning with anti-T cell mAbs and 3 Gy whole body irradiation. Transplantation 61: 477-485, 1996

36. Tomita Y, Sachs DH, Khan A, Sykes M: Additional mAb injections can replace thymic irradiation to allow induction of mixed chimerism and tolerance in mice receiving bone marrow transplantation after conditioning with anti-T cell mAbs and 3 Gy whole body irradiation. Transplantation 61: 469-477, 1996

37. Vincenti F: What's in the Pipeline? New Immunosuppressive Drugs in Transplantation. Am J Transplant 2: 898-903., 2002

38. Wekerle T, Kurtz J, Bigenzahn S, Takeuchi Y, Sykes M: Mechanisms of transplant tolerance induction using costimulatory blockade. Curr Opin Immunol 14: 592-600, 2002

39. Wekerle T, Kurtz J, Ito H, Ronquillo JV, Dong V, Zhao G, Shaffer J, Sayegh MH, Sykes M: Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. Nature Med 6: 464-469, 2000

40. Wekerle T, Nikolic B, Pearson DA, Swenson KG, Sykes M: Minimal conditioning required in a murine model of T cell depletion, thymic irradiation and high-dose bone marrow transplantation for the induction of mixed chimerism and tolerance.

Transplant Int 15: 248-253, 2002

41. Wekerle T, Sayegh MH, Hill J, Zhao Y, Chandraker A, Swenson KG, Zhao G, Sykes M: Extrathymic T cell deletion and allogeneic stem cell engraftment induced with costimulatory blockade is followed by central T cell tolerance. J Exp Med 187: 2037-2044, 1998

42. Wekerle T, Sykes M: Mixed chimerism and transplantation tolerance. Annu Rev Med 52: 353-370, 2001

43. Wells AD, Li XC, Li Y, Walsh MC, Zheng XX, Wu Z, Nunez G, Tang A, Sayegh M, Hancock WW, Strom TB, Turka LA: Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. Nature Med. 5: 1303-1307, 1999

TABLES

Table 1

Protocol	TBI (Gy)	HSCT (cells/mouse)	CB	TCD	DST	IS
A	2, 3	20x10^6 BMC	+	-	-	-
B	0, 1, 1.5, 2, 3	20x, 75x, 200x10^6 PBSC	+	-	-	-
C	3	25x10^6 BMC	-	*in vivo* d -5, -1	-	-
D	3	75x10^6 PBSC	-	*in vivo* d -5, -1	-	-
E	10	40x10^6 PBSC	-	*in vitro*	-	-
F	3	20x10^6 BMC (d0) *plus* 2x, 5x, 10x, 20x, 60x 10^6 PBSC (d0)	+	-	-	-
G	3	20x10^6 BMC (d0) *plus* 60x10^6 PBSC (d94)	+	-	-	-
H	3	20x10^6 BMC plus 60x10^6 PBSC (d0)	+	*in vitro*	-	-
I	3	20x10^6 BMC plus 60x10^6 PBSC (d0)	+	*in vivo* d 0	-	-
J	3	75x, 200x10^6 PBSC	+	-	+	+ / -

Table 1. Experimental protocols (A-J). Groups of B6 mice received TBI one day before being transplanted with the indicated doses of unseparated Balb/c BMC alone, PBSC alone, or BMC together with PBSC (d0 or d94). Costimulation blockade (CB), *in vivo* or *in vitro* T cell depletion (TCD), donor-specific transfusion (DST) and transient immunosuppression (IS) were added to the specific HSCT protocols as shown. CB consisted of 1mg anti-CD154 mAb (MR1) on day 0, and 0.5mg CTLA4Ig on day 2. In two (of four) experiments of protocol J (200x10^6 PBSC) higher doses of MR1 and CTLA4Ig were used, without a significant effect on outcome (MR1: 1mg on day 0, 0.5mg on days 2, 4 and 6; CTLA4Ig: 0.5mg on days 2, 4, 6 and 8). *In vivo* TCD consisted of a depleting anti-CD8 mAb (2.43, 1.4 mg) and a depleteing anti-CD4 mAb (GK1.5, 1.8 mg) injected at d -5 and -1 (protocols C, D) or only d 0 (protocol I). For *in vitro* TCD of PBSC anti-CD90 (Thy1.2) MACS separation was employed (protocols E, H). In protocol

I additional *in vitro* TCD was performed which, however, was inefficient. DST in protocol J consisted of $40x10^6$ splenocytes from Balb/c mice injected on d-6, together with MR1 (1mg on d-6 and 0.5mg on d-4). For transient IS rapamycin, mycophenolate mofetil and methylprednisolone were injected in groups of protocol J for 21 or 28 days following HSCT.

Table 2

Protocol	TBI (Gy)	HSCT (cells/mouse)	CB	TCD	Rate of chimeras	Rate of skin graft acceptance	MLR results	
							SI vs. donor	SI vs. 3rd party
A	2, 3	$20x10^6$ BMC	+	-	6/6, 10/10	-	2.3, 1.8	1.3, 1.6
B	2, 3	$20x10^6$ PBSC	+	-	0/6, 0/6	-	-	-
	1, 1.5, 2, 3	$75x10^6$ PBSC	+	-	0/4, 0/6, 0/6, 0/6	-	-, -, 6.6, 6.5	2.5; 1.5
	3	$200x10^6$ PBSC	+	-	2/9 early, 0/9 late	-	4.7	2.5
	0	$200x10^6$ PBSC	+	-	0/3	-	2	0.9
C	3	$25x10^6$ BMC	-	*in vivo* d -5, -1	4/6	2/3	0.8	0.9
D	3	$75x10^6$ PBSC	-	*in vivo* d -5, -1	5/5 early, 0/5 late	0/4	2.9	0.9
A vs. B ($20x10^6$ PBSC)					p<0.001	-	-	
A vs. B ($75x10^6$ PBSC)					p<0.001	-	p<0.005, p=n.s.	
A vs. B ($200x10^6$ PBSC)					p<0.001	-	p<0.005	
C vs. D					p<0.01	p=n.s.	p<0.05	

Table 2. PBSC are less tolerogenic compared to BMC in protocols employing costimulation blockade or *in vivo* T cell depletion. Groups of mice were treated as shown in Table 1. In contrast to transplantation of $20x10^6$ BMC (A), the transplantation of up to $200x10^6$ PBSC (B) after non-myeloablative TBI and costimulation blockade did not lead to long-term chimerism or donor-specific hyporesonsiveness in MLR assays. Similarly transplantation of PBSC after global *in vivo* T cell depletion (without

costimulation blockade; D) did not lead to long-term chimerism or tolerance, whereas BMC did (C). Chimerism rates in table are given for the following time points; protocol A at w8 and w10 (6/6 and 10/10), for protocol B early at w2 (undetectable chimerism in majority of mice) and late at w8 after the HSCT (group with $200x10^6$ mPBSC under 3Gy TBI lost chimerism). For protocol C at w7 and for protocol D at w2 for early and w7 for late chimerism determination after the HSCT. MLR assay was performed in protocol A at w11 and w7, in protocol B at w7 ($75x10^6$ under 1, 1.5, and 2Gy and for all other groups of protocol B at w11). For protocol C and D chimerism is given for the time point of 27w after the HSCT.. In generally, low response against C3H 3rd party stimulator SPLs was notice for both naïve Balb/c and B6 or experimental B6 animals in MLRs. In opposite, this was not the case with MLRs performed with SJL/JCrl as 3rd party stimulators.

Table 3

Protocol	TBI (Gy)	HSCT (cells/mouse)	CB	TCD	Rate of chimeras	Rate of skin graft acceptance	MLR results	
							SI vs. donor	SI vs. 3rd
A	3	20x10^6 BMC	+	-	19/22	14/19	0.7	0.9
F	3	20x10^6 BMC (d0) *plus* 60x10^6 PBSC (d0)	+	-	5/18	1/13	2.4	0.9
F	3	20x10^6 BMC (d0) *plus* 2x, 5x, 10x, 20x 10^6 PBSC (d0)	+	-	2/3, 2/5, 4/5, 3/4	- , - , -, 0/4	-	-
G	3	20x10^6 BMC (d0) *plus* 60x10^6 PBSC (d94)	+	-	5/5 early, 4/4 late	5/5 early, 4/4 late	-	-
H	3	20x10^6 BMC plus 60x10^6 PBSC (d0)	+	*in vitro*	12/14	12/14	-	-
I	3	20x10^6 BMC plus 60x10^6 PBSC (d0)	+	*in vivo* d0	7/7	7/7	-	-
A vs. I					p=n.s.	p=n.s.	-	
A vs. H					p=n.s.	p=n.s.	-	
A vs. G					p=n.s.	p=n.s.	-	
A vs. F (60x10^6 PBSC)					p<0.001	p<0.001	p<0.001	
H vs. F (60x10^6 PBSC) pooled data					p<0.01	p<0.001	-	
G vs. F (60x10^6 PBSC) pooled data					p<0.01	p<0.001	-	
I vs. F					p<0.01	p<0.001	-	

Table 3. Co-transplantation of PBSC with BMC. When 20x10^6 BMC were transplanted together with 60x10^6 PBSC (3 Gy TBI and costimulation blockade, protocol F) chimerism and tolerance induction were abrogated in most mice (compared to recipients of BMC alone, protocol A). MLR assays showed reactivity against donor-type stimulator cells in mice co-transplanted with PBSC. When lower doses of PBSC were co-transplanted, the negative effect was diminished or absent, respectively. When injection of 60x10^6 PBSC was delayed until 94 days post-BMT, no detrimental effect was observed (protocol G). The co-transplantation of *in vitro* T cell depleted-PBSC (60x10^6) with 20x10^6 BMC (both on day 0, protocol H) led to chimerism and donor skin graft acceptance in most recipients. Similarly, co-transplantation of PBSC (60x10^6) with

20x10^6 BMC (protocol I) together with *in vivo* T cell depletion led to chimerism and tolerance. In generally, low response against C3H 3rd party stimulator SPLs was notice for both naïve Balb/c and B6 or experimental B6 animals in MLRs. In opposite, this was not the case with MLRs performed with SJL/JCrl as 3rd party stimulators.

FIGURE LEGENDS

Figure 1. Co-transplantation of PBSC with BMC abrogates the induction of chimerism. Groups of mice received either $20x10^6$ BMC alone (lower row) or $20x10^6$ BMC plus $60x10^6$ PBSC (upper row) (with 3 TBI and costimulation blockade, protocols A and F). One week (day 6) after the HSCT two color FCM analysis of WBC revealed approximately 4% chimerism in the B cell and myeloid lineages in a BMC-only recipient, whereas no chimerism was detectable after simultaneous transplantation of BMC plus PBSC. Data from one representative mouse per group are shown.

Figure 2. Rejection triggered by co-transplantation of PBSC with BMC can be prevented by T cell depletion. Co-transplantation of $60x10^6$ PBSC with $20x10^6$ BMC prevents chimerism induction in 3 of 4 experiments (3 of them shown in this figure; B, E, H; protocol F). When *in vitro* T cell depleted-PBSC were co-transplanted (A, D; protocol H), chimerism levels were observed which were higher than in the corresponding groups transplanted with BMC alone (C, F; protocol A). Likewise, *in vivo* T cell depletion prevented rejection (G, protocol I). Late administration of PBSC into BMT chimeras augments chimerism (I, protocol G). All groups received 3 Gy TBI and costimulation blockade. Two-color FCM was used to determine chimerism among WBC at multiple time points post-HSCT. Chimerism levels are shown as mean. The fractions on each panel indicate the fraction of analyzed mice showing chimerism at the time point below. Details of experimental protocols are shown in Table 1. Each row shows groups of one particular experiment. *$p<0.05$ indicates a significant difference in chimerism levels of a particular lineage between the indicated groups.

Figure 3. *In vitro* or *in vivo* T cell depletion allows induction of skin graft tolerance. After co-transplantation of $20x10^6$ BMC plus $60x10^6$ PBSC donor skin grafts were rejected in almost all recipients (pooled results of three experiments, A), whereas co-transplantation of *in vitro* T cell depleted PBSC, and transplantation of BMC alone led to long-term acceptance of donor grafts (pooled results of three experiments). Likewise, co-transplantation of *in vivo* T cell depleted PBSC allowed donor-specific skin graft tolerance (B). Late administration of PBSC into BMT chimeras did not lead to rejection of healed-in donor skin (C). Donor and 3rd party skin was grafted 3-6 weeks after HSCT. *$p<0.001$ for comparison of BMC+PBSC vs. BMC+PBSC *in vitro* TCD or vs. BMC.

Figure 4. PBSC contain significantly higher percentage of T cells and do not trigger an evident cytokine shift. A) Cytokine levels were measured in serum 5 days after transplantation of BMC alone or BMC together with PBSC (n=4-6). Wide variations of cytokine levels among individual mice of the same group were observed, without clear evidence for a major shift in cytokine response between the groups. B) Percentages of cell populations contained in either BMC or PBSC were determined by FCM. Pooled results from 13 experiments are shown as mean plus standard deviation.

Figure 5. Additional immunosuppression and DST allow induction of chimerism and tolerance after transplantation of PBSC with costimulation blockade. Transplantation of $200x10^6$ (A, C) or $75x10^6$ (B, D) PBSC leads to high levels of chimerism and donor-specific skin graft tolerance when DST and transient immunosuppression are added to costimulation blockade and 3 Gy TBI (protocol J).

However, GVHD occurred in a large fraction of mice that had to be sacrificed. Panel A shows representative chimerism data from one of four separate experiments, panel B shows pooled skin graft data from all four experiments. Two-color FCM was used to determine chimerism among WBC at multiple time points post-HSCT. Chimerism levels are shown as mean. Donor and 3rd party skin were grafted 7-8 weeks after HSCT.

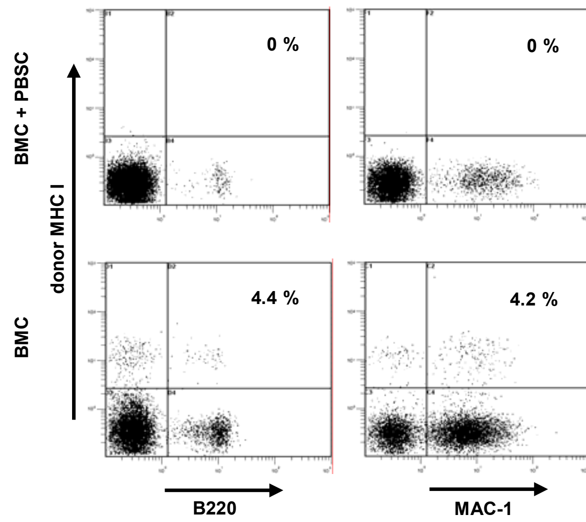

Figure 1

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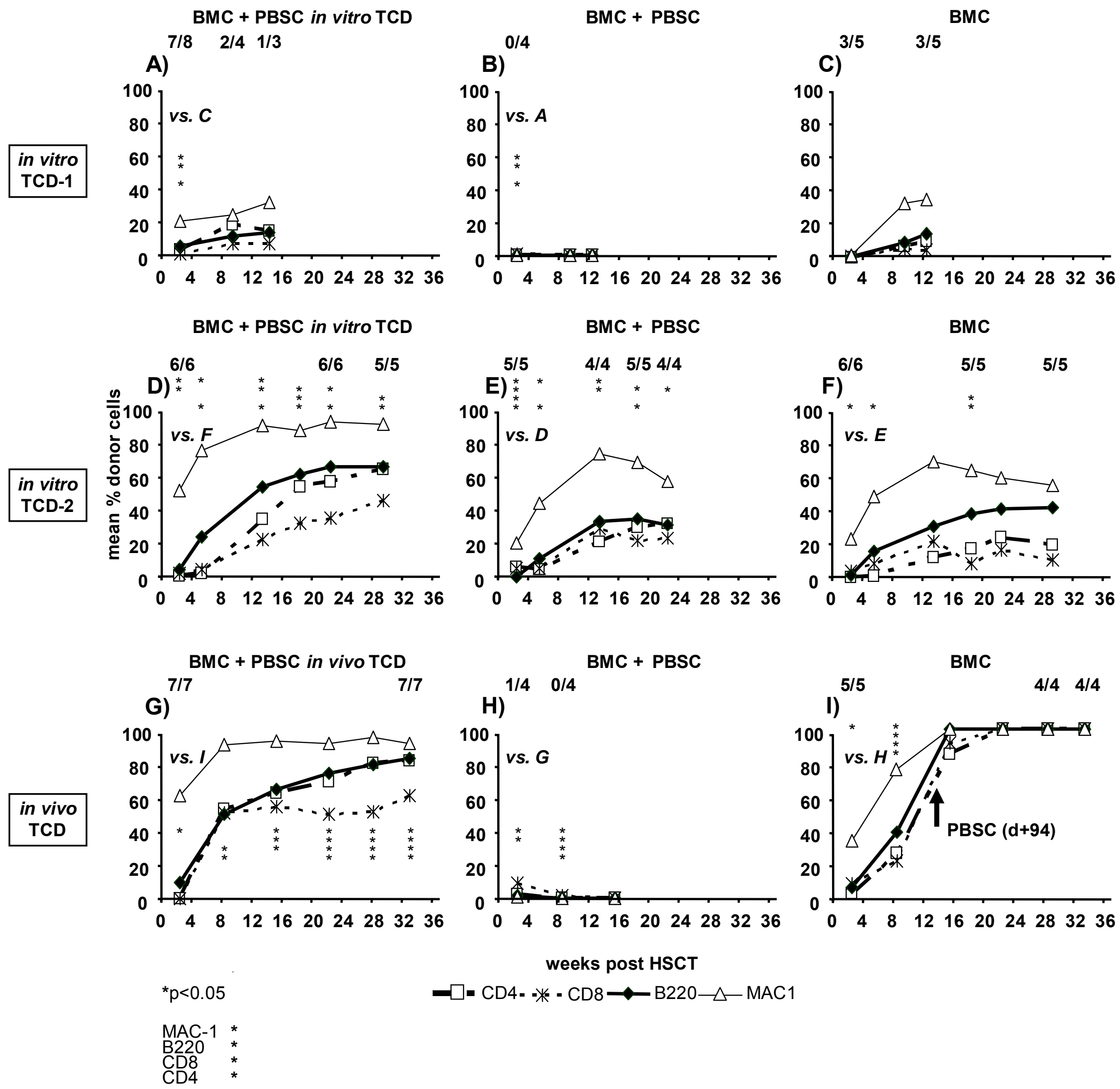

Figure 2

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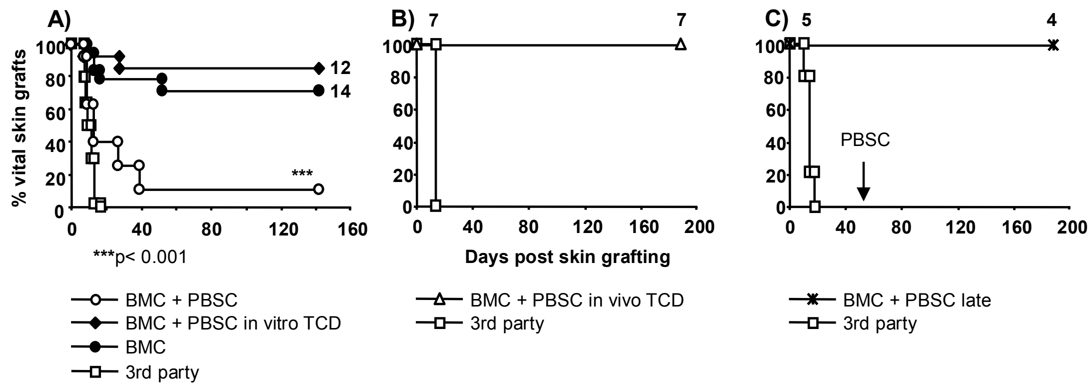

Figure 3

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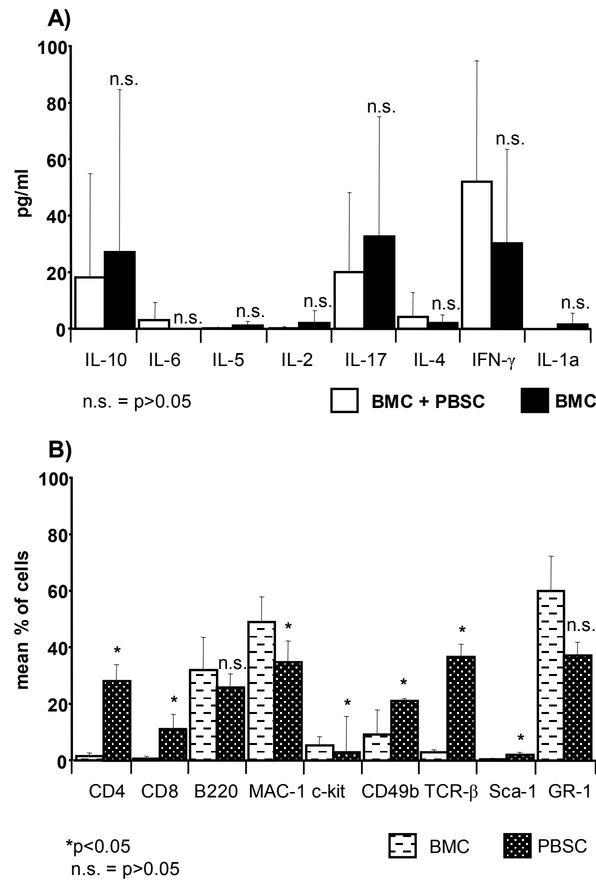

Figure 4

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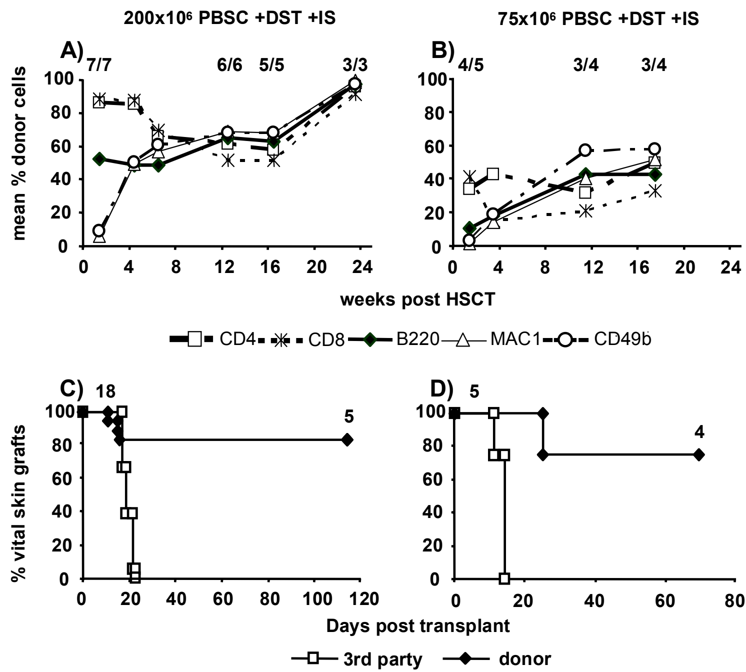

Figure 5

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4

CONCLUSIONS AND SHORT DISSCUSSION

4.1. CONCLUSIONS

4.1.1 mobilized peripheral blood stem cells transplantation (mPBSCT) in the murine syngeneic model (CD45.2 donor-CD45.1 recipient):

- Sufficient numbers of HSC are contained in $20x10^6$ mPBSC to allow successful engraftment and lasting reconstitution under non-myeloablative conditions, as evidenced by multilineage macrochimerism remaining stable for more than 6 months in the murine CD45 congeneic model.
- Under all tested doses of TBI, chimerism induced with mPBSC was lower among all lineages than chimerism achieved with the same number of BMC.
- BMC contain approximately three to four times as many progenitors as mPBSC.
- Under non-myeloablative conditions murine progenitor cells contained in either mPBSC or BMC have similar engraftment characteristics, leading to comparable multi-lineage macrochimerism.
- Use of immunoupression did not detectably increase engraftment, thus indicating that alloreactivity is not responsible for the lower chimerism achieved with mPBSC in syngeneic study.

4.1.2 mPBSCT in the fully MHC mismatched murine allogeneic model (C57BL/6 recipient – Balb/c donor):

- Tolerogenic potential of mPBSC is lower than that of BMC in recipients of costimulatory blockade as even the transplantation of $20x10^7$ mPBSC under 3Gy of TBI together with costimulatory blockade failed to induce long term chimerism in any of transplanted mice.

- Rejection of mPBSC in protocols employing the costimulatory blockade does not appear due to the possible cointeraction between mPBSC and co.bl. because this cells failed to induce mixed chimerism and tolerance in non-co.bl. model as well.
- It could not be confirmed that mPBSC induce Th2 type of immune rejection.
- mPBSC contain significantly more $CD49b^+$, $CD4^+$, $CD8^+$, TCR-β^+ and less MAC-1^+, c-kit^+ (CD117) cells.
- Only the intensified conditioning which employs both DST and IS induces mixed chimerism and tolerance, although for the price of heavy GVHD.
- Addition of $60x10^6$mPBSC to the standard BMT protocol ($20x10^6$ BMC, 3Gy TBI + co.bl.) abrogate the induction of mixed chimerism and tolerance shortly after the HSCT. This effect is depended on dose of mPBSC mixed with BMC.
- Late injection of $60x10^6$mPBSC to the chimeras established with standard BMT protocol does not led to abrogation of established chimerism, turning the recipient animals in to the full chimeras.
- T cells present in useparated mPBSC represent the subpopulation which causes the rejection, as the abrogation of mixed chimerism is not seen when *in vitro* T cell depleted mPBSC were mixed with standard BMT protocol. Similarly, injection of in vivo T cell depletion mAb at d0 (αCD4 and αCD8) to the mixture of unseparated $60x10^6$ mPBSC and standard BMT protocol prevents abrogation of chimerism leading to the high levels of stable multilineage chimerism and tolerance.

4.2. SHORT DISSCUSSION

The main emphasis of this work was to expand the knowledge about the use of mPBSC in an experimental murine non-myeloablative setting, employing the costimulatory blocking antibodies, what should possibly represent a potential base for further clinical application of this protocol.

In my thesis I clearly show that mPBSC can successfully engraft in murine syngeneic non-myeloablative model (with 1-3 Gy TBI), inducing stable and long lasting chimerism. The levels of induced chimerism were significantly lower in animals transplanted with mPBSC in comparison with BMC recipients, under all used doses of TBI. Although the composition analysis of BMC and mPBSC showed typically that mPBSC contains significantly more T cells and less c-kit$^+$ (CD117$^+$) cells, found and proven reason for the development of the lower chimerism levels in mPBSC recipients was the lower level of progenitor cells contained in mPBSC (4,5 times less according to the performed CFU-assay) than in BMC.

Unlike expected, examination of the mPBSCT in a murine non-myeloablative allogeneic model with or without costimulatory blockade, clearly demonstrated that mPBSC were less tolerogenic inducing the fast immunological rejection (as shown with MLR assays) in a very short time after the transplantation (usually in the first week post HSCT).
This result obtained from allogeneic model was in contrast with those achieved with standard doses of BMC ($20x10^6$) where all the transplanted mice developed long term chimerism. Even the transplantation of high dose of mPBSC (10 times more than standard dose) did not succeed to induce long term chimerism. Interestingly, the transplantation of high dose of BMC together with costimulatory blockade completely avoids additional need on cytoreduction [1]. Fast rejection of transplanted cells shortly after the allotransplantation, couldn't be prevented even when immunosuppressive drugs were used, what normally improves the outcome of BMT in similar experimental settings [96]. Since the experiments performed in CD45 congenic model showed that these cells can successfully engraft inducing long lasting chimerism, it was obvious that the transplanted mPBSC were immunologicaly rejected. Several MLR assays showed typically higher

donor reactivity in recipients of mPBSC in comparison with recipients of BMC, confirming strong immunological rejection. Final prove came from experiment, where the standard protocol for routine induction of mixed chimerism and tolerance, employing the BMC ($20x10^6$ BMC, co.bl. + 3Gy TBI) was mixed with 60×10^6 mPBSC (d0) leading to the full abrogation of chimerism induction. This "mixture protocol" clearly demonstrates that mPBSC were less tolerogenic than BMC inducing prompt immunological rejection.

Unexpectedly, when the mPBSC were not involved in previously mentioned "mixture model" at day 0 but at day 94, there was no abrogation of previously established chimerism. Contrarily, the recipients become full chimeras and no signs of GVHD were noticed. This results correlates with a previously published (Sykes et al, Blood 2004) [2].

Only heavy conditioning of the mPBSC transplantation protocol performed in a target to overcome high immunological barrier allowed induction of mixed chimerism. Crucial part of this heavy conditioning protocol was involvement of donor specific transfusion (DST) together with IS treatment (d0-28). Unfortunately, shortly after the HSCT, all chimeric recipients of mPBSC started to develop typical signs of GVHD.

Possible speculation that mPBSC were rejected due to the interaction of mPBSC with costimulation blockade was also taken in the consideration. As the prompt rejection of transplanted mPBSC appeared in experimental model without costimulations blockade as well (employing the *in vivo* Tc depletion antibodies αCD4 and αCD8 mAb at d-5 and d-1), it became clear that the rejection kinetics was independent of the possible co-interaction between mPBSC and costimulatory blockade.

Further, as the focus of interest was placed on immunological properties of these cells, several interesting issues were determined. First, exploration of previously published data [that T cells mobilized to peripheral blood with granulocyte colony forming unit (G-CSF) and contained in mPBSC induce Th2 type of immune response][118] by the use of Bendermed Systems microbeads, could not be confirmed. Secondly, by the addition of

αCD4 and αCD8 depletion antibodies at d0 to the “mixture protocol” ($20x10^6$ BMC + $60x10^6$mPBSC, 3Gy TBI + co.bl.), abrogation of chimerism induction normally seen in this model was fully prevented, and recipient animals were turned in to “full chimeras” shortly after the HSCT, without any trace of GVHD.

Using the “mixture model”, further it was possible to clearly determine that T-cells contained in mPBSC were responsible for immunological rejection. When the mPBSC were *in vitro* T cell depleted by the use of MACS separation column and αCD90 microbeads, and so used in “mixture model”, mice developed long term chimerism and tolerance, showing the significant difference when compared to the recipients of non-depletion mixture protocol.

These findings of behaviour of mPBSC in recipients of c.b., warrant consideration in the development of (pre-) clinical tolerance protocols employing mPBSC.

5

REFERENCES

5 REFERENCES

1. Wekerle T, Kurtz J, Ito H, Ronquillo JV, Dong V, Zhao G, Shaffer J, Sayegh MH, Sykes M: Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. Nature Med 6: 464-469, 2000

2. Kim, Yong-Mi, Mapara, Markus Y., Down, Julian D., Johnson, Kevin W., Boisgerault, Florence, Akiyama, Yoshinobu, Benichou, Gilles, Pelot, Michele, Zhao, Guiling, and Sykes, Megan. Graft-versus-host-reactive donor CD4 cells can induce T cell-mediated rejection of the donor marrow in mixed allogeneic chimeras prepared with nonmyeloablative conditioning. Blood 103(2), 732-739. 2004.

3. Kuwaki K, Tseng Y-L, Dor FJMF, Shimizu A, Houser SL, Sanderson TM, Lancos CJ, Prabharasuth DD, Cheng J, Moran K, Hisashi Y, Mueller N, Yamada K, Greenstein JL, Hawley RJ, Patience C, Awwad M, Fishman JA, Robson SC, Schuurman H-J, Sachs DH, Cooper DKC: Heart transplantation in baboons using [alpha]1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. 11: 29-31, 2005

4. Wekerle T, Sykes M: Induction of tolerance. Surgery 135: 359-364, 2004

5. Facon T, Mary J-Y, Pegourie B, Attal M, Renaud M, Sadoun A, Voillat L, Dorvaux V, Hulin C, Lepeu G, Harousseau J-L, Eschard J-P, Ferrant A, Blanc M, Maloisel F, Orfeuvre H, Rossi J-F, Azais I, Monconduit M, Collet P, Anglaret B,

Yakoub-Agha I, Wetterwald M, Eghbali H, Vekemans M-C, Maisonneuve H, Troncy J, Grosbois B, Doyen C, Thyss A, Jaubert J, Casassus P, Bataille R: Dexamethasone-based regimens versus melphalan-prednisone for elderly multiple myeloma patients ineligible for high-dose therapy. Blood 2005-04-1588, 2005

6. Schuurman HJ, Jambroes G, Borleffs JC, Slootweg PJ, Meyling FH, de Gast GC: Acute humoral rejection after heart transplantation. Transplantation 46: 603-5, 1988

7. Kino T, Hatanaka H, Hashimoto M, Nishiyama M, Goto T, Okuhara M, Kohsaka M, Aoki H, Imanaka H: FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics. J Antibiot (Tokyo) 40: 1249-55, 1987

8. Martel RR, Klicius J, Galet S: Inhibition of the immune response by rapamycin, a new antifungal antibiotic. Can J Physiol Pharmacol 55: 48-51, 1977

9. Almawi WY, Melemedjian OK: Clinical and mechanistic differences between FK506 (tacrolimus) and cyclosporin A. Nephrol. Dial. Transplant. 15: 1916-1918, 2000

10. Jiang H, Kobayashi M: Differences between cyclosporin A and tacrolimus in organ transplantation. Transplantation Proceedings 31: 1978-1980, 1999

11. Morris RE, Meiser B: A new pharmacologic action for an old compound. Med Sci Res 17: 877, 1989

12. Calne RY: Rapamycin for immunosuppresion in organ allografting. Lancet 2: 227, 1989

13. Cosimi AB, Colvin RB, Burton RC, Rubin RH, Goldstein G, Kung PC, Hansen WP, Delmonico FL, Russell PS: Use of monoclonal antibodies to T-cell subsets for immunologic monitoring and treatment in recipients of renal allografts. N Engl J Med 305: 308-14, 1981

14. Kuypers DRJ, Vanrenterghem YFC: Monoclonal antibodies in renal transplantation: old and new. Nephrol. Dial. Transplant. 19: 297-300, 2004

15. Hale G, Waldmann H: CAMPATH-1 monoclonal antibodies in bone marrow transplantation. J.Hematother. 3: 15-31, 1994

16. Garland RJ, Groves SJ, Diamanti P, West SE, Winship KL, Virgo PF, Robinson SP, Oakhill A, Cornish JM, Pamphilon DH, Marks DI, Goulden NJ, Steward CG: Early emergence of PNH-like T cells after allogeneic stem cell transplants utilising CAMPATH-1H for T cell depletion. 36: 237-244, 2005

17. Salama AD, Remuzzi G, Harmon WE, Sayegh MH: Challenges to achieving clinical transplantation tolerance. J. Clin. Invest. 108: 943-948, 2001

18. Matthews JB, Ramos E, Bluestone JA: Clinical Trials of Transplant Tolerance: Slow But Steady Progress. American Journal of Transplantation 3: 794-803, 2003

19. Womer KL, Sayegh MH: Donor Antigen and Transplant Tolerance Strategies: It Takes Two to Tango! J Am Soc Nephrol 15: 1101-1103, 2004

20. Kissmeyer-Nielsen F, Pslen S, Petersen VP, Fjeldborg O: Hyperacute rejection of kidney allografts, associated with pre-existing humoral antibodies against donor cells. Lancet 2: 662-665, 1966

21. Starzl TE, Lerner RA, Dixon FJ, Groth CG, Brettschneider L, Terasaki PI: Shwartzman reaction after human renal homotransplantation. N Engl J Med. 278: 642-8, 1968

22. Williams GM, Hume DM, Hudson RP Jr, Morris PJ, Kano K, Miligrom F: "Hyperacute" renal-homograft rejection in man. N Engl J Med 279: 611-8, 1968

23. Myburgh JA, Cohen I, Gecelter L, Meyers AM, Abrahams c, Furman KI, Goldberg B, van Blerk PJ: Hyperacute rejection in human-kidney allografts-shwartzman or arthus reaction. N Engl J Med 281: 131-5, 1969

24. Chapman JR, Taylor C, Ting A, Morris PJ: Hyperacute rejection of a renal allograft in the presence of anti-HLA-Cw5 antibody. Transplantation 42: 91-3, 1986

25. Jordan SC, Yap HK, Sakai RS, Alfonso P, Fitchman m: Hyperacute allograft rejection mediated by anti-vascular endothelial cell antibodies with a negative monocyte crossmatch. Transplantation 46: 585-7, 1988

26. Medawar PB: The behaviour and fate of skin autografts and skin homografts in rabbits. J Anat 78: 176-199, 1944

27. Mason DW, Morris PJ: Effector Mechanisms in Allograft Rejection. Annual

Review of Immunology 4: 119-145, 1986

28. Paul LC: Chronic rejection of organ allografts:magnitude of the problem. Transplant Proc 25: 2024-5, 1993

29. Wekerle T: Transplantation tolerance induced by mixed chimerism. The Journal of Heart and Lung Transplantation 20: 816-823, 2001

30. Lafferty KJ, Woolnough J: The origin and mechanism of the allograft rejection. Immunol.Rev 35: 231, 1977

31. Schwartz RH: Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. Cell 71: 1065-1068, 1992

32. Schwartz RH: A cell culture model for T lymphocyte clonal anergy. Science 248: 1349-1356, 1990

33. Boussiotis VA, Barber DL, Nakarai T, Freeman GJ, Gribben JG, Bernstein GM, D`Andrea AD, Ritz J NL: Prevention of T cell anergy by signaling through the gamma c chain of the IL-2 receptor. Science 266: 1039-42, 1994

34. Brinster C, Shevach EM: Bone Marrow-Derived Dendritic Cells Reverse the Anergic State of CD4+CD25+ T Cells without Reversing Their Suppressive Function. J Immunol 175: 7332-7340, 2005

35. Wood KJ, Sakaguchi S: REGULATORY T CELLS IN TRANSPLANTATION TOLERANCE. Nat Rev Immunol 3: 199-210, 2003

36. Graca L, Thompson S, Lin C-Y, Adams E, Cobbold SP, Waldmann H: Both CD4+CD25+ and CD4+CD25- Regulatory Cells Mediate Dominant Transplantation Tolerance. J Immunol 168: 5558-5565, 2002

37. Fontenot JD, Gavin MA, Rudensky AY: Foxp3 programs the development and function of $CD4^{+}CD25^{+}$ regulatory T cells. Nature Immunology 4: 330-336 , 2003

38. Deborah JK, Droin N, Soper DM, Ramsdell F, Douglas RG, Ziegler SF: Dynamic regulation of FoxP3 expression controls the balance between CD4(+) T cell activation and cell death. European Journal of Immunology 35: 3424-3432, 2005

39. Gilliet M, Liu Y-J: Generation of Human CD8 T Regulatory Cells by CD40 Ligand-activated Plasmacytoid Dendritic Cells. J. Exp. Med. 195: 695-704, 2002

40. Zhou J, Carr RI, Liwski RS, Stadnyk AW, G. Lee TD: Oral Exposure to Alloantigen Generates Intragraft CD8+ Regulatory Cells. J Immunol 167: 107-113, 2001

41. Ciubotariu R , Colovai AI, Pennesi G, Liu Z, Smith D, Berlocco P, Cortesini R, Suciu-Foca N: Specific Suppression of Human CD4+ Th Cell Responses to Pig MHC Antigens by CD8+CD28- Regulatory T Cells. J Immunol 161: 5193-5202, 1998

42. Zeng D, Hoffmann P, Lan F, Huie P, Higgins J, Strober S: Unique patterns of surface receptors, cytokine secretion, and immune functions distinguish T cells in the bone marrow from those in the periphery: impact on allogeneic bone marrow transplantation. Blood 99: 1449-1457, 2002

43. Seino K, Fukao K, Muramoto K, Yanagisawa K, Takada Y, Kakuta S, Iwakura Y, Van Kaer L, Takeda K, Nakayama T, Taniguchi M, Bashuda H, Yagita H, Okumura K: Requirement for natural killer T (NKT) cells in the induction of allograft tolerance. PNAS 98: 2577-2581, 2001

44. Battaglia M, Gianfrani C, Gregori S, Roncaroloa MG: IL-10-Producing T Regulatory Type 1 Cells and Oral Tolerance. Ann NY Acad Sci 1029: 142-153, 2004

45. Cosmi L, Liotta F, Lazzeri E, Francalanci M, Angeli R, Mazzinghi B, Santarlasci V, Manetti R, Vanini V, Romagnani P, Maggi E, Romagnani S, Annunziato F: Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes. Blood 102: 4107-4114, 2003

46. Maggi E, Cosmi L, Liotta F, Romagnani P, Romagnani S, Annunziato F: Thymic regulatory T cells. Autoimmunity Reviews 4: 579-586, 2005

47. Colovai AI, Liu Z, Ciubotariu R, Lederman S, Cortesini R, Suciu-Foca N: Induction of xenoreactive CD4+ T-cell anergy by suppressor CD8+CD28- T cells. Transplantation 69: 1304-10, 2000

48. Manavalan JS , Kim-Schulze S, Scotto L, Naiyer AJ, Vlad G, Colombo PC, Marboe C, Mancini D, Cortesini R, Suciu-Foca N: Alloantigen specific CD8+CD28- FOXP3+ T suppressor cells induce ILT3+ ILT4+ tolerogenic endothelial cells, inhibiting alloreactivity. Int. Immunol. 16: 1055-1068, 2004

49. Rocha B, Von Boehmer H: Peripheral selection of the T cell repertoire. Science

251: 1225-1228, 1991

50. Webb SR, Morris C, Sprent J: Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. Cell 63: 1249-1256, 1990

51. Kawabe Y, Ochi A: Programmed cell death and extrathymic reduction in Vβ8+ CD4+ T cells in mice tolerant to Staphylococcus aureus enterotoxin B. Nature 349: 245-248, 1991

52. Moskophidis D, Lechner F, Pircher H, Zinkernagel RM: Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. Nature 362: 758-761, 1993

53. Wekerle T, Sayegh MH, Hill J, Zhao Y, Chandraker A, Swenson KG, Zhao G, Sykes M: Extrathymic T cell deletion and allogeneic stem cell engraftment induced with costimulatory blockade is followed by central T cell tolerance. J Exp Med 187: 2037-2044, 1998

54. Wekerle T, Sayegh MH, Chandraker A, Swenson KG, Zhao Y, Sykes M: Role of peripheral clonal deletion in tolerance induction with bone marrow transplantation and costimulatory blockade. Transplant.Proc. 31: 680, 1999

55. Bertolino P, Trescol-Biemont M-C, Thomas J, de St Groth BF , Pihlgren M, Marvel J, Rabourdin-Combe C: Death by neglect as a deletional mechanism of peripheral tolerance. Int. Immunol. 11: 1225-1238, 1999

56. Salaun J, Bandeira A, Khazaal I, Calman F, Coltey M, Coutinho A, Le Douarin

NM: Thymic epithelium tolerizes for histocompatibility antigens. Science 247: 1471-1474, 1990

57. Sprent J, Gao E-K, Webb SR: T cell reactivity to MHC molecules: immunity versus tolerance. Science 248: 1357-1363, 1990

58. Blackman M, Kappler J, Marrack P: The role of the T cell receptor in positive and negative selection of developing T cells. Science 248: 1335-1341, 1990

59. Von Boehmer H, Kisielow P: Self-nonself discrimination by T cells. Science 248: 1369-1373, 1990

60. Nikolic B, Sykes M: Clonal deletion as a mechanism of transplantation tolerance. J Heart Lung Transplant 15: 1171-1178, 1996

61. Kappler JW, Roehm N, Marrack P: T cell tolerance by clonal elimination in the thymus. Cell 49: 273-280, 1987

62. Robey E, Fowlkes BJ: Selective events in T cell development. Ann.Rev.Immunol. 12: 675-705, 1994

63. Onciu M, Lorsbach RB, Henry E.C., Behm F.G.: Terminal Deoxynucleotidyl Transferase-Positive Lymphoid Cells in Reactive Lymph Nodes From Children With Malignant Tumors. American Journal of Clinical Pathology Volume 118: 248-254 , 2002

64. Surh CD, Sprent J: T-cell apoptosis detected in situ during positive and negative selection in the thymus. 372: 100-103, 1994

65. Venanzi E S, Benoist C, Mathis D: Good riddance: thymocyte clonal deletion prevents autoimmunity. Current Opinion in Immunology 16: 197-202, 2004

66. Manilay JO, Pearson DA, Sergio JJ, Swenson KG, Sykes M: Intrathymic deletion of alloreactive T cells in mixed bone marrow chimeras prepared with a non-myeloablative conditioning regimen. Transplantation 66: 96-102, 1998

67. Tomita Y, Khan A, Sykes M: Role of intrathymic clonal deletion and peripheral anergy in transplantation tolerance induced by bone marrow transplantion in mice conditioned with a non-myeloablative regimen. J.Immunol. 153: 1087-1098, 1994

68. Colson YL, Lange J, Fowler K, Ildstad ST: Mechanism of cotolerance in nonlethally conditioned mixed chimeras: negative selection of the Vbeta T-cells receptor repertoire by both host and donor bone marrow-derived cells. Blood 88: 4601-4610, 1997

69. Wekerle T, Sykes M: Mixed chimerism and transplantation tolerance. Annu Rev Med 52: 353-370, 2001

70. Ildstad ST, Sachs DH: Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. Nature 307: 168-170, 1984

71. Sharabi Y, Sachs DH: Mixed chimerism and permanent specific transplantation tolerance induced by a non-lethal preparative regimen. J Exp Med 169: 493-502, 1989

72. Colson YL, Li H, Boggs SS, Patrene KD, Johnson PC, Ildstad ST: Durable mixed allogeneic chimerism and tolerance by a nonlethal radiation-based cytoreductive approach. J Immunol 157: 2820-2829, 1996

73. De Vries-van der Zwan A, Besseling AC, De Waal LP, Boog CJP: Specific tolerance induction and transplantation: a single-day protocol. Blood 89: 2596-2601, 1997

74. Mayumi H, Good RA: Long-lasting skin allograft tolerance in adult mice induced across fully allogeneic (multimajor H-2 plus multiminor histocompatibility) antigen barriers by a tolerance-inducing method using cyclophosphamide. J Exp Med 169: 213-238, 1989

75. Tomita Y, Sachs DH, Sykes M: Myelosuppressive conditioning is required to achieve engraftment of pluripotent stem cells contained in moderate doses of syngeneic bone marrow. Blood 83: 939-948, 1994

76. Durham MM, Bingaman AW, Adams AB, Ha J, Waitze S-Y, Pearson TC, Larsen CP: Administration of anti-CD40 ligand and donor bone marrow leads to hematopoietic chimerism and donor-specific tolerance without cytoreductive conditioning. J Immunol 165: 1-4, 2000

77. Waldmann H, Cobbold S: The use of monoclonal antibodies to achieve immunological tolerance. Immunol.Today 14: 247-251, 1993

78. Larsen CP, Elwood ET, Alexander DZ, Ritchie SC, Hendrix R, Tucker-Burden C, Rae Cho H, Aruffo A, Hollenbaugh D, Linsley PS, Winn KJ, Pearson TC: Long-

term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. Nature 381: 434-438, 1996

79. Kirk A, Burkly L, Batty D, Baumgartner R, Berning J, Buchanan K, Fechner J, Germond R, Kampen R, Patterson N, Swanson S, Tadaki D, TenHoor C, White L, Knechtle SJ, Harlan DM: Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. Nature Med. 5: 686-693, 1999

80. Sayegh MH, Turka LA: The role of T cell costimulatory activation pathways in transplant rejection. N Engl J Med 338: 1813-1821, 1998

81. Wekerle T, Kurtz J, Bigenzahn S, Takeuchi Y, Sykes M: Mechanisms of transplant tolerance induction using costimulatory blockade. Curr Opin Immunol 14: 592-600, 2002

82. Waldmann H, Cobbold S: Regulating the immune response to transplants: A role for $CD4^+$ regulatory cells? Immunity 14: 399-406, 2001

83. Wood KJ, Sakaguchi S: Regulatory T cells in transplantation tolerance. Nature Rev Immunol 3: 199-210, 2003

84. Hodes RJ: T-cell-mediated regulation: Help and suppression, in Paul WE (ed): Fundamental Immunology. New York, Raven Press Ltd., 1989, pp 587-620

85. Maurik Av, Herber M, Wood KJ, Jones ND: Cutting Edge: CD4+CD25+ alloantigen-specific immunoregulatory cells that can prevent CD8+ T cell-

mediated graft rejection: implications for anti-CD154 immunotherapy. J Immunol 169: 5401-5404, 2002

86. Lee RS, Rusche JR, Maloney ME, Sachs DH, Sayegh MH, Madsen JC: CTLA4Ig-induced linked regulation of allogeneic T cell responses. J Immunol 166: 1572-82, 2001

87. Williams MA, Trambley J, Ha J, Adams AB, Durham MM, Rees P, Cowan SR, Pearson TC, Larsen CP: Genetic characterization of strain differences in the ability to mediate CD40/CD28-independent rejection of skin allografts. J Immunol 165: 6849-57, 2000

88. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB: Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. Nature Med. 5: 1298-1302, 1999

89. Markees TG, Phillips NE, Gordon EJ, Noelle RJ, Shultz LD, Mordes JP, Greiner DL, Rossini AA: Long-term survival of skin allografts induced by donor splenocytes and anti-CD154 antibody in thymectomized mice requires $CD4^+$ T cells, Interferon-γ, and CTLA4. J.Clin.Invest. 101: 2446-2455, 1998

90. Sayegh MH, Akalin E, Hancock WW, Russell ME, Carpenter CB, Linsley PS, Turka LA: CD28-B7 blockade after alloantigenic challenge in vivo inhibits Th1 cytokines but spares Th2. J Exp Med 181: 1869-1874, 1995

91. Wekerle T, Sykes M: Mixed chimerism as an approach for the induction of transplantation tolerance. Transplantation 68: 459-467, 1999

92. Seung E, Iwakoshi N, Woda BA, Markees TG, Mordes JP, Rossini AA, Greiner DL: Allogeneic hematopoietic chimerism in mice treated with sublethal myeloablation and anti-CD154 antibody: absence of graft-versus-host disease, induction of skin allograft tolerance, and prevention of recurrent autoimmunity in islet-allografted NOD/Lt mice. Blood 95: 2175-2182, 2000

93. Salam A, Aitouche A, Kuddus R, Peach R, Fung JJ, Rao AS: The combined use of sublethal irradiation and costimulatory blockade to generate mixed bone marrow chimerism. Transplant.Proc. 31: 1197-1198, 1999

94. Kurtz J, Ito H, Wekerle T, Shaffer J, Sykes M: Mechanisms involved in the establishment of tolerance through costimulatory blockade and BMT: Lack of requirement for CD40L-mediated signaling for tolerance or deletion of donor-reactive $CD4^+$ cells. Am J Transplant 1: 339-349, 2001

95. Bigenzahn S, Blaha P, Koporc Z, Pree I, Selzer E, Bergmeister H, Wrba F, Heusser C, Wagner K, Muehlbacher F, Wekerle T: The role of non-deletional tolerance mechanisms in a murine model of mixed chimerism with costimulation blockade. Am J Transplant 5: 1237-1247, 2005

96. Blaha P, Bigenzahn S, Koporc Z, Schmid M, Langer F, Selzer E, Bergmeister H, Wrba F, Kurtz J, Kiss C, Roth E, Muehlbacher F, Sykes M, Wekerle T: The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. Blood 101: 2886-2893, 2003

97. Trambley J, Bingaman AW, Lin A, Elwood ET, Waitze SY, Ha J, Durham MM,

Corbascio M, Cowan SR, Pearson TC, Larsen CP: Asialo GM1(+) CD8 (+) T cells play a critical role in costimulation blockade-resistant allograft rejection. J Clin Invest 104: 1715-1722, 1999

98. Bensinger WI, Martin PJ, Storer B, Clift R, Forman SJ, Negrin R, Kashyap A, Flowers ME, Lilleby K, Chauncey TR, Storb R, Appelbaum FR: Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. N Engl J Med 344: 175-81, 2001

99. Ringden O, Remberger M, Runde V, Bornhauser M, Blau IW, Basara N, Holig K, Beelen DW, Hagglund H, Basu O, Ehninger G, Fauser AA: Peripheral blood stem cell transplantation from unrelated donors: a comparison with marrow transplantation. Blood 94: 455-64, 1999

100. Elfenbein GJ, Sackstein R: Primed marrow for autologous and allogeneic transplantation: A review comparing primed marrow to mobilized blood and steady-state marrow. Experimental Hematology 32: 327-339, 2004

101. Dreger P, Schmitz N: Allogeneic transplantation of blood stem cells: coming of age? Ann Hematol 80: 127-36, 2001

102. Iking-Konert C, Ostendorf B, Foede M, Fischer-Betz R, Jung G, Haensch MG, Schneider M: Granulocyte colony-stimulating factor induces disease flare in patients with antineutrophil cytoplasmic antibody-associated vasculitis. J Rheumatol 8: 1655-8, 2004

103. Stroncek DF , Dittmar K, Shawker T, Heatherman A, Leitman SF: Transient

spleen enlargement in peripheral blood progenitor cell donors given G-CSF. J Transl Med 2: 25, 2004

104. Rowley SD, Donaldson G, Lilleby K, Bensinger WI, Appelbaum FR: Experiences of donors enrolled in a randomized study of allogeneic bone marrow or peripheral blood stem cell transplantation. Blood 97: 2541-8, 2001

105. Neipp M, Zorina T, Domenick MA, Exner BG, Ildstad ST: Effect of FLT3 ligand and granulocyte colony-stimulating factor on expansion and mobilization of facilitating cells and hematopoietic stem cells in mice: kinetics and repopulating potential. Blood 92: 3177-3188, 1998

106. Hasegawa M, Baldwin TM, Metcalf D, and Foote SJ. Progenitor cell mobilization by granulocyte colony-stimulating factor controlled by loci on chromosomes 2 and 11. Blood 95(5), 1872-1874. 2000.

107. de Haan G, Ausema A, Wilkens M, Molineux G, Donthe B: Efficient mobilization of haematopoietic progenitors after a single injection of pegylated recombinant human granulocyte colony-stimulating factor in mouse strains with distinct marrow-cell pool sizes. Br J Haematol 110: 638-46, 2000

108. Bensinger WI, Martin PJ, Storer B, Clift R, Forman SJ, Negrin R, Kashyap A, Flowers ME, Lilleby K, Chauncey TR, Storb R, Appelbaum FR: Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. N Engl J Med 344: 175-81, 2001

109. Champlin RE, Schmitz N, Horowitz MM, Chapuis B, Chopra R, Cornelissen JJ,

Gale RP, Goldman JM, Loberiza FR Jr, Hertenstein B, Klein JP, Montserrat E, Zhang MJ, Ringden O, Tomany SC, Rowlings PA, Van Hoef ME, Gratwohl A: Blood stem cells compared with bone marrow as a source of hematopoietic cells for allogeneic transplantation. IBMTR Histocompatibility and Stem Cell Sources Working Committee and the European Group for Blood and Marrow Transplantation (EBMT). Blood 95: 3702-9, 2000

110. Powles R, Mehta J, Kulkarni S, Treleaven J, Millar B, Marsden J, Shepherd V, Rowland A, Sirohi B, Tait D, Horton C, Long S, Singhal S: Allogeneic blood and bone-marrow stem-cell transplantation in haematological malignant diseases: a randomised trial. Lancet 355: 1231-7, 2000

111. Storek J, Dawson MA, Storer B, Stevens-Ayers T, Maloney DG, Marr KA, Witherspoon RP, Bensinger W, Flowers ME, Martin P, Storb R, Appelbaum FR, Boeckh M: Immune reconstitution after allogeneic marrow transplantation compared with blood stem cell transplantation. Blood 97: 3380-9, 2001

112. Tayebi H, Tiberghien P, Ferrand C, Lienard A, Duperrier A, Cahn JY, Lapierre V, Saas P, Kuentz M, Blaise D, Herve P, Robinet E: Allogeneic peripheral blood stem cell transplantation results in less alteration of early T cell compartment homeostasis than bone marrow transplantation. Bone Marrow Transplant 27: 167-75, 2001

113. Lapierre V, Oubouzar N, Auperin A, Tramalloni D, Tayebi H, Robinet E, Kuentz M, Blaise D, Hartmann O, Herve P, Tiberghien P: Influence of the hematopoietic stem cell source on early immunohematologic reconstitution after allogeneic

transplantation. Blood 97: 2580-6, 2001

114. Noborio K, Muroi K, Izumi T, Toshima M, Kawano-Yamamoto C, Otsuki T, Nagai T, Komatsu N, Ozawa K: Massive immune hemolysis after non-myeloablative allogeneic peripheral blood stem cell transplantation with minor ABO-incompatibility. Leuk Lymphoma 44: 357-9, 2003

115. Pan L, Delmonte J, Jalonen CK, Ferrara JL: Pretreatment of donor mice with granulocyte colony-stimulating factor polarizes donor T lymphocytes toward type-2 cytokine production and reduces severity of experimental graft-versus-host disease. Blood 86: 4422-9, 1995

116. Krenger W, Cook KR, Crawford JM, Sonis ST, Simmons R, Pan L, Delmonte J, Karandikar M, Ferrara JLM: Transplantation of polarized type 2 donor T cells reduces mortality caused by experimental graft-vs-host disease. Transplantation 62: 1278-1285, 1996

117. Sloand EM, Kim S, Maciejewski JP, Van Rhee F, Chaudhuri A, Barrett J, Young NS: Pharmacologic doses of granulocyte colony-stimulating factor affect cytokine production by lymphocytes in vitro and in vivo. Blood 95: 2269-74, 2000

118. Arpinati M, Green CL, Heimfeld S, Heuser JE, Anasetti C: Granulocyte-colony stimulating factor mobilizes T helper 2-inducing dendritic cells. Blood 95: 2484-90, 2000

119. Klangsinsirikul P and Russell NH. Peripheral blood stem cell harvests from G-CSF-stimulated donors contain a skewed Th2 CD4 phenotype and a

predominance of type 2 dendritic cells . Experimental Hematology 30(5), 495-501. 2002.

120. Nawa Y, Teshima T, Sunami K, Hiramatsu Y, Maeda Y, Yano T, Shinagawa K, Ishimaru F, Omoto E, Harada M: G-CSF reduces IFN- and IL-4 production by T cells after allogeneic stimulation by indirectly modulating monocyte function. Bone Marrow Transplantation 25: 1175-1184, 2000

121. Flowers MED, Parker PM, Johnston LJ, Matos AVB, Storer B, Bensinger WI, Storb R, Appelbaum FR, Forman SJ, Blume KG, and Martin PJ. Comparison of chronic graft-versus-host disease after transplantation of peripheral blood stem cells versus bone marrow in allogeneic recipients: long-term follow-up of a randomized trial. Blood 100(2), 415-419. 2002.

122. Storek J, Gooley T, Siadak M, Bensinger WI, Maloney DG, Chauncey TR, Flowers M, Sullivan KM, Witherspoon RP, Rowley SD, Hansen JA, Storb R, Appelbaum FR: Allogeneic peripheral blood stem cell transplantation may be associated with a high risk of chronic graft-versus-host disease. Blood 90: 4705-9, 1997

123. Urbano-Ispizua A, Garcia-Conde J, Brunet S, Hernandez F, Sanz G, Petit J, Bargay J, Figuera A, Rovira M, Solano C, Ojeda E, de la Rubia J, Rozman C: High incidence of chronic graft versus host disease after allogeneic peripheral blood progenitor cell transplantation. The Spanish Group of Allo-PBPCT. Haematologica 82: 683-9, 1997

124. Tanimoto TE , Yamaguchi T, Tanaka Y, Saito A, Tajima K, Kasai M, Kishi K, Mori T, Maseki N, Morishima S, Miyakoshi S, Kasai M, Ohno Y, Kim SW, Numata A, Kami M, Takaue Y, Mori S, Harada M: Comparative analysis of clinical outcomes after allogeneic bone marrow transplantation versus peripheral blood stem cell transplantation from a related donor in Japanese patients. Br J Haematol 125: 480-93, 2004

125. Mohty M, Bilger K, Jourdan E, Kuentz M, Michallet M, Bourhis JH, Milpied N, Sutton L, Jouet JP, Attal M, Bordigoni P, Cahn JY, Sadoun A, Ifrah N, Guyotat D, Faucher C, Fegueux N, Reiffers, Maraninchi D, Blaise D: Higher doses of CD34+ peripheral blood stem cells are associated with increased mortality from chronic graft-versus-host disease after allogeneic HLA-identical sibling transplantation. Leukemia 17: 869-75, 2003

126. Cutler C, Giri S, Jeyapalan S, Paniagua D, Viswanathan A, Antin JH: Acute and Chronic Graft-Versus-Host Disease After Allogeneic Peripheral-Blood Stem-Cell and Bone Marrow Transplantation: A Meta-Analysis. J Clin Oncol 19: 3685-3691, 2001

127. Fuchimoto Y, Huang CA, Shimizu A, Kitamura H, Colvin RB, Ferrara V, Murphy MC, Sykes M, White-Sharf ME, Neville DM, Sachs DH: Mixed chimerism without whole body irradiation in a large animal model. J Clin Invest 105: 1779-1789, 2000

128. Colby C, Chang Q, Fuchimoto Y, Ferrara V, Murphy M, Sackstein R, Spitzer TR, White-Sharf ME, Sachs DH: Cytokine-mobilized peripheral blood progenitor

cells for allogeneic reconstitution of miniature swine. Transplantation 69: 135-140, 2000

6

LIST OF PUBLICATIONS

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1. **Koporc Z**, Bigenzahn S, Blaha P, Fariborz E, Selzer E, Sykes M, Muehlbacher F, Wekerle T: Induction of mixed chimerism through transplantation of CD45-congenic mobilized peripheral blood stem. *In press, Biology of Blood and Marrow Transplantation, 2006*
2. **Koporc Z**, Pree I, Bigenzahn S, Blaha P, Nierlich P, Fariborz E, Sykes M, Muehlbacher F, Wekerle T: Transplant tolerance induced by use of mPBSC in murine allogeneic model. *In preparation, 2006*
3. Blaha P, Bigenzahn S, **Koporc Z**, Schmid M, Langer F, Selzer E, Bergmeister H, Wrba F, Kurtz J, Kiss C, Roth E, Muehlbacher F, Sykes M, Wekerle T: The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. Blood 101: 2886-2893, 2003
4. Wekerle T, Blaha P, **Koporc Z**, Bigenzahn S, Pusch M, Muehlbacher F: Mechanisms of tolerance induction through the transplantation of donor hematopoietic stem cells; central versus peripheral. Transplantation 75: 21S-25S, 2003
5. Bigenzahn S, Blaha P, **Koporc Z**, Pree I, Selzer E, Bergmeister H, Wrba F, Heusser C, Wagner K, Muehlbacher F, Wekerle T: The Role of Non-Deletional Tolerance Mechanism in a Murine Model of Mixed Chimerism with Costimulation Blockade. American Journal of Transplantation 5: 1-11,2005
6. Blaha P, Bigenzahn S, **Koporc Z**, Sykes M, Muehlbacher F, Wekerle T: Short-term immunosuppression facilitates induction of mixed chimerism and tolerance after bone marrow transplantation without cytoreductive conditioning. Transplantation 80:

237-43, 2005

Abstracts and Proceedings:

1. Bigenzahn S, Blaha P, **Koporc Z**, Schmid M, Selzer E, Bergmeister H, Heusser C, Wagner K, Mühlbacher F, Wekerle T: Influence of an anti-CD25 mAb on tolerance induction through BMT plus costimulation blockade. European Surgery 34 (Suppl. 188): 17, 2002

2. Blaha P, Langer F, Bigenzahn S, **Koporc Z**, Selzer E, Bergmeister H, Mühlbacher F, Sykes M, Wekerle T: Minimal irradiation dose required for tolerance induction through bone marrow transplantation with costimulation blockade. European Surgery 34 (Suppl. 188): 18, 2002

3. Bigenzahn S, Blaha P, **Koporc Z**, Schmid M, Selzer E, Bergmeister H, Heusser C, Wagner K, Muehlbacher F, Wekerle T: Influence of an anti-CD25 mAb on tolerance induction through BMT with costimulation blockade. J Heart Lung Transplant 22: S81, 2003

4. Bigenzahn S, Blaha P, **Koporc Z**, Selzer E, Bergmeister H, Heusser C, Wagner K, Muehlbacher F, Wekerle T: Anti-CD25 mAb treatment inhibits tolerance induction through BMT with costimulation blockade. Am J Transplant 3 (Suppl. 5): 455, 2003

5. Bigenzahn S, Blaha P, **Koporc Z**, Selzer E, Heusser C, Wagner K, Muehlbacher F, Wekerle T: The role of regulation for induction and maintenance of tolerance through BMT plus costimulation blockade. Proc. Basic Sciences Symposium 2003 of The Transplantation Society 2003

6. Blaha P, Bigenzahn S, **Koporc Z**, Muehlbacher F, Sykes M, Wekerle T: Transient immunosuppression allows a reduction in the cell dose required for tolerance induction through BMT with costimulation blockade without cytoreduction. Proc. Basic Sciences Symposium 2003 of The Transplantation Society 2003

7. **Koporc Z**, Blaha P, Bigenzahn S, Muehlbacher F, Sykes M, Wekerle T: Use of mobilized peripheral blood stem cells for the induction of mixed chimerism. Proc. Basic Sciences Symposium 2003 of The Transplantation Society 2003

8. Blaha P, Bigenzahn S, **Koporc Z**, Muehlbacher F, Sykes M, Wekerle T: Short-course immunosuppression allows a reduction in the cell dose required for tolerance induction in a model using BMT with costimulation blockade without cytoreduction. European Surgery 35 (Suppl. 194): 12, 2003

9. **Koporc Z**, Blaha P, Bigenzahn S, Muehlbacher F, Sykes M, Wekerle T: Use of mobilized peripheral blood stem cells for the induction of mixed chimerism and tolerance. European Surgery 35 (Suppl. 194): 12, 2003

10. Blaha P, Bigenzahn S, **Koporc Z**, Muehlbacher F, Takeuchi Y, Sykes M, Wekerle T: Peripheral deletion of alloreactive T cells in a tolerance model using BMT and costimulation blockade cannot be elicited by the transplantation of separated B cells or dendritic cells. European Surgery 35 (Suppl. 194): 26, 2003

11. Bigenzahn S, Blaha P, **Koporc Z**, Selzer E, Wrba F, Heusser C, Wagner K, Muehlbacher F, Wekerle T: The role of regulation for induction and maintenance of tolerance through BMT plus costimulation blockade. European Surgery 35 (Suppl.

194): 27, 2003

12. Bigenzahn S, Blaha P, **Koporc Z**, Muehlbacher F, Wekerle T: Progress towards clinical tolerance induction through mixed chimerism. European Surgery 35 (Suppl. 195): 7, 2003

13. **Koporc Z**, Bigenzahn S, Blaha P, Wrba F, Muehlbacher F, Sykes M, Wekerle T: Mobilized peripheral blood stem cells show lower tolerogenic potential than bone marrow cells in recipients of costimulation blockade. American Transplantation Congress, Boston, USA 2004 (accepted) 2004

14. **Koporc Z**, Bigenzahn S, Blaha P, Muehlbacher F, Sykes M, Wekerle T: The use of Mobilized peripheral blood stem cells for the induction of mixed chimerism and tolerance. XX international congress of the transplantation society, Vienna-Austria Congress Abstracts Supplement to Transplantation: 27 July 2004, O336

15. Blaha P, Bigenzahn S, **Koporc Z**, Muehlbacher F, Sykes M and Wekerle T: Short-term immunosuppressive therapy improves chimerism and tolerance development in a model using bone marrow transplantation plus costimulation blockade without cytoreduction. XX international congress of the transplantation society, Vienna-Austria Congress Abstracts Supplement to Transplantation: 27 July 2004, O108

16. Biegenzahn S, Pree I, Nierlich P, **Koporc Z**, Blaha P, Selzer E, Muehlbacher F, WekerleT: CTLA4Ig promotes induction of long-term chimerism and tolerance after BMT plus anti-CD154mAb in a fully allogeneic mouse model.18th Congress of Austrian Society for Transplantation, Transfusion and Genetic, Austrotransplant

2004 Graz (accepted-oral presentation)

17. Blaha P, Bigenzahn S, **Koporc Z**, Schmid M, Langer F, Selzer E, Bergmeister H, Wrba F, Kurtz J, Kiss C, Roth E, Muehlbacher F, Sykes M, Wekerle T: The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. European Surgery 36 (Suppl. 198): 7, 2004

7

CURRICULUM VITAE

7 CURRICULUM VITAE

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EDUCATION

1978-1986:	Primary school, Zagreb
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Juli 1999	Österreichischen akademischen Grad "Dipl.-Ing.", BMWV
October 1999	TU – Wien, Ph.D. study of technical chemistry
1999-2000	TU - Wien, Team of Dr. Richard Gapes, science work on project "Aceton Butanol Fermentation" and project of "Biodisel"
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APPENDIX

APPENDIX
Proof

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Induction of Mixed Chimerism through Transplantation of CD45-Congenic Mobilized Peripheral Blood Stem Cells after Nonmyeloablative Irradiation

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ABSTRACT

Clinical translation of the mixed-chimerism approach for inducing transplantation tolerance would be facilitated if mobilized peripheral blood stem cells (mPBSCs) could be used instead of bone marrow cells (BMCs). Because the use of mPBSCs for this purpose has not been investigated in nonmyeloablative murine protocols, we explored the engraftment potential of mPBSCs in a CD45-congenic model as a first step. After 2, 1.5, or 1 Gy of total body irradiation, CD45.1 B6 hosts received unseparated granulocyte colony-stimulating factor-mobilized CD45.2 B6 PBSCs or unseparated CD45.2 B6 BMCs. The same total cell numbers, or aliquots, of mPBSCs and BMCs containing similar numbers of c-kit$^+$ cells, were transplanted both with and without a short course of rapamycin-based immunosuppression (IS). Transplantation of mPBSCs induced long-term multilineage macrochimerism, but chimerism levels were significantly lower than among recipients of the same number of BMCs. Transplanting aliquots containing similar numbers of c-kit$^+$ cells reduced the difference between mPBSCs and BMCs, but lower levels of chimerism were nonetheless observed in mPBSC recipients. Chimerism levels correlated more closely with the number of transplanted progenitor cells as determined by colony-forming unit assays. IS did not affect chimerism levels, indicating that the donor CD45 isoform or other minor disparities do not pose a major barrier to engraftment. Our findings indicate that under nonmyeloablative conditions, progenitor cells contained in mPBSCs have an engraftment capacity similar to progenitor cells from BMCs, allowing induction of lasting mixed chimerism with moderate cell numbers. On a cell-per-cell basis, unseparated BMCs have some advantages that may be minimized if the number of progenitor cells is equalized. These results are expected to facilitate the development of mPBSC-based allogeneic tolerance protocols.
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KEY WORDS
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INTRODUCTION

Induction of donor-specific tolerance is a major research goal of transplantation medicine. Mixed chimerism, established through the transplantation of donor hematopoietic stem cells (HSCs), is an attractive tolerance strategy for clinical development [1]; however, a major obstacle in the clinical translation of this approach is the required recipient conditioning.

Although mild conditioning protocols using costimulation blockers have been developed for the induction of lasting allogeneic mixed chimerism using relatively realistic bone marrow cell (BMC) doses (approximately 15×10^6 BMCs per mouse), they require

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nonmyeloablative total body irradiation (TBI) or cytotoxic drug treatment [2-5]. Many would still consider such conditioning requirements too toxic for routine use in organ transplantation recipients. We and others have shown that allogeneic bone marrow transplantation (BMT) can be achieved experimentally without any cytoreductive conditioning if extremely high doses of BMCs are transplanted (approximately 200×10^6 BMCs per mouse) [6-9]. Although these models provide proof of principle that completely noncytoreductive BMT regimens are possible, they are not clinically practicable, because such high numbers of BMCs cannot be routinely obtained from a single human donor.

In the clinic, the highest numbers of HSCs are obtained through the collection of mobilized peripheral blood stem cells (mPBSCs) from a living donor. Transplantation of granulocyte colony-stimulating factor (G-CSF)–mobilized PBSCs has an established role in the treatment of hematologic disease [10,11], with the cell yield of a mPBSC collection typically being 5- to 10-fold higher than that from a conventional BM harvest from the iliac crests. But although BMCs and mPBSCs are used for similar clinical indications, they have several important biologic and immunologic differences [12-16] that can have a significant influence on the induction of mixed chimerism and tolerance. In particular, it is unknown whether co-stimulation blockade retains the same tolerance-inducing effect if allogeneic PBSCs are transplanted instead of BMCs.

Before investigating allogeneic mPBSCs in combination with co-stimulation blockade, however, we deemed it necessary to establish the behavior of murine mPBSCs after nonmyeloablative conditioning in the absence of alloreactivity. For this purpose, we transplanted congenic mPBSCs after nonmyeloablative TBI.

Engraftment properties of murine PBSCs mobilized with G-CSF and/or other growth factors have been evaluated in lethally irradiated recipients, and their reconstituting capability has been established [17-24]. Although mostly allogeneic models were used in these studies, congenic systems have been investigated as well, albeit after myeloablative conditioning [22,24]. One study evaluated the graft-versus-leukemia effect of allogeneic G-CSF–mobilized PBSCs after 7 Gy of sublethal TBI, leading to high levels of chimerism ($>$ 75%) [25]. The therapeutic effect of transplantation of a mixture of allogeneic and syngeneic murine mPBSCs was demonstrated in a myeloablative systemic lupus erythematosus–like autoimmune disease model (BXSB) [19]. To the best of our knowledge, however, murine mPBSCs have not yet been investigated in detail after low-dose (1–3 Gy) nonmyeloablative TBI, and thus, their engraftment properties under these conditions remain unknown.

Consequently, although mPBSCs are an attractive candidate for tolerance induction through co-stimulation blockade and mixed chimerism due to the large number of hematopoietic cells obtainable from a single donor, nonmyeloablative or noncytoreductive murine protocols using mPBSCs have not been explored for this purpose. As an initial step toward the development of mPBSC-based mixed-chimerism protocols for tolerance induction, we show here that CD45-congenic mPBSCs transplanted after nonmyeloablative TBI led to lasting multilineage mixed chimerism. Consistent with the frequency of progenitor cells, transplantation of unseparated mPBSCs resulted in significantly lower levels of chimerism than transplantation of the same number of unseparated BMCs, but mPBSC and BMC transplantation had a comparable engraftment efficiency per number of progenitor cells transplanted. These results should facilitate the development of allogeneic mPBSC-based tolerance protocols.

MATERIALS AND METHODS

Animals

Female B6.SJL-Ptprc^{a}Pep3^b/BoyJ (CD45.1, hosts) were purchased from the Jackson Laboratory (Bar Harbor, ME), and congenic female C57BL/6NCrl (B6: H-2^b) mice (CD45.2, donors) were purchased from the Charles River Laboratories (Sulzfeld, Germany). Animals were kept under specific pathogen-free conditions and were used between 6 and 10 weeks of age. All experiments were approved by the local review board of the University of Vienna and were performed in accordance with national and international guidelines for laboratory animal care.

BMT and mPBSC Transplantation

CD45.1 hosts received nonmyeloablative TBI (2, 1.5 or 1 Gy, as indicated) 1 day before either mPBSC transplantation or BMT. To avoid pooling of mPBSCs in spleen, donor CD45.2 mice were splenectomized at least 14 days before the mobilization procedure, as described by Weissman et al. [22]. Thereafter, 5μg of human G-CSF (approx. 250 μg/kg; filgrastim, Neupogen; Amgen Europe BV, Breda, The Netherlands) were injected subcutaneously for 5 consecutive days. Two hours after the final injection, the mice were maximally bled (using tail bleeding and heart puncture), and the heparinized blood was pooled and diluted with phosphate-buffered saline (PBS) (1:1). Subsequently, the same volume of 2% dextran T500 solution was added, to give a final concentration of 1% dextran. Red blood cells were separated by sedimentation for 45 minutes at 37°C before the supernatant fraction containing the mobilized leukocytes (mPBSCs) was collected. BM was harvested from tibiae, femura, and humeri as described in detail previously [26]. Both

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BMCs and mPBSCs were filtered through a 70-μm filter. BMCs were counted manually (by trypan blue exclusion of dead cells), and mPBSCs were counted both manually and automatically. Cells were diluted with cold BM media (500 mL of Medium 199 [Sigma, Vienna, Austria], supplemented with 5 mL of HEPES buffer [ICN; Biomedica, Vienna, Austria], 5 mg of DNAse [Sigma], and 2 mg of gentamycin [Sigma]), then injected in a volume of 1 mL into a tail vein of recipient mice (d0). BMCs and mPBSCs were used without further manipulation (without enrichment or depletion).

Flow Cytometric Analysis

Two-color flow cytometric analysis was used to distinguish donor and host cells of particular lineages, by staining with fluorescein isothiocyanate (FICT)-conjugated antibodies against CD4, CD8, B220, MAC-1, NK1.1, and biotin-conjugated CD45.2 (developed with phycoerythrin-avidin). Mice were considered chimeric if they demonstrated at least 2% of donor cells within the myeloid lineage and at least 1 lymphoid lineage. To analyze cell composition, BMCs and mPBSCs were stained with FITC-conjugated antibodies against CD4, CD8, B220, MAC-1, CD117 (c-kit), CD49b, TCR-β, and biotin-conjugated CD45.2 (developed with phycoerythrin-avidin). The percentage of subpopulations among $CD45.2^+$ live cells was calculated. Irrelevant isotype controls were included, and propidium iodide staining was used to exclude dead cells.

Colony-Forming Unit Assay

BMCs and mPBSCs were suspended at a cell concentration of 1×10^6/mL in BM medium. In addition, 1×10^5 of either BMCs or mPBSCs (in 100 μL) were cultivated in duplicates inside Petri plates (Falcon Nr. 3003) with 1 mL of cultivating medium prepared by mixing of 75 μL of recombinant mouse IL-3 (25 ng/mL; R&D Systems,) and 2.3 mL of MyeloCult H5100 medium (StemCell Technologies,). After 14 days of incubation, colony-forming unit granulocytes/macrophages (CFU-GM) were counted by an experienced hematologist.

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Chimerism in Transplanted Progenitor Cells

The total number of progenitor cells transplanted per mouse was calculated by multiplying the number of CFU colonies grown from 1×10^5 plated cells (288 for BMCs and 64 for mPBSCs) by 100 for BMCs (because 10×10^6 cells were transplanted) and by 300 for mPBSCs (because 30×10^6 cells were transplanted). Accordingly, 28,800 progenitors per mouse were transplanted in the BMC group, and 19,200 progenitors per mouse were transplanted in the mPBSC group. The level of chimerism per 1×10^4 transplanted progenitor cells was then obtained by multiplying the measured percentage of donor chimerism (as determined by flow cytometry [FCM]) by 1×10^4 divided by the total number of transplanted progenitors (chimerism per 1×10^4 transplanted progenitor cells = % measured chimerism × [1×10^4/total number of transplanted progenitors]). Thus, the percentage of measured chimerism was multiplied by a factor of 0.35 for BMCs (1×10^4/28,800) and by a factor 0.52 for mPBSCs (1×10^4/19,200).

Immunosuppression

In the indicated groups, mice were injected with immunosuppressive drugs daily from day 0 to day 27. Drugs were used at following doses: rapamycin, 0.2 mg/kg/day; methylprednisolone (MP), 10 mg/kg/day; and mycophenolate mofetil (MMF), 20 mg/kg/day. Drugs were diluted and administered as described previously [26]. Rapamycin was kindly provided by Wyeth-Ayerst (?, NJ), MMF was kindly provided by Roche (Vienna, Austria), and MP was purchased from Aventis (Vienna, Austria).

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Statistics

A 2-tailed Student *t*-test was used for comparing chimerism levels between groups. *P* values < .05 were considered statistically significant.

RESULTS

Unseparated mPBSCs Successfully Engraft after Nonmyeloablative Conditioning, Although with Reduced Efficiency Compared with BMCs

Because to date only BMCs have been investigated for the purpose of tolerance induction through nonmyeloablative hematopoietic stem cell transplantation (HSCT) and mixed chimerism, and because little is known about the engraftment properties of murine mPBSCs under nonmyeloablative conditions, the aim of this study was to investigate the capability of "syngeneic" (ie, congenic) mPBSCs for the induction of mixed chimerism after nonmyeloablative TBI. To compare the engraftment of mPBSCs and BMCs in the absence of alloreactivity, we chose a CD45-congenic model. CD45.1 recipients received 2, 1.5, or 1 Gy TBI 1 day before transplantation of 20×10^6 unseparated mPBSCs or BMCs (6 mice per group).

All mice receiving either 20×10^6 mPBSCs or BMCs after 2, 1, or 1.5 Gy of TBI developed long-term multilineage macrochimerism (chimerism rates: mPBSC, 6/6, 6/6, 6/6; BMC, 6/6, 6/6, 6/6) (Figure 1). Chimerism was stable for 29 weeks in all groups. Levels of chimerism obtained both with mPBSCs and BMCs were correlated with the dose of TBI. At all tested TBI doses, mPBSC transplantation led to significantly lower levels of hematopoietic chimerism compared with those from transplantation of the same

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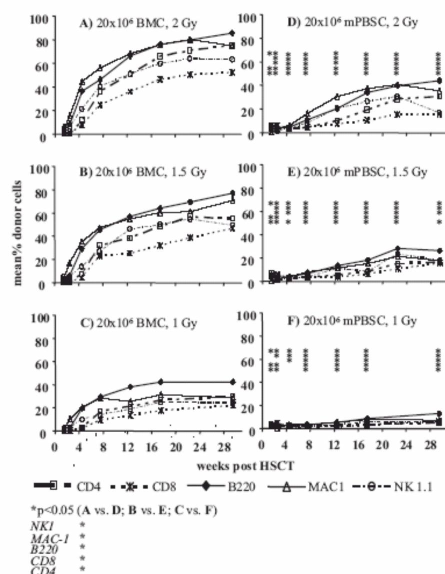

Figure 1. Transplantation of PBSCs leads to significantly lower levels of long-term chimerism in comparison with BMT after nonmyeloablative TBI. CD45.1 mice (n = 6/group) received either 20×10^6 CD45.2 BMC (A, B, C) or 20×10^6 CD45.2 mPBSCs (D, E, F) after various doses of TBI (2 Gy [A,D], 1.5 Gy [B, E], or 1 Gy [C, F). The percentages of donor-derived $CD4^+$ cells, $CD8^+$ cells, B cells, monocytes/granulocytes, and NK cells among white blood cells were evaluated by FCM at different time points and are shown as means. Levels of chimerism induced with mPBSCs were significantly lower than those induced with BMCs treated with the same TBI dose (A vs D, B vs E, and C vs F; $P < .05$).

number of BMCs. For example, at the end of follow-up, this difference (expressed as BMC vs mPBSC chimerism after 2 Gy TBI) was 75% versus 31% for $CD4^+$ cells ($P < .0005$), 52% versus 16% for $CD8^+$ cells ($P < .0005$), 86% versus 44% for B cells ($P < .0005$), 75% versus 35% for myeloid cells ($P < .0005$), and 63% versus 17% for NK cells ($P < .0005$); see Figure 1. Similar significant differences were also observed in the groups treated with 1.5 and 1 Gy TBI.

We also determined chimerism levels in BM and spleen (SPL) of randomly selected mice (2 per group, at 1 Gy and 2 Gy TBI) at the end of follow-up (32 weeks posttransplantation); the results are given in Table 1. mPBSC recipients showed substantially lower levels of chimerism compared with BMC recipients (eg, in BM, 78% vs 42% for myeloid cells [$P < .05$] and 79% vs 40% for B cells [$P < .005$]; in SPL, 65% vs 29% for $CD4^+$ cells [$P < .005$], 59% vs 27% for $CD8^+$ cells [$P < .05$], and 86% vs 47% for B cells [$P < .05$], using 2 Gy TBI).

FCM analysis of BMC and mPBSC composition revealed that, as expected, mPBSCs contained higher percentages of $CD4^+$ and $CD8^+$ cells (17% vs 2% for $CD4^+$ and 12% vs 2% for $CD8^+$ cells; Table 2) and B cells (39% vs 28%), but a lower percentage of MAC-1^+ cells (32% vs 65%), than BMCs. Of note, the percentage of c-kit^+ (CD117) cells was higher in BMCs than in mPBSCs (11.1% vs 3.7%).

The foregoing findings indicate that CD45-congenic mPBSCs can successfully engraft and induce stable long-term multilineage macrochimerism after nonmyeloablative TBI. Furthermore, the same number of unseparated mPBSCs resulted in significantly lower chimerism levels compared with BMCs in all lineages tested and at all 3 TBI doses used). The lower engraftment of unseparated mPBSCs was associated with a lower percentage of c-kit^+ cells. These results suggest the reduced per-cell potential of unseparated murine mPBSCs to induce mixed chimerism under nonmyeloablative conditions.

The Chimerism Achieved with mPBSCs and BMCs Correlates with the Number of Transplanted Progenitor Cells

The foregoing results suggest that the difference in chimerism levels might be due to the higher percentage of c-kit^+ cells in BMCs, implying a higher number of progenitors [27]. Consequently, we next transplanted aliquots of mPBSCs and BMCs containing similar numbers of c-kit^+ cells. BMCs contain approximately 3 times as many c-kit^+ cells as mPBSCs (11.1% vs 3.7%; Table 2), so based on this ratio, we transplanted 30×10^6 unseparated mPBSCs and 10×10^6 unseparated BMCs (with 1.5 Gy TBI).

Although the transplantation of unseparated mPBSCs and BMCs containing similar numbers of c-kit^+ cells again led to lower levels of chimerism in the recipients of mPBSCs than in recipients of BMCs, the overall differences were substantially smaller and reached statistical significance in only certain lineages and at certain time points (Figure 2). For instance, chimerism levels at 26 weeks post-HSCT were (shown as BMCs vs mPBSCs) 36% vs 17% for $CD4^+$ cells ($P < .05$), 26% vs 11% for $CD8^+$ cells ($P < .05$), 42% vs 31% for B cells (P = not significant [NS]), 29% vs 20% for myeloid cells (P = NS), and 17% vs 11% for NK cells (P = NS) (Figure 2A and 2B). Furthermore, CFU assays of donor BMCs and mPBSCs performed in this experiment demonstrated that 4.5 times as many colonies grew from BMCs than from mPBSCs (mean of 2 plates, 288 vs 64 CFU-GM/1×10^5 plated cells for BMCs vs mPBSCs), suggesting that the actual difference in the frequencies of progenitors might be larger than estimated based on the percentages of c-kit^+ cells.

We hypothesized that the determined percentages of c-kit^+ cells underestimate the true difference in

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Table 1. *Chimerism Levels in BM and Spleen Correlated With Chimerism Levels in Blood After BMT or mPBSCT*

	CD4	CD8	B220	MAC1	NK 1.1
I: 2 Gy TBI					
20×10^6 BMCs					
Mean % of donor cells in BM	—	—	78.7	78.0	—
Mean % of donor cells in SPL	65.4	58.4	86.2	81.0	45.9
20×10^6 mPBSCs					
Mean % of donor cells in BM	—	—	39.7	41.9	—
Mean % of donor cells in SPL	29.1	27.2	46.7	48.8	23.1
II: 1 Gy TBI					
20×10^6 BMCs					
Mean % of donor cells in BM	—	—	41.5	43.6	—
Mean % of donor cells in SPL	28.6	26.2	48.1	39.3	22.5
20×10^6 mPBSCs					
Mean % of donor cells in BM	—	—	12.5	11.7	—
Mean % of donor cells in SPL	6.3	5.0	12.9	10.5	5.4

Chimerism was analyzed by FCM in BM and spleen (SPL) of recipients transplanted with 20×10^6 BMCs or mPBSCs under 2 or 1 Gy of TBI (33 weeks post-HSCT). Chimerism levels in BM and SPL correlated with chimerism in peripheral blood. Two randomly selected mice per group were analyzed.

progenitor content. Thus we calculated whether chimerism levels correlate more closely with the number of transplanted progenitor cells as estimated by CFU assay. Because we transplanted only 3 times as many mPBSCs as BMCs (following the percentages of c-kit$^+$ cells), but 4.5 times as many colonies grew from BMCs than from mPBSCs, there still may have been 50% more progenitor cells transplanted in the BMC group than in the mPBSC group (28,800 progenitor cells contained in 10×10^6 BMCs vs 19,200 progenitor cells in 30×10^6 mPBSCs). Consistent with this assessment, we found that in BMC recipients, chimerism levels were 49% higher in CD4$^+$ cells, 40% higher in CD8$^+$ cells, and 57% higher in B cells (in SPL at 29 weeks post-HSCT; results from the 2 best chimeras from each group; Table 3). The differences in chimerism levels among various lineages in blood over time were generally of similar magnitudes.

We thus also calculated levels of measured chimerism in relation to the estimated number of progenitor cells transplanted (according to CFU results; details of the calculation given in Methods); the results are shown in Figure 3. Levels of donor chimerism per 1×10^4 transplanted progenitors determined in this way were very similar for recipients of BMCs and recipients of mPBSCs (Figure 3). Higher levels of T-cell chimerism detected among the mPBSC recipients during the first few weeks after HSCT were likely due to the higher percentage of CD4$^+$ and CD8$^+$ cells contained in mPBSCs.

Thus the lower levels of multilineage chimerism among recipients of unseparated mPBSCs compared with recipients of BMCs correlate with a lower number of progenitor cells contained in transplanted unseparated mPBSCs. On a per-cell basis, progenitor cells from mPBSCs and BMCs seem to have very similar engraftment potential under nonmyeloablative conditions, leading to similar levels of long-lasting multilinear macrochimerism.

Short-Course Immunosuppression Does Not Significantly Improve Engraftment

Although CD45-congenic strain combinations are frequently thought of as essentially syngeneic systems that allow the tracking of donor cells, it has been recognized that CD45 isoforms can elicit immune responses [28]. Furthermore, the possibility that minor transplantation antigen differences between the recipient and donor strains develop over time due to spontaneous mutations during breeding cannot be ruled out. Consequently, we explored the possibility that the lower levels of mPBSC chimerism were due to an increased alloresponse compared with that elicited by BMC. We thus treated recipients with a combination of immunosuppressive drugs (rapamycin, mycophenolate mofetil, and methylprednisolone, from day 0 to day 27), which has been shown to effectively increase engraftment of allogeneic BMCs in combination with costimulation blockade [26,29]. As shown in Figure 2, transient immunosuppression

Table 2. *FCM Analysis of Various Lineage Markers Among BMCs and mPBSCs*

Cell Marker	CD4	CD8	B220	MAC1	CD117	CD49b	TCR-β
% in BM	1.9	1.8	27.8	64.5	11.1	11.9	4.9
% in mPBSC	16.8	11.7	38.5	32.3	3.7	33.8	31.6

mPBSCs contain markedly more CD4$^+$ and CD8$^+$ cells. Almost 3 times as many c-kit$^+$ (CD117) cells are found in BMCs than in mPBSCs. One representative result of two similar experiments is shown.

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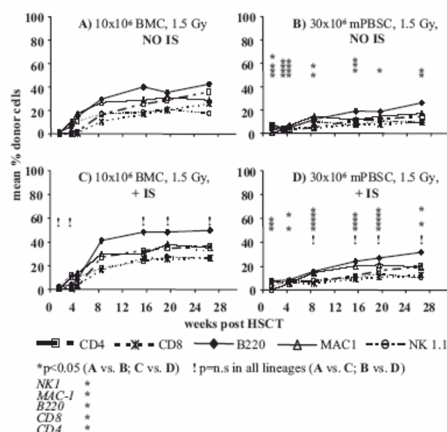

Figure 2. Transplantation of similar numbers of c-kit$^+$ mPBSCs and c-kit$^+$ BMCs still led to differences in chimerism levels. CD45.1 mice (n = 6/group) received either 10×10^6 CD45.2 BMCs (A, C) or 30×10^6 CD45.2 mPBSCs (B, D) with 1.5 Gy of TBI. In this manner, similar numbers of c-kit$^+$ cells were transplanted in both groups (according to the percentage of c-kit$^+$ cells as determined by FCM analysis among unseparated mPBSCs and BMCs). To evaluate whether the lower chimerism induced in mPBSCT recipients is due to a remaining alloresponse against CD45.2, or to other antigenic differences, some groups received immunosuppression (A, B) (rapa, MP, and MMF for 4 weeks), whereas others remained untreated (C, D). Chimerism levels in various lineages as determined by FCM analysis over time are shown as means. $^*P < .05$ indicates a significant difference in chimerism between the BMT and mPBSC transplantation groups (A vs B and C vs D). IS treatment did not significantly improve levels of chimerism (P = NS in any lineage) by either BMT or mPBSC transplantation (A vs C or B vs D).

(IS) did not significantly affect chimerism rates or levels after either BMT or mPBSC transplantation. With 1.5-Gy TBI, after mPBSC transplantation, chimerism developed in 4 of 4 mice treated with IS versus 5 of 6 mice without, and after BMT transplantation, chimerism developed in 5 of 5 mice with IS and in 5 of 5 without. Chimerism levels were similar between the groups with and without IS for the 26-week observation period. At the end of follow-up, the mean percentage of donor chimerism in peripheral blood of BMC recipients was (with IS vs without IS) 36% versus 36% for CD4$^+$ cells, 26% versus 26% for CD8$^+$ cells, 42% versus 50% for B cells, 29% versus 35% for myeloid cells, and 17% versus 26% for NK cells, and that of mPBSC recipients was 17% versus 20% for CD4$^+$ cells, 11% versus 13% for CD8$^+$ cells, 31% versus 32% for B cells, 20% versus 19% for myeloid cells, and 11% versus 11% for NK cells.

Because additional IS did not significantly improve chimerism, the reduced engraftment of mPBSCs is most likely not due to an alloresponse against CD45 or other putative antigenic strain differences.

DISCUSSION

Although tolerance induction through mixed chimerism has been investigated for many years, its translation into the routine clinical practice of organ transplantation has not yet been achieved [1]. A recent pilot trial of transplanting kidney and BM grafts simultaneously from the same donor to HLA-identical recipients suffering from end-stage kidney failure and multiple myeloma has provided proof of principle that tolerance can indeed be achieved with this approach [30]. At the same time, application of this protocol to patients without concomitant malignancy might be problematic. Substantially milder BMT protocols are needed to facilitate widespread use of this tolerance strategy. The clinically unattainable number of required donor BMCs is one reason why the mildest regimens developed in murine studies have not been translated to the clinic so far [6-9,31]. The use of mPBSCs would allow transplantation of substantially higher numbers of hematopoietic cells from a single donor, thus potentially permitting the application of milder regimens. We thus began to investigate whether mPBSCs could be substituted for BMCs in costimulation-based mixed-chimerism protocols designed for the induction of transplantation tolerance.

As a first step toward developing murine mPBSC-based tolerance regimens, we investigated the engraftment of unseparated murine mPBSCs in the absence of alloreactivity. To the best of our knowledge, the transplantation of murine mPBSCs after low-dose nonmyeloablative TBI has not yet been investigated in detail; most previous studies used lethal conditioning of the recipient [17-24]. Our results demonstrate that sufficient numbers of HSCs are contained in 20×10^6 mPBSCs to allow successful engraftment and lasting reconstitution under nonmyeloablative conditions, as evidenced by multilineage macrochimerism remaining stable for more than 6 months. As expected, chimerism levels correlated with the dose of irradiation. Notably, at all tested TBI doses and in all tested lineages, chimerism induced with mPBSCs was lower than that achieved with the same number of BMCs. This difference correlates with the lower percentage of c-kit$^+$ cells and CFU-generating cells contained in mPBSCs. Our data suggest that murine BMCs contain approximately 3–4 times as many progenitors as mPBSCs, consistent with data of Glass et al. [20] showing approximately 4 times as many CFU-GMs in BMCs than in mPBSCs of DBA/2 mice. Likewise, the percentage of CD34$^+$ cells is higher in human BMCs than in human mPBSCs [32]. Thus, under nonmyeloablative conditions, murine progenitor cells contained in either mPB-

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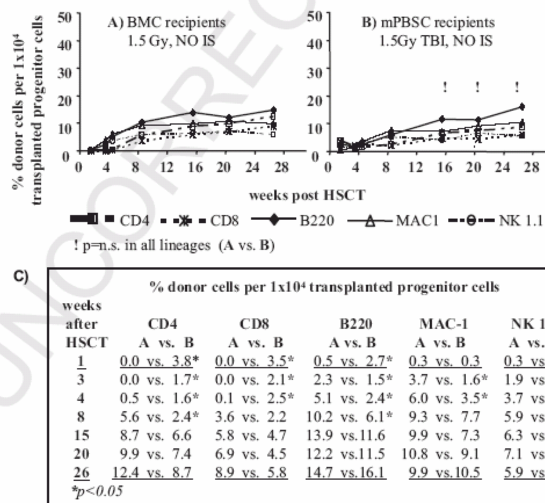
Table 3. *Chimerism Levels in BM and Spleen Correlated With Chimerism Levels in Blood After BMT or mPBSC Transplantation*

	CD4	CD8	B220	MAC1	TCR-β
I: 1.5 Gy TBI					
10 × 10⁶ BMC					
Mean % of donor cells in BM	—	—	37.8	43.9	—
Mean % of donor cells in SPL	47.2	36.5	54.3	—	—
Mean % of donor cells in THY	—	65.7	—	—	69.0
30 × 10⁶ mPBSC					
Mean % of donor cells in BM	—	—	32.3	32.5	—
Mean % of donor cells in SPL	31.6	26.1	34.5	—	—
Mean % of donor cells in THY	—	57.4	—	—	61.1
II: 1.5 Gy TBI, IS					
10 × 10⁶ BMC					
Mean % of donor cells in BM	—	—	55.9	48.2	—
Mean % of donor cells in SPL	37.9	33.9	51.7	—	—
Mean % of donor cells in THY	—	53.7	—	—	57.4
30 × 10⁶ mPBSC					
Mean % of donor cells in BM	—	—	36.9	23.8	—
Mean % of donor cells in SPL	26.1	24.8	45.4	—	—
Mean % of donor cells in THY	—	37.4	—	—	41.3

Chimerism was determined by FCM in BM, spleen (SPL), and thymus (THY) of recipients undergoing transplantation with 10×10^6 BMCs or 30×10^6 mPBSCs after 1.5 Gy of TBI, with IS (I) or without (II) IS (29 weeks post-HSCT). Similar differences in chimerism between recipients of BMCs and recipients of mPBSCs were found in these tissues as in blood. IS did not significantly influence chimerism. Two best chimeras were analyzed per group.

SCs or BMCs have similar engraftment characteristics, leading to comparable multilineage macrochimerism.

Our results can serve as a rough indicator for the maximum possible chimerism levels that can ideally be expected after transplantation of a given number of unseparated murine allogeneic mPBSCs if alloreactivity is completely overcome. According to our data, ~4.5 times as many murine mPBSCs as BMCs would have to be transplanted to achieve similar chimerism. In the clinic, the cell yield of a mPBSC collection is typically 5- to 10-fold greater than that from a conventional BM harvest. Thus, although substantially



% donor cells per 1x10⁴ transplanted progenitor cells

weeks after HSCT	CD4 A vs. B	CD8 A vs. B	B220 A vs. B	MAC-1 A vs. B	NK 1.1 A vs. B
1	0.0 vs. 3.8*	0.0 vs. 3.5*	0.5 vs. 2.7*	0.3 vs. 0.3	0.3 vs. 1.5*
3	0.0 vs. 1.7*	0.0 vs. 2.1*	2.3 vs. 1.5*	3.7 vs. 1.6*	1.9 vs. 1.6
4	0.5 vs. 1.6*	0.1 vs. 2.5*	5.1 vs. 2.4*	6.0 vs. 3.5*	3.7 vs. 3.0
8	5.6 vs. 2.4*	3.6 vs. 2.2	10.2 vs. 6.1*	9.3 vs. 7.7	5.9 vs. 5.1
15	8.7 vs. 6.6	5.8 vs. 4.7	13.9 vs.11.6	9.9 vs. 7.3	6.3 vs. 4.1
20	9.9 vs. 7.4	6.9 vs. 4.5	12.2 vs.11.5	10.8 vs. 9.1	7.1 vs. 6.5
26	12.4 vs. 8.7	8.9 vs. 5.8	14.7 vs.16.1	9.9 vs.10.5	5.9 vs. 5.7

*$p<0.05$

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Figure 3. Levels of chimerism induced in BMC and mPBSC recipients correlated with the number of transplanted progenitor cells as estimated from CFU assays. Chimerism per transplanted progenitor cell was calculated as described in detail in the Methods section. A significant difference in percentage of chimerism per 1×10^4 transplanted progenitor cells, between BMC and mPBSC recipients (A vs B) is noticeable only in the first few weeks after HSCT (C), probably due to the larger percentage of CD4⁺ and CD8⁺ cells contained in mPBSC.Data are from the experiment shown in Figure 3.

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higher chimerism levels can be expected from transplantation of an mPBSC harvest, these results raise doubts as to whether the advantage of harvesting mPBSCs is great enough to make mPBSCs a viable source of sufficient donor hematopoietic cells to induce chimerism without cytoreduction [6,29]. This question is currently being addressed in our laboratory.

One caveat that must be considered when attempting to extrapolate these results from a syngeneic model to tolerance protocols pertains to the role of donor T cells in promoting engraftment in the allogeneic setting. Theoretically, mPBSCs, which are enriched for T cells, might lead to better engraftment when transplanted across an allogeneic barrier.

The CD45.1 isoform may induce a weak T-cell–mediated immune response [28] that is nonetheless strong enough to reduce BM engraftment [33]. This immunogenicity is of concern, especially when minimal recipient conditioning leaves the host immune system largely intact. Even though significant immunogenicity was not found in the (reverse) strain combination that we used (CD45.2 into CD45.1) [34,35], we nevertheless wanted to rule out that mPBSCs elicit a stronger immune response against CD45.2 than BMCs, or any other minor antigenic differences between the donor and recipient strains. We thus treated recipients with a combination of immunosuppressive drugs that promotes chimerism in allogeneic models [26,29]. IS did not detectably increase engraftment, indicating that alloreactivity is not responsible for the lower chimerism achieved with mPBSCs in our studies.

mPBSCs have already been used in a limited number of large-animal models to induce chimerism and tolerance [36-38]. In a haploidentical swine model, for instance, transplantation of megadoses of mPBSCs after thymic irradiation and recipient T-cell depletion led to long-term chimerism and tolerance. Although substantial levels of chimerism were achieved, it remains unclear how the engraftment potential of swine mPBSCs compares with that of BMCs, because no direct comparison is available within the same model. For numerous reasons, large-animal models of tolerance generally are less successful and require more intense protocols than murine models. These regimens are usually developed in mice using BMCs, but not mPBSCs [39]. Nonetheless, it has not been convincingly shown that allogeneic mPBSCs behave sufficiently similarly to allow substitution without requiring modification of the protocols. Given the numerous known immunologic differences between BMCs and mPBSCs [12-16], it appears likely that their properties associated with the ability to induce tolerance may differ. This is of particular concern when costimulation blockers are used in combination with HSCT, because these blockers' mechanisms of action depend critically on the details of a specific experimental protocol. Based on the results of the congenic models presented herein, studies are currently under way in our laboratory to develop costimulation-based allogeneic murine PBSC protocols.

From the present study, we conclude that in the absence of alloreactivity, transplantation of unseparated murine mPBSCs effectively leads to stable, long-term multilineage chimerism. However, due to the lower frequency of progenitor cells, mPBSCs induce lower chimerism levels than the same number of unseparated BMCs. A typical mPBSC harvest in the clinic yields 5-10 times as many hematopoietic cells as a BM harvest [40,41], however, more than offsetting this difference and allowing the transplantation of more HSCs. Thus, because our data show that very similar chimerism levels can be expected per transplanted progenitor, they support the evaluation of allogeneic mPBSCs for the purpose of developing clinically more relevant mixed-chimerism models.

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REFERENCES

1. Wekerle T, Sykes M. Mixed chimerism and transplantation tolerance. *Annu Rev Med*. 2001;52:353-370.
2. Wekerle T, Sayegh MH, Hill J, et al. Extrathymic T cell deletion and allogeneic stem cell engraftment induced with costimulatory blockade is followed by central T cell tolerance. *J Exp Med*. 1998;187:2037-2044.
3. Adams AB, Durham MM, Kean L, et al. Costimulation blockade, busulfan, and bone marrow promote titratable macrochimerism, induce transplantation tolerance, and correct genetic hemoglobinopathies with minimal myelosuppression. *J Immunol*. 2001;167:1103-1111.
4. Taylor PA, Lees CJ, Waldmann H, et al. Requirements for the promotion of allogeneic engraftment by anti-CD154 (anti-CD40L) monoclonal antibody under nonmyeloablative conditions. *Blood*. 2001;98:467-474.
5. Quesenberry PJ, Zhong S, Wang H, et al. Allogeneic chimerism with low-dose irradiation, antigen presensitization, and costimulator blockade in H-2 mismatched mice. *Blood*. 2001;97:557-564.
6. Wekerle T, Kurtz J, Ito H, et al. Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. *Nat Med*. 2000;6:464-469.
7. Seung E, Mordes JP, Rossini AA, et al. Hematopoietic chimerism and central tolerance created by peripheral-tolerance induction without myeloablative conditioning. *J Clin Invest*. 2003;112:795-808.
8. Durham MM, Bingaman AW, Adams AB, et al. Administration of anti-CD40 ligand and donor bone marrow leads to hematopoietic

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chimerism and donor-specific tolerance without cytoreductive conditioning. *J Immunol*. 2000;165:1-4.

9. Lambert JF, Colvin GA, Zhong S, et al. H2-mismatched transplantation with repetitive cell infusions and CD40 ligand antibody infusions without myeloablation. *Br J Haematol*. 2002;119:155-163.
10. Dreger P, Schmitz N. Allogeneic transplantation of blood stem cells: coming of age? *Ann Hematol*. 2001;80:127-136.
11. Korbling M, Anderlini P. Peripheral blood stem cell versus bone marrow allotransplantation: does the source of hematopoietic stem cells matter? *Blood*. 2001;98:2900-2908.
12. Arpinati M, Green CL, Heimfeld S, Heuser JE, Anasetti C: Granulocyte-colony stimulating factor mobilizes T-helper 2–inducing dendritic cells. *Blood*. 2000;95:2484-2490.
13. Cutler C, Antin JH. Peripheral blood stem cells for allogeneic transplantation: a review. *Stem Cells*. 2001;19:108-117.
14. Gyger M, Stuart RK, Perreault C. Immunobiology of allogeneic peripheral blood mononuclear cells mobilized with granulocyte-colony stimulating factor. *Bone Marrow Transplant*. 2000;26:1-16.
15. Pan L, Delmonte J, Jalonen CK, et al. Pretreatment of donor mice with granulocyte colony-stimulating factor polarizes donor T lymphocytes toward type-2 cytokine production and reduces severity of experimental graft-vs-host disease. *Blood*. 1996;86:4422-4429.
16. Sloand EM, Kim S, Maciejewski JP, et al. Pharmacologic doses of granulocyte colony-stimulating factor affect cytokine production by lymphocytes in vitro and in vivo. *Blood*. 2000;95:2269-2274.
17. Neipp M, Zorina T, Domenick MA, et al. Effect of FLT3 ligand and granulocyte colony-stimulating factor on expansion and mobilization of facilitating cells and hematopoietic stem cells in mice: kinetics and repopulating potential. *Blood*. 1998;92:3177-3188.
18. Yamamoto Y, Yasumizu R, Amou Y, et al. Characterization of peripheral blood stem cells in mice. *Blood*. 1996;88:445-454.
19. Yamamoto Y, Wang B, Fukuhara S, et al. Mixed peripheral blood stem cell transplantation for autoimmune disease in BXSB/MpJ mice. *Blood*. 2002;100:1886-1893.
20. Glass B, Uharek L, Zeis M, et al. Allogeneic peripheral blood progenitor cell transplantation in a murine model: evidence for an improved graft-versus-leukemia effect. *Blood*. 1997;90:1694-1700.
21. Wagers AJ, Allsopp RC, Weissman IL: Changes in integrin expression are associated with altered homing properties of Lin-/loThy1.1loSca-1+c-kit+ hematopoietic stem cells following mobilization by cyclophosphamide/granulocyte colony-stimulating factor. *Exp Hematol*. 2002;30:176-185.
22. Morrison SJ, Wright DE, Weissman IL. Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. *Proc Natl Acad Sci USA*. 1997;94:1908-1913.
23. Brasel K, McKenna HJ, Charrier K, et al. Flt3 ligand synergizes with granulocyte-macrophage colony-stimulating factor or granulocyte colony-stimulating factor to mobilize hematopoietic progenitor cells into the peripheral blood of mice. *Blood*. 1997;90:3781-3788.
24. Szilvassy SJ, Meyerrose TE, Ragland PL, et al. Differential homing and engraftment properties of hematopoietic progenitor cells from murine bone marrow, mobilized peripheral blood, and fetal liver. *Blood*. 2001;98:2108-2115.
25. Weiss L, Or R, Slavin S, et al. Immunotherapy of murine leukemia following non-myeloablative conditioning with naive or G-CSF mobilized blood or bone marrow stem cells. *Cancer Immunol Immunother*. 2004;X:358-362. AQ: 4
26. Blaha P, Bigenzahn S, Koporc Z, et al. The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. *Blood*. 2003;101:2886-2893.
27. Okada S, Nakauchi H, Nagayoshi K, et al. Enrichment and characterization of murine hematopoietic stem cells that express c-kit molecule. *Blood*. 1991;78:1706-1712.
28. Xu H, Exner BG, Chilton PM, et al. CD45 congenic bone marrow transplantation: evidence for T cell–mediated immunity. *Stem Cells*. 2004;22:1039-1048.
29. Blaha P, Bigenzahn S, Koporc Z, et al. Short-term immunosuppression facilitates induction of mixed chimerism and tolerance after BMT without cytoreductive conditioning. *Transplantation*. 2005;80:237-243.
30. Bühler LH, Spitzer TR, Sykes M, et al. Induction of kidney allograft tolerance after transient lymphohematopoietic chimerism in patients with multiple myeloma and end-stage renal disease. *Transplantation*. 2002;74:1405-1409.
31. Graca L, Le Moine A, Lin C-Y, et al. Donor-specific transplantation tolerance: the paradoxical behavior of CD4+CD25+ T cells. *Proc Natl Acad Sci USA*. 2004;101:10122-10126.
32. To L, Haylock D, Dowse T, et al. A comparative study of the phenotype and proliferative capacity of peripheral blood (PB) CD34+ cells mobilized by four different protocols and those of steady-phase PB and bone marrow CD34+ cells. *Blood*. 1994;84:2930-2939.
33. van Os R, Sheridan T, Robinson S, et al. Immunogenicity of Ly5 (CD45) antigens hampers long-term engraftment following minimal conditioning in a murine bone marrow transplantation model. *Stem Cells*. 2001;19:80-87.
34. Sykes M, Chester CH, Sundt TM, et al. Effects of T cell depletion in radiation bone marrow chimeras. III: characterization of allogeneic bone marrow cell populations that increase allogeneic chimerism independently of graft-vs-host disease in mixed marrow recipients. *J Immunol*. 1989;143:3503-3511.
35. Ito H, Takeuchi Y, Shaffer J, et al. Local irradiation enhances congenic donor pluripotent hematopoietic stem cell engraftment similarly in irradiated and nonirradiated sites. *Blood*. 2004;103:1949-1954.
36. Fuchimoto Y, Huang CA, Shimizu A, et al. Mixed chimerism without whole body irradiation in a large animal model. *J Clin Invest*. 2000;105:1779-1789.
37. Colby C, Chang Q, Fuchimoto Y, et al. Cytokine-mobilized peripheral blood progenitor cells for allogeneic reconstitution of miniature swine. *Transplantation*. 2000;69:135-140.
38. Bühler L, Awwad M, Treter S, et al. Pig hematopoietic cell chimerism in baboons conditioned with a nonmyeloablative regimen and CD154 blockade. *Transplantation*. 2002;73:12-22.
39. Sykes M, Szot GL, Swenson K, et al. Induction of high levels of allogeneic hematopoietic reconstitution and donor-specific tolerance without myelosuppressive conditioning. *Nat Med*. 1997;3:783-787.
40. Bensinger WI, Martin PJ, Storer B, et al. Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. *N Engl J Med*. 2002;344:175-181.
41. Ringden O, Remberger M, Runde V, et al. Peripheral blood stem cell transplantation from unrelated donors: a comparison with marrow transplantation. *Blood*. 1999;94:455-464.

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