Editorial Commentary

Towards sustained human platelet production for therapeutic use

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Platelet concentrates are transfused for life-saving prophylactic or therapeutic purposes. These include treatment for those thrombocytopenic patients with acute or chronic blood loss, or those undergoing hematopoietic stem cell (HSC) or organ transplantation (1). In 2017, European blood banks issued over 2.9 million platelet concentrates, of which just over 2.28 million were transfused into 317,898 recipients (2). More recent estimates [although these figures are affected by and evolving during the current 2020 SARS-CoV2 pandemic (3)] indicate that over 2 million platelet concentrates (around 7,000 per day) are administered per annum to patients in the USA alone (4,5).

Although guidelines have been developed for platelet transfusions (1,5,6), and revised recently in the light of a potential scarcity of platelets during the SARS-CoV2 pandemic (3), controversies still exist as to best practice. In less challenging times, one such controversy relates to defining or developing the best platelet product for transfusion. While such platelets are generally sourced from healthy blood donors by apheresis or after isolation and pooling from whole blood (1), they possess a short shelf-life (5–7 days at room temperature), have restrictive storage conditions, rely on donor availability, are susceptible to pathogen contamination, and can lead to allo-immunisation to HLA Class I antigens (1,3,5).

The prospect that platelet donations may not meet future demands has spurred on research to generate platelets from alternative sources. These include the production of megakaryocytes and platelets from a variety of stem and progenitor cells *ex vivo* and the development of technologies to manufacture artificial platelets (7-9). Needless to say, platelets once generated *ex vivo* from stem and progenitor cells would undoubtedly face similar, or possibly even further, limitations related to shelf-life and storage conditions as those sourced directly from donors. However, advantages cited include a lack of reliance on immediate blood donor availability, reduction in the potential risk of pathogen (e.g., viral or variant Creutzfeldt-Jakob disease) transmission, and the potential to reduce their immunogenicity through, for example, genetic modification of the originating stem and progenitor cells (7,8).

A number of stem cell studies have concentrated on the use of ES (embryonic stem) or iPS (induced pluripotent stem) cells or immortalised cell lines with megakaryocytic potential for sustained platelet production, incorporating such techniques as forward programing to enhance megakaryocyte and platelet production (7,8). Safety concerns include the propensity for tumor formation through cross-contamination of the platelet products by the originating lines (7,8). An alternative approach is to manufacture and amplify megakaryocytes and their precursors *ex vivo* after sourcing hematopoietic stem and progenitor cells (HSPCs) from healthy human donors prior to infusion into patients, with platelet production then proceeding *in vivo*. Indeed, modelling studies in murine or non-human primate models indicate that megakaryocytes

derived from peripheral blood (PB) or umbilical cord blood (UCB) can generate platelets *in vivo* (7,10-12). Furthermore, phase I clinical trials suggest that human UCB or autologous PB-derived megakaryocytes infused into patients are well tolerated (13-15). Keeping this in mind, Patel *et al.* (16) have recently taken an interesting approach, with a focus on developing a large scale, cryopreservable Good Manufacturing Practice (cGMP) cell product of megakaryocytes at different maturation stages, derived from human CD34+ donor UCB cells. The ultimate aim is that, once transplanted, these cells would allow the extended release of platelets within human *in vivo* microenvironments, via processes known to be more efficient than the *ex vivo* production of platelets, while overcoming other limitations associated with the generation *ex vivo* of human platelets from immortalized or other cell lines (7,8).

In their article "Pre-clinical development of a cryopreservable megakaryocytic cell product capable of sustained platelet production in mice", Patel *et al.* (16) cultured human UCB CD34+ cells for 7 days in stem cell factor (SCF) and thrombopoietin (TPO), followed by 3 days in TPO alone. They then functionally assessed the cultured megakaryocytes before and after cryopreservation in the cGMP-grade CS10 cryoprotectant. Assessments included (I) *in vitro* production of megakaryocytes and their precursors, (II) hematological engraftment and PB platelet recovery *in vivo* in NSG mice pre-treated with human TPO (a single 0.3 μg i.p. injection), and (III) functional characterization (activation and incorporation into thrombi) of human platelets produced over an 8-week period. Accordingly, as proof of concept, Patel and colleagues isolated and expanded 5-donor cryopreserved clinical-grade UCB units *ex vivo* (16). From whole clinical grade UCB units, they recovered 2.3×10^6 CD34+ cells (median; range, $1.5–4.3\times10^6$) with a CD34+ purity of 81.2–95.3%, of which 0.3% (range, 0.2–2.8%) were CD41+. This equated to 0.94×10^4 (median; range, $0.8–5\times10^4$) CD41+ cells. Using a minimal cytokine combination of TPO with or without SCF for 10 days, they demonstrated enhanced *ex vivo* expansion of human megakaryocyte subsets. Commencing with 2.3×10^6 (median; range, $1.5–4.3\times10^6$) human UCB CD34+ cells, 30×10^6 (range, $21–34\times10^6$) CD41+CD42b+ mature megakaryocytes and 33×10^6 (range, $20–47\times10^6$) total CD41+ megakaryocytes were generated, making up over 50% of the expanded TNC. On average, 1.5×10^6 CFU-MK colonies were present in the expanded UCB units. Patel *et al.* (16) calculated that this would equate to a median dose for *in vivo* transplantation of 4.1×10^5 (range, $2.4–5.3\times10^5$) CD41+ cells/kg body weight for an 80-kg patient. Proceeding with transplantation of ~1.5×10^6 CD41+ human cells into sublethally irradiated female NSG mice, by week 1 they detected human platelets, which peaked by week 4 and then remained constant to at least week 6 post-transplant.

An important issue addressed by Patel *et al.* (16) was comparison of platelets derived from fresh versus cryopreserved expanded human megakaryocyte products. Comparisons were also made with donor platelets and unexpanded human CD34+ HSPCs. Notably, cryopreservation and thawing significantly reduced the recovery of viable TNC (to 66%±9%) from the expanded megakaryocyte product. This was accompanied by a transient delay in the week 2 post-transplant human platelet production in NSG mice for the cryopreserved and thawed megakaryocyte product as opposed to the fresh megakaryocyte product, but, from 3 to 8 weeks' post-transplant, human platelet production recovered and was similar to that observed for the freshly expanded product. This extended human platelet production in NSG mice *in vivo* was in contrast to the short survival of human donor platelets, which were detected at 1 hour, but had declined by day 5 post-infusion, and the delayed reconstitution with unexpanded CD34+ cells (16). Platelet activation and thrombus formation of the *in vivo* human platelets were similar whether the platelets originated from the cryopreserved and thawed or the freshly expanded megakaryocyte products. Post-transplant, human CD34+ and CD41+ cells were detected in low numbers in both the bone marrow and lung, the proposed sites for platelet production, as well as the spleen.

This protocol raises several interesting points. First, is it possible, given the limited cell content of human UCB units, to reduce cell loss during the cGMP manufacturing process; secondly, is there a consensus about the best factor combinations to stimulate optimal megakaryocyte generation and hence their production from donor derived HSPCs *ex vivo* and does this differ according to human CD34+ cell source; and thirdly, what are the safety, efficacy and potency criteria for cGMP-grade CD41+ cell transplantation in patients of differing ages and with differing disorders?

Standardizing cGMP protocols is extremely time consuming and not easy to achieve. However, the magnetic bead separation technology applied by Patel *et al.* (16) for isolating human UCB CD34+ HSPCs is relatively rapid and simple to use, provides good to high purity, viability

and reasonable recovery rates, and is a standardized cGMP compliant procedure, with results essentially consistent with earlier studies (17). There will undoubtedly be cell losses on thawing the clinical grade banked UCB units, as well as some diminution in CD34+ cell recovery after magnetic bead enrichment when starting with cryopreserved rather than freshly sourced UCB units (17). Although this information would be useful given the inherent variability of TNC content in UCB units, these losses cannot easily be assessed in the studies presented by Patel *et al.* (16), but will nevertheless have impacted on the numbers of megakaryocytic lineage cells that could be generated *ex vivo*. Additional cell loss during the second cryopreservation (i.e., of the expanded product), was observed and, although not statistically different from freshly expanded cells, may impact to some extent on potential megakaryocyte and platelet yield. Maximizing yield at every step of the process of *in vivo* megakaryocyte production is likely to be extremely important given the modest numbers of human platelets (10^3–10^4/μL Of mouse PB) detectable in the circulation after infusion of the *ex vivo* expanded products. Furthermore, it is not yet clear how long transfused CD41+ cells will persist and will continue to generate platelets. In part, this reflects uncertainties about the nature of the cells captured within the CD41+ population as this will include both megakaryocytes, their antecedent progenitors and possibly CD41+ 'megakaryocyte-biased' HSC, as discussed below.

With regard to the cytokines available for the megakaryocyte expansion, Patel *et al.* (16) chose a combination of SCF and TPO. Both are early-acting factors for human HSCs, with TPO also capable on its own of promoting the differentiation of CD41+ cells from CD34+ HSPCs (11,18). Recent studies in adult mice suggest that 30% of HSCs are megakaryocyte-biased and that these generate 50–60% of all megakaryocyte progeny *in vivo* (19). Whether this is the case for human HSCs or their immediate downstream progeny MPP (myeloid-primed progenitors) has not as yet been firmly established, although Psaila *et al.* (20) have also recently demonstrated that it is possible to isolate CD41$^+$HSC/MPP (CD34$^+$Lin$^-$CD38$^-$ CD45RA$^-$CD41$^+$) from human PB of healthy donors. While these CD41$^+$ HSC/MPPs and their CD41$^-$ counterparts have similar myeloid potentials *in vitro*, the former, when cultured in the presence of SCF and TPO, show accelerated megakaryocyte differentiation (20). Interestingly, a higher fraction of cultured CD41+ HSC/MPPs expressed CD42 (the mature megakaryocyte marker) and these cells were larger and appeared earlier in the cultures than those derived from CD41$^-$ HSCs and MPPs (20). This points to the existence, in the human, of a TPO-dependent megakaryocyte-biased HSC subset, which may be the subset responsible for the megakaryocyte expansion described by Patel and colleagues (16). Whether this is the same megakaryocyte-biased HSC subset that rapidly responds with platelet production to conditions of hematological stress, infectious agents and/or inflammation is unknown and deserves further investigation (21). Other *ex vivo* expansion studies have used TPO alone or in combination with other cytokines (e.g., SCF, FL, IL-6, IL-11, IL-3, GM-CSF, EPO), and/or other factors including SR-1 and epigenetic modifiers, to direct or modulate megakaryocyte differentiation from human HSPCs (7,10,11,18,22,23). Whether some of these factors target particular subsets of HSCs or modulate lineage potential of HSCs to enhance megakaryocyte differentiation and to perhaps generate megakaryocytes and platelets with differing functions is not fully understood and is also worthy of further research. A further consideration is the extent to which the different culture conditions and cytokines generate myeloid and lymphoid cells, in addition to megakaryocyte lineage cells, with attendant potential risks of alloimmunization. Thus, there is still no general consensus as to the best cytokine and factor combinations for optimal generation of megakaryocytes from human UCB, PB or bone marrow CD34+ HSPCs for different clinical indications in patients.

Other questions that remain incompletely resolved in relation to translating such processes *ex vivo* into manufacturing strategies for clinical use include differences in megakaryopoiesis during ontogeny, the appropriate source of human HSPCs for adult versus pediatric patients, the different *in vivo* microenvironmental influences that impact on platelet production post-transplantation of megakaryocytes, the dose of expanded megakaryocytes that can be safely and effectively infused into patients of different ages, and whether the addition of certain factors to the *ex vivo* cultures creates an '*in vitro* inflammatory' or altered environment that modulates the function and characteristics of the megakaryocytes and platelets generated (7,8,21,24,25). This may be a particular problem if large numbers of megakaryocytes lodge or engraft in the lung. The data from Patel *et al.* (16) are encouraging in that there was no evidence of microthrombi in the lungs of mice infused with expanded CD41+ cells and, indeed, no evidence of a hypercoagulable state which is another potential risk of transfusion of these cells.

A strength of this study by Patel and colleagues (16) is its use of donor derived megakaryocytes from human CD34+ HSPCs, which are potentially safer and less onerous to prepare than human ES, iPS, AT (adipose tissue) or immortalised cell-derived megakaryocyte products (7), also making it easier to meet cGMP and regulatory requirements. In particular, the former can be derived from pre-screened, HLA- and blood group-typed healthy donors in a shorter time, without reprograming, forward programing or immortalization with genes that may potentially promote selective cell survival or growth advantages and increase the risk of tumor formation (7,8). There is also the added advantage of over 798,500 banked, tested and typed cGMP-grade UCB donations registered with the WMDA Search and Match registry (https://statistics.wmda.info/; November 20, 2020) that are potentially accessible for clinical use. Another strength, albeit using an immunodeficient mouse model, is that it has already been demonstrated previously that co-transplanting TPO expanded human UCB CD34+ HSPCs with unmanipulated human UCB CD34+ HSPCs from a second donor results in accelerated earlier platelet recovery from the TPO-treated graft until the untreated CD34+ cell graft makes a delayed but dominant contribution to platelet engraftment over the intermediate to longer term (11). Furthermore, there were no significant adverse events reported when megakaryocytes derived from donated human CD34+ HSCs were tested by others in safety clinical trials in man (13-15). One disadvantage, depending on clinical usage, remains the limited cell numbers in UCB units. Additionally, megakaryocytes derived from human UCB CD34+ HSPCs are smaller and demonstrate lower ploidy than those derived from adult human CD34+ HSPCs (24,25). These parameters are associated with reduced and smaller, but cytoplasmically mature, platelet production *in vivo*. While this is thought to mostly represent cell-intrinsic behaviour, it is possible that such parameters can be modulated by microenvironmental influences, thereby enhancing platelet production (7,21-25).

In conclusion, Patel *et al.* (16) have taken a significant step forward in the cGMP development of megakaryocyte manufacturing from healthy human donors. There are still challenges to be met. These include determining optimal cell numbers and content of the cellular products for transplantation and ensuring safety and functional efficacy of these cellular products *in vivo* in human clinical trials. Furthermore, it seems inevitable that further advances in basic research will significantly increase our knowledge of human megakaryopoiesis during ontogeny and more clearly define the newer functions being attributed to HSC, megakaryocyte and platelet subsets. This will also contribute to selecting the best sources of such cells for transplantation of particular patient cohorts in defined age groups and it is hoped will eventually allow the cGMP manufacturing processes to mimic the huge blood cell production achieved under daily homeostatic conditions *in vivo*.

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