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Spring Seminars 2009

Cellular therapies: insights and new horizons

16 & 17 April 2009
Amsterdam, The Netherlands

Royal Tropical Institute, Amsterdam, The Netherlands

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Sanquin Spring Seminars

Cellular Therapies: Insights and new horizons

Conference chair: C Ellen van der Schoot, Amsterdam, The Netherlands

April 16 & 17, 2009

Royal Tropical Institute, Amsterdam, The Netherlands

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Words of welcome

On behalf of Sanquin we welcome you to the third biennial Sanquin Spring Seminar. This year's theme – cellular therapies – is not only timely, it is also 'hot'. Many new findings will be presented, with possibilities for treatment in the near future and speculations about what may become possible further ahead. Sanquin, as a blood establishment, strongly feels that it has an important role to play within the cycle of cellular therapies from donor to patient. Similarities between the logistics and technologies for blood products and cellular therapies have already led to inclusion of cells, tissues and transplants in various blood services, as for example in the United Kingdom and in Quebec. Therefore, Sanquin is expanding its research lines on cellular therapies in order to be able to contribute to the new developments.

We are extremely pleased that so many top notch scientists agreed to speak during our conference and to share their knowledge and views with you. We are also pleased to be able to offer a podium for the younger generation of scientists in this field, with a number of selected oral presentations and the poster sessions.

We think that the Scientific Committee succeeded in putting together an exciting scientific and social program, we'll hope you will enjoy.

C. Ellen van der Schoot,
Conference Chair

Ernest Briët,
Sanquin Executive Board

Scientific Committee

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Scientific program, Thursday 16 April 2009

09:00 am	Registration, Coffee & Tea
10:30 am	Welcome & opening Ernest Briët, Sanquin Executive Board, The Netherlands

Session I: Cancer immunotherapy

Chair:	Anja ten Brinke, Amsterdam, The Netherlands
10.35-11.10:	Engineering T cell Immunity Ton Schumacher, Amsterdam, The Netherlands
11.10-11.30:	Identification of HLA Class II restricted antigens by screening a recombinant bacteria cDNA library Anita Strumpf-Falkenburg-Griffioen, Leiden, The Netherlands
11.30-12.05:	Migration and effectiveness of tumor antigen loaded dendritic cells in melanoma patients Jolanda de Vries, Nijmegen, The Netherlands
12.05-12.25:	Costimulatory ligand CD70 allows induction of CD8+ T cell immunity by immature dendritic cells in a vaccination setting Jannie Borst, Amsterdam, The Netherlands
12.25-13.45:	Lunch break / Visit to the exhibition and posters

Session II: Basic Hematopoietic Stem Cell Research

Chair:	Carlijn Voermans, Amsterdam,The Netherlands
13.45-14.05:	CD27 on self-renewing hematopoietic stem cells induces proliferation and affects hematopoietic differentiation Martijn Nolte, Amsterdam, The Netherlands
14.05-14.40:	Wnt signaling regulates critical steps in hematopoiesis and lymphopoiesis Frank Staal, Leiden, The Netherlands
14.40-15.15:	Rho GTPase regulation of normal and abnormal hemapoietic stem functions David Williams, Boston, United States of America
15.15-15.35:	Differentiation-dependent regulation of hematopoietic cell migration by Slit proteins Paula van Hennik, Amsterdam, The Netherlands
15.35-16.05:	Coffee & Tea break / Visit to the exhibition and posters

Session III: Cord blood and mesenchymal stromal cells

Chair:	Yvette van Hensbergen, Leiden,The Netherlands
16.05-16.40:	Reduced risk of leukemia relapse after double UCB transplantation Michael Verneris, Minneapolis, USA
16.40-17.15:	Umbilical cord blood transplantation in children - the potential role of MSC Franco Locatelli, Pavia, Italy
17.15-17.35:	Co-transplantation of HLA-haploidentical, bone marrow derived mesenchymal stromal cells overcomes graft failure and provides a potential platform for additional immune therapeutic interventions Lynne Ball, Leiden, The Netherlands
17.35-21.00:	Visit to the Posters & Drinks; Conference Buffet and Poster Award Ceremony

Scientific program, Friday 17 April 2009

08.45 – 09.30:	Registration, coffee & tea

Session IV: Cellular therapy for cardiovascular disorders

Chair:	Jan-Jaap Zwaginga, Leiden, The Netherlands
09.30-10.05:	Progenitor cell therapy for cardiovascular disease Stefan Janssens, Catholic University Leuven, Leuven, Belgium
10.05-10.40:	Immune modulation in neovascularisation: the role of the NK cells Paul Quax, Leiden, The Netherlands
10.40-11.00:	Coffee & Tea break / Visit to the exhibition and posters

Session V: Pluripotency

Chair:	Wim Fibbe, Leiden, The Netherlands
11.00-11.35:	Cardiomyocytes and vascular endothelial cells from pluripotent stem cells Christine Mummery, Leiden, The Netherlands

11.35-11.55:	Pluripotent stem cells from adult human testis Ans van Pelt, Amsterdam, The Netherlands
11.55-12.30:	Mechanisms underlying increased potency of postnatal cells Catherine Verfaillie, Leuven, Belgium
12.30-13.50:	Lunch break / visit to the exhibition and posters

Session VI: Tolerance induction

Chair:	Bart Roep, Leiden University Medical Center, The Netherlands
13.50-14.25:	Dendritic cells and transplant tolerance Angus Thomson, Pittsburgh, USA
14.25-15.00:	Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans Maria-Grazia Roncarolo, Milan, Italy
15.00-15.20:	Human CD25highFoxp3pos regulatory T-cells differentiate into IL-17 producing cells Hans Koenen, Nijmegen, The Netherlands
15.20-15.40:	De Novo generation and enhanced suppression of human CD4+CD25+ regulatory T cells by retinoic acid Jun Wang, Leiden, The Netherlands
15.40:	Closing remarks & Farewell reception

Abstracts of Sessions I-VI

Session I Thursday 16 April 2009, 10.35

Ton Schumacher
Netherlands Cancer Institute, Amsterdam, The Netherlands

Engineering T cell immunity

Adoptive therapy with allogeneic or tumor-specific T cells has shown substantial clinical effects for several human tumors, but the widespread application of this strategy remains a daunting task. As an alternative to the adoptive transfer of T cells, we and others have examined the feasibility of transfer of T cell receptor genes into recipient T cells. We have previously described the genetic introduction of antigen-specific T cell receptors into peripheral T cells in mouse model systems. These experiments reveal that cytotoxic T cells that are redirected by TCR gene transfer expand dramatically upon *in vivo* antigen encounter and efficiently home to effector sites. While these data demonstrated that redirected CD8+ T cells canfunction *in vivo*, a number of issues that are essential for the application of this strategy in the human setting remained unaddressed:

- Can redirected T cells be used to target tumor-associated antigens in settings where the endogenous T cell repertoire fails due to self-tolerance?
- Which strategies for immunomodulation are most effective in promoting the *in vivo* persistence and anti-tumor activity of TCR-modified T cells?
- What are the risks of TCR gene therapy and how can such risks be avoided?

We have recently addressed these issues in murine model systems. The resulting data and their possible implications for clinical trial design will be discussed.

Anita Stumpf, Edith van der Meijden, Cornelis van Bergen, Roel Willemze, Frederik Falkenburg, Marieke Griffioen
Leiden University Medical Center, Leiden, The Netherlands

Identification of HLA Class II restricted antigens by screening a recombinant bacteria cDNA library

Over the last decades anti-tumor immunotherapy focused on CD8+ cytotoxic T cells because of their capability to effectively lyse tumor cells. Great emphasize was therefore successfully placed on identification of HLA class I restricted tumor antigens. Nevertheless, CD4+ T cells can exert direct cytolytic activity as effector cells against HLA class II+ tumor cells and are indispensable as helper cells for the induction and maintenance of CD8+ T cells to achieve a potent and permanent anti-tumor response. Thus far, most HLA class II restricted epitopes have been identified by screening antigens that were known targets for CD8+ T cells or antibodies.
Although this approach led to the identification of useful targets for T cell based therapies, HLA class II epitopes from unknown and perhaps clinically more relevant antigens have been missed.
We developed a method to identify antigens as targets for CD4+ T cells by screening recombinant bacteria cDNA expression libraries. In a patient with relapsed chronic myeloid leukemia (CML) after HLA-matched stem cell transplantation who experienced long-term complete remission after donor lymphocyte infusion (DLI), activated CD4+ T cells were sorted from bone marrow obtained 5 weeks after DLI. By screening a bacterial cDNA library from patient-derived EBV-transformed B cells, we could identify 4 new minor histocompatibility antigens (mHags). This illustrates the efficacy of this technique to identify unknown HLA class II restricted antigens as targets for CD4+ T cells.

Two mHags were restricted by DRB1 and encoded by the methylene-tetrahydrofolate dehydrogenase 1 gene (MTHFD1; DRB1*0301) and lymphocyte antigen 75 gene (LY75; DRB1*1301), respectively.
The other two mHags were restricted by DRB3 and encoded by the protein tyrosine kinase 2 beta gene (PTK2B; DRB3*0101) and the major histocompatibility complex, class I related gene (MR1; DRB3*0202), respectively. All newly identified HLA class II restricted antigens show selective (LY75) or predominant expression in cells of hematopoietic origin.
All CD4+ T cell clones recognized *in vitro* cultured leukemic cells with antigen-presenting phenotype as well as CD33+ AML progenitor

cells. The clone recognizing MTHFD1 also showed direct recognition of CD34+ CML precursor cells. Screening recombinant bacteria cDNA libraries is an extremely efficient technique leading to the identification of four new HLA class II restricted antigens recognized by CD4+ T cells isolated during a potent anti-tumor response. Our data therefore illustrate the broad value of this method for characterization of unknown targets for CD4+ T cells in anti-tumor or auto-immunity.

I. Jolanda M. de Vries, Cornelis J.A. Punt, Gosse J. Adema, Carl G. Figdor
Department of Tumor Immunology and Medical Oncology, Nijmegen Centre for Molecular Life Sciences (NCMLS), Nijmegen, The Netherlands

Migration and effectiveness of tumor antigen loaded dendritic cells in melanoma patients

We exploited dendritic cells (DCs) to vaccinate melanoma patients. We recently demonstrated a statistical significant correlation between favorable clinical outcome and the presence of vaccine-related tumor antigen specific T cells in delayed type hypersensitivity (DTH) skin biopsies. However, favorable clinical outcome is only observed in a minority of the treated patients. We study in small *proof of principle* trials the fate, interactions and effectiveness of the injected DCs.
Firstly, we compared DC loaded with tumor antigen specific MHC class I binding peptides alone, in combination with MHC class II binding peptides or with defined tumor antigen mRNA (gp100 and tyrosinase). In each group of patients, tumor antigen specific T cells were detected in the DTH skin biopsies. Also CD4 antigen specific T cells could be detected with MHC class II tetramers. This T helper response might be beneficial for the clinical outcome in these patients. Secondly, we compared different routes of administration. Our data clearly indicate that the cells that reach the lymph nodes are fully mature DCs that are able to induce an immune response *in vivo*. Currently we study whether pre-conditioning of the vaccination site results in enhanced migration.
This work was supported by grants 1999-1950, 2000-2301, 2003-2893, 2003-2917 and 2004-3127 from the Dutch Cancer Society, and EU projects DC-THERA, and DC-VACC, the TIL-foundation and the NOTK.

Jannie Borst, Anna Keller, Yanling Xiao, Victor Peperzak, Shalin Naik
NKI-AVL, Amsterdam, The Netherlands

Costimulatory ligand CD70 allows induction of CD8+ T cell immunity by immature dendritic cells in a vaccination setting

Use of dendritic cells (DCs) as anti-cancer vaccines holds promise for therapy, but requires optimization. We have explored the potential of costimulatory ligand CD70 to boost the capacity of DCs to evoke a tumor-eradicating CD8+ T cell response. We demonstrate thatimmature conventional DCs, when endowed with CD70 expression by transgenesis, are converted from a tolerogenic state into an immunogenic state. CD70-expressing immature DCs could prime CD8+ T cells - via CD27 - to become cytolytic effectors that controlled pre-established tumors, as well as memory cells with a capacity for robust secondary expansion. The CD8+ T cell response was independent of CD4+ T cell help, since adoptively transferred immature DCs were loaded with MHC class I-restricted peptide only. Without CD70 expression, the DCs generated abortive clonal expansion, dysfunctional CTL responses and no CD8+ T cell memory. In our experimental set-up, CD70 expressing CD8+ DCs were responsible for CD8+ T cell priming and performed comparably to fully matured DCs. These data highlight the importance of CD27/CD70 interactions at the T cell/DC interface and indicate that CD70 should be considered in the design of DC vaccination strategies.

Martijn Nolte, Alex de Bruin
Academic Medical Center, Amsterdam, The Netherlands

CD27 on self-renewing hematopoietic stem cells induces proliferation and affects hematopoietic differentiation

The intricate process of hematopoiesis is tightly controlled by local feedback mechanisms, as growth factors and stromal cells regulate bone marrow output. However, we have found evidence that this process can also be regulated by cues from outside the bone marrow, as HSC and progenitor cells express the TNF-R superfamily member CD27 and can therefore interact with activated immune cells expressing CD70, the unique ligand for CD27.
We found that CD27 is expressed on both long-term (LT) and short-term (ST) self-renewing HSC, as well as on more differentiated progenitors such as MPP, CLP, CMP and GMP, but not MEP. CD27-deficiency does not affect the presence of HSC/progenitor populations, but CD27-/- HSC were found to be more capable in generating myeloid colonies *in vitro* as well as leukocytes *in vivo*. On the other hand, stimulation of CD27 strongly affected the fate of HSC, as CD70-transgenic mice, in which CD70 is overexpressed on B cells, have increased absolute numbers of LT- and ST-HSC, but a strong reduction of all downstream progenitor populations. We found that CD27-triggering induces proliferation of HSC, which correlates with fewer senescent HSC present in bone marrow of CD70TG mice and an increased sensitivity of these mice to 5-FU treatment. Microarray analysis of CD70TG mice revealed high expression of cell-cycle related genes. Finally, when the potency of HSC was tested both *in vitro* and *in vivo*, we found that CD27-stimulated HSC were less capable in giving rise to progeny, both in myeloid and in lymphoid direction.
These data reveal that CD27 is expressed on a large variety of early bone marrow progenitor cells and can induce proliferation of HSC. However, this does not benefit hematopoietic differentiation as such, which might indicate that this receptor can serve as a negative regulator of hematopoiesis during immune activation.

Frank J.T. Staal
Leiden University Medical Center, Leiden, The Netherlands

Wnt signaling regulates critical steps in hematopoiesis and lymphopoiesis

The Wnt signaling pathway has been implicated in regulation of hematopoiesis through a plethora of studies from many different laboratories. However, different inducible gain- and loss-of-function approaches retrieved controversial and some times contradictory results. Different levels of activation of the pathway, dosages of Wnt signaling required and the interference by other signals in the context of Wnt activation collectively explain these controversies. Gain-of-function or *in vitro* exposure to Wnt proteins and more specifically WNT3a was shown to enhance hematopoietic stem cell (HSC) activity but its exact role was still not completely understood. In a recent study we analyzed the hematopoietic system of mice deficient for this specific Wnt gene. Wnt3a deficiency results in early embryonic lethality around embryonic day 12.5 (E12.5), precluding analysis in adult mice, but allowing hematopoiesis to be studied in fetal liver and in the just colonized thymic rudiment. Notably, we showed that long-term HSCs and multipotent progenitors are reduced in fetal liver *in situ* and have severely reduced long-term reconstitution capacity as observed in serial transplantation assays. Of interest, deficiency in Wnt3a leads to complete abolishment of canonical Wnt signaling in fetal liver hematopoietic stem and progenitor cells. This HSC deficiency is not explained by altered cell cycle or survival and is irreversible since it cannot be restored by transplantation into Wnt3a competent mice. In addition, Wnt3a deficiency differentially affects myeloid and B-lymphoid lineages with myeloid cells being affected at the progenitor level, while B lymphopoiesis is apparently unaffected. Immature thymocytes however were reduced in cell numbers due to lack of Wnt3a production by the thymic micro environment. Our results show that while in the thymus Wnt3a provides cytokine-like, proliferative stimuli to developing thymocytes, Wnt3a regulates cell fate decisions of fetal liver HSC in a non-redundant way.

David Williams
Dana Faber Harvard Cancer Center, Boston, United States of America

Rho GTPase regulation of normal and abnormal hemapoeitic stem functions

Hematopoietic stem and progenitor cells (HSC/P) reside in the bone marrow (BM) cavity during post-natal life and may be localized to specific 'niches' within the hematopoietic microenvironment (HM). A tiny fraction of HSC, of unknown physiological relevance, are found circulating in the blood and the number of these cells in circulation can be increased in a process termed 'mobilization'. Infusion of HSC into the blood during BM transplantation procedures leads to engraftment of these cells in the marrow space and subsequent reconstitution of multi-lineage hematopoiesis. Using gene-targeted mice, we have found that signals involved in engraftment and mobilization of HSC/P are intimately associated with cytoskeleton rearrangement regulating cell shape, adhesion and migration. These pathways directly or indirectly also affect gene transcription, cell survival, cell cycle progression and may be important in transformation and changes seen in HSC during aging. These cytoskeleton rearrangements are controlled by members of the Rho GTPase family, particularly Rac GTPases. In addition, Rac and a unique GTPase-deficient GTPase called RhoH appear dysregulated in chronic leukemias. Development and utilization of a small molecule inhibitor of Rac has shown that Rac GTPases are novel targets for the manipulation of normal and transformed HSC behavior.

Paula van Hennik[1], Sacha Geutskens[1], William Andrews[2], Sandra de Haan[1], John Parnavelas[2], Peter Hordijk[1]
[1] Sanquin Research, Amsterdam, The Netherlands
[2] University College London, London, United Kingdom

Differentiation-dependent regulation of hematopoietic cell migration by Slit proteins

Slit is an extracellular matrix molecule originally identified in the developing central nervous system (CNS) of *Drosophila*. Here it transmits a repulsive cue to neuronal progenitors via the transmembrane receptor Roundabout (Robo), preventing axonal outgrowth. Four Robo and three Slit homologues have been identified in vertebrates. While Slit1 is confined to the CNS, Slit2 and Slit3 are widely expressed and have been reported to inhibit the CXCL12-induced migration of breast cancer cells, myocardial progenitors and of mature hematopoietic cells, i.e. T-lymphocytes and monocytes.

CXCL12 and its receptor CXCR4 are critical in the regulation of hematopoietic stem/progenitor cell (HSPC) migration to the bone marrow (BM) upon transplantation. We set out to investigate whether a negative migratory cue, i.e. Slit and its receptor Robo, can modulate HSPC migration.

We show that Slit2 and -3 are expressed in primary BM stroma and BM-derived endothelial and stromal cell lines, whereas Robo1 is expressed by CD34+ HSPC from different sources. Interestingly, Robo1 mRNA and surface protein expression levels were significantly higher in HSPC as compared to CD14+ monocytes. In line with this, we found that Robo1 expression was reduced during differentiation of HSPC towards CD14+ cells *in vitro*. These data suggest a role for Robo1 in hematopoietic stem cell differentiation. Furthermore, Slit3 inhibited the CXCL12-induced migration of Robo1-expressing HL60 and U937 cells, while it enhanced the directional migration of primary monocytes. HSPC migration remained unaffected by the same dose of Slit3, but lower Slit3 concentrations decreased HSPC chemotaxis. This may be related to the relatively high expression of Robo1 by HSPC compared to monocytes or to the expression of other, (un)known Slit receptor(s) by HSPC.

To establish the effect of Slit3 on HSPC migration *in vivo*, HSPC were pretreated with Slit3 and subsequently transplanted into immunodeficient NOD/SID mice. In contrast to the *in vitro* migration data, the homing to the BM at 24 hours after transplantation was 2-fold increased by Slit3.

Finally, in order elucidate the molecular mechanism underlying the

Slit response of hematopoietic cells, myc-tagged Robo1 (mutant) constructs were used to identify (novel) Robo1 interacting proteins. We were able to confirm the interaction of the adaptor protein NCK with Robo1, as was described earlier in Drosophila. This interaction involves the CC3 and CC2 domain of the intracellular tail of the Robo1 receptor and the SH3-domain of NCK. Interestingly, our results further show that there may be a role for the CC0 domain of Robo1 and the SH2-domain of NCK as well. Whether these different types of interaction are differentially regulated and are biologically relevant is currently being studied.In summary, Slit proteins may inhibit or promote leukocyte chemotaxis, depending on the cell type, its state of differentiation, relative expression of the Robo1 receptor and environmental, possibly tissue-specific, components. These findings uncover previously unrecognized complexity in the regulation of CXCL12-induced chemotaxis of hematopoietic (stem) cells.

Michael Verneris
University of Minnesota, Minneapolis, USA

Reduced risk of leukemia relapse after double UCB transplantation

Allogenic hematopoietic cell transplantation (allo-HCT) is potentially curative therapy for patients with high-risk or relapsed acute leukemia. In the absence of a matched sibling donor, hematoipoietic progenitor cells can be obtained from either the bone marrow (BM), peripheral blood (PB) or umbilical cord blood (UCB) of volunteer donors. Compared to BM and PB, UCB has a number of benefits including the lack of collection risk to the donor, the absence of donor attrition and rapid availability. UCB also has less stringent requirements for HLA matching and in the majority of patients a suitably matched donor can be identified. However, the widespread applicability of UCB transplantation has been constrained by the limited volume of blood contained in within the umbilical cord. This small volume of blood translates into limited numbers of mononuclear cells and CD34+ progenitor cells and the dose of these (per kg recipient body weight) is the single most import variable for UCB outcome. Thus, UCB has been reserved mainly for children.

Recently we have demonstrated that the addition of a second, partially HLA matched UCB unit can augment the cell dose; increasing both the rate and frequency of donor engraftment in patients who would otherwise have an inadequate cell dose with in a single UCB unit. Interestingly, the majority (>60%) of recipients of double UCB transplantation showed engraftment of a single UCB unit. By day 21 after transplant, the second unit was not detected and presumably eradicated due to immunological mechanisms. To date it is unknown whether the addition of a second, partially matched UCB unit has any impact on leukemia recurrence. We retrospectively reviewed the outcomes of patients with acute leukemia who received myeloablative conditioning chemotherapy followed by either single or double UCB transplantation.

Patients were selected to receive either single or double UCB unit transplantation based on the cell dose within the donor unit. Patients transplanted with two UCB units had a number of transplant related variables which differed from recipients of one UCB unit. Not surprisingly, double unit recipients were older and weighed more. The disease types (AML vs. ALL) and stage (CR1-2 vs. CR3-relapse) were not different between the two groups. The total dose of infused MNCs or CD34+ cells was not different between the two groups (for double UCB transplants this was the total of *both* UCB units). Two conditioning regimens were used during the course

of this study. Single unit recipients received either TBI/Cytoxan/ATG followed by CSA/Methylprednisone or Fludarabine/CTX/TBI followed by CSA/MMF. Recipients of double unit transplantation received only the fludarabine containing regimen.

The kinetics of engraftment and incidence of TRM was not different between the two groups. Recipients of two UCB transplants had a significantly higher rate of aGVHD than recipients of single unit transplantation. There was no effect on the conditioning regimen or GVHD prophylaxis on aGVHD in the single cord group (a similar comparison could not be made in the double unit recipients since all patients in this group received fludarabine/CTX/TBI regimen). The rate of cGVHD was not different between recipients of single and double UCB transplantation. Patients in early stages of disease (CR1-2) that received double UCB transplantation had a lower rate of disease recurrence compared to recipients of a single unit. Multivariate analysis showed that the use of double UCB transplants resulted in a significantly lower rate of disease recurrence.

Session III Thursday 16 April 2009, 16.40

Franco Locatelli
San Matteo, Pavia, Italy

Umbilical cord blood transplantation in children - the potential role of MSC

Lynne Ball[1], Maria Ester Bernardo[2], Arjan Lankester[1], Helene Roelofs[1], Angela Cometa[2], Jaap Jan Zwaginga[1], Maarten Egeler[1], Franco Locatelli[2], Willem Fibbe[1]

[1] **Leiden Univeristy Medical Center, Leiden, The Netherlands**

[2] **Fondazione IRCCS Policlinico S. Matteo, University of Pavia, PAVIA, Italy**

Co-transplantation of HLA-haploidentical, bone marrow derived mesenchymal stromal cells overcomes graft failure and provides a potential platform for additional immune therapeutic interventions

Haploidentical stem cell transplantation is a feasible option for children lacking a suitable HLA matched donor. To avoid fatal graft versus host disease (GvHD) associated with transplantation across major HLA incompatibility, intensive T cell depletion is necessary. This can be achieved either by positive CD34 selection of GCSF mobilized stem cells using magnetic bead techniques (CliniMACs). Exploitation of post transplant immune modulation may offer future opportunities to induce anti-tumor/ leukemia specific activity. The advantage of positive selection in relation to depletion is that no post immune supression, which could interfer with post-transplant cellular therapies, is necessary. The disadvantage however is the risk of graft rejection, which can to some extent be overcome but not entirely eliminated by infusing high doses of CD34 positive cells (mega dose concept). In order to provide a safe platform for haploidentical transplantation we chose to investigate the 'co-transplantation' of bone marrow derived mesenchymal stromal cells (MSC). Animal models suggest that MSC may promote engraftment after hematopoietic stem cell transplantation (HSCT) but their role in overcoming graft failure in human HSCT remains unclear. Here we report our series of 28 pediatric patients scheduled for haploidentical peripheral blood stem cell transplantation (PBSCT) all of whom were eligible to participate in an ethically approved study. MSCs were isolated from marrow of PBSCT donors and expanded under GMP conditions using same batch FCS containing medium and a standardized protocol, approximately 4-5 weeks before transplantation. MSC (fresh or cryopreserved) were administered i.v. 4 hours before the allograft. Conditioning depended on underlying disease. No pharmacological GvHD prophylaxis was given after PBSCT. All donors tolerated the procedure well. There were no failures of MSC expansion and all cultures fulfilled strict release criteria including sterility and karyotypic analysis. MSC were not associated with infusional toxicities. In comparison to historical controls (20% graft failure),

all patients given MSC showed successful and stable engraftment (p=0.001). (Follow up 2-50 months). Hematological recovery was accelerated, albeit immune recovery other than a faster NK cell recovery at one month, did not differ from historical controls. Viral reactivations were common but incidence did not differ between study patients and controls. Death due to infection was not increased in patients receiving MSC. Acute GvHD occurred in only 2/28 study patients (grade I-II) compared to 12/52 historical controls (p=0.006). Follow up is too short to comment on relapse risk. Our extended analysis shows a highly statistically significant ($p<0.001$) reduction in graft failure rates for patients co-transplanted with MSC, in the absence of a GvHD and post transplant immune suppression. Co-transplantation of MSC in the haploidentical setting has established a safe alternative platform for transplantation, which will permit future strategies aimed at post transplant immune modulation in children with poor outcome malignant disease.

Session IV Friday 17 April 2009, 09.30

Stefan Janssens
Catholic University Leuven, Leuven, Belgium

Progenitor cell therapy for cardiovascular disease

Paul Quax
TNO-Quality of Life, Leiden, The Netherlands

Immune modulation in neovascularisation: the role of the NK cells

Christine Mummery
Leiden University Medical Center, Leiden,
The Netherlands

Cardiomyocytes and vascular endothelial cells from pluripotent stem cells

Methods have been developed for efficient and reproducible differentiation of human embryonic stem cells to cardiomyocytes. This has allowed production of genetically marked cardiomyocytes in sufficient numbers for transplantation to mouse hearts with view to tracking their fate in control mice and in mice that have undergone myocardial infarction over several months. The results show that there is selective survival of cardiomyocytes and that they mature *in vivo* but that the fibrosis can develop around grafts which may isolate them from the host heart tissue. Significant improvements in cardiac function are observed by non-invasive imaging (MRI) 4 weeks after injection in mice with myocardial infarction compared with mice receiving (non-cardiomyocyte) differentiated HESC controls. At 14-16 weeks, these differences are no longer significant and grafts resulting from single injections may remain too small to contribute contractility to the myocardium long term. This indicates that claims of short term functional improvement may not reflect meaningful long term effects and that multiple graft may be required.

The efficient differentiation protocol has also facilitated micro array analysis of gene expression patterns during human cardiomyocyte differentiation. Apart from genes that would have been expected on the basis of studies of heart development in multiple species, 15 novel or unique genes were identified. Functional analysis of these in zebra fish and mice has indicated several that might be associated with congenital heart defects in humans. This is currently being investigated in DNA databases of patients with heart defects with view to developing new paradigms for prenatal genetic diagnosis.

A.M. van Pelt
S.C. Mizrak, J.V. Chikhovskaya, H. Sadri Ardekani, F. Van der Veen, D.G. de Rooij, S. Repping
University of Amsterdam, Amsterdam, The Netherlands

Pluripotent stem cells from adult human testis

The search for adult pluripotent stem cells has accelerated because of the drawbacks of ES cell applications in regenerative medicine including immune rejection, low generation efficiency and ethical concerns.

Recent reports in mice indicate that male germ stem cells can be propagated in culture for as long as two years. These long term cultured mouse spermatogonial stem cells (SSC) are able to repopulate the testis after transplantation and generate new offspring. In addition, these SSCs can transform into pluripotent cells, i.e. they are able to differentiate *in vitro* into derivatives of all three germ layers, form teratomas when injected into immune deficient mice and can generate chimeras upon blastocyst injection.

We and others have now shown the generation of pluripotent cells from adult human testis. These cells have the ability to differentiate *in vitro* into cells of all three germ layers, including neuronal, osteogenic and pancreatic cells, the latter being able to secrete insulin. Their easy accessibility, ethical acceptability, and autogenic nature, make these cells an attractive alternative to human ES cells or induced pluripotent stem cells (iPS) for future stem cell therapies.

Catherine M Verfaillie and colleagues
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Mechanisms underlying increased potency of postnatal cells

During the last 5-7 years we and others demonstrated that cells can be cultured from bone marrow, cord blood, testis and perhaps other postnatal tissues that have the ability to differentiate into multiple cell types, including mesoderm, endoderm and ectoderm. We termed these cells multipotent adult progenitor cells (MAPC). However, the greater potency of MAPC and other cells from somatic tissues, but perhaps not from testis, is still less than that of Embryonic Stem Cells (ESC). Transcriptome analysis demonstrated that MAPC differ significantly from other adult multipotent stem cells, such as NSC and MSC, but that MAPC express a number, but not all, genes specifically expressed in ESC, which are known to be important for their pluripotency (MAPC express e.g. Oct4, Sall4, Tbx3, Rex1; not Nanog and Sox2), even though MAPC also express genes known to be associated with endoderm and mesoderm or primitive endoderm specification. Noteworthy, MAPC express four of the six factors recently identified to be capable of reprogramming mouse and human fibroblasts to IPs cells. Studies aimed at further delineating the mechanism underlying the greater potency of MAPC, whether pre-existing *in vivo* or induced in culture, will be discussed, as well as studies aimed at identifying the developmental stage with which MAPC can be compared.

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Dendritic cells and transplant tolerance

In recent years, there has been a shift from the traditional perception of bone marrow-derived dendritic cells (DCs) solely as inducers of immune reactivity and transplant rejection. The contemporary view is that these cells are crucial regulators of immunity, including their ability to induce and maintain transplant tolerance. Advances in our understanding of the phenotypic and functional plasticity of DCs, and the ability to manipulate their development and maturation *in vitro* and *in vivo*, has provided a basis for the promotion of their inherent tolerogenicity.

Considerable insight has been gained into the role of DC subsets in central and peripheral tolerance and into the molecular pathways that regulate the outcome of DC–T cell interactions. Both DC-based cell therapy and *in situ* targeting of DCs have shown promise for the promotion of transplant tolerance. Much of this information has been obtained using *in vitro* and small animal model systems, and many questions remain regarding the relevance of these findings to humans. Early non-human primate work indicates that DCs can be used to regulate alloimmune reactivity *in vivo*. A major challenge in this emerging area of applied DC biology is how the accumulated knowledge can be exploited successfully to improve the outcome of clinical organ or bone marrow transplantation.

Proof of principle that human DCs can induce T cell tolerance to model antigens has been demonstrated in volunteers. Challenges facing tolerogenic DC therapy in transplantation include the optimum type of DCs, the form of alloantigen to be delivered to host-derived DCs, the optimal timing, route, dose and frequency of delivery of the cells, optimal use of DCs with immunosuppressive agents, their interaction with memory T cells, and defining outcomes that can be adequately monitored.

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Session VI Friday 17 April 2009, 14.25

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Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans

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Human CD25highFoxp3pos regulatory T-cells differentiate into IL-17 producing cells

The effector T-cell lineage shows great plasticity. Th17 cells are acknowledged to be instrumental in the response against microbial infection, but are also associated with autoimmune inflammatory processes. Here, we report that human regulatory T-cells (CD4posCD25highFoxp3posCD127negCD27pos) can differentiate into IL-17 producing cells, when stimulated by allogeneic antigen presenting cells, especially monocytes, in the presence of rhIL-2/rhIL-15. These Treg derived IL-17 producing cells showed high expression of the Th17-related transcription factor RORγt and were positively identified by CCR6 expression. This differentiation process was enhanced by exogenous IL-1β, IL-23, IL-21, while IL-6 or TGFβ did not affect the emergence of IL-17 producing cells. The addition of IL-1 receptor antagonist (IL-1Ra), but not anti-IL-23 antibody, reduced IL-17 producing cell numbers. When an histone deacetylase (HDAC) inhibitor Trichostatin A (TSA) was evaluated, we found a profound negative effect on the emergence of IL-17 producing cells from Treg, implying that Treg differentiation into IL-17 producing cells depends on histone/protein deacetylase activity. Thus, the data suggest that epigenetic modification underlies the phenomenon of Treg plasticity here described. The ability of Treg to convert into IL-17-producing cells may be exploited to facilitate vaccination strategies and tumor immunotherapy. Conversely, it may have implications for the clinical application of *ex vivo* expanded Treg as part of tolerance inducing strategies.

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De Novo generation and enhanced suppression of human CD4+CD25+ regulatory T cells by retinoic acid

Therapies based on CD4+CD25+FOXP3+ T regulatory (Treg) cells offer promise for the restoration of tolerance in many immune-mediated disorders. However, it has been proven difficult to obtain large numbers of Treg cells with potent and stable suppressive ability from human peripheral blood due to the lack of specific markers, compromised function of isolated CD4+CD25++ T cell populations and the difficulty to convert conventional T cells, for example by TGF-beta, into Treg cells in a consistent manner. Here we show that:
1) in the presence of TGF-beta, all-trans retinoic acid (ATRA) efficiently converts adult human peripheral blood naive CD4+ T cells into FOXP3+ Treg cells with stable and potent suppressive ability;
2) memory CD4+ T cells are resistant to FOXP3 induction and, moreover, inhibit Treg-conversion of naive T cells;
3) treatment of isolated CD4+CD25++ T cells with ATRA/TGF-beta enhances their suppressive potential during expansion. Our results indicate that ATRA/TGF-beta can be employed to generate highly suppressive CD4+FOXP3+ Treg cells from adult human peripheral blood, and are relevant for the development for Treg-based therapies.

Posters

Numbers refer to the poster board

1. *Ex vivo* generation of human alloantigen-specific regulatory T cells from CD4posCD25high T cells for immunotherapy

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Background
Regulatory T cell (Treg) based immunotherapy is a potential treatment for several immune disorders. By now, this approach proved successful in preclinical animal transplantation and auto-immunity models. In these models the success of Treg based immunotherapy crucially depends on the antigen-specificity of the infused Treg population. For the human setting, information is lacking on how to generate Treg with direct antigen-specificity *ex vivo* to be used for immunotherapy.

Methodology/Principle Findings
Here, we demonstrate that in as little as two stimulation cycles with HLA mismatched allogeneic stimulator cells and T cell growth factors a very high degree of alloantigen-specificity was reached in magnetic bead isolated human CD4posCD25high Treg. Efficient increases in cell numbers were obtained. Primary allogeneic stimulation appeared a prerequisite in the generation of alloantigen-specific Treg, while secondary allogeneic or polyclonal stimulation with anti-CD3 plus anti-CD28 monoclonal antibodies enriched alloantigen-specificity and cell yield to a similar extent.

Conclusions/Significance
The *ex vivo* expansion protocol that we describe will very likely increase the success of clinical Treg-based immunotherapy, and will help to induce tolerance to selected antigens, while minimizing general immune suppression. This approach is of particular interest for recipients of HLA mismatched transplants.

2. Clinical grade treg: GMP isolation, improvement of purity by CD127pos depletion, treg expansion, and treg cryopreservation

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Background
Treg based immunotherapy is of great interest to facilitate tolerance in autoimmunity and transplantation. For clinical trials, it is essential to have a clinical grade Treg isolation protocol in accordance with Good Manufacturing Practice (GMP) guidelines. To obtain sufficient Treg for immunotherapy, subsequent *ex vivo* expansion might be needed.

Methodology/Principle Findings
Treg were isolated from leukapheresis products by CliniMACS based GMP isolation strategies, using anti-CD25, anti-CD8 and anti-CD19 coated microbeads. CliniMACS isolation procedures led to 40-60% pure CD4posCD25highFoxP3pos Treg populations that were anergic and had moderate suppressive activity. Such CliniMACS isolated Treg populations could be expanded with maintenance of suppressive function. Alloantigen stimulated expansion caused an enrichment of alloantigen-specific Treg. Depletion of unwanted CD19pos cells during CliniMACS Treg isolation proved necessary to prevent B-cell outgrowth during expansion.
CD4posCD127pos conventional T cells were the major contaminating cell type in CliniMACS isolated Treg populations. Depletion of CD127pos cells improved the purity of CD4posCD25highFoxP3pos Treg in CliniMACS isolated cell populations to approximately 90%. Expanded CD127neg CliniMACS isolated Treg populations showed very potent suppressive capacity and high FoxP3 expression. Furthermore, our data show that cryopreservation of CliniMACS isolated Treg is feasible, but that activation after thawing is necessary to restore suppressive potential.

Conclusions/Significance
The feasibility of Treg based therapy is widely accepted, provided that tailor made clinical grade procedures for isolation and *ex vivo* cell handling are available. We here provide further support for this approach by showing that a high Treg purity can be reached, and that isolated cells can be cryopreserved and expanded successfully.

3. Glycosylation changes on the epithelial mucin MUC1 affect dendritic cell maturation

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C-type lectin receptors (CLR) on dendritic cells (DC) play a crucial role in immune responses to glycosylated antigens. Tumor cells exhibit significantly altered glycan structures on their cell surface and how they influence immune cells is unknown. We have demonstrated that the human Macrophage Galactose-type lectin (MGL) expressed by DC interacts with the epithelial mucin MUC1, containing Tn-antigens (Ser/Thr-N-acetylgalactosamine). To study the influence of tumor glycans on DC, recombinant MUC1-Fc containing Tn-antigens or sialylated Thomsen-Friedenreich antigens were incubated with monocyte-derived DC before addition of lipopolysaccharide. Maturation markers and cytokine production were studied. MUC1 induced a slight maturation of DC in the absence of TLR-stimulus as observed by staining of the maturation markers: CD80, CD83, and CD86. No induction of cytokines was observed. Interestingly, MUC1 had no influence on DC maturation in the presence of LPS, but production of IL-10, IL-6 and IL12p70 was significantly reduced in the presence of MUC1. Both glycoforms had an effect on cytokine production. We are currently investigating whether the glycans present on MUC1 influence DC function and whether the C-type lectin MGL plays a role in these responses. Our data suggest that the highly glycosylated tumor antigen, MUC1, may influence the function of antigen presenting cells to benefit tumor survival.

4. Fine-tuning of a CFSE- and PKH-based suppression assay with induced regulatory T cells generated by IL-10-treated alternatively activated dendritic cells

Martine Boks, Gijs van Schijndel, Jaap Jan Zwaginga, Marieke van Ham, Anja ten Brinke
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Tolerogenic dendritic cells (DCs) have a strong potential to form an important type of cellular therapy to treat autoimmune disorders, or to prevent undesired immune responses against allogeneic transplants or therapeutic proteins. Key players for the induction of tolerance are regulatory T cells (Tregs), which may be induced by tolerogenic DCs. The aim of this study is to generate tolerogenic DCs and analyze their capacity of inducing Tregs. Therefore, we have set up a suppression assay in which the suppressive capacity of DC-induced Tregs can be measured.

DCs that are matured in the presence of IL-10 have a tolerogenic phenotype and induce Tregs with suppressive capacity. (Steinbrink et al., J Immunol, 1997) From literature it is known that 'alternatively activated' DCs, i.e. DCs that are matured with an immuno-activatory stimulus and a tolerogenic stimulus, will have both tolerogenic properties and a stable phenotype.

We have generated alternatively activated (aa)DCs from monocyte-derived immature DCs. Immature DCs are pretreated with IL-10 and subsequently with a maturation cocktail, consisting of TNFα, IL-1β and PGE2. Alternatively, immature DCs are treated with the maturation cocktail only to generate mature immuno-activatory DCs. To study Treg inducing capacity of IL-10 aaDCs, DCs are first incubated with allogeneic naïve T cells for priming. Next, the suppressive capacity of the primed T cells on syngeneic responder T cells is determined in a CFSE- and PKH-based suppression assay. Our data clearly shows that IL-10 aaDC-primed T cells suppress proliferation of responder T cells in a dose-dependent manner, whereas control T cells primed by mature immuno-activatory DCs do not induce suppression. Furthermore, we found that the strength of stimulation in the suppression assay is important for an optimal read-out of suppression by induced Tregs.

Induced Tregs can mediate suppression either via cell contact-dependent mechanisms or via soluble mediators, like cytokines, or both. With transwell assays we have shown that cell contact is necessary for suppression by IL-10 aaDC-induced Tregs. In addition, with blocking antibodies we have shown that IL-10 and TGFβ are not important for the suppressive capacity of IL-10 aaDC-induced Tregs.

In conclusion, DCs that are matured in the presence of IL-10 have a tolerogenic phenotype and induce Tregs with suppressive capacity. We have set up and optimized a suppression assay in which the suppression of proliferation of responder T cells by primed T cells is

a measure of Treg induction. Furthermore, we have shown that cell contact is an important mechanism for the suppressive capacity of IL-10 aaDC-induced Tregs. In the future, we will compare different types of tolerogenic DCs, generated with various compounds, to see which is best suited for tolerance-inducing cellular therapy.

5. Monocyte derived dendritic cells matured with a clinical grade maturation cocktail consisiting of MPLA plus IFN-gamma induce superior CTL responses

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In clinical trials *ex vivo* generated autologous monocyte-derived dendritic cells (DCs) are used as a cellular vaccine against cancer for the *in vivo* generation or boosting of antigen-specific T cell mediated immunity.

Ex vivo, immature DCs can be generated from monocytes and subsequently differentiated into mature DCs by various cocktails of maturation factors. Clinical studies require protocols where a sufficient number of well characterized highly immunogenic DCs are produced according to current Good Manufacturing Practice (cGMP) Guidelines. Besides, in order to be able to induce an efficient tumour-specific CTL response during immunotherapy, DCs have to be able to migrate to the lymph node and produce the Th1 polarizing cytokine, IL-12p70, upon encounter of T cells in the lymph node.

However, a common used PGE2-based maturation cocktail in clinical trials induces very good maturation, but no IL-12p70 production. Other maturation cocktails, which include TLR ligands, induce IL-12p70 production, but render these DCs less migratory. Thus, IL-12 production and migration by DCs seem to be inversely correlated processes. However Mailliard et al. (Cancer Research, 2004) have found a cocktail which induces DCs which can both migrate and produce IL-12, but because of its 5 components this cocktail is quite expensive.

We have developed a cost-effective, new clinical grade maturation cocktail which fulfils the requirements for induction of type 1 immunity. Monophosphoryl lipid A (MPLA) and IFNγ matured DCs combine intermediate migration with very high IL-12 production upon CD40L stimulation. Subsequently these DCs induce mainly Th1 cells *in vitro*. Furthermore, MPLA plus IFNγ-matured DCs induce a superior CTL response compared to DCs matured with the PGE2-based maturation cocktail. The Mart1 (MelanA) specific CTLs, induced by Mart1-peptide loaded MPLA plus IFNγ DCs, are capable to kill melanoma derived cell lines.

In conclusion, MPLA plus IFNγ is a simple clinical grade maturation cocktail to generate immunostimulatory DCs with superior capacity to induce type 1 immunity and CTL responses. Therefore DCs matured with MPLA and IFNγ are very promising for anti-cancer therapy.

6. Exploiting *ex vivo* generated natural killer cells in cancer immunotherapy

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Donor natural killer (NK) cells displaying killer immunoglobulin-like receptor (KIR)-ligand mismatches with malignant cells of the recipient play a significant role in the anti-tumor effect following haploidentical stem cell transplantation. Therefore, infusion of alloreactive NK cells from an HLA-haploidentical donor could be an attractive approach for cancer immunotherapy. However, the success of NK cell-based immunotherapy depends on robust isolation and expansion methods for obtaining sufficient numbers of pure alloreactive NK cell products. We have established an efficient cytokine-based culture system for the *ex vivo* expansion of fully functional NK cells from CD34+ hematopoietic stem and progenitor cells isolated from umbilical cord blood (UCB) or bone marrow (BM). The developed NK cell generation procedure consists of two steps. The first step involves the expansion of CD34+ cells in 8-10 days of culture using specific modified glycosaminoglycans and growth factors including SCF, Flt3-L, IL-7 and TPO. The second step consists of the differentiation of the expanded CD34+ cells into the NK cell lineage, which requires an additional 4-week culture period using cytokines like IL-2 and IL-15. Systematic refinement of our system resulted in a clinically applicable protocol enabling the *ex vivo* expansion and differentiation of CD34+ UCB cells to more than 4-logs into CD56+CD3- NK cells of very high purity. NK cell expansion rates from CD34+ BM cells were lower and required prolongation of the differentiation step for several days. These *ex vivo*-generated CD56+ cell products contain NK cell subsets differentially expressing CD94/NKG2A and KIR. Furthermore, such CD56+ cell products uniformly express high levels of activating NKG2D and NKp receptors. Functional analysis showed that CD56+ cell products containing alloreactive NK cells efficiently kill the classical K562 target as well as MHC class I expressing myeloid leukemia and melanoma cell lines. These data point towards the likelihood of a breakthrough in producing pure NK cell products from limited numbers of CD34+ cells for cancer immunotherapy. Our first aim is to explore the feasibility of adoptive transfer of *ex vivo*-generated NK cell products in elderly patients with acute myeloid leukemia following reduced intensity conditioning immunosuppression.

7. Recruitment and activation of NK cells by TLR2/4 agonist and IFN-y matured DC enhances TH1 responses

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Introduction

Besides their prominent role in the destruction of altered self-cells, natural killer (NK) cells have been shown to potentiate T cell responses by interacting with dendritic cells (DC). In the context of dendritic cell-based vaccines it remains to be determined how NK-DC interactions depend on differential DC maturation.

Materials and methods

Monocyte-derived DC are maturated either in the presence of a maturation cocktail containing TNF-α/PGE-2 or FMKp (TLR2,4)/ IFN-γ. The recruitment of NK cells by these DC is analyzed by a trans-well migration assay and activation of NK cells by IFN-γ ELISA. Chemokine (CXCL10, CCL19, RANTES) and cytokine (IL-12, IL-18) production of the differently matured DC is investigated by ELISA. Th1 polarization is analyzed by intercellular IFN-γ flow cytometry after coculture with SEB coated NK-DC cultures.

Results

By comparing different DC differentiation (IL-4/GM-CSF and IL-13/ GM-CSF) and maturation cocktails (IFN-γ/FMKp and PGE2/TNF-α), we show that the ability of human DC to attract NK cells is imprinted during DC maturation. Only FMKP/IFN-γ maturated DC have the capacity to actively recruit NK cells *in vitro* and our data indicate that CCR5 is the dominant chemokine receptor in this recruitment. Furthermore, in contrast to PGE2/TNF-α matured DC, FMKP/IFN-γ maturated DC activate NK cells to produce IFN-γ in a IL-12/IL-18 dependent manner and this activation contributes to strong TH1 polarization. In addition upon contact with these DC, NK cells upregulate their lymph node homing receptors, possibly inducing secondary migration to the lymph nodes.

Conclusion

In conclusion, besides the identification of a superior DC maturation cocktail which enhances NK-DC interactions, we identified a novel recruitment mechanism for peripheral human NK cells which may contribute to secondary, central DC-NK interactions and strong TH1 polarization.

8. Targeting of murine DCs expressing MGL-1 and -2 to enhance antigen presentation and CD4 / CD8 T cell responses

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The aim of this study is to determine the potential to use MGL for DC targeting for cancer immunotherapy. In humans, the C-type lectin MGL is expressed on dendritic cells (DC) and macrophages. MGL recognizes terminal N-acetylgalactosamine (GalNAc)-containing glycans on self-antigens and pathogens and plays a role in antigen uptake. MGL orthologues have been found in mice and rats. In mice two functional copies of MGL are present, MGL-1 and MGL-2, that are both expressed on tissue DC, macrophages and bone marrow derived dendritic cells (BM-DC).

In this project, We chemically modified OVA with MGL-1 and MGL-2 specific glycans. With this OVA-glycoconjugates we investigated their potency to be processed by BM-DC.
We found expression of MGL-1 and -2 in various tissues of the mice, such as spleen, lymphnodes and skin. We cloned murine MGL-1 and MGL-2 and determined their glycan specificity. Cellular mMGL-1 displays exclusive specificity for Lewis X and Lewis A structures whereas mMGL-2 prefers Gal/GalNAc structures. We targeted BM-DC with glycan modified OVA (OVA-lewis X and OVA-GalNAc) and studied their effect on the maturation of BM-DC *in vitro*. There were no differences seen in their ability to mature DCs in comparison to non-modified OVA. We furthermore analyzed antigen presentation *in vitro*, using OVA-Lewis X and OVA-GalNAc loaded DCs. The targeting of BM-DC with OVA-Lewis X resulted into increased OT-1 responses, whereas OVA-GalNAc resulted in less enhanced OT-1 proliferation but increased OT-2 T cell proliferation.

Our results demonstrate that MGL may be a receptor for DC targeting and immunotherapy. Both its expression on APC in skin, spleen and lymphnodes, as well as its function to facilitate antigen uptake are in favor for enhanced presentation. We observed that glycan-modified antigen can differentially induce CD4 and CD8 responses depending on the the glycan-receptor they target.

9. Adoptive T cell progenitor therapy for patients undergoing haploidentical HSC-transplantation

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Introduction

The delay in T cell recovery after haploidentical hematopoietic stem cell (HSC) transplantation - a method that can cure patients with leukemia in a very high rate - causes that patients suffer from opportunistic infections. An *in vitro* generated T cell or a progenitor supplement may facilitate recovery of T cell levels, and decrease morbidity.

Aim

Determine (1) if mobilized CD34+ HSC can be differentiated efficiently towards T and/or T/NK lineage, (2) confirm T cell potential using model-systems.

Methods

T and NK-depleted CD34+ HSC (n=3) were obtained from G-CSF treated volunteers. Purity ranged from 90-92% CD34+ HSC. HSC were seeded on monolayers of TSt-4 thymic storma cells expressing Notch ligands DLL1 or DLL4, and co-cultured for 4-8 weeks in the presence of growth-factors.

Results

After 4 weeks on TSt-4/DLL1 or DLL4 cells, cell numbers were increased by 500-600x (all donors), with 50-60% of the cells stably arrested at a CD5+CD7+iCD3+CD56-sTCR- stage - T/NK progenitors. Following sorting, T/NK progenitors were propagated for another 4 weeks on DLL-expressing TSt-4 monolayers, without phenotypical changes. Preliminary data of transplantation experiments where these progenitors were transferred to RAG1 -/-, cγ-chain -/- mice show that these cells can develop into mature single positive CD4 and CD8 T cells. On TSt4 monolayers without DLL, these bipotent progenitors became CD56+ NK-lineage cells. No eCD3+ or sTCR+ cells could be detected.

Conclusion

The TSt-4/DLL1 or -4 system is very efficient and reliable in inducing T/NK lineage commitment from various HSC sources, including mobilized HSC from adult donors. To maintain their phenotype, T/NK progenitors require contact with DLL. These cells seem very attractive for adoptive therapy.

10. Regulatory requirements for development and marketing of cellular therapies

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So-called advanced therapy medicinal products, i.e cell-therapy, gene therapy and tissue engineering products, lately received much attention from regulatory authorities in Europe and the United States. Various initiatives take place to ensure a good regulatory environment for these innovative therapies. For instance, both the European Medicines Evaluation Agency (EMEA) and the US Food and Drug Administration (FDA) have issued guidelines to address the specific aspects of these products. And the EMEA has installed a new Committee for Advanced Therapies which will be involved amongst others in the review of marketing authorisation applications of these products and in scientific advice.
This presentation will focus on cellular therapies. An overview of applicable regulatory requirements and related regulatory initiatives will be given, as well as information on the practical implications of this regulatory information in the development of a cell therapy medicinal product.

11. Haploidentical *ex vivo* expanded mesenchymal stromal cells are not immunogenic

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Cord blood and haploidentical stem cells are alternative sources of stem cells for transplantation. In children undergoing cord blood and haploidentical stem cell transplantation, bone marrow derived *ex vivo* expanded mesenchymal stromal cells (MSCs) have been reported to overcome delayed engraftment and rejection.
MSCs are multipotential non-hematopoietic progenitor cells capable of differentiating into multiple mesenchymal lineages. They are immunomodulatory showing clear *in vitro* effects on cells of both the innate and adaptive immune systems. Both contact-dependent mechanisms and soluble factors are thought to be involved in this induction of MSC-mediated, immune-suppression. Additionally, MSCs, being poor antigen presenting cells and lacking MHC class II or co-stimulatory molecule expression, are considered hypoimmunogenic.

On the other hand, MSC may function as antigen presenting cells and activate immune responses under appropriate conditions. Indeed, MSCs are able to take up antigens, and after stimulation with IFNγ they induce T-cell responses to recall antigens. Infusion of allogeneic MSC can prime naive T cells in immunocompetent mice inducing donor graft rejection. These different *in vitro* findings clearly show that only clinical studies will be able to determine the immunogenicity of MSCs.
In Leiden and Pavia, children undergoing cord blood stem cell transplantation or haploidentical peripheral blood stem cell transplantation (PBSCT) are co-transplanted with haploidentical bone marrow-derived, *ex vivo* expanded MSCs to respectively improve hematological engraftment and reduce the risk of graft rejection.
We found evidence on the low immunogenicity of haploidentical MSC in two children that underwent an initial unrelated cord blood transplantation and later following rejection, a haploidentical PBSCT. Both transplants were preceded by myeloablative conditioning and undertaken with co-administration of MSC, derived from the same parent who later acted as the hematopoietic stem cell donor for the second transplant.
Patients' details and graft characteristics will be summarized. Although both patients were able to reject cord blood derived stem cells, however, the simultaneous re-exposure to paternal derived

MSC antigens did not induce T-cell responses to recall antigens during the second transplant. HLA antibodies against the father were not detected which allowed for subsequent successful second haploidentical engraftment. Engraftment (defined as leukocytes >1.0 x 10^9/l, neutrophils > 0.5 x 10^9/l and platelets > 20 x 10^9/l and 100% donor chimerism) moreover, was rapid in both patients (+12 and 15 days respectively) and in agreement with results on co-transplantation of haploidentical PBSC and MSCs, as previously reported by our group.

In conclusion potential immunogenicity of MSCs needs to be considered as a risk for immunisation *in vivo* especially if this immunisation might lead to rejection of later administered hematopoietic stem cells sharing HLA expression with the MSCs. We were, however, able to demonstrate that, despite previous exposure to the hematopoietic stem cell donor derived MSC's, further exposure to same donor MSC's and hematopoietic stem cells did not result into rejection of the donor graft. We are presently investigating if MSCs expressed antigens merely escape immunisation or that MSCs even induce specific tolerance against these antigens.

12. The enhancement of short-term engraftment in NOD/Scid mice after double cord blood transplantation is mediated by CD34+ cells

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Cord blood (CB) is an attractive source of hematopoietic stem cells, however, the low number of cells hampers its use in adult patients. Transplantation of two unrelated CB units seems to overcome the cell dose limitations for adult patients. Pre-clinical studies show an important role for total cell numbers in the graft, but also CD34+ stem cells or CD34- cells may play a facilitating role. In this study we explored the role of the CD34+ and CD34- cells in facilitating engraftment in NOD/Scid mice after double CB transplantation. Cohorts (ch.) of irradiated NOD/Scid mice (n=9-18/cohort; 3 experiments) were transplanted with a combination of 1.105 CD34+ cells and 1.107 CD34- cells from one CB unit. In addition, mice received a combination of CD34+ and CD34- cells, or CD34+, or CD34- cells, from a second CB unit. BM engraftment was assessed by the percentage of human CD45+ cells (>10% hCD45+) at 6 weeks. In addition, the distribution of human lymphoid and myeloid cells in the BM was evaluated and the kinetics of human platelet (hPLT) and hCD45+ recovery in blood was measured. Contribution of both CBs to the engraftment was determined with HLA-allele-specific-MoAbs.

At 6 weeks, 67% of the mice in the single CB cohort (ch.1), 75% in the cohort co-transplanted with CD34- cells (ch.4), 83% in the cohort co-transplanted with CD34+ cells (ch.3) and 89% in the cohort co-transplanted with the combination of CD34+ and CD34- cells of the second donor (ch.2), showed human engraftment in the BM. Mice that were co-transplanted with only CD34+ cells (ch.3) had a significantly higher level of engraftment in the BM than mice receiving no second CB unit (ch.1) (54% vs. 22%; p=0.011). A similar engraftment enhancing effect was observed in mice co-transplanted with a combination of CD34+ and CD34- cells (ch.2) (50% p=0.016 vs. ch.1). In contrast, no significant enhancing effect was observed after co-transplantation of only CD34- cells (ch.4) (36%). The distribution between human lymphoid and myeloid cells in BM was similar between al cohorts. In most experiments a stable mixed chimerism (6 weeks-3 months) was observed in the BM without pre-dominance of one CB unit. hPLT recovery was significantly increased in mice co-transplanted with CD34+ cells, either alone (ch.3; 607plt/µl) or in combination with CD34- cells (ch.2; 1076 plt/µl) compared to mice receiving no second CB (ch.1; 183 plt/µl) (p=0.04 and p<0.001 resp.). The observed mean hPTL concentration in cohort-2 and -3 was greater than expected by the sum of the single CBs (305 plt/µl), indicating a synergistic effect. In mice that were co-transplantated with CD34- cells from a second donor (ch.4) no significant effect on hPLT recovery (253 plt/µl) was observed.

Similar results were obtained for hCD45. The engraftment facilitating effect of co-transplantation of CD34+ cells only are similar compared to co-transplantation of both CD34+ and CD34- cells. Therefore, it is suggested that enhancement of short-term engraftment in NOD/Scid mice by double cord blood transplantation is mainly mediated by the CD34+ cells of the second CB.

13. Autologous bone marrow transplantation for muscle improvement in traumatic brachial plexus injuries; results of a phase-1 clinical study

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Background
Traumatic brachial plexus (BP) injuries may cause loss of elbow flexion. After nerve surgery active elbow flexion often remains insufficient. Muscle strength improvement via cell therapy would be a potential option and could avoid muscle transfer surgery. The primary objective of this pilot study was to assess the safety and feasibility of autologous bone marrow (BM)-derived mononuclear cell (MNC) injection in partly denervated m. biceps brachii of BP patients. Secondary, this study has focused on the myogenic potential of BM-derived MNC by assessing the morphological and functional improvement of the biceps.

Methods
Nine adult BP patients with insufficient force recovery of elbow flexion were included. Three escalating doses (0.9, 4 and 8 * $10e^8$) of MNCs were injected in the m. biceps brachii (group A, B and C). To obtain the MNC dosis, BM was aspirated under local anesthesia (60 ml in group A) or in combination with a muscle tendon transfer (Steindler flexorplasty) under general anesthesia (350 ml in group B and 650 ml in group C). A muscle biopsy was performed before and 3 months after transplantation. Furthermore, quantitative needle EMG, CT-scan and clinical function was obtained at pre-transplantation and at 3 and 6 months follow-up. The EMG and CT-scan data were blinded for analysis. The data analysis was performed using a paired t-test (muscle biopsies) and a mixed model analysis (EMG, CT and clinical function).

Results
No negative side effects were observed. Biopsies showed an increase of 80% in myofiber diameter (hematoxylin-and-eosin, $P = 0.007$), 51% in satellite cells (pax7, $P = 0.045$), 83% in capillary to myofiber ratio (von Willebrand factor, $P < 0.001$) and a decrease of 51% in fibrosis (masson trichrome, $P = 0.012$). Histological changes were most apparent in group B with an increase of 126% in myofiber diameter ($P = 0.019$), 100% in capillary to myofiber ratio ($P = 0.027$), and a decrease of 70% in fibrosis ($P = 0.023$). No changes in the amount of reinnervated myofibers were observed (CD56). EMG demonstrated an increase of 36% in amplitude ($P = 0.045$), 29% in duration ($P = 0.005$) and 29% in number of phases of the motor unit potentials ($P = 0.002$). CT-scan analysis showed a decrease of 48% in mean muscle density ($P = 0.009$). An increase in elbow flexion range

of motion and strength was observed in the patients who received the muscle tendon transfer (88 degrees in group B (P = 0.002) and 67 degrees in group C (P = 0.036)), but not in the patients without this surgery (23 degrees in group A (P = 0.16)).

Discussion

This study shows that BM-derived MNC transplantation in a partly denervated muscle of traumatic PB patients is safe and feasible. Muscle improvement was observed in muscle biopsies. Furthermore, changes in EMGs and CT-scans were also suggestive for muscle regeneration. The BM dose applied in group B could represent the optimal dose to enhance partly denervated muscles. The results of the present study require confirmation in a controlled clinical study.

14. Cell based approaches for the treatment of age-related macular degeneration. Differentiation of adult human progenitor cells

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Introduction

Age-Related Macular Degeneration (AMD) is the main cause of irreversible vision loss in people of 65 years and older in Europe and the USA and as the population ages the incidence of AMD is increasing alarmingly. For a vast majority of AMD patients there is no treatment available. It is known that deficiencies in the support tissue, the retinal pigment epithelium (RPE), under the macula (which is responsible for our central vision) are pivotal to development of the disease. The function of the macula can be preserved if it is placed on healthy rather than the diseased support tissue. We are therefore investigating whether a recently identified population of adult progenitor/stem cells located within the eye, have the ability to differentiate into the RPE cells and thus act as a potential source of autologous cells for transplantation.

Methods

Post mortem interval (PMI) eyes ranged from 30 to 48 hours, and donor age ranged from 55 to 97 years. The ciliary body and iris of human donor eyes was dissected out and submitted to a 20 min digestion with dispase followed by a 20 min digestion with 1.33 mg/ml trypsin, 0.67 mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid at 37°C to yield a suspension of cells from the pigmented epithelium layer. The cells were plated in serum free medium containing FGF2. To induce differentiation, single spheres were transferred to medium containing 10% serum and the resulting adherent cultures grown in the presence of transforming growth factor (TGF)beta superfamily members with reduced serum levels (2%) for 7 days. Cytokeratin (RPE marker) and Nestin (neural stem cell marker) expression was analysed by immunocytochemical staining.

Results

No spheres were obtained from the iris but pigmented spheres were obtained from the ciliary body of several donors, some with post mortem intervals of up to 48 hours and aged up to 92 years. The spheres survived in serum free culture for several months. Removal of FGF2 was sufficient to reduce nestin expression but the additional presence of BMP4 was required to produce cytokeratin expression in these retinal progenitor cells (RPCs) (an increased from less then 1% to 21%) in a concentration dependent manner. The effect of Activin A was less pronounced and TGFbeta had no noticeable effect.

Conclusion

Pigmented structures that appear like neurospheres can be obtained from human eyes with PMIs between 30 and 48 hours and from donor ages up to 92 years. These spheres can be expanded to form monolayer cultures of RPCs and BMP4 significantly increases the number of cells differentiating into cytokeratin positive cells.

15. Modulation of activity human plasmacytoid dendritic cells by cigarette smoke

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Myeloid and plasmacytoid dendritic cells (mDCs, pDC) are crucial immune cells detecting microorganisms and linking innate and adaptive immunity. mDC are antigen presenting cells and pDC are intermediate cells. pDCs produce large amounts of IFN-α after stimulation with bacterial DNA containing unmethylated CpG motifs and are also antigen presenting cells. The antiviral effect exerted by IFN-α is due to the induction of IFN response genes. Chronic airway inflammation is a cardinal feature of chronic obstructive pulmonary disease (COPD), a destructive cigarette smoke-induced lung disease. COPD patients are more susceptible to viral infections. Previously, we have demonstrated that exposure of mDC to cigarette smoke extract (CSE) lead to release of chemokines from these cells. Not much is known about the number and role of pDC in smokers with COPD. In this study, we addressed several key questions with respect to the mechanism of action of CSE on human pDC in an in vitro model. Human pDCs were isolated from normal healthy volunteers and subjected to fresh CSE. The release of protein and expression of mRNA of CXCL8, TNF-α, CXCL10, IL-6, IL-1, IL-12 and IL-10 and IFN-α were studied by ELISA and real time PCR methods, respectively. We observed that CSE augmented the production of CXCL8 and suppressed release of TNF-α, IL-6 and IFN-IFN-α. Moreover, CSE suppressed PI3K/Akt signalling in pDC. Thus, our data indicate that cigarette smoke by suppression of releases of IFN- IFN-α and increasing CXCL8 production may play role in diminishing anti-viral immunity and increasing recruitment of neutrophils in pathogenesis of COPD.

16. Mobilization of granulocytes by G-CSF and dexamethasone does not affect their basics functions, although it does influence their transcriptome and prolongs life-span

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Patients with prolonged periods of chemotherapy-induced neutropenia may suffer from severe fungal and bacterial infections. When such patients appear refractory to antibiotic treatment granulocyte transfusions (GTX) may be a valuable alternative. To mobilize adequate number of granulocytes, donors are treated with G-CSF and dexamethasone, and after the leukapheresis procedure the concentrates are irradiated. First we assessed the functional capacity *in vitro* of the mobilized granulocytes. Granulocytes from the leukapheresis products were tested for their phenotypic activation, NADPH oxidase activity, microbial killing capacity, rolling over endothelial cell monolayers, adhesion and chemotaxis, as well as survival. Despite some phenotypic changes mobilized granulocytes showed relatively normal functional behavior, and remarkably prolonged life-span when compared to control cells (Drewniak et al., Haematologica, July 2008).

To study more in detail the influence of mobilizing agents on granulocytes, we used oligonucleotide microarrays to identify genes that are differentially expressed in mobilized granulocytes as compared to control granulocytes. More than 1000 genes displayed a differential expression pattern, with at least a three-fold difference. Since mobilized granulocytes have a prolonged life span, we focused on genes involved in the regulation of apoptosis. One of the most prominent among these was calpastatin (CAST), the endogenous inhibitor of calpains, a family of calcium-dependent cysteine proteases recently shown to be involved in neutrophil apoptosis. Messenger-RNA expression of the CAST gene was induced by G-CSF/dexamethasone treatment, both *in vivo* and *in vitro*, while the protein expression of CAST was stabilized during culture. Therefore, granulocytes mobilized with G-CSF and dexamethasone can be obtained in sufficient numbers, with appropriate functional capacity and a prolonged life span. Clearly, the next challenge will be to firmly establish the clinical relevance of granulocyte transfusions in patients.

17. A new method for enrichment of MNC and progenitor stem cells from whole blood and bone marrow

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Introduction

Within a number of cell therapy applications a need exists for a rapid, simple method for the preparation of mononuclear cells (MNC), hematopoietic, and mesenchymal stem cells (MSC) from human whole blood (WB) and bone marrow (BM). Use of a functionally closed system providing high cell recovery is also highly desirable within the translational and clinical research environment. We have developed a gravity filtration-based system called Purecell Select' for enrichment of MNC from 20-120 ml of WB with MNC recovery greater than 70% and a total processing time of less than 15 minutes. In addition, we present progenitor cell and cellular recovery results from a similar filtration system for MNC enrichment from human BM. Proliferation and differentiation of mesenchymal stem cell (MSC) is demonstrated with colony forming unit (CFU-F) assays and cellular differentiation.

Materials and Methods

50 ml aliquots of WB (n=6) and BM (n=5) are processed with our filtration system, with the resultant cellular product collected into ~18 ml volume. All analytical measures are conducted, both pre- and post- blood sample filtration, to gain an assessment of filtration performance. Ficoll® gradients (GE Healthcare) are carried out according to manufacturer's instructions for all samples as a direct comparison for cell recovery. Cell counts, in triplicate, are carried out using a CELL-DYN® 1800 instrument (Abbott Labs). Three-part differential determined from the forward and side scatter plots from flow cytometry (BD Biosciences). CD34+ cell enumeration is carried out using the BD ProCOUNT™ kit (BD Biosciences) and ISHAGE gating. All flow cytometry analyses are done with BD CellQuest™ Pro software (BD Biosciences). Percent recoveries for each subpopulation are determined by calculating the number of cells before and after filtration as follows: ((cells) after filtration*volume / (cells) before filtration*50 ml)*100. CFU-F proliferation assays and adipocyte, chondrocyte and osteogenic differentiation kit assays are carried out using manufacturer's instructions (STEMCELL Technologies).

Results

WB and BM cells enriched by filtration exhibit greater than 95% viability indicating that the event does not harm the resultant cell population.

Hematopoetic progenitor cells (HSC) colony forming unit (CFU) assay indicates that efficiency and proliferation capacity of HSCs enriched by filtration is statistically the same as input bone marrow sample and Ficoll prepared cells. Differentiation capacity of the BM MSCs enriched by filtration is illustrated by culture of these cells with appropriate differentiation inducing factors (adipogenic, osteogenic and chondrogenic).

Conclusions

We demonstrate high recoveries of MNC directly from WB and BM processed in less than 15 minutes using the Purecell Select system. Under appropriate conditions, BM derived cells can proliferate and differentiate (into adipocytes, chrondrocytes and osteocytes) reflecting the properties of human MSCs. Purecell Select has potential to meet the need of a clinical research environment for a rapid, robust method for cell enrichment while eliminating open tube processing.

18. Regulation of CXCR4 trafficking by the small GTPase Rac1

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The chemokine Stromal Cell Derived Factor-1 (SDF-1/CXCL12) and its receptor CXCR4 are critical for homing of hematopoietic stem and progenitor cells to the bone marrow after transplantation. This chemokine/receptor pair is also involved in the metastasis of tumor cells and in the tissue infiltration of inflammatory cells. The initiation of directional migration in response to a chemokine is dependent on the level of cell surface expression of the chemokine receptor as well as the cell's ability to properly initiate signalling upon ligand binding. In the present study, we demonstrate that Rho-like GTPase Rac1 specifically regulates the expression of CXCR4 in a ligand-independent manner, thereby determining the initial chemokine responsiveness of the cell.

By making use of a cell-permeable Rac1 peptide that inhibits Rac1 signaling, we found that the surface-expression of CXCR4 was significantly reduced by 65% (SD±7.5%) resulting in a blockade of CXCL12-induced chemotaxis. Peptide-mediated inhibition of a related GTPase, CDC42, did not result in a decrease of surface CXCR4 expression, showing the specificity of Rac1 for the regulation of steady-state CXCR4 surface expression. Furthermore, the Rac1 peptide-induced downregulation of surface CXCR4 expression occurred within a minute in various cell types and was specific for CXCR4.

Experiments using various pharmacological Rac inhibitors confirmed these results and showed that Rac inhibition significantly reduced CXCR4 cell surface expression on U937 cells to 50% (SD±11%). Importantly, preliminary results indicated that these inhibitors did not affect the surface expression of the alternative CXCL12 receptor, CXCR7, or the unrelated adhesion molecule, LFA-1, and therefore appear to specifically target CXCR4.

Finally, pull-down experiments with a biotinylated CXCR4 C-terminal peptide showed an interaction with endogenous Rac1. This suggests that Rac1 physically interacts with CXCR4 and that this interaction may be necessary for regulating the surface expression levels of CXCR4.

Thus, we have shown that Rac1 functionally interacts with the chemokine receptor, CXCR4, and it regulates CXCR4 surface expression in a ligand-independent manner. Future experiments are aimed at determining whether the reduced levels of surface CXCR4 after Rac1 inhibition is caused by enhanced receptor endocytosis or by impaired exocytosis.

19. A novel interaction between the small GTPase Rac1 and the adapter protein CD2AP

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Rho-family small-GTPases, including Rac1, modulate the actin cytoskeleton and thereby influence cellular processes such as cell adhesion and migration. Active Rac1 localizes to the leading edge of migrating cells, and stimulates lamellipodia formation. For correct intracellular localization of Rho-family GTPases, the hypervariable C-terminus is critical.

We identified the adapter protein CD2AP/CMS (CD2 associated protein/p130Cas ligand with multiple SH3 domains) by mass spectrometry, as a binding partner of the Rac1 C-terminus. CD2AP is known for its role in linking cell-cell adhesion structures to the cytoskeleton. CD2AP also influences actin polymerisation by interacting with actin-capping proteins.

We found that CD2AP specifically associates to the C-terminus of the small-GTPase Rac1. This association occurred in leukocytes, as well as in adherent cells, and required the proline-rich and poly-basic region in the Rac1 C-terminus, and the N-terminal domain of CD2AP. This suggests an interaction via one of the N-terminal CD2AP SH3 domains. Rac1-Q61 and GFP-CD2AP also co-localize in membrane ruffles at the leading edge of polarized cells. Finally, we observed an increase in membrane integrity after reducing CD2AP levels in HUVEC, using CD2AP siRNA. This suggests that CD2AP in endothelial cells is involved in the dynamics of cell-cell contacts.

20. The role of MEIS1 in the megakaryocytopoiesis

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MEIS1 (myeloid ecotropic integration site 1) is a transcription factor that plays key roles in definitive hematopoiesis, nervous system development, and leukemogenesis. MEIS1 knockout mice are completely devoid of megakaryocytes (MKs) and platelets, but still produce cells of the myeloerythroid lineages, albeit in lesser numbers. Recently, we measured genome wide expression across nine mature blood cell elements including erythroblasts, and identified that MEIS1 is uniquely restricted to MKs and platelets. Although these observations highlight the significance of MEIS1, little is known about its involvement in directing the establishment of the MK lineage. We therefore aim to elucidate the role of MEIS1 in the megakaryocytopoiesis and platelet formation.

In primary hematopoietic progenitor (CD34+) cells we measured MEIS1 mRNA and protein expression and detected two splice variants, with (WT) and without exon 12 (12del). The mRNA expression of MEIS1 WT in CD34+ derived from mobilized peripheral blood (PB) was 2-fold higher than in CD34+ cells from cord blood (CB) and bone marrow (BM) CD34+ cells. In PB CD34+ cells the expression of MEIS1 WT was twice that of 12del, whereas the expression of the splice variants was equal in CD34+ cells from BM and CB. *In vitro* culturing towards megakaryocytes of CD34+ cells with thrombopoietin and IL-1b showed an up-regulation of both MEIS1 transcripts although the increase in MEIS1 WT was twice that of 12del. The up-regulation was restricted to the megakaryocytic (CD41+) population.

To identify putative MEIS1 regulated genes, MEIS1 was knocked down in the megakaryocytic cell line DAMI, using lentiviral short-hairpin RNAs (shRNA) directed against MEIS1 or scrambled shRNAs. Downregulation of MEIS1 resulted in an initial 50% reduced proliferation rate compared to control. Comparative transciptional profiling, with a threshold at 2-fold change, showed a total of 255 and 273 up- and down-regulated genes, respectively. Consistent with the role of MEIS1 in MK differentiation, down-regulated genes were significantly enriched with functional categories such as response to wounding, hemostasis, and coagulation (all with $p<0.001$, Fischer's exact test). Moreover, the promoter regions of these genes were significantly enriched with MEIS1:HOXA9 binding motifs ($p=0.008$ permutation test following computational prediction of binding

sites). Interestingly, up-regulated genes appeared depleted for these binding sites (p=0.06), suggesting that MEIS1 primarily acts as a transcriptional activator. To further identify possible MEIS1 targeted genes, we selected genes whose expression was closely correlated with that of MEIS1 across MKs and three MK cell lines (DAMI, CHRF and MEG01) with potential MEIS1 binding sites in their promoter regions. Together with the differentially expressed genes from the knockdown experiments, we have identified potential MEIS1 controlled genes. Among these genes are several regulators of platelet volume, recently identified in genome wide association studies.

In conclusion, MEIS1 shows a distinct developmental stage-specific expression pattern during megakaryocytopoiesis. By combining the above results with ChIP-Seq in megakaryocytes and silencing of MEIS1 in Danio rerio thrombocytes, we will be able to predict with some confidence the MEIS1 regulated genes.

21. βig-h3 regulates adhesion of hematopoietic stem/progenitor cells and accelerates megakaryocytic differentiation

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Adult hematopoietic stem cells (HSC) reside in the bone marrow (BM) in so-called niches. Within this specialized microenvironment, the interactions of HSC with adhesion molecules on neighboring cells and extracellular matrix components are thought to be critical for the maintenance of the HSC population.
Comparative gene-expression profiling of HSC in homeostatic and regenerative conditions allowed the identification of a set of differentially expressed adhesion molecules and extracellular matrix proteins. The objective of our research is to unravel the mechanism by which these proteins regulate the differentiation and self-renewal of HSC in homeostatic and regenerative hematopoiesis.
We analyzed the mRNA expression of these differentially expressed genes on CD34+ hematopoietic stem/progenitor cells (HSPC) isolated from BM, mobilized peripheral blood (MPB) and umbilical cord blood (UCB). The results show a significant lower expression of Alcam (CD166) and Embigin in BM CD34+ cells as compared to MPB-derived CD34+ cells. The expression of βig-h3 and Biglycan was found to be significantly higher in BM CD34+ cells as compared to MPB CD34+ cells, suggesting a role for these extracellular matrix molecules in retaining HSC in the BM.
Furthermore, the highest βig-h3 mRNA expression is measured in monocytes, dendritic cells and mesenchymal stromal cells (MSC), while its expression in megakaryocytes and HUVEC is comparable to that in HSPC. Cell surface expression of the βig-h3 protein was determined by flowcytometry. Preliminary results indicate that βig-h3 is expressed on a subpopulation of BM derived CD34+ cells (0.5%), monocytes (5%), MSCs (11%) and megakaryocytes (30%).
Adhesion experiments show a significant two-fold increased adhesion of MPB CD34+ cells to βig-h3 compared to a BSA coating (mean 40% (SEM ± 9.8%) and 23% (SEM ± 5.0%), respectively, (n=3)). Preliminary results suggest that adhesion of CD34+ cells to βig-h3 is mediated by both β1- and β2- integrins.
The functional relevance of the target proteins in HSC differentiation and self-renewal is studied by lentiviral mediated overexpression. We used the βig-h3-SIN-GFP vector or the control SIN-GFP vector to transduce CD34+ cells isolated from MPB, UCB or BM and cultured them towards a megakaryocytic lineage. Overexpression of βig-h3 in MPB and UCB CD34+ cells resulted in an increased percentage of mature megakaryocytes cells (i.e. CD41+) two weeks after transduction, compared to the control.
In conclusion, CD34+ cells show increased adhesion towards βig-h3

and overexpression of βig-h3 in these cells accelerates differentiation towards megakaryocytes. Furthermore, the effect of βig-h3 on the differentiation of CD34+ cells towards other lineages, like monocytes and neutrophils, will be analyzed as well.

22. Storage and regulated secretion of factor VIII by blood outgrowth endothelial cells

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Background

Gene therapy provides an attractive alternative for protein replacement therapy in Hemophilia A patients. However, human clinical trials have shown disappointing results so far. Recent insights have suggested to direct factor (F)VIII gene delivery to cells that also express its natural carrier protein von Willebrand factor (VWF) e.g. platelets and endothelial cells. VWF synthesized in endothelial cells is stored in storage organelles called Weibel-Palade bodies (WPBs) that release their content upon agonist-induced stimulation. Co-storage of the VWF/FVIII complex in WPBs and subsequent release of this complex upon agonist-induced stimulation has the potential of secreting large amounts of FVIII at sites of vascular injury as well as directly increasing FVIII half-life by protecting FVIII from proteolytic attack and premature clearance.

Aim

In this study, we explored the feasibility of Blood Outgrowth Endothelial Cells (BOECs) as a cellular FVIII delivery device with particular reference to long-term production levels, intracellular storage in Weibel-Palade bodies and agonist-induced regulated secretion.

Methods

Human BOECs were isolated from citrated venous blood collected from healthy donors, transduced at passage 5 with a single exposure to a lentiviral vector encoding human B-domain deleted FVIII-GFP. Transduced BOECs were characterized by FACS and immunohistochemical analysis. Intracellular distribution analysis was performed by subcellular fractionation followed by a density gradient. Secretion pathways of VWF and FVIII were determined by brefeldin A (BFA) treatment, which blocks constitutive release of newly synthesized proteins.

Results

Transduction with the lentiviral vector encoding FVIII-GFP resulted in 80% positive cells. The FVIII-GFP transduced BOECs expressed 1.6±1.0 pmol/1x10^6 cells/24 h FVIII antigen and 0.45±0.23

pmol/1x10^6 cells/24 h endogenous VWF. Immunohistochemical analysis demonstrated that FVIII-GFP was stored in WPBs together with VWF and P-selectin. Although agonist-induced (PMA) secretion resulted in an 8-fold increase of VWF, FVIII levels were only slightly increased compared to the non-stimulated control. Subcellular fractionation revealed that the ratio of FVIII:VWF in the WPBs was on average 1:15. Treatment of transduced BOECs with BFA demonstrated that basal secretion is responsible for the majority of the VWF secreted without stimulation, whereas FVIII is mainly released via the constitutive pathway.

Conclusions

Lentiviral transduction of BOECs results in long-term FVIII-GFP expression and secretion of high FVIII levels. FVIII co-localizes with VWF and P-selectin in the WPBs, however, regulated secretion of FVIII from the WPBs seems to be restricted. The limited FVIII increase after stimulation seems to result from low sorting efficiency of FVIII to the WPBs. Therefore, the potential of regulated FVIII secretion from endothelial cells remains to be established.

23. Human mesenchymal stromal cell migration

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Mesenchymal stromal cells (MSC) are a potential cell source for cellular therapies, in which recruitment and migration of MSC towards injured tissue is crucial. However, the processes in which MSC migrate towards their destiny is poorly understood and their *in vivo* homing capacity is limited. With respect to future cell therapy, we are studying the process of migration of various human mesenchymal stem cell sources.

Our data show that MSC from various tissues are able to migrate *in vitro*, but the optimal stimulus differs among sources, which is most likely due to niche-induced characteristics. The migratory MSC fraction was still able to differentiate after migration, and maintained an increased migratory capacity in secondary migration experiments compared to the MSC that did not migrate in the first run. Cell cycle is a process that has been demonstrated to be involved in homing, engraftment and mobilization of HSC. Therefore, it was evaluated whether cell cycle would also influence MSC migration. It was observed that the migratory MSC fraction contained significantly less cells in S- and G2/M-phase as compared to the non-migrating MSC. Expression of the Ki67 antigen, which enables discrimination between G0- and G1-phase, revealed a trend of more cells in G1-phase in migratory MSC, however this did not reach significance. Thus cell cycle also affects MSC migration.

The migratory MSC could not be defined as a specific subpopulation by surface marker expression. Thus, migratory MSC may not be very different from non-migrating MSC, but specific (intracellular) signaling in these MSC may induce a status that enables translation of migratory cues into migratory behavior.

To further study the molecular background of MSC migration, a micro array was performed on migrating and non-migrating fetal bone marrow MSC. MSC that were only exposed to a gradient of SDF-1α and cultured fetal bone marrow MSC were included as controls. Genes that are differentially expressed between migrating and non-migrating MSC include NR4A1, NR4A2, CYR61, SMAD7, AXIN1, ID3 and HIST1H2AK. SDF-exposure induced large differences compared to cultured MSC, and the data was enriched for genes involved in the (regulation of) cell cycle, response to wound healing and regulation of cell differentiation. These results indicate that besides promoting MSC migration, SDF may also induce other (paracrine) functions MSC may have in the injured niche.

In conclusion, our results have identified a role for cell cycle in MSC migration. Similar to HSC, S- and G2/M-phase negatively influences MSC migration. Functional confirmation of the micro array results will contribute to unraveling the processes involved in MSC migration and offers perspectives for modulation of MSC in order to ultimately improve future cellular therapies.

24. The factors produced by mesenchymal stromal cells (MSC), which activate endothelial cells (EC) towards a proangiogenic state, are VEGF and HGF

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Mesenchymal stem cells (MSC) secrete multiple paracrine factors, which might have therapeutic potential by enhancing angiogenesis and inhibiting fibrosis and apoptosis. We examined the angiogenic inducing potential of fetal human MSC. To elucidate the paracrine factors involved in activating endothelial cells towards a pro-angiogenic state, candidate factors were blocked and its effect on proliferation and *in vitro* angiogenesis studied.

The expression of angiogenic genes and concentration of angiogenic factors produced in culture by fetal lung (FL) derived-MSC were examined by a PCR array and by ELISA, respectively. *In vitro* angiogenesis of TNF-activated MVEC stimulated with MSC-conditioned medium (CM) was studied in 3D fibrin matrices. Proliferation of stimulated MVEC, as determined by 3H-thymidine incorporation was used as angiogenic read-out. To determine which of the angiogenic factors as measured in FL-MSC-CM might be involved in proliferation and *in vitro* angiogenesis, neutralizing antibodies against bFGF, CXCL-1, HGF, IL-6, IL-8, MDK, SDF-1α, or VEGF were tested for its ability to block the effect of FL-MSC-CM. FL-MSC-CM significantly induced angiogenic tube formation of TNFα-stimulated MVEC. A selection was made of genes expressed by FL-MSC encoding soluble growth factors with relevant transcript levels. Levels of some of their protein products in MSC-CM were increased, i.e. for CXCL1, HGF, IL-6, SDF-1α, TGF1, VEGF and uPA, as compared to CM from MVEC or control medium.

Proliferation experiments showed that MSC-CM induced a 14-fold increase in proliferation of MVEC. High concentrations of VEGF or HGF (10ng/ml) in control medium resulted in a proliferation rate of only 3-4 fold. Adding neutralizing antibodies against the measured proteins to MSC-CM, resulted in a reduced proliferation only after blocking HGF or VEGF, although this was maximally 50% when both factors were neutralized. The effect of MSC-CM neutralized for HGF or VEGF was more impressive on *in vitro* angiogenesis; this reduced tube formation by 30% and 90%, respectively. Blocking both VEGF and HGF in MSC-CM resulted in a complete inhibition of *in vitro* angiogenesis. In summary, we have demonstrated that MSC of foetal origin produce angiogenic factors which are able to induce *in vitro*

angiogenesis. VEGF and HGF in MSC-CM were for 50% responsible for the enhancement in MVEC proliferation and entirely for inducing tube formation in fibrin matrices.

List of participating companies – medical technical exhibition

1. Fresenius Kabi
Runde ZZ 41
7881 HM Emmer Compascuum
The Netherlands

2. Hemocue
Gestelsestraat 15h
5582 HH Waalre
The Netherlands

3. Roche Diagnotics Nederland B.V.
Transitorstraat 41
1322 CK Almere
The Netherlands

4. Caridian BCT
Ikaroslaan 41
1930 Zaventem
Belgium

5. Cell Genix
Am Flughafen 16
79108 Freiburg
Germany

6. Miltenyi Biotec
P.O. Box 85183
3508 AD Utrecht
The Netherlands

7. Sanquin Blood Supply Foundation / Sanquin Reagents
P.O. Box 9892
1006 AN Amsterdam
The Netherlands

Social programme

Drinks and ‘East Meets West’ buffet - Thursday 16 April

The drinks and congress buffet will be held in the main hall of the Royal Tropical Institute. This enables you to discuss the sessions and posters during your buffet.
The Poster Award Ceremony will be held during the buffet.

Farewell drinks - Friday 17 April

On Friday after the last session a farewell reception will be organized in the main hall of the Royal Tropical Institute. You are most welcome to join this reception.

General Information

(in alphabetical order)

Accreditation and certificate of attendance

The following societies have rewarded accreditation points.
Please, ask at the reception desk for certificates of attendance You may be asked to sign a list of attendance for the society in question.

- Dutch Society for Internal Medicine: 12 points for 2 days
- Dutch Society for Hematology: 12 points for 2 days
- Dutch Society for Vascular Medicine: 12 points for 2 days
- Dutch Society for Cardiology: 2 points
- Dutch Society for Clinical Chemistry: 12 points for 2 days
- Dutch Society for Immunology: 12 points for 2 days

Accreditation applied for:

- European Accreditation Council for Continuing Medical Education (EACCME). AMA conversion applies
- Dutch Society for Pediatrics

Badges

All participants will receive a personal badge upon registration. You are kindly requested to wear your name badge when attending any meeting or social gathering during the conference.

Banking Facilities

The official currency in The Netherlands is the Euro. It is recommended that foreign currencies will be converted to Euros at Dutch chartered banks, which are usually open from Monday through Friday from 09.00-16.00 hours. Exchange of foreign money and travellers' cheques is also possible in most hotels.

Cloakroom and luggage

At the Royal Tropical Institute a cloakroom is located near the registration area.

Electricity

In the Netherlands, electricity is supplied at 220 V - 50 Hz AC.

Insurance

In registering for the Sanquin Spring Seminars, participants agree that neither the Organizing Committee nor the Seminar Secretariat assume any liability whatsoever. Participants are requested to make their own arrangements for health and travel insurance.

Language

The official language of the Sanquin Spring Seminars is English.

Registration Desk

The registration desk will be open at the following times:

Thursday, 16 April 2009	07.30-18.00 hours
Friday, 17 April 2009	07.30-16.00 hours

Shops

Most shops in Amsterdam are open from 09.00 to 18.00 hours. On Thursdays, shops are open till 21.00 hours.

Taxis

Numerous taxi stands are located throughout Amsterdam. The telephone number of the central taxi service is 020-7777777 (country code 31).

Weather

While April may offer lovely spring weather, it can be quite unpredictable and might be chilly in the evening. Temperatures range from 8 to 14°C. As showers might occur, we advise you to bring raincoat or umbrella.

Sanquin

Spring Seminars 2011

Advances in Clinical Transfusion Science

14 & 15 April 2011
Amsterdam, The Netherlands

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Blood and Beyond