PRODUCTION OF HUMAN MYELOID PROGENITOR CELLS FROM HEMATOPOIETIC STEM CELLS FOR THE TREATMENT OF NEUTROPENIA

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CELLERANT THERAPEUTICS

Introduction

To this day, neutropenia represents a life threatening clinical condition due to the increased risk of lethal fungal and bacterial infections. Neutropenia often occurs as a result of pre-transplantation conditioning, radiotherapy or chemotherapy and despite many clinical advances including the development of growth factors and new anti-microbial drugs the problem is still prevailing. Cellerant has chosen a novel approach and established culture conditions using serum-free medium and a defined cytokine cocktail to expand purified hematopoietic stem cells (HSC) *in vitro* and direct their development to the myeloid lineage. These *ex vivo* derived myeloid progenitor cells (MPC) can be cryopreserved and are primarily aimed to generate neutrophils once infused into a patient. Most importantly, the protective efficacy of *ex vivo* derived myeloid progenitors does not require major histocompatibility (MHC) matching between donor and recipient and allows us to develop an universal off-the-shelf product for the treatment of neutropenia.

Background

Myeloid progenitors are defined as the direct precursors of mature myeloid cell lineages including granulocytes, monocytes/macrophages and dendritic cells. Compared to hematopoietic stem cells (HSC) they lack lymphoid potential, have only a limited self-renewal capacity and therefore, will only transiently give rise to any progeny[1]. We and the laboratory of Dr. Janice Brown at Stanford University have shown before that myeloid progenitors, either freshly isolated from mouse bone marrow[2,3] or *ex vivo* derived from sorted hematopoietic stem cells (see poster TUE-362 at this meeting), can be used to rescue infected mice from an otherwise lethal fungal or bacterial dose. Also, it has been shown that the function of myeloid progenitors is not MHC restricted[4] and an allogeneic product derived from pooling of multiple donors is as effective as a syngeneic product (see poster# TUE-362).

Materials & Methods

$CD34^+$ cells were isolated from G-CSF mobilized volunteer donors using the Baxter ISOLEX330i magnetic cell separation system. The CD34 enriched fraction was stained with anti-CD34 (PR3A, Cellerant) and anti-CD90 (PR13, Cellerant) antibodies and $CD34^+CD90^+$ HSC purified by fluorescence activated cell sorting using a Dako MoFlo High Performance cell sorter. Cryopreserved HSCs from multiple donors were individually seeded and grown up in Vuelife culture bags (American Fluoroseal, Gaithersburg, MD) using serum-free XVivo15 medium supplemented with recombinant human SCF, Flt3L, IL-3 and mimetic TPO. After 8 days of culture cells were harvested and analyzed or cryopreserved in CS10 using a controlled rate freezer (CBS Freezer 2100) and stored in vapor phase of liquid nitrogen. For flow cytometry cells were stained with the human specific antibodies, data collected using a FACSAria and analyzed using FlowJo software. All animal experiments were done in NOD/scid mice conditioned with 2 doses of anti-Asialo GM1 and a single dose of 275 rad of irradiation using a CP-160 Faxitron. All cells were intravenously transplanted via the retro-orbital plexus.

Results

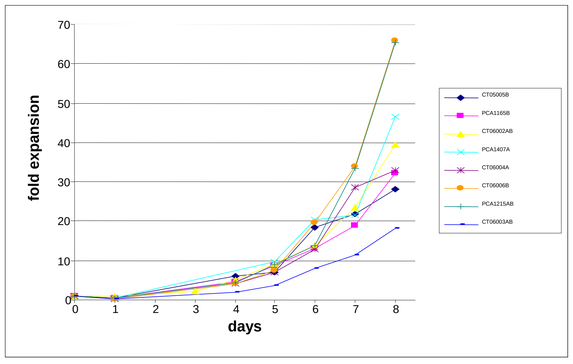

Figure 1: Average expansion. Purified HSC were seeded at a density of $2x10^5$ cells per ml in Vuelife bags of various sizes (32 ml - 197 ml). Cultures were frequently assessed for total cell counts and viability. Bags were regularly fed with fresh complete medium to keep the cell density between 5-10 x 10^5 cells/ml. Shown are the expansion profiles of 8 individual donors over time. The average expansion of total cells among these 8 donors was 41-fold (range: 18.1-66; st. dev.: 17.3).

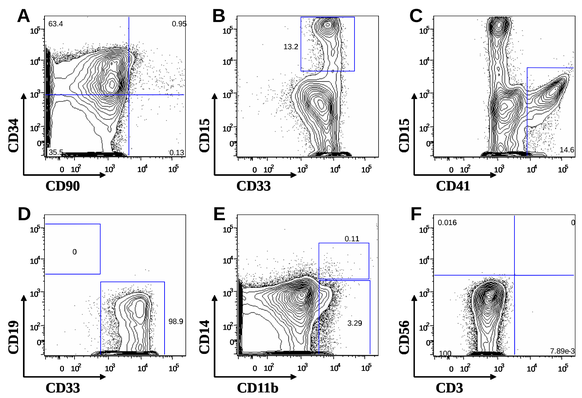

Figure 2: Composition of human MP cultures. MPC cultures were routinely analyzed by flow cytometry to determine their cellular components based on surface marker expression. Our goal was to drive most $CD34^+CD90^+$ cells out of the stem cell compartment and optimize the yield of $CD34^+CD38^+CD90^-$ cells, which correlates with the naïve phenotype of myeloid progenitors in the bone marrow[5]. As seen in panel A by day 8 most cells had lost expression of CD90/Thy and on average only 1% of MPC resembled HSC by phenotype. More than 50% of the cells still expressed CD34 and CD38 and therefore had the desired phenotype of myeloid progenitors. Notably, almost all cells expressed CD33 (see panel B), which marks early hematopoietic progenitor and myeloid cells. In all cultures distinct populations of $CD15^+$, $CD41^+$ and $CD11b^+$ cells were found. In contrast, no expression of lymphoid markers (CD3, CD19, and CD56) were detectable and only a few $CD14^+$ cells were present.

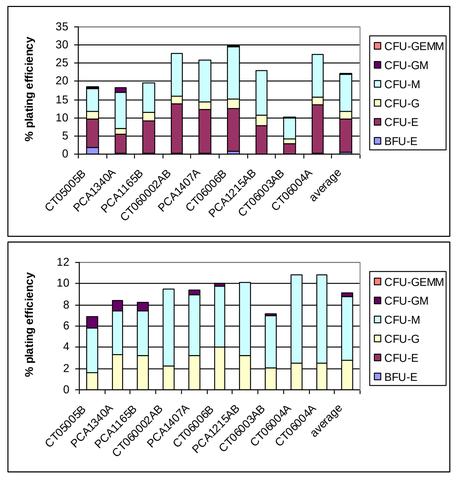

Figure 3: Colony Formation Units. Results from colony formation assays of *ex vivo* derived human MPC on day 8 of their culture. 500 cells were plated in triplicates in methylcellulose containing either recombinant human SCF, Flt3L, IL-3, IL-6, IL-11, Epo, Tpo, and GM-CSF (upper panel) or a limited cocktail of just SCF and G-CSF (lower panel). Whereas the 8 cytokine cocktail allows simultaneous growth of myeloid, erythroid and megakaryocytic colonies the latter is aimed to determine better the granulocyte colony forming potential. Colonies were counted and scored based on their morphology after 14 days of culture. The average number of CFUs using the two different cytokine cocktails was 21% and 8.5%, respectively.

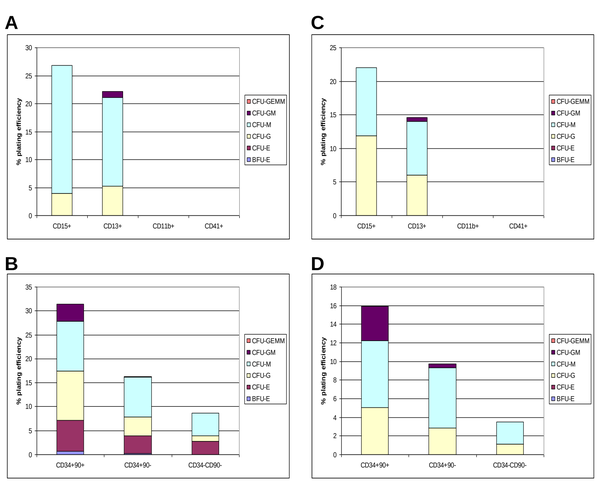

Figure 4: Colony Formation of purified sub fractions. Since phenotypes *in vitro* can differ from their naïve counterparts in bone marrow we wanted to demonstrate the importance of certain surface marker and tried to correlate the FACS surface phenotype data with a specific developmental potential. Therefore, we sorted the following populations from a day 8 MPC cultures: $CD15^+CD33^+$, $CD11b^+CD33^+$, $CD13^+CD33^+$, $CD41^+$ (A and C) and $CD34^+CD90^+Lin^-$, $CD34^+CD90^-Lin^-$, $CD34^-CD90^-Lin^-$ (B and D) and analyzed their colony formation potential in the two above described methylcellulose assays (A and B using the 8 cytokine cocktail; C and D using SCF and G-CSF only; see legend Figure 3). The data presented demonstrate that $CD15^+$ and $CD13^+$ cells contain at a high frequency myeloid committed progenitor. In contrast, $CD41^+$ cells have no colony forming potential and cytospins confirmed that they mostly consist of megakaryoblasts and promegakaryoblasts (not shown). Similarly, $CD11b^+$ did not give rise to colonies and based on their morphology are most likely immediate precursor of monocytes/macrophages. All Lin^-CD34^+ sub fractions contain at a high frequency myeloid/erythroid progenitor. Interestingly, even the $CD34^-$ fraction shows colony forming potential, although to a lower degree than the $CD34^+$ fractions. Shown is one representative experiment out of three using MPC from 3 different donors.

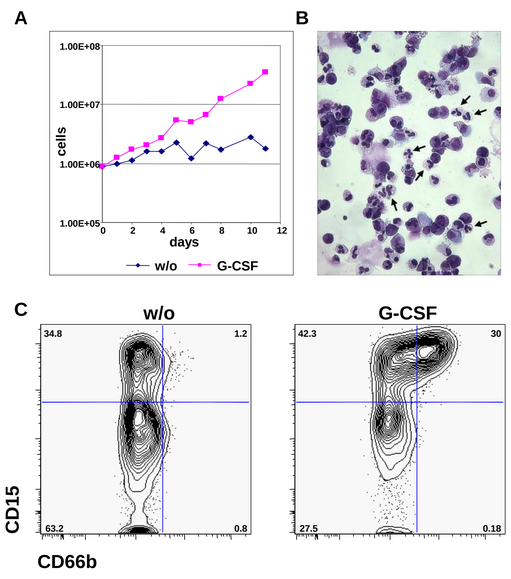

Figure 5: Granulocyte potential. Since the primary function of our allogeneic cell therapeutic CLT-008 is to provide rapid and substantial production of neutrophils we wanted to demonstrate that *ex vivo* derived MPC can generate functional neutrophils. MPC were harvested on day8 and re-plated in fresh complete medium containing either no or 300 ng/ml rhG-CSF. As the growth curve in Figure 5A demonstrates, MPC respond very well to G-CSF and continue to proliferate in response to G-CSF over the course of 10 days. As shown on a cytospin stained with May-Grünwald/Giemsa within 4 days of culture neutrophils become clearly visible by their distinct polymorphic shaped nucleus (see arrows in Figure 5B). Cells cultured in the presence of G-CSF also show up regulation of CD15 and CD66b consistent with the surface phenotype of mature neutrophils (Figure 5C). As functional tests we also demonstrated phagocytosis of fluorescent particles and extracellular release of reactive oxygen species in response to bacterial (LPS) or fungal (zymosan) stimuli (data not shown). Identical results were obtained when cryopreserved MPC were used to start the G-CSF cultures.

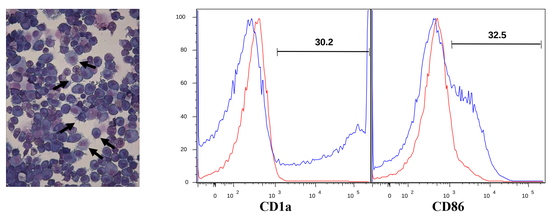

Figure 6. Dendritic cell potential. To evaluate the potential of MPC to give rise to dendritic cells (DC) we cultured day 8 MPC for additional 5 days in the presence of GM-CSF and IL-4 and added TNF-α for the last 24 hrs to the cultures. Cytospins reveal that a high proportion of cells acquired multiple dendrites and a morphology typical for DC. We also detected an upregulation of many DC-typical surface marker by flow cytometry including CD1a and CD86 (see histograms above) as well as CD40, CD80, CD209, CMRF-44 and HLA-DR (data not shown).

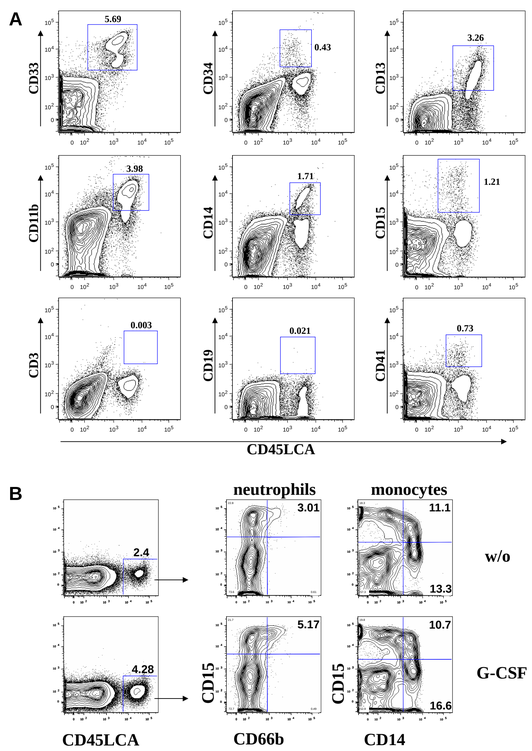

Figure 7: *In vivo* developmental potential. FACS analysis of bone marrow from NOD/SCID mice transplanted with $1x10^7$ cryopreserved human MPC pooled from 3 individual donors. Panel A shows the surface phenotype of MPC derived cells 2 weeks after transplantation into conditioned NOD-SCID mice. Human cells were identified using a human specific anti-CD45 antibody and were found to reside mostly in the bone marrow. Flow cytometry revealed that almost all cells expressed CD33 characteristic of hematopoietic progenitors and myeloid cells and some cells retained CD34 expression. Many cells expressed myeloid typical markers including CD11b, CD13, CD14 and CD15 but no T cells and only few B cells were detectable using anti-CD3 or anti-CD19 antibodies. Consistent with our *in vitro* data we found robust readout of $CD41^+$ megakaryocyte progenitors indicating the potential of MPC to generate platelets. When treated with rhG-CSF over the course of 12 days (Figure 7B) relatively more MPC derived cells expressed either CD15/CD66b or CD14 in conjunction with CD33. We found that G-CSF did not extend the persistence of human MPC but merely accelerated their maturation process. We also performed long term persistence and tumorigenicity studies in NOD/scid mice and found no adverse events caused by the infusion of human MPC.

Conclusion

Here we show that human myeloid progenitors can reproducibly be generated *ex vivo* from $CD34^+CD90^+$ purified stem cells isolated from mobilized blood. Our chosen culture conditions lead to comparable cellular compositions in a donor independent fashion that contains mostly early myeloid progenitor and are void of any lymphoid cells. We have provided data demonstrating that human MPC give rise to functional granulocytes *in vitro* and *in vivo*. Furthermore, these cells can be cryopreserved and as our mouse data demonstrate (see poster# TUE-362) can be used as an universal allogeneic pooled cell product to protect neutropenic patients from lethal bacterial and fungal infections.

References

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Disclosure: All authors are employees of and have stock options in Cellerant Therapeutics, Inc.