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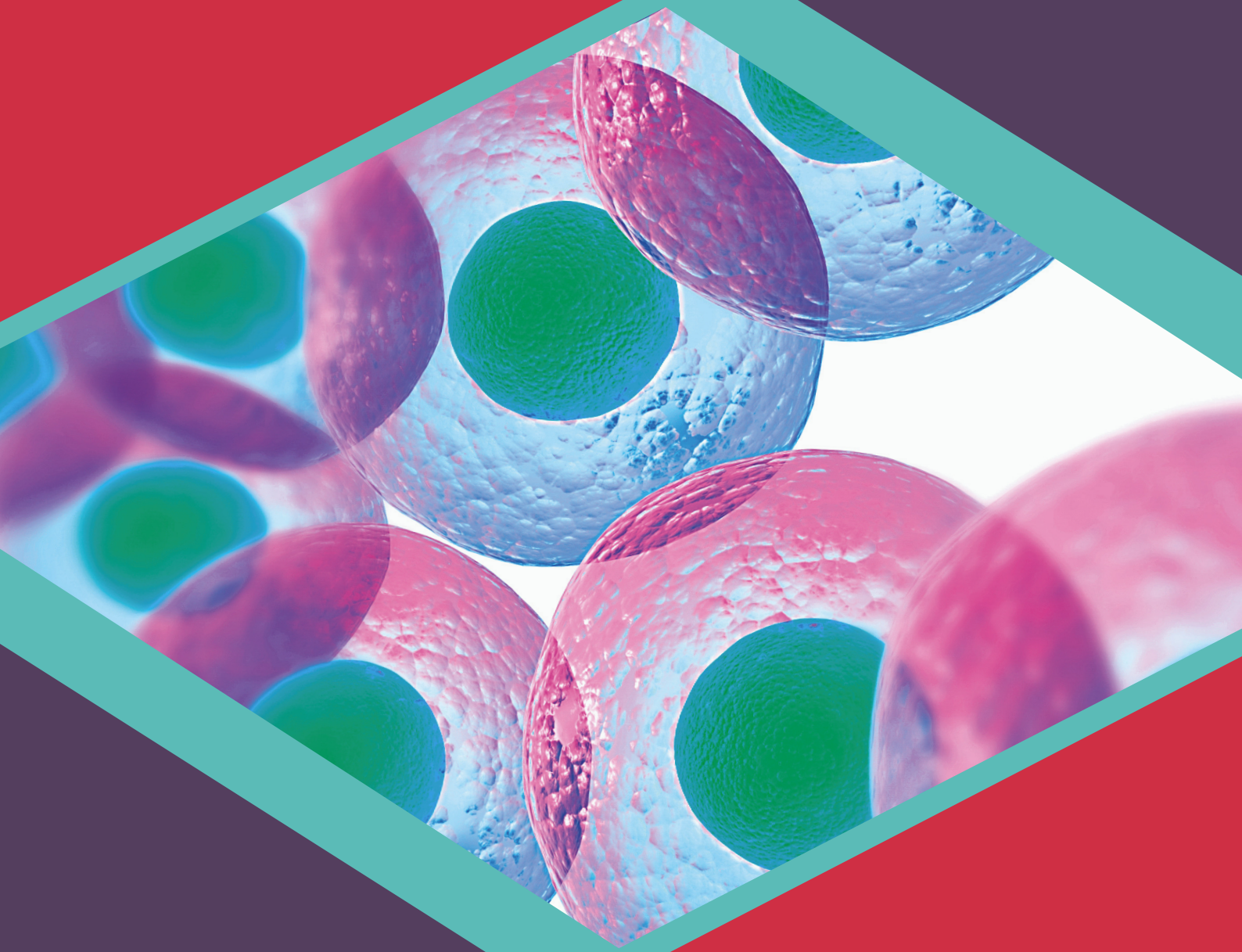

# Cell therapy: allogeneic

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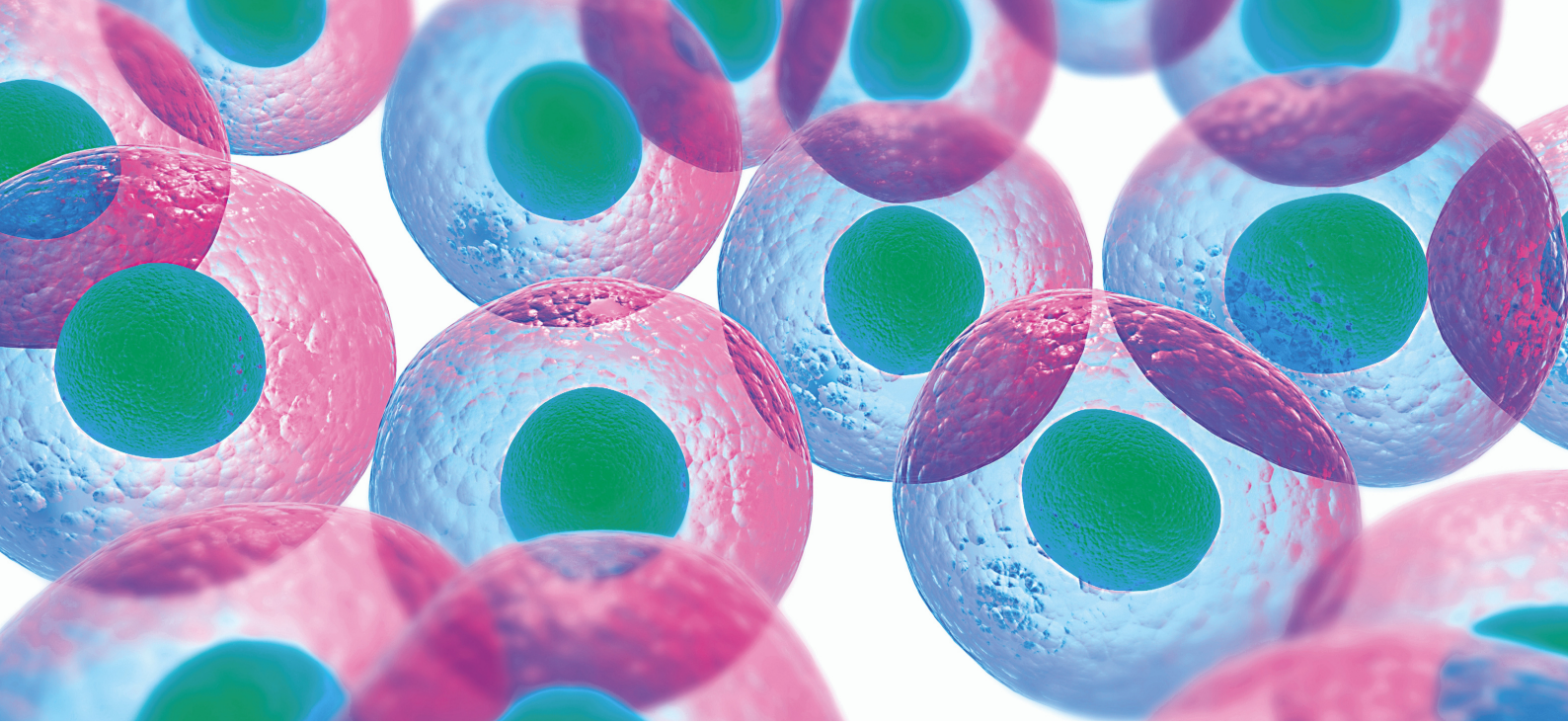

# CONTENTS



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# Developing allogeneic CAR-Tregs: an interview with Sangamo Therapeutics

*In this interview, Jason Fontenot, SVP, Head of Cell Therapy, Sangamo Therapeutics, Inc. (WA, USA), discusses the challenges in producing therapeutic CAR-Treg cells and the opportunities presented by a new partnership with UK-based Mogrify.*

## 1 Could you introduce yourself and tell us about your role?

I am the Head of Cell Therapy at Sangamo Therapeutics. I lead the cell therapy efforts at Sangamo including the CAR-Treg portfolio, the Sangamo collaboration with Kite/Gilead (CA, USA) to develop next generation T and NK cell therapies for oncology and other early-stage cell therapy programs. I am a T-cell immunologist by training and my previous roles included the Head of Exploratory Research at Juno Therapeutics (WA, USA), Chief Scientific Officer at Immusoft (WA, USA) and Group Leader at Biogen (MA, USA).

## 2 What are CAR-Treg cells and how can they be utilized therapeutically?

CAR-Tregs are regulatory T-cells (or Tregs), which are genetically re-programmed ex vivo (outside the body) to add a chimeric antigen receptor (CAR). Tregs are a type of white blood cell and are a sub-population of T-cells. They act as the key regulators of the immune system and have a targeted immunosuppressive function. They inhibit the inflammation that can be caused by other immune and non-immune cells. They have also been shown to promote tissue healing and regeneration. At Sangamo, we describe them with the metaphor of 'peacekeepers' because they can direct other T-cells to ceasefire to ensure the immune system does not mistakenly harm healthy organs, whilst still allowing the immune system to protect the body from foreign intruders (such as viruses and bacteria).

CARs are artificial receptors, which give Tregs the ability to target a specific protein, called an antigen. By engineering a Treg to express a CAR, CAR-Tregs can recognize and accumulate in specific tissues where this antigen is being expressed and an immune-mediated disorder is occurring.

Our preclinical research shows that CAR-Tregs can inhibit overactive immune cells within the body and have the potential to induce long-term immune tolerance. We are investigating CAR-Tregs in diseases where an excessive inflammatory or immune reaction is at the root of the disease. We aim to develop therapies that can induce and restore immune tolerance to address a wide range of inflammatory and autoimmune diseases.

## 3 How do you create a CAR-Treg therapy?

To create a CAR-Treg therapy, you need to engineer Tregs to add a CAR. These Tregs can either come from the patient, in which case we talk about autologous CAR-Treg therapy, or they can come from a different person (a healthy donor), in which case it is an allogeneic CAR-Treg therapy.

For our most advanced CAR-Treg program in transplantation, we are currently using a lentivirus to engineer autologous Tregs with a CAR. We are also working on additional genetic engineering approaches using our proprietary zinc finger nuclease (ZFN) technology to enhance the function of Tregs and bring Treg cell therapy to as many patients as possible.

# Developing allogeneic CAR-Tregs: an interview with Sangamo Therapeutics

## 4 What are some of the added challenges in producing a CAR-Treg therapy compared to a CAR-T therapy?

CAR-T cells are made using effector T-cells, or Teffs. The quantity of Treg cells in the blood is much lower than for Teffs, since Tregs represent only 1—2% of circulating lymphocytes. Therefore, the starting material is smaller for CAR-Tregs than for CAR-Ts, which is an added production challenge. In contrast to Teffs, Tregs are also more challenging to obtain at high purity and to expand *ex vivo*.

## 5 You've recently started a collaboration with Mogrify (Cambridge, UK). What obstacles will their cell conversion technology help you overcome? Have there been any extra challenges in creating allogeneic CAR-Treg cells?

The collaboration with Mogrify gives us access to their cell conversion technology, which enables the transformation of any cell type into another. Our collaboration is focused on developing methods for converting induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) into Tregs. This agreement expands our options as we work to develop 'off-the-shelf' allogeneic CAR-Treg cell therapies. We are also exploring other avenues to create allogeneic CAR-Tregs and this new option will complement the approaches we were already working on.

Allogeneic cell therapies can be produced from terminally differentiated cells derived from a healthy donor or, alternatively, from a renewable cell source, such as an iPSC or an ESC. Each approach has its challenges and benefits. We believe the allogeneic approach to CAR-Treg therapies has great potential and so we are exploring both of these avenues.

The collaboration with Mogrify is focused on deriving Tregs from renewable cell sources and we believe Mogrify's technology will help us identify a path to derive Tregs from iPSCs and ESCs.

## 6 How is quality ensured when working with iPSCs and ESCs as a starting material?

When using iPSCs and ESCs as a starting material it is critical to develop attributes that define the health and quality of the cells both in their base state and as they differentiate into the cell type of interest. These attributes must be defined and confirmed through detailed experimental analysis and testing.

## 7 What are the major bottlenecks to CAR-T treatments becoming widely commercially available?

Developing cell therapy manufacturing processes that are scalable and uniform is an important goal. Such processes will allow us to produce therapies that are accessible to all patients who can benefit from them.

## 8 How do you foresee CAR technology evolving?

Two important areas of innovation will be; 1) the development of 'off-the-shelf' allogeneic therapies and; 2) the development of more advanced engineering strategies to allow more precise programing and control of cell therapies such as gene control and synthetic biology.
Our collaboration with Mogrify is focused on the first. We have additional internal efforts focused on both of these areas. Sangamo's genome engineering platform is a critical tool in both of these areas.

## 9 When can we expect to see Sangamo's CAR-Treg therapy in clinical trials?

# Developing allogeneic CAR-Tregs: an interview with Sangamo Therapeutics

Our most advanced CAR-Treg candidate is TX200, which is being studied for the prevention of immune-mediated rejection following mismatched kidney transplantation. The patient's Tregs are collected before transplant, genetically engineered with a CAR designed to bind to HLA-A2 and then injected back into the same patient after transplantation.

We will evaluate TX200 in a clinical study to determine its safety, tolerability and mechanism of action in patients who have received a kidney transplant. The STEADFAST clinical study will help us understand how TX200 works in humans and may provide broader proof-of-concept for genetically modified cell therapy using Tregs. Our Clinical Trial Application (CTA) has been approved in the UK and we are working towards other regulatory approvals. The next step will be to initiate first clinical sites in Europe.

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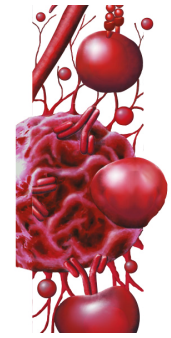

# Use of chimeric antigen receptor T cells in allogeneic hematopoietic stem cell transplantation

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**The chimeric antigen receptor T (CAR-T) cells play an antileukemia role, and can be used to treat or prevent relapse by targeting minimal residual disease for patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, the infusion of allogeneic CAR-T cells may also cause graft-versus-host disease, which limited their applications during and after allo-HSCT. In this review, we discuss the clinical trials that applying CAR-T cells before allo-HSCT and the use of donor-derived CAR-T cells as conditioning regimen during allo-HSCT. At last, we analyzed the effect of donor-derived CAR-T cells on preventive infusion after allo-HSCT.**

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**Keywords:** acute lymphoblastic leukemia • acute myeloid leukemia • allogeneic CAR-T cells • allogeneic hematopoietic stem cell transplantation • CAR-T cells • conditioning regimen • donor-derived CAR-T cells • hematological malignancies • preventive infusion

The use of chimeric antigen receptor T (CAR-T) cells is an excellent example of applying basic research concept to clinical treatment. The CAR-T cells have shown a strong function in antileukemia [1,2]. With the advancement of technology, there are four generations of CAR-T cells available now [3]. At present, the second generation is extensively used in the treatment of hematological malignancies.

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is one of the most effective methods to treat refractory/relapsed acute lymphoblastic leukemia (ALL) [4]. The high relapse rate has been the main cause of death for patients with ALL after allo-HSCT [5,6]. And how to decrease the relapse rate after allo-HSCT is an important matter in treating ALL. Applications of CAR-T cells have resulted in good outcomes in refractory/relapsed ALL [7]. However, the adverse events, especially cytokine release syndrome (CRS), still need to be addressed for the application of CAR-T cells in clinical treatments [8–10]. CAR-T cells can treat or prevent relapse by targeting minimal residual disease (MRD) for B-cells malignancies. However, the infusion of allogeneic CAR-T cells could also cause the graft-versus-host disease (GVHD), which limits the use of CAR-T cells during and after allo-HSCT. The outcome is very poor for patients with refractory/relapsed hematological malignancies undergone allo-HSCT without reaching complete remission (CR) [11]. In this review, we discuss using CAR-T cells with allo-HSCT for treating refractory/relapsed B-cells malignancies, especial for B-cell ALL. Further, we discuss the use of donor-derived CAR-T cells as a part of conditioning regimen during allo-HSCT. Finally, we discuss the role of donor-derived CAR-T cells on preventive infusion after allo-HSCT for preventing relapse and improving long-term survival of patients.

## Production of allogeneic CAR-T cells

The production of allogeneic CAR-T cells needs several steps. The first step is leukapheresis of leukocytes from the donors. Then, the T cells are separated from the leukocytes [12]. The third step is the separation of CD4/CD8 T-cell subsets by using specific markers or antibody–bead conjugates. Then, the T cells were activated by co-culturing

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with anti-CD3/anti-CD28 monoclonal antibodies coated beads; autologous antigen-presenting cells from donors; anti-CD3 antibodies in combination with feeder cells and growth factors, such as IL-2 [13,14].

## CAR-T cells as a bridge of allogeneic transplantation

Many studies showed that using CAR-T cells can achieve approximately 70–90% CR in relapsed/refractory B-cell malignancies [15–20]. However, CAR-T cells only can survive in the body for a short period of time, some of the patients relapsed quickly [21,22]. In our center, a total of 14 patients with refractory/relapsed B-ALL were treated with CD19-CAR-T cells. Among them, seven cases use autologous CAR-T cells and seven cases use cells from the donors. Eight patients achieved CR, but three of these patients relapsed within 3 months (Zhang C *et al.,* Unpublished Data). It is noteworthy that we have treated a case of B-ALL patients with CNS recurrence. After the treatment of CAR-T cells, the patient remains negative for CNS leukemia. Only one patient died on day 10. The main adverse reactions were granulocyte deficiency, anemia, thrombocytopenia and thrombocytopenia. Thus, further studies should be carried out to improve the long-term outcomes.

Some researchers showed that CAR-T cells treatment with allo-HSCT may be the best way to treat relapsed/refractory B-cell malignancies. Zhang *et al.* had showed that the patients with relapsed/refractory B-cell malignancies showed no sign of relapse if they received CAR-T cells treatment followed by allo-HSCT at the last follow-up [23]. In personal communication, Tong also showed that the survival is better for patients with refractory/relapsed B-cells ALL who received allo-HSCT after CD19-CAR-T cells treatment compared with the patients only received CAR-T cells [24].

However, it is controversial whether patients with refractory/relapsed B-cell malignancies should receive allo-HSCT after CAR-T cells. A recent study showed that there is no significant difference between patients who underwent allo-HSCT and those who did not (79 vs 80%) after CAR-T cells treatment in terms of 6-month overall survival [25]. The 6-months overall survival rates were 64% in patients who did not receive allo-HSCT after CD19-CAR-T cells treatment, and 70% in patients who underwent allo-HSCT post-CAR-T cells treatment without statistical difference.

A few studies showed good outcomes for relapsed/refractory B-cell malignancies for those who received CAR-T cells treatment followed by allo-HSCT. However, there are some concerns on these studies. First, all of these studies were not randomized clinical trials and the follow-up time is short, along with a small case number, thus make it difficult to draw the definitive conclusions. Second, the controlled trials should be performed to compare the outcomes of CAR-T cells treatment followed with or without allo-HSCT for a longer follow-up time.

## CAR-T cells as conditioning regimen

After allo-HSCT, disease relapse remains a significant cause of treatment failure in acute leukemia [26]. It is crucial to eliminate the leukemia cells as much as possible during allo-HSCT. Although myeloablative conditioning can eliminate most leukemia cells, procedure-related toxicities restrict its use, especially for older and weak patients with high leukemia burden [27,28]. Reduced-intensity conditioning allo-HSCT has less procedure-related toxicities and provides adequate immunosuppression. But the relapse incidence remains high [29–32]. Thus, it is vital to know how to further eliminate MRD and prevent the relapse in these patients.

Although the CAR-T cells have been used in relapsed/relapse B cells malignancies with good outcomes, the outcome is still poor for nonresponse patient treated by donor lymphocyte infusion (DLI) after allo-HSCT [33]. The outcome is also poor for a patient with a higher burden of disease [34]. In addition, it is very difficult to collect sufficient T cells for the construction of CAR-T cells from patients who have an extremely high leukemia burden [23,35].

One study showed that the donor-derived anti-CD123-41BB-CAR-T cells have graft-versus-leukemia (GVL) effect after infusion in an acute myeloid leukemia xenograft model with NSG mice [36]. In this study, the CAR was constructed with a third-generation self-inactivating lentiviral vector plasmid, pRRL-SIN-CMV-eGFP-WPRE, in which the cytomegalovirus promoter was replaced by the EF-1α promoter. The study showed that CAR-T cells have GVL effect [36]. In another murine haploidentical HSCT model, donor-derived CD19-CAR-T cells constructed with retroviral vector can induce GVL effect and diminish GVHD effect at day 1 [37].

A recent study systematically analyzed GVL effect and GVHD complication for 72 patients with relapsed ALL after allo-HSCT and received donor-derived CAR-T cells [38]. In this study, only 5 of 72 patients (6.9%) developed slight GVHD without other serious side effects such as CRS and CNS toxicity. The authors indicated that donor-derived CAR-T cells are highly effective for relapse prophylaxis, salvaging relapse or MRD clearance.

**Table 1. Clinical trial registrations on allogeneic chimeric antigen receptor T cell in allogeneic transplantation.**

| Center | Clinical trial number | Disease | CAR-T type | Status |
|---|---|---|---|---|
| Xinqiao Hospital, Army Medical University, Chongqing, China | ChiCTR1800015353 | ALL | CD19 | Recruiting |
| Affiliated Hospital of Xuzhou Medical University, China | ChiCTR-OIC-17012374 | B-cell hematologic malignancies | CD19 | Recruiting |
| Xinqiao Hospital, Army Medical University, Chongqing, China | ChiCTR-OOC-16008447 | ALL | CD19 | Recruiting |
| Fred Hutchinson Cancer Research Center | NCT01475058 | ALL | CD19 | Completed |
| Affiliated Hospital to Academy of Military Medical Sciences, Beijing Shi, China | NCT03114670 | Adult AML | 41BB- CD123 | Recruiting |
| Biotherapeutic Department and Hematology Department of Chinese PLA General Hospital, Beijing, China | NCT03463928 | B-cell leukemia | Bispecific CD19/22 or CD19 | Recruiting |
| National Cancer Institute, USA | NCT01087294 | B-cell cancer | CD19 | Recruiting |
| University of Pennsylvania, USA | NCT01551043 | ALL | CD19 | Completed |

The trials are registered at www.chictr.org.cn/ and ClinicalTrials.gov. Search performed on 3rd September 2018.
ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; CAR-T: Chimeric antigen receptor T cell.

**Table 2. Chimeric antigen receptor T cells used as conditioning regimens and preventive infusion after allogeneic hematopoietic stem cell transplantation.**

| Center | Target | No. | Disease | Dose of CAR-T cells | Role of CAR-T cells | GVHD | Serious CRS | Survival at last follow-up |
|---|---|---|---|---|---|---|---|---|
| Department of hematology, Xinqiao Hospital, Army Medical University, Chongqing, China | CD19 | 3 | ALL | $6.4 \times 10^6$/kg | Conditioning regimens | No | No | 100 days |
| Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas, USA | CD19 | 2 | ALL | $9.7 \times 10^7$ and $5.8 \times 10^7$, respectively | Preventive infusion | No | No | 2 weeks to 8 months |
| The University of Texas MD Anderson Cancer Center, Houston, Texas, USA | CD19 | 19 | B-cell malignancies | $10^6/m^2$ to $10^8/m^2$ | Preventive infusion | Three patients with GVHD. One died from liver failure | No | 12-month OS: 63% |

ALL: Acute lymphoblastic leukemia; allo-HSCT: Allogeneic hematopoietic stem cell transplantation; CAR-T cell: Chimeric antigen receptor T cell; CRS: Cytokine-release syndrome; GVHD: Graft-versus-host disease; OS: Overall survival.

Therefore, the donor-CAR-T cells may be safe and effective in allo-HSCT. Recently, Ai *et al.* reported an older patient with refractory ALL, who was treated with low numbers of donor-derived CD19-CAR-T cells and with low dose of recombinant human granulocyte colony-stimulating factor-mobilized peripheral blood stem cells [35]. This patient developed serious GVHD and by treating with anti-GVHD drugs, the related complication was controlled. However, this patient died at last because of severe infection on day 31. It is still not clear that whether donor-derived CAR-T cells should be used during allo-HSCT as a part of conditioning regimens because of the related complications, such as CRS and GVHD, especially for patients with high leukemia burden who can collect enough T cell for the construction of CAR-T cells. In our center, a 12-year-old girl with refractory/relapsed ALL was treated with donor-derived CD19-CAR-T cells as a reduced-intensity conditioning regimen for haploidentical transplantation [39]. This patient received a reduced-intensity conditioning regimen, then infusion with equal dose

of the donor-derived CD19-CAR-T cells for 4 days infusion with total dose of $6.4 \times 10^6$/kg. The granulocyte-mobilized peripheral blood stem cells and granulocyte colony-stimulating factor-mobilized bone marrow was infused on days 1 and 2, respectively. The tacrolimus and microphenolate motetil were used on day 1, and the antithymocyte globulin (ATG) was used on days +14 and +15. The highest level of the donor-derived CD19-CAR-T cells in the peripheral blood was reached on day 8 and gradually dropped until undetectable on day +40. The patient completely engrafted without serious complications and is disease free at the last follow-up.

Although it is easier to get sufficient T cells from healthy donor in construction of CAR-T cells, the proliferation rate of allogeneic CAR-T cells is slower than that of the autologous CAR-T cells. Therefore, it must be careful when using the immunosuppressors because of their potential effect in killing CAR-T cells and inhibiting the proliferation of CAR-T cells during transplantation. In this study, the immunosuppressors of mycophenolate mofetil and tacrolimus were changed from day 7 to day 1 after the 7th day of the first infusion of CAR-T cells, which was adopted to avoid or alleviate the killing effect and the inhibition of the proliferation of the CAR-T cells [39]. The ATG) can also kill and inhibit the proliferation of CAR-T cells. In this protocol, the ATG was used only two-times on days +14 and +15 after 2 weeks of the infusion of CAR-T cells. Then we further applied this treatment in another two patients, which also showed good outcomes (Zhang C *et al.*, Unpublished Data). Nevertheless, a trial with larger number of patients is still needed to confirm the curative effect of CAR-T cells.

## CAR-T cells in preventing relapse after transplantation

The relapse rate has been high for patients with ALL. Even with allo-HSCT, the long-term survival is still poor for patients with refractory/relapsed ALL [40–42]. So it is vital to prevent relapse for patients with ALL after allo-HSCT.

One of the main methods used to prevent or treat relapse for patients with ALL after allo-HSCT is DLI through the GVL effect [43]. Severe acute GVHD developed in approximately a third of DLI [44]. Besides, the curative effect is very limited for relapse ALL after allo-HSCT or with preventive DLI infusion after transplantation [45–47]. Preventing relapse for patients with ALL remains an unmet clinical challenge after allo-HSCT. Therefore, new treatment strategies are still needed.

Although allogeneic CAR-T cells have been successfully used on patients with refractory/relapsed ALL without serious complications, the long-term survival is still needed to be improved [48]. Pre-emptive therapy should be performed upon the detection of MRD via molecular or immunophenotypic methods [49]. Better detection of MRD can be developed with more advanced molecular analyses, such as single-cell technology and microfluidic devices [50,51]. As a new method of immunotherapy, CAR-T cells could be applied for preventive infusion after allo-HSCT. Recent study showed that between days +55 and +200 after allo-HSCT is the best time for immunotherapy [23].

The infused donor-derived virus-specific T cells expanded *in vivo* can persist long term and display antiviral activity without GVHD after allo-HSCT [52–54]. Therefore, Dotti *et al.* used the donor-derived CD19.CAR-virus-specific T cells to treat B-cell malignancies in a Phase I study in 2003 [55]. In this study, one patient remains in CR for >8 months after CD19-CAR-T cells infusion, and another patient remains in CR for 8 weeks after CAR-T cells treatment. The two patients did not develop GVHD and serious CRS. In this study, whether the patients received immunosuppressors after CAR-T cells infusion is unclear.

Cooper *et al.* reported a Phase I clinical trial for 19 patients with B-cell malignancies who received a CD19-CAR-T cells infusion after allo-HSCT [56]. The CAR-T cells were constructed using the Sleeping Beauty transposon/transposase system to express a CD19-specific CAR. The tacrolimus and minidose methotrexate were used as GVHD prophylaxis for matched-sibling HSCT, and tacrolimus, mycophenolate mofetil and post cyclophosphamide were used for haploidentical HSCT. The drugs of GVHD prophylaxis were tapered and discontinued 6 months after allo-HSCT. The CAR-T cells were administered during 6–12 weeks after allo-HSCT based on a median time occurrence of acute GVHD is 6 weeks after allo-HSCT. The white blood cells in most of these patients were normal ($1.2 \times 10^9$/l to $9.2 \times 10^9$/l) when performed CAR-T cells infusion. The numbers of CAR-T cells were $10^6/m^2$ to $10^8/m^2$. The CAR-T cells persisted *in vivo* for 51 days in average, and in one patient, the CAR-T cells still can be detected even after 1 year. No acute infusion or delayed toxicities were developed. Three patients developed GVHD. One of them with grade 1 acute skin GVHD was resolved with topical steroids, one with chronic skin GVHD responded to systemic steroids, and one patient died from liver failure with a component of liver GVHD due to a prior history of drug-induced liver toxicity. The 12-month progression-free and overall survivals rates were 53 and 63%, respectively. This study suggested that CAR-T cells infusion is safe and effective, and the immunosuppressors did not affect the proliferation of CAR-T cells and may inhibit the release of cytokines.

In our center, we performed a prospective clinical trial to explore the safety and efficacy of preventive infusion with donor-derived CD19.CAR-T after allo-HSCT at day +60 (ChiCTR1800015353). In this study, the GVHD prophylaxis was continually used according to the standard protocol of allo-HSCT. Our preliminary results showed that the CAR-T cells persisted, and they are safe and effective without serious adverse effects, such as CRS and GVHD. The reason that the infusion of allogeneic CAR-T cells post-transplant seldom induced GVHD may be the continual use of immunosuppressor, and that the donor-derived CAR-T cells are partial tolerance forms because the immune system origins from the donors after hematopoietic reconstitution with full chimerism, which is similar to the patients' autologous CAR-T cells. At present, there are several clinical trials on the use of allogeneic CAR-T cells (Table 1).

## Conclusion

Some questions still need to be solved for the use of allogeneic CAR-T cells. The sequential CAR-T cells or dual CAR-T cells should be deeply explored to escape off-target effect and further improve the curative effect and long-term survival. Universal CAR-T cells may be ideal CAR-T cells in the future. New CAR-T cells those can combat relapse, mitigate overactivation and enhance specificity should be produced.

Altogether, the application of CAR-T cells before allo-HSCT showed good outcome for refractory/relapsed B-cells malignancies, suggesting a safe and effective role of CAR-T cells as conditioning regimens (Table 2). However, further randomized and double-blinded clinical trials should be performed to systematically evaluate the role of CAR-T cells in conditioning regimens and preventive infusion after allo-HSCT.

## Future perspective

Although allogeneic or donor-derived CAR-T cells showed good outcomes at present, there are still some questions need to be solved. First, the sequential CAR-T cells or dual CAR-T cells such as CD19CD22 CAR-T cells, CD5CD19 CAR-T cells and CD33CD123 CAR-T cells should be deeply explored in order to escape off-target effect and increase long-term survival rate with improved curative effect. Second, although the use of donor-derived allogeneic CAR-T cells showed good outcomes during and after transplantation with little complications, the production period of CAR-T cells is still too long; the universal CAR-T cells that can be used immediately may be one of the best CAR-T cells in future. Third, the new CAR-T cells that can combat relapse, mitigate overactivation and enhance specificity should be developed. A recent study reported a split, universal and programmable CAR system, which simultaneously encompasses multiple critical 'upgrades', such as finely tune T-cell activation strength, sense and logically respond to multiple antigens, and the ability to switch targets without re-engineering the T cells [57]. Due to the persistence and engraftment related to the state of T-cell differentiation, limiting differentiation

### Executive summary

**Background**

- Relapse is still the main factor of death after allogeneic hematopoietic stem cell transplantation (allo-HSCT).
- At present, there are no effective methods to prevent the relapse of disease after allo-HSCT.
- The chimeric antigen receptors T (CAR-T) cells can treat or prevent relapse by targeting minimal residual disease for B cells malignancies; however, whether the CAR-T cells can be safely be used with allo-HSCT is still unclear.

**Production of allogeneic CAR-T cells**

- The production of allogeneic CAR-T cells needs several steps.
- The allegeneic CAR-T cells are usually produced from the donors.

**CAR-T cells as bridge of allogeneic transplantation**

- CAR-T cells can make a good outcome for refractory/relapsed B cells malignancies; however, relapse rate is very high.
- It may be the best way to treat relapsed/refractory acute lymphoblastic leukemia with CAR-T cells followed by allo-HSCT.

**CAR-T cells as conditioning regimen**

- CAR-T cells can be used as a part of conditioning regimens in allo-HSCT.
- The immunosuppressors did not affect the proliferation of CAR-T cells and may inhibit the release of cytokines.

**CAR-T cells in preventing relapse after transplantation**

- It is safe and effective for preventive infusion of CAR-T cells after allo-HSCT.
- The immunosuppressors did not affect the proliferation of CAR-T cells and may inhibit the release of cytokines by CAR-T cells.

may enhance the efficacy of CAR-T cell therapy. A recent study showed that producing CAR-T cells with less time can improve effector function [58].

### Financial & competing interests disclosure

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Research Article

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Regenerative Medicine

# Intra-articular injections of allogeneic human adipose-derived mesenchymal progenitor cells in patients with symptomatic bilateral knee osteoarthritis: a Phase I pilot study

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**Aim:** This study investigated the safety and clinical outcomes of expanded allogeneic human adipose-derived mesenchymal progenitor cells injected into patients with symptomatic, bilateral knee osteoarthritis. **Design:** In this single-site, randomized, double-blind, dose-ranging, Phase I study, patients were randomized to three treatment groups (low dose, 1 x $10^7$ cells; medium dose, 2 x $10^7$ cells; high dose, 5 x $10^7$ cells). All patients received two bilateral intra-articular injections: week 0 (baseline) and week 3. The primary end point was adverse events within 48 weeks. Secondary end points were measured with Western Ontario and McMaster Universities Osteoarthritis index, visual analog scale, short form-36 at weeks 12, 24 and 48. Quantitative MRI measurements of cartilage volume were compared from baseline and week 48. **Results:** A total of 22 subjects were enrolled of which 19 (86%) completed the study. Adverse events were transient, including mild to moderate pain and swelling of injection site. Improvements from baseline were measured in the secondary end points. MRI assessments showed slight improvements in the low-dose group. **Conclusion:** Safety and improvements in pain and function after intra-articular injections of allogeneic human adipose-derived mesenchymal progenitor cells into arthritic patients was demonstrated.

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**Keywords:** allogeneic • human adipose-derived mesenchymal progenitor cells • intra-articular injection • knee osteoarthritis • magnetic resonance imaging • mesenchymal stem cells

## Background

Knee osteoarthritis (OA) is a degenerative and highly debilitating joint disease; characterized by a loss of articular cartilage, subchondral sclerosis and marginal osteophyte formation [1]. The pathology of knee OA results in increased pain and impaired joint function as well as an increased risk of developing comorbidities such as cardiovascular disease, metabolic disorders and obesity [2].

Current pharmacological therapies of knee OA are limited to analgesics, NSAIDs, corticosteroids and hyaluronic acid [3–6]. Current options provide temporary pain relief, but many patients are unable to take them due to either comorbidities or adverse events. Recent literature and clinical practice guidelines have also questioned the use of these standard therapies [7,8]. Moreover, there are no approved pharmacological options that provide disease modifying capabilities.

Mesenchymal stem cells (MSCs) have been identified as a potential therapeutic option for knee OA [9,10]. MSCs, also known as mesenchymal progenitor cells (MPCs), have immunomodulatory properties by secreting of a wide array of cytokines that modulate an anti-inflammatory environment in the OA joint [11]. In addition, they may have a unique capacity to promote new cartilage-like cells *in vitro* [12], direct cell engraftment and differentiation

Future Medicine

to boost repair, regenerate cartilage [13] and stimulate type II collagen production [14]. MPCs originate from either an autologous or allogeneic source and are most commonly harvested from bone marrow and adipose tissue. Subcutaneous adipose tissue remains an easy, reproducible source for MPCs [15]. The process involves a simple aspiration procedure to derive an approximately 500–1000-fold greater numbers of fresh MPCs from adipose tissue over bone marrow [15,16].

From 2013 to 2017, two clinical trials using an autologous, adipose-derived, *in vitro* expanded MPCs therapy (Re-Join®, Cellular Biomedicine Group, Shanghai, China) was conducted in adults with knee OA [17]. The results of the first clinical trial with 18 patients suggested the safe administration of the autologous product, Re-Join. The exploratory study showed reduced pain, increased function and halted the degeneration of cartilage in knee OA patients 48 and 96 weeks after Re-Join treatment [17]. Autologous MPCs offer immunogenic advantages; however, the need for cell expansion makes the procedure slow and expensive. The allogenic product would create a cheaper and a logistically more convenient cellular therapy.

AlloJoin® (Cellular Biomedicine Group) is an allogenic, adipose-derived, *in vitro* expanded mesenchymal progenitor cell therapy. AlloJoin is delivered via intra-articular injection. Previous animal studies in rats, rabbits and sheep have shown an intra-articular injection of AlloJoin to be safe and have possible regenerative properties [18–20]. After 14 weeks, MPCs within AlloJoin were detected in the joint by MRI and histology revealed MPCs remained in the synovium [19].

In summary, preclinical studies with AlloJoin have demonstrated the safety and provide potential disease modifying properties. The primary purpose of this pilot study was to evaluate the safety and dosage for intra-articular injections of AlloJoin in patients with mild to moderate knee OA.

## Materials & methods

### Study design

This was a Phase I, single-site (Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, China), randomized, double-blind (blinded patient/blinded evaluator), dose-ranging, 48 weeks follow-up study to assess the safety of two intra-articular bilateral injections of AlloJoin. The primary objective was to characterize the safety of AlloJoin. Secondary objectives included the use of clinical outcomes as an exploratory measurement for pain and function as well as MRI assessment of the cartilage volume to measure the potential effects on disease modification.

The trial was conducted between November 2015 and November 2017, in accordance with the good clinical practice guidelines and the Declaration of Helsinki. The Shanghai Jiao Tong University and Ren Ji Hospital Institutional Review Board approved the study protocol and written informed consents were obtained from all participants before screening. The study was registered at http://ClinicalTrials.gov (NCT02641860).

### Inclusion/exclusion criteria

The study included patients aged 18–70 years old with a Kellgren-Lawrence grade II–III [21] primary diagnosis of bilateral knee OA according to the American College of Rheumatology [22]. Subjects were required to stop using analgesics for at least 3 days before screening and have a baseline visual analog scale (VAS)-pain level between 3 and 8 points.

Female subjects were excluded if they were pregnant, breastfeeding or planning to conceive within the next 6 months. Subjects were excluded if they had a history of allergies, allergic constitutions, concomitant or severe infection (i.e., HIV, hepatitis virus, etc), malignant tumor, coagulation disorder, uncontrolled or unmanageable systemic or infectious diseases or any other types of arthritis except OA. Subjects were also excluded if they presented any other types of arthritis except OA. Further exclusions included the treatment with an intra-articular injection of hyaluronic acid or corticosteroid in the preceding 6 months, participation in other clinical trials in the preceding 3 months, local knee infection in the preceding 3 months, arthroscopic surgeries in the preceding 6 months and a history of alcoholism, drug abuse or mental illness in the preceding 3 years before enrollment into the trial.

### Tissue & human adipose-derived MPC processing

Adipose tissue was obtained from the subcutaneous abdomen of three healthy donors by aspiration with local anesthetic. Isolation and culture of human adipose-derived MPCs was performed under Good Manufacturing Practices (GMP) conditions. As described in the previous Phase I/IIa study [17], the same standard operating procedures were used to purify and culture-expand cells. MSCs were defined in accordance with the International

Society for Cellular Therapy criteria for 'mesenchymal stromal cells' [23]. The human adipose-derived mesenchymal progenitor cells were tested for viability, population doublings, morphology, potency, identity, purity and sterility.

### Randomization & intervention

Randomization (block size 6) was performed by a biostatistician using PROC PLAN in SAS. All study-related case report forms were recorded by blinded randomization number. AlloJoin was shipped in a vaccine box to the clinical trial site (temperature 4–8°C). A total of 22 patients were randomized into three groups, low-dose ($1 \times 10^7$ cells, n = 7), mid-dose ($2 \times 10^7$ cells, n = 8) and high-dose ($5 \times 10^7$ cells, n = 7) groups and were given a sitting anterior knee (knee flexed at 90°) 'Medial Portal' injection of 3 ml of AlloJoin into each knee joint at weeks 0 (baseline) and week 3. Understanding that MSCs can remain viable intraarticularly for up to 10 weeks [20], this study evaluated the effects and safety of an additional intra-articular injection. With administration of each injection, synovial fluid was aspirated to ensure a correct intra-articular injection. Patients were advised to rest for 24 h following each injection. Pain medications were recorded together with adverse events (AEs) and serious AEs (SAEs), graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE). Clinical assessments were performed at screening (prior to the first injection) and at weeks 1, 3, 4, 8, 12, 24, 36 and 48. This study attempted to closely monitor the initial patient response for the first 2 months following by 3, 6, 8 and 11 months. The number and percentages of patients who experienced AEs and SAEs were recorded.

### Laboratory assessments for safety

Samples were obtained for urinalysis, hematology, chemistry and immunology. The measurement at weeks 1, 3, 4, 8 and 12 were compared with baseline values. The following tests were performed and measured: complete blood count (CBC), urine routine, liver function, renal function, blood lipids, blood sugar, immunology, electrocardiogram, 12-lead electrocadiogram (ECG) and vitals.

### Exploratory efficacy assessments

Exploratory efficacy assessments included the administration of a patient reported VAS pain in the left and right knee, Western Ontario and McMaster Universities Osteoarthritis index (WOMAC) and short form-36 (SF-36). Patients completed exploratory assessments at baseline, 1, 3, 4, 8, 12, 24, 36 and 48 weeks. The score at each time point of follow-up was compared with baseline scores.

### Magnetic resonance assessments

Knee MRIs were obtained with a 16-channel knee coil on a 3.0 T MRI machine using a standard diagnostic protocol (resolution 1.2 mm, RadCore/BioTelemetry). Cartilage volume measurements were made using a 3D spoiled gradient recalled echo (SPGR) with a fat suppression sequence, oblique sagittal orientation, 110 slices, repetition time 13.2 ms, echo time 4.5 ms, flip angle 15° and $0.56 \times 0.46 \times 0.60$ mm$^3$ voxel resolution. MRI evaluations were completed at screening and week 48. All imaging was standardized with study-specific imaging acquisition guidelines.

Knee cartilage volume (including the femur, tibia and patella) was graded by two blinded, independent radiologists according to the methods of Bae *et al.*, a semi-automated segmentation method using a graph-cuts algorithm [24]. The first step included the manual placement of initial seeds to segment and indicate cartilage versus noncartilage. Then, an automated computational segmentation of the cartilage occurred using a graphs cut-algorithm[25]. When the segmentation was not satisfactory, the radiologist repeated these steps to accurately quantify the cartilage volume as a whole and into three distinct compartments: femur, tibia and patella.

In addition, the two experienced radiologists performed a semiquantitative scoring of the fat-suppressed sequences using a modified Whole Organ Magnetic Resonance Imaging Score (WORMS) [25] (Supplement 1).

### Statistical methods

The primary objective of this study was to determine the safety of AlloJoin using physical examinations, laboratory, MRI assessments and the reporting of adverse events. Safety data was collected from all subjects who received AlloJoin. End points were collected as a descriptive feature; providing a summary of reactions and the general safety profile of AlloJoin. Laboratory measures were compared with their corresponding normal ranges and their relative shifts from baseline. Clinically significant abnormalities [17] were listed for each participant and their respective

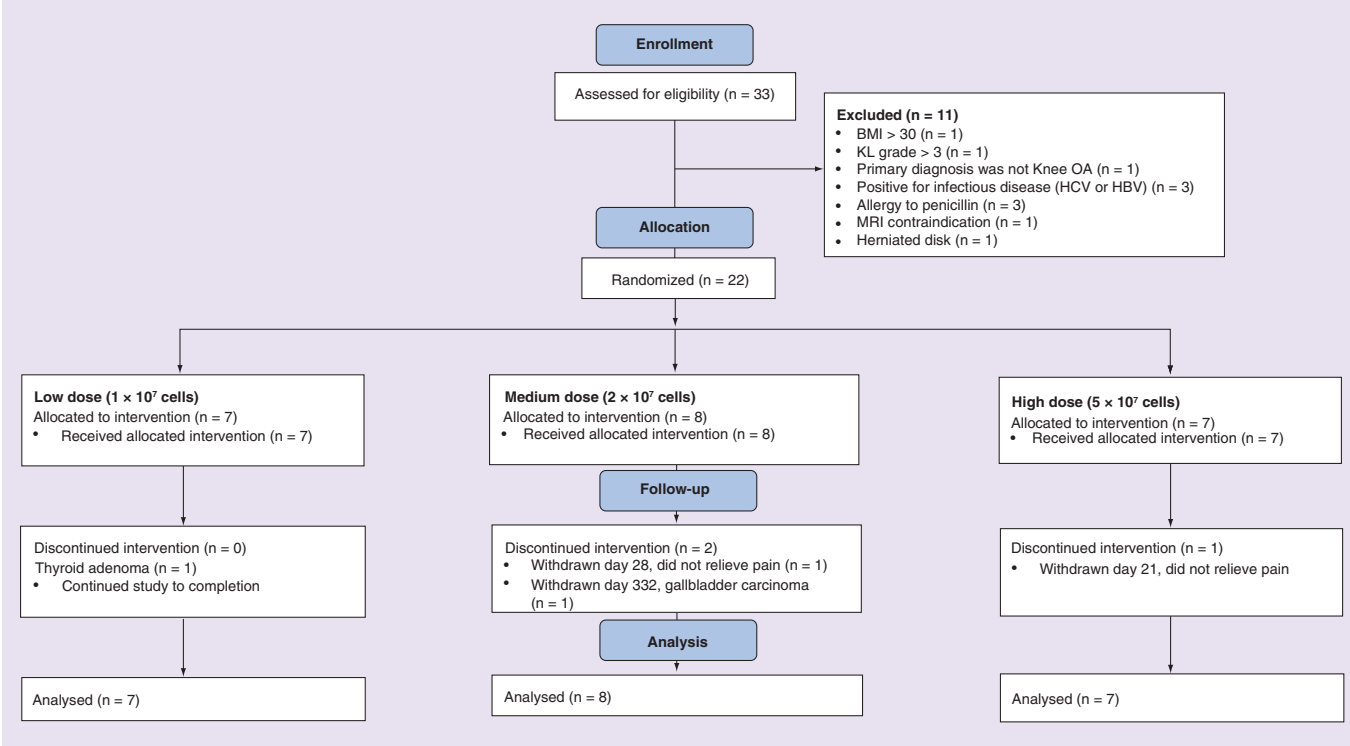


**Figure 1. Consort diagram of Phase I safety of AlloJoin®.** Subjects were randomized to a 1:1:1 ratio. A total of three patients withdrew from the study, two from the medium dose and one from the high dose.
HBV: Hepatitis B virus; HCV: Hepatitis C virus; KL grade: Kellgren-Lawrence grade; OA: Osteoarthritis.

time point for a safety review. Vital signs and ECGs were analyzed for their respective changes from baseline values for each dose level.

The sample size for this pilot study was based on the precedent set by other Phase I safety and pharmacokinetic studies [26,27]. No formal *a priori* power calculation was conducted for secondary exploratory end points. The preliminary efficacy data presented was performed on an intent-to-treat population, defined as all patients randomized to treatment. All data presented were from an intent-to-treat/last observation carried forward basis. Clinical improvement was evaluated by established minimal clinical improvement differences (MCID) and the instruments used to measure patient results (WOMAC, SF-36, VAS).

## Results

### Patient profiles

The flow chart of the clinical trial is shown in Figure 1. Of 33 patients screened at study entry, a total of 22 patients met the criteria and were randomized: seven to low-, eight to middle- and seven to high-dose group. A total of three patients withdrew from the study, two from the medium dose and one from the high dose.

The demographic characteristics of the study population were broadly consistent between the low-, medium- and high-dose cohorts (Table 1). A majority of the patients enrolled were females (19/22) with an average age and BMI of 57.93 years and 26.32 kg/m$^2$ and had experienced more than 5 years of symptomatic knee OA. Patients in each group showed similar baseline characteristics for age, height, weight, vital signs, BMI, radiographic grade of osteoarthritis and cartilage volume of both knees by MRI.

### Characterization of the adipose-derived mesenchymal stem cells

Based on the product specification, the characterization of the adipose-derived mesenchymal stem cells from three donors is given in Table 2.

**Table 1. Demographic and baseline characteristics.**

| Demographic | Low dose (n = 7) ($1 \times 10^7$ cells) | | Medium dose (n = 8) ($2 \times 10^7$ cells) | | High dose (n = 7) ($5 \times 10^7$ cells) | |
|---|---|---|---|---|---|---|
| Age, years (SD) | 59.29 (4.14) | | 57.30 (7.84) | | 57.20 (5.61) | |
| Gender (%) | | | | | | |
| – Female | 7 (100%) | | 6 (75%) | | 6 (85.71%) | |
| – Male | 0 (0%) | | 2 (25%) | | 1 (14.29%) | |
| BMI, kg/m$^2$ (SD) | 27.77 (1.93) | | 26.69 (2.63) | | 24.51 (2.49) | |
| Course of disease, months (SD) | 62.57 (38.11) | | 80.13 (25.19) | | 41.71 (36.70) | |
| KL grade | | | | | | |
| | Right | Left | Right | Left | Right | Left |
| – Level 2 | 4 | 3 | 3 | 4 | 4 | 4 |
| – Level 3 | 3 | 4 | 4 | 3 | 4 | 4 |
| – Level 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| VAS (left knee) | 6.57 (2.07) | | 5.63 (1.92) | | 5.57 (1.90) | |
| VAS (right knee) | 5.71 (1.60) | | 6.00 (1.07) | | 5.93 (1.24) | |
| WOMAC | 48.00 (20.20) | | 42.13 (14.08) | | 40.14 (12.02) | |
| SF-36 | 91.86 (12.80) | | 86.25 (10.91) | | 89.00 (13.17) | |
| WORMS (left knee) | 14.21 (4.17) | | 12.44 (3.40) | | 11.18 (3.20) | |
| WORMS (right knee) | 13.32 (3.67) | | 12.88 (3.63) | | 12.25 (4.05) | |
| Total cartilage volume (mm$^3$) | 10.34 (0.15) | | 10.43 (0.19) | | 10.26 (0.21) | |
| Femur end cartilage volume (mm$^3$) | 9.91 (0.19) | | 9.98 (0.20) | | 9.84 (0.22) | |
| Tibial end cartilage volume (mm$^3$) | 8.83 (0.21) | | 9.01 (0.18) | | 8.77 (0.26) | |
| Knee cartilage volume (mm$^3$) | 8.18 (0.46) | | 8.27 (0.40) | | 8.01 (0.37) | |

SF-36: Short form-36; KL grade: Kellgren-Lawrence grade; SD: Standard deviation; VAS: Visual analog scale; WOMAC: Western Ontario and McMaster Universities Osteoarthritis index; WORMS: Whole Organ Magnetic Resonance Imaging Score.

**Table 2. Characterization of the adipose-derived mesenchymal stem cells from three donors.**

| Description | Characterization | | |
|---|---|---|---|
| **Donor** | | | |
| n | 1# | 2# | 3# |
| Sex | Male | Female | Female |
| Age (years) | 32 | 23 | 26 |
| Volume of adipose (ml) | 150 | 100 | 100 |
| **Cell** | | | |
| Morphology | Cells are adherence to plastic and in spindle shape with large oval nuclei | | |
| Cell marker | Positive marker (CD90, CD73, CD105) >95% +; negative marker (HLA-DR, CD14, CD45) <2%- | | |
| Potency of chondrogenic differentiation | Positive | | |
| Viability | >80% | | |
| Endotoxin | <4 EU/ml | | |
| Sterility | Negative | | |
| Mycoplasma | Negative | | |

# refers to different donors.
EU: Endotoxin Unit.

## Safety

No changes were observed in electrocardiogram, vital signs and physical examinations. The incidence of adverse events were 71.42% (5/7), 87.50% (7/8), 100% (7/7) in low-, middle- and high-dose group, respectively (Table 3). The most adverse events were transient including mild to moderate pain and swelling of injection-site which resolved within 3 days of the treatment. A number of symptoms associated with the treatment were relieved with acetaminophen. There were three incidents of treatment discontinuation; two were due to knee pain and was one

Table 3. Summary of adverse events.

| Adverse event | Low dose (n = 7) (1 × 107 cells) | Medium dose (n = 8) (2 × 107 cells) | High dose (n = 7) (5 × 107 cells) | All subjects (n = 22) |
|---|---|---|---|---|
| Patients with no AE | 2 (28.57%) | 1 (12.50%) | 0 (0%) | 3 (13.64%) |
| Patients with at least one AE | 5 (71.43%) | 7 (87.50%) | 7 (100%) | 19 (86.36%) |
| Patients with SAE | 1 (14.29%)† | 1 (12.50%)† | 0 (0%) | 2 (9.09%) |
| **Right knee** | | | | |
| Knee pain | 4 (57.14%) | 8 (100%) | 6 (85.71%) | 18 (81.82%) |
| Knee kobility | 0 (0%) | 2 (25.00%) | 3 (42.86%) | 5 (22.73%) |
| Knee effusion | 1 (14.29%) | 1 (12.50%) | 1 (14.29%) | 3 (13.64%) |
| Knee edema | 0 (0%) | 3 (37.50%) | 3 (42.86%) | 6 (27.27%) |
| **Left knee** | | | | |
| Knee pain | 3 (42.86%) | 5 (62.50%) | 6 (85.71%) | 14 (63.63%) |
| Knee mobility | 0 (0%) | (0%) | 0 (0%) | 0 (0%) |
| Knee effusion | 1 (14.29%) | (0%) | 0 (0%) | 1 (4.55%) |
| Knee edema | 3 (42.89%) | 2 (25.00%) | 4 (57.14%) | 9 (40.90%) |
| **Other adverse reactions** | | | | |
| Dizziness and nausea | 0 (0%) | 2 (25.00%) | 0 (0%) | 2 (9.09%) |
| Right chest pain† | 1 (14.29%) | 0 (0%) | 0 (0%) | 1 (4.55%) |
| Hyperlipidemia† | 1 (14.29%) | 0 (0%) | 0 (0%) | 1 (4.55%) |
| Right thyroid adenoma† | 1 (14.29%) | 0 (0%) | 0 (0%) | 1 (4.55%) |
| Chronic venous insufficiency† | 1 (14.29%) | 0 (0%) | 0 (0%) | 1 (4.55%) |
| Gastrointestinal dysfunction† | 0 (0%) | 0 (0%) | 1 (14.29%) | 1 (4.55%) |
| Neurological disorder† | 0 (0%) | 0 (0%) | 1 (14.29%) | 1 (4.55%) |
| Cervical spondylosis† | 0 (0%) | 0 (0%) | 1 (14.29%) | 1 (4.55%) |
| Acute respiratory tract infection† | 0 (0%) | 1 (14.29%) | 0 (0%) | 1 (4.55%) |
| Cold† | 0 (0%) | 1 (14.29%) | 0 (0%) | 1 (4.55%) |
| Abdominal cavity metastasis of gallbladder carcinoma† | 0 (0%) | 1 (14.29%) | 0 (0%) | 1 (4.55%) |
| Toothache† | 0 (0%) | 0 (0%) | 1 (14.29%) | 1 (4.55%) |

†Investigator-assessed relationship deemed to be 'unrelated to treatment'. AEs related to treatment emerged 1–3 days post cell injection and recovered in 3 weeks; AEs unrelated to treatment occurred 4–48 weeks post cell injection.
AE: Adverse event; SAE: Serious adverse event.

due to a SAE that was determined unrelated to the study treatment by the investigator. No deaths were reported during the study.

Two SAEs (2/22) occurred in the clinical trial. At a routine outpatient visit, a 61-year old female (medium dose, $2 \times 10^7$ cells) was diagnosed with an abdominal metastasis of gallbladder carcinoma at week 45. The diagnosis did not require hospitalization and the patient was withdrawn from the study. In another routine outpatient visit a 58-year old female (low dose, $1 \times 10^7$ cells) was diagnosed with thyroid cancer at week 23. The patient's thyroid cancer was cured by surgery and she completed all protocol visits without the need to unmask her treatment group. Both SAEs were assessed by the investigator and determined to be unrelated to the therapy.

All and patient laboratory measurements were within acceptable limits and no abnormal lab tests were noted when comparing recorded timepoints (baseline, weeks 1, 3, 4, 8 and 12).

## Clinical outcomes

Analysis from the exploratory dataset using VAS-pain in the left and right knee; SF-36; WOMAC Total; and WOMAC subscale scores were shown in Table 4 and Figure 2. At week 12 and 24, improvements can be observed in all cohorts. In addition, at week 48 within-cohort improvements in the low, medium and high groups were maintained with outcome measurements showing improvements from the baseline. Many statistically significant improvements were met as seen in Table 4.

In the low-dose cohort, improvements were seen with respect to -22.71 in SF-36, -23.71 in WOMAC-total, -17.14 in WOMAC-function, -2.29 in WOMAC-stiffness and -4.29 in WOMAC-pain. In the medium-dose cohort, improvements were seen with respect to -2.25 in left knee VAS, -2.13 in right knee VAS, -16.50 in

Table 4. Clinical and MRI outcome measurement differences.

| Timepoint | Outcome measure | Low dose (n = 7) ($1 \times 10^7$ cells) | Medium dose (n = 8) ($2 \times 10^7$ cells) | High dose (n = 7) ($5 \times 10^7$ cells) |
|---|---|---|---|---|
| 12 weeks | Left knee VAS (0–10) | -1.43 (2.83) | -1.38 (2.07) | -1.43† (1.27) |
| | Right knee VAS (0–10) | -1.36 (2.50) | -1.81† (1.41) | -2.36† (1.25) |
| | SF-36 (1–100) | -15.57‡ (7.46) | -8.00 (17.92) | -12.00 (17.20) |
| | WOMAC (0–96) | -18.57 (21.35) | -9.88 (15.80) | -10.29 (14.08) |
| | WOMAC-function (0–68) | -14.00† (13.94) | -6.75 (10.48) | -6.57 (9.00) |
| | WOMAC-stiffness (0–8) | -2.14† (2.19) | -1.57† (1.40) | -2.00† (1.79) |
| | WOMAC-pain (0–20) | -2.43 (6.05) | -1.88 (4.22) | -2.29 (3.40) |
| 24 weeks | Left knee VAS (0–10) | -1.86 (2.75) | -1.75‡ (1.28) | -1.14 (1.35) |
| | Right knee VAS (0–10) | -1.71 (2.41) | -1.88† (1.36) | -1.79† (1.73) |
| | SF-36 (1–100) | -22.86† (17.02) | -12.25 (17.74) | -9.57 (21.02) |
| | WOMAC (0–96) | -24.00† (21.83) | -14.88† (13.98) | -9.29 (14.50) |
| | WOMAC-function (0–68) | -18.14† (14.60) | -10.88† (9.93) | -5.43 (9.27) |
| | WOMAC-stiffness (0–8) | -2.29† (2.06) | -1.57† (1.27) | -1.67 (1.86) |
| | WOMAC-pain (0–20) | -3.57 (5.62) | -2.75† (3.20) | -2.71 (3.68) |
| 48 weeks | Left knee VAS (0–10) | -2.19 (2.65) | -2.25† (1.49) | -1.36‡ (1.70) |
| | Right knee VAS (0–10) | -1.89 (2.04) | -2.13‡ (1.36) | -2.07† (1.30) |
| | SF-36 (1–100) | -22.71‡ (13.14) | -12.63 (17.11) | -10.57 (17.14) |
| | WOMAC (0–96) | -23.71† (21.27) | -16.50† (15.20) | -10.71 (14.40) |
| | WOMAC-function (0–68) | -17.14† (15.16) | -11.88† (10.91) | -6.71 (9.36) |
| | WOMAC-stiffness (0–8) | -2.29† (2.14) | -1.71† (1.60) | -1.67 (1.86) |
| | WOMAC-pain (0–20) | -4.29† (4.23) | -3.25† (3.49) | -2.86 (3.48) |
| | WORMS-left | -0.36 (0.69) | 0.00 (0.63) | -0.42 (0.92) |
| | WORMS-right | -0.86 (0.69) | 0.08 (0.97) | 0.08 (0.74) |
| | Total cartilage volume ($mm^3$) | 54.58 (778.74) | -224.42 (1692.41) | -210.25 (919.10) |
| | Knee femur end cartilage volume ($mm^3$) | 38.63 (557.25) | -502.17 (773.43) | -214.14 (754.14) |
| | Knee tibial end cartilage volume ($mm^3$) | -23.74 (165.44) | 243.32 (655.15) | 122.92 (216.28) |
| | Knee cartilage volume ($mm^3$) | 39.69 (170.14) | 34.44 (372.35) | -119.03 (287.79) |

The differences are calculated from baseline to follow-up time point.
†$p < 0.05$
‡$p < 0.005$
SF-36: Short form-36; VAS: Visual analog scale; WOMAC: Western Ontario and McMaster Universities Osteoarthritis index; WORMS: Whole Organ MRI Score.

WOMAC-total, -11.88 in WOMAC-function, -1.71 in WOMAC-stiffness and -3.25 in WOMAC-pain. In the high-dose cohort, statistically significant improvements were seen in -1.36 in left knee VAS and -2.07 in right knee VAS.

## MRI outcomes

Observations from exploratory MRI imaging using a WORMS protocol and a semi-automated segmentation method to calculate cartilage are shown in Table 4. At week 48, the low-dose group exhibited a mean change from baseline of -0.36 and -0.86 in both the left and right knee using the WORMS method, respectively. Additionally, the mean changes in the low-dose group for the total cartilage volume, knee femur end cartilage volume and knee patellar cartilage volume were 54.58, 38.63 and 39.69 $mm^3$, respectively. In the medium-dose group, improvements were seen in the knee tibial end cartilage volume and knee cartilage volume 243.32 and 34.44 $mm^3$, respectively. In the high-dose group, increases were seen in the left knee WORMS and knee femur end cartilage volume -0.42 and 122.92 $mm^3$.

No radiographic abnormalities consistent with ectopic bone formation were identified at follow-up.

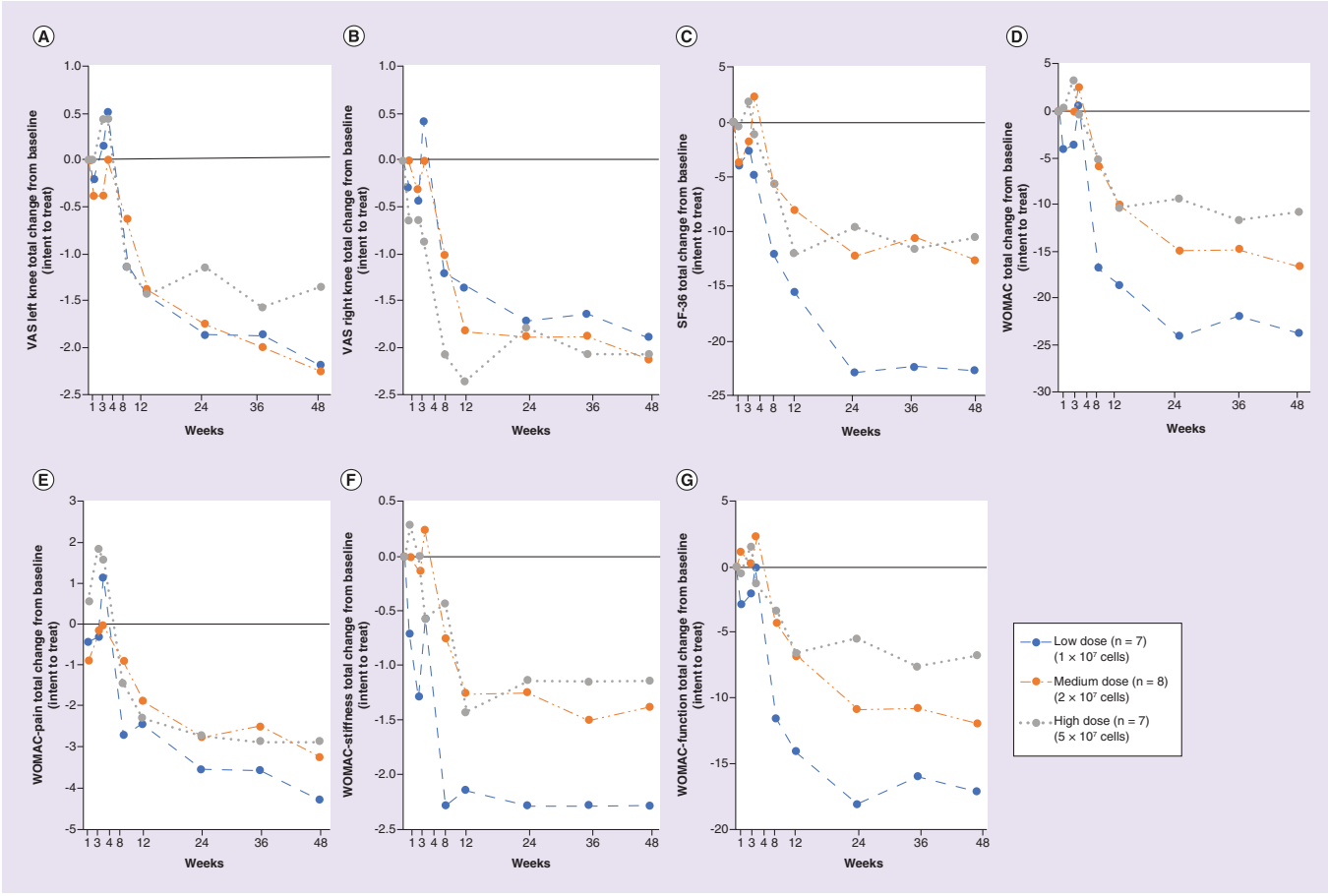


**Figure 2. Clinical outcome.** Mean change from baseline was taken over time for all cohorts in terms **(A)** VAS left knee, **(B)** VAS right knee, **(C)** SF-36, **(D)** WOMAC total, **(E)** WOMAC-pain, **(F)** WOMAC-stiffness and **(G)** WOMAC-function. The elevation in pain levels at week 3 and week 4 can be attributed to the second injection and the transient postinjection pain associated with an intra-articular knee injection.
SF-36: Short form-36; VAS: Visual analog scale; WOMAC: Western Ontario and McMaster Universities Osteoarthritis index.

## Discussion

The results from this Phase I trial suggested that two intra-articular (IA) injections of AlloJoin at baseline and after 3-week follow-up in bilateral knee OA patients was well tolerated, with no evidence of systemic exposure and demonstrated clinically meaningful differences in WOMAC score and VAS.

The adverse events associated with AlloJoin appeared to be mild to moderate. A higher incidence of injection site pain and swelling were reported in the medium and high-dose groups. All laboratory measurements were within acceptable limits and did not present signs of systemic exposure. At 3 months, no differences in adverse event rates were identified between low, medium and high-dose groups. Furthermore, there was no evidence of ectopic bone formation based on MRI evaluation at 48 weeks.

There were two SAEs in this study and both were found to be unrelated to the cellular therapy. The SAEs of both cases emerged in a prolong timeframe after the completion of the injections, week 23 and 45. In preclinical rat models, MPCs labeled with fluorescent dye persisted locally in the joint for up to 10 weeks and became undetectable thereafter [20]. In addition, the SAEs are inconsistent with all preclinical animal studies which showed no evidence of systemic exposure [18–20]. Furthermore, the administration of intra-articular adipose-derived MPC has been demonstrated to be safe with previous human studies [28–30]. Caution will be applied when designing and implementing the Phase II clinical trial.

Although the primary purpose of this study was safety, some exploratory end points to assess the potential efficacy was explored. The VAS, SF-36, WOMAC and WOMAC subscales suggested symptomatic improvement in all cohorts at 48 weeks. MCIDs are important for usage in clinical trials because statistically significant difference is mostly a matter of sample size and does not always translate into clinically importance. The published averages of

MCID for the WOMAC with knee OA [31] ranges between 9.1 and 19.9 mm for the WOMAC functional score which showed that this study's WOMAC scores showed meaningful clinical improvement for the total WOMAC functional (17.1) for both the left and right knees score at 48 weeks for two of the doses [32–35]. Similarly, MCID scores for SF36 have been demonstrated to be around 10% which was surpassed by this study's data [33,36,37]. The study's reported VAS MCID score of 30.0 mm [38,39] showed our VAS data demonstrated clinical improvement with these cell injections. A reduction in pain and an improvement in knee function was suggested at 48 weeks when applying these established MCID values; however, these findings should be interpreted with caution due to the small number of subjects enrolled in this pilot study.

MRI assessments were used to assess cartilage morphology and evaluated the potential for disease-modifying osteoarthritis drug (DMOAD) efficacy [40]. MRI assessments provided a more accurate depiction of the articular cartilage degeneration and change in position of the menisci over radiographic x-rays [41]. Exploratory MRI assessments measuring average cartilage volumes and average WORMS from baseline at week 48 suggested no change in the medium- ($2 \times 10^7$ cells) and high-dose ($5 \times 10^7$ cells) groups and an improvement in the low-dose group ($1 \times 10^7$ cells). These findings must be interpreted with caution due to the small number of subjects enrolled into this trial and a possible type II statistical error. A period of 48 weeks is a rather short follow-up time point for OA, nevertheless, low-dose AlloJoin ($1 \times 10^7$ cells) showed slight improvements across the total cartilage volume, knee femur end cartilage volume, knee patellar cartilage volume and the WORMS evaluation of the right and left knee. These early limited results suggested that AlloJoin may have a potential utility as a DMOAD in knee OA.

This preclinical work with AlloJoin incorporated two bilateral intra-articular knee injections, each three weeks apart (18–20 days). Bilateral knee OA was evaluated due to the high prevalence in the treatment population [42,43]. This treatment protocol allowed a greater clinical opportunity to evaluate a larger number of knee responses to these progenitor cells. Future clinical trials will evaluate synovial fluid assays to get a better understanding of the mechanism of the intra-articular cellular response.

There are a number of limitations within the current study. The primary focus of this study was to establish a safe dose of AlloJoin and therefore the number of participants enrolled into the trial was small. Any indication or suggestion of efficacy in the exploratory outcome measurements needs to be determined by adequately powered randomized clinical trials. A majority of the patients enrolled within this study were females. Women are more severely impacted by OA due to knee anatomy, kinematics, previous knee injuries and hormonal influences [6,44]. Due to the small samples size and primary safety end point, a covariate analysis of the effect of gender on AlloJoin was not evaluated. Gender differences must be evaluated in the larger clinical trials.

## Conclusion & future perspective

These initial results showed the safety and exploratory clinical findings of a Phase I trial with AlloJoin. The primary goal of safety was accomplished and the secondary end points suggested positive trends of improvement in pain and function by historical MCID levels for VAS, SF-36 and WOMAC for articular evaluation. Additionally, exploratory MRI assessments suggested DMOAD properties. This Phase I data supported further trials of safety and efficacy investigating AlloJoin injections for knee osteoarthritis. Future studies will involve larger clinical trials to further test the safety and efficacy of AlloJoin.

### Summary points

- We performed a single-site, randomized, double-blind Phase I study that investigated the safety and efficacy of patients with osteoarthritis receiving intra-articular injections of AlloJoin®.
- Patient-reported outcomes included the Western Ontario and McMaster Universities Osteoarthritis index score, visual analog scale-pain and short form-36.
- Patients in all three dosing groups demonstrated improvements from baseline. The scores passed the threshold for minimally clinically important differences. MRI assessment suggested an improvement in cartilage volumes in the low-dose group.
- These results are promising and showed trends in improvement in this patient study population.
- Future studies will involve larger clinical trials to further test the safety and efficacy of AlloJoin.

### Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/rme-2019-0106

### Author contributions

The authors designed the studies and analyzed and interpreted the data. All authors have made substantial contributions: the conception and design of the study or acquisition of data or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; and final approval of the version to be submitted. C Bao (email: baochunde_1678@126.com) takes responsibility for the integrity of the work as a whole.

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No writing assistance was utilized in the production of this manuscript.

### Data sharing statement

The authors certify that this manuscript reports original clinical trial data (NCT02641860). The datasets used or analyzed during the current study will be made available from the corresponding author upon reasonable request.

### Ethical conduct of research

The protocol of the study was approved by the Ethics Committee of Ren Ji Hospital, Shanghai Jiao Tong University. Researchers adhered to the principles of the Helsinki Declaration throughout the study and written informed consent was obtained from the patients. The study was registered at http://ClinicalTrials.gov (NCT02641860).

### Clinical trial registration number

NCT02641860 (clinicaltrials.gov). Registered 21 December 2015.

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Regenerative Medicine

# Characterization of an umbilical cord blood sourced product suitable for allogeneic applications

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**Aim:** Umbilical cord blood (UCB) sourced allografts are promising interventions for tissue regeneration. As applications of these allografts and regulations governing them continue to evolve, we were prompted to identify parameters determining their quality, safety and regenerative potential. **Materials & methods:** Flow-cytometry, mass-spectrometry, protein multiplexing, nanoparticle tracking analysis and standard biological techniques were employed. **Results:** Quality attributes of a uniquely processed UCB-allograft (UCBr) were enumerated based on identity (cell viability, immunophenotyping, proteomic profiling, and quantification of relevant cytokines); safety (bioburden and microbiological screening), purity (endotoxin levels) and potency (effect of UCBr on chondrocytes and mesenchymal stem cells derived exosomes). These attributes were stable up to 24 months in cryopreserved UCBr. **Conclusion:** We identified a comprehensive panel of tests to establish the clinical efficacy and quality control attributes of a UCB-sourced allograft.

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**Keywords:** endotoxin • exosomes • graft versus host disease • quality control • tissue regeneration

The field of regenerative medicine holds abundant promise for the treatment of diseases afflicting millions worldwide. Thousands of clinical trials are underway for trauma and degenerative conditions in the USA alone (www.clinicaltrials.gov). The interest in the field is further amplified by the aging population's need to find alternatives to surgery, effectively offloading the financial burden from the healthcare system as well as patients [1]. An in-depth understanding of stem cell biology and therapeutic benefits of cytokines, along with improved techniques for optimizing these factors are helping regenerative medicine to grow at an unprecedented pace.

Recent research has demonstrated that UCB is a convenient source of adult stem cells containing hematopoietic (HSC) as well as mesenchymal stem cells (MSC) [2,3]. HSC in cord blood prompted its application in certain hematological diseases [4], whereas the presence of multipotent MSC that can differentiate into other structural tissues like bone and cartilage has further expanded the potential applications of UCB [5–7]. While the cellular characteristics of UCB have been extensively studied for the purposes of clinical applications, not enough has been reported on regenerative potential of the associated biomolecules. This is partly because regenerative medicine has long been associated with stem cell therapy. However, it has been shown that regenerative potential is often derived from the paracrine effect of the interventions and the activated host stem cells, rather than the direct involvement of exogenous cells administered at the site of injury [8]. We have previously demonstrated that a uniquely processed UCB product contains cytokines and growth factors, which are integral to bone regeneration [9]. These factors initiate signaling crosstalk with target cells that positively impacts the molecular milieu of the tissue microenvironment [10].

Exosomes are extracellular vesicles (EVs) comprising of several factors including cytokines. MSC release EVs packed with proteins and genomic materials which induce biological changes in the target cells to accomplish tissue regeneration [11]. Translational research on exosomes derived from the MSC have demonstrated that they can mimic the therapeutic attributes of the MSC themselves [12–14]. Clinical trials on rheumatic pathologies, cartilage

Future Medicine

development, brain injury and cardiovascular diseases are also currently testing this hypothesis [15]. Based on these findings, clinical suitability of cord blood allografts must account for the noncellular components while evaluating its promising role in tissue regeneration.

As UCB allografts are increasingly used in the clinics for tissue regeneration, the regulatory framework continues to evolve. Such products may be classified and regulated differently from cell and gene-based therapies. The quality control guidelines for cyto/genetic therapy includes cell identity, viability, purity, sterility and potency to minimize lot-to-lot variability [16]. Since UCB allografts are a complex embodiment of cellular and noncellular components, a quality control approach recommended for such products should be appropriately modified to address suitability and release criteria. Claims to regenerative function of these allografts should be based on characterization of identity and consistency of the noncellular components. UCB allografts have heterogeneous cellular and molecular composition due to inherent donor variability and alterations in the processing methodologies will add to this factor [17]. Variability can however be largely reduced, if not completely eliminated, by careful optimization of the manufacturing process and ensuring a stringent quality control mechanism.

The present study is aimed to address the unmet need for a uniform quality control framework to determine clinical suitability and safety of cord blood allografts. A uniquely processed UCB sourced allograft (UCBr) reportedly used for soft tissue and ligament repair application was investigated for identity, purity, safety and stability. Additionally, it is important to determine the biological potency of UCBr to establish a comprehensive quality control regime. The potency assays provide the mechanistic insight into how noncellular components of UCBr support biological processes, which may positively impact cartilage repair. Finally, the long-term stability of UCBr was over a period of 24 months. Taken together, to the best of our knowledge, this is the first study that identifies a comprehensive panel of tests to adequately address the quality attributes, clinical suitability and efficacy of UCB-sourced allografts.

## Materials & methods

### UCB-sourced allograft

UCB obtained from consenting donors was processed by the patent pending method following US FDA's regulatory guidelines. UCB-sourced allografts, UCBr (BioBurst Rejuv, Burst Biologics, ID, USA) were stored at -80°C until further use.

### Preparation of UCBr lysate

UCBr was sonicated on ice (three pulses of 10 s each), centrifuged at 12,000 g for 15 min at 4°C and supernatant was collected and used for downstream experiments. Supernatant was not subjected to more than one freeze thaw cycle. When appropriate, supernatant was diluted in serum free media (SFM) at 1:5 or 1:10 dilution.

### Cells & tissue culture media

Human Knee Articular Chondrocytes (HC, Lonza CC-2550) were cultured in the basal media supplemented with growth kit (cell applications; 410PR-500 and 411-GS). Bone marrow derived mesenchymal stem cells (MSC, ATCC, PCS-500-012) were also cultured in the respective basal media supplemented with growth kit (ATCC; PCS-500-030 and PCS-500-041). Cells were maintained in a humidified incubator at 37°C with 5% $CO_2$. Cells between passages 3 and 5 were used for all experiments.

### Cell viability measurement

UCBr was passively thawed and mixed in equal volumes with acridine orange/propidium iodide (AO/PI) staining solution. The mixture was loaded onto the SD100 cell counting chamber slides, and cells were visualized and counted using Cellometer K2 (Nexcelom, MA, USA). Data were analyzed using the K2 software optimized for frozen peripheral blood mononuclear cells (PBMC) using 15-micron cutoff for nonviable cells.

### Fluorescence activated cell sorting

UCBr was thawed and added dropwise into RPMI supplemented with 10% FBS and 400 units of DNAse. The cell suspension was centrifuged at 300 g for 5 min, and the cell pellet was resuspended in 10% FBS/RPMI containing 50 units of DNAse. Cells were sequentially washed with PBS and FACS Buffer (2% FBS/HBSS, 2 mM EDTA). Cells were stained with antibodies (listed in Supplementary Material). Cells stained with all the fluorophores minus one fluorophore (FMO) were used as controls to determine the gating boundaries of positive and negative

populations. Unstained and single-stained beads were used to calculate compensation values using the automated calculation function. Flow cytometry was performed on an ACEA NovoCyte with blue and red lasers and detectors for six color analysis. 10,000 events at 14 ml/min were collected for compensation controls and 100,000 events at 35 ml/min were collected for UCBr cells.

### Mass spectrophotometry & proteomic analysis

LC-MS/MS was performed using methods established previously with modifications [18]. Briefly, UCBr lysates were run for approximately 1 cm in 10% Bis-Tris gel and excised gel pieces were reduced, alkylated and digested with trypsin overnight at 37°C (Thermo Fisher Scientific, MA, USA). LC–MS/MS analysis of the resulting tryptic peptides was conducted on a Velos Pro Dual-Pressure Linear Ion Trap mass spectrometer equipped with a nano electrospray ionization source and coupled with an Easy-nLC II nano LC system (Thermo Fisher Scientific). Raw spectrum data were searched against the UniProtKB/Swiss-Prot protein database for Humans (acquired from www.uniprot.org on 12 February 2019). Further analysis was done using Database for Annotation, Visualization and Integrated Discovery web tool to analyze candidate genes and pathways [19].

### Cytokine measurement

Cytokine concentration was measured in UCBr lysate (prepared from 31–64 donors) using multiplex ProcartaPlex Panel (Invitrogen, EPX450-12171-901). Luminex xMAP magnetic-bead fluorescent immunoassays (Invitrogen) were run on MAGPIX™ and measurements were done as per the manufacturer's protocol. Universal assay buffer provided baseline values for the assay. For each standard, percent recovery values outside 90–110% were invalidated using xPonent Analysis software and concentration of cytokines were calculated using a standard curve with $R^2 \geq 0.9$.

### Maternal peripheral blood serology & UCB bioburden testing

Maternal peripheral blood samples were tested for infectious diseases using antibody-based, nucleic acid based and PCR-based detection methods. Serology testing was performed at VRL (TX, USA), a Clinical Laboratory Improvement Amendments certified laboratory. UCB samples were tested for anerobic and aerobic bacterial contamination prior to processing as per current Good Tissue Practice (cGTP) guidelines. To test possible contamination of UCBr, it was passively thawed and inoculated in either TSB, Sabdex or FTH broth and corresponding agar plates. For all tests appropriate positive and negative controls were included. Growth was monitored every 72 h up to 14 days.

### Endotoxin test

Endotoxin levels were determined in UCBr directly using EndoSafe® nexgen PTS™ and US FDA licensed cartridges (PTS2005F or PTS5505F, sensitivity – 0.05 EU/ml, Charles River, MA, USA) following manufacture's protocol.

### Mixed lymphocyte reaction

PBMCs were isolated from the buffy coat of healthy donors (PBMC Donor #36, male, age 46 years, White; Donor #50, male, age 25 years, White; Donor #42, female, age 24years, African American) by Ficoll density gradient centrifugation and cryopreserved. Prior to use, the cryopreserved samples were thawed and rested at 37°C overnight in RPMI-1640 medium supplemented with 10% fetal bovine serum. Cryopreserved UCBr were thawed and plated at $10^5$ cells/well in a 96-well plate. Rested PBMC cells were co-cultured with $1 \times 10^5$ UCBr cells or PBMCs from unrelated donor at a density of $2 \times 10^5$ cells/well in the mixture of 200 µl AIM-V. For mitogenic stimulation, the PBMC cells were stimulated with 2 µg/well phytohemagglutinin. Background controls were performed for all UCBr samples and each institutional review board donor. Cells were incubated at 37°C, in a 5% $CO_2$ incubator for 4 days. Bromo-deoxy uridine (BrdU) was added on day 3, and proliferative responses were determined by absorbance based BrdU ELISA assay on day 4 (Roche, Basel, Switzerland). BrdU optical density was recorded at 450 nm. The assay was performed by Xeno Diagnostics, LLC (IN, USA) and PBMC used were approved through their institutional review board program.

### Cell proliferation

HC and MSC were seeded at density of 5000 cells/well in a 96-well plate and incubated overnight. Cells were starved for 2 h and then grown in SFM, complete media or diluted UCBr lysates (1:10 or 1:5) for additional

24 h. Cell proliferation was measured by CyQUANT (Thermo Fisher Scientific) assay according to manufacturer's protocol.

### Transwell cell migration

Transwell inserts were coated with 5 μg/ml of fibronectin (Sigma–Aldrich, MO, USA) and left overnight for surface adsorption. HC were serum-starved for 2 h, suspended in SFM and $4 \times 10^4$ cells were added to the upper chamber of a 24-well, 8 μm pore membrane transwell insert. Complete media, SFM or UCBr lysate (1:10) were added to the lower chamber as a chemoattractant. After 24 h, cells on the upper surface were removed, cells on the lower surface were fixed in 5% glutaraldehyde (Sigma–Aldrich, MO, USA), stained with 1% (w/v) crystal violet (Sigma–Aldrich) and visualized at 20× magnification under stereoscope (Leica, NJ, USA).

### RNA isolation & reverse transcriptase PCR

Cells were treated with UCBr lysates (1:5) for 18–20 h, total RNA was extracted using Quick RNA kit (Zymo Research, CA, USA) and 1 μg was reverse transcribed into cDNA and subsequently amplified with appropriate primers (listed in Supplementary Material) using SuperScript™ VILO™ cDNA Synthesis Kit and Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) respectively. All reactions were run in QuantStudio 3 (Thermo Fisher Scientific), and data are represented as relative mRNA expression with 18 s rRNA as internal control.

### Western blot analysis

Proteins were extracted in RIPA buffer (Thermo Fisher Scientific) from cells treated with UCBr lysates (1:5) for 24 h and estimated by BCA assay. Total 20–60 μg of proteins were separated in 10% Bis-Tris gels by electrophoresis, transferred onto nitrocellulose membrane and immunoprobed with antibodies to detect HMOX1, Cu/ZnSOD, MnSOD, Catalase, Thioredoxin 1 (Cell Signaling Technologies, MA, USA) and Hsp60 (SCBT, TX, USA). Chemiluminescence was captured with iBright FL1000 (Thermo Fisher Scientific). Hsp60 was used as loading control and was used for normalization of band intensities using ImageJ software.

### Exosome isolation & characterization

MSC in 100 mm culture plates with 80–90% confluency were briefly subjected to 2 h of serum starvation. Cells were subsequently treated with UCBr lysate (pooled from 6 or more donors) for 18 h. Exosomes were extracted by Total Exosome Isolation (TEI) kit according to manufacturer's protocol (Thermo Fisher Scientific) or Exo-Quick kit (SBI, CA, USA). Isolated exosomes were first characterized by fluorescent nanoparticle tracking analysis (performed by System Biosciences, CA, USA). For downstream proliferation assay on HC and MSC, cells were treated with isolated exosomes diluted 1:2 for 24 h. Cytokine level was measured as described previously. All experiments, including the exosome analysis, were repeated twice independently, each time at least in triplicates.

### Data analysis & statistics

All experiments, unless otherwise stated, were repeated independently three-times. All cell biological assays were performed with UCBr lysates obtained from at least three donors. Data are expressed as mean ± standard deviation or standard error of the mean as indicated. Student's t-test and ANOVA were used for statistical analysis between two groups, and among three or more groups respectively. Statistical significance was set at $p < 0.05$ (*).

## Results

### Characterization of the cellular components of UCBr

Our previous publication shows that intact cells in a cord blood allograft are not directly involved in the regeneration process, and possibly act as reservoirs of the intracellular and membrane bound cytokines that are released by autolysis or passive secretion. We specifically demonstrated that the cytokine concentration in the conditioned media (CM) derived from intact UCB allografts was not significantly different from the CM derived from lysed UCB allografts [9]. However, it is critical that we evaluate the cellular components in UCBr from a quality control perspective, as the reproducibility of cell viability and characteristics will indicate a consistent processing technique.

#### *Cell viability assay*

Total 21 random lots of UCBr, stored at -80°C for a period of at least 30 days post processing were passively thawed and viability of the primary cell population was measured. Cell count, viability and diameter were determined by

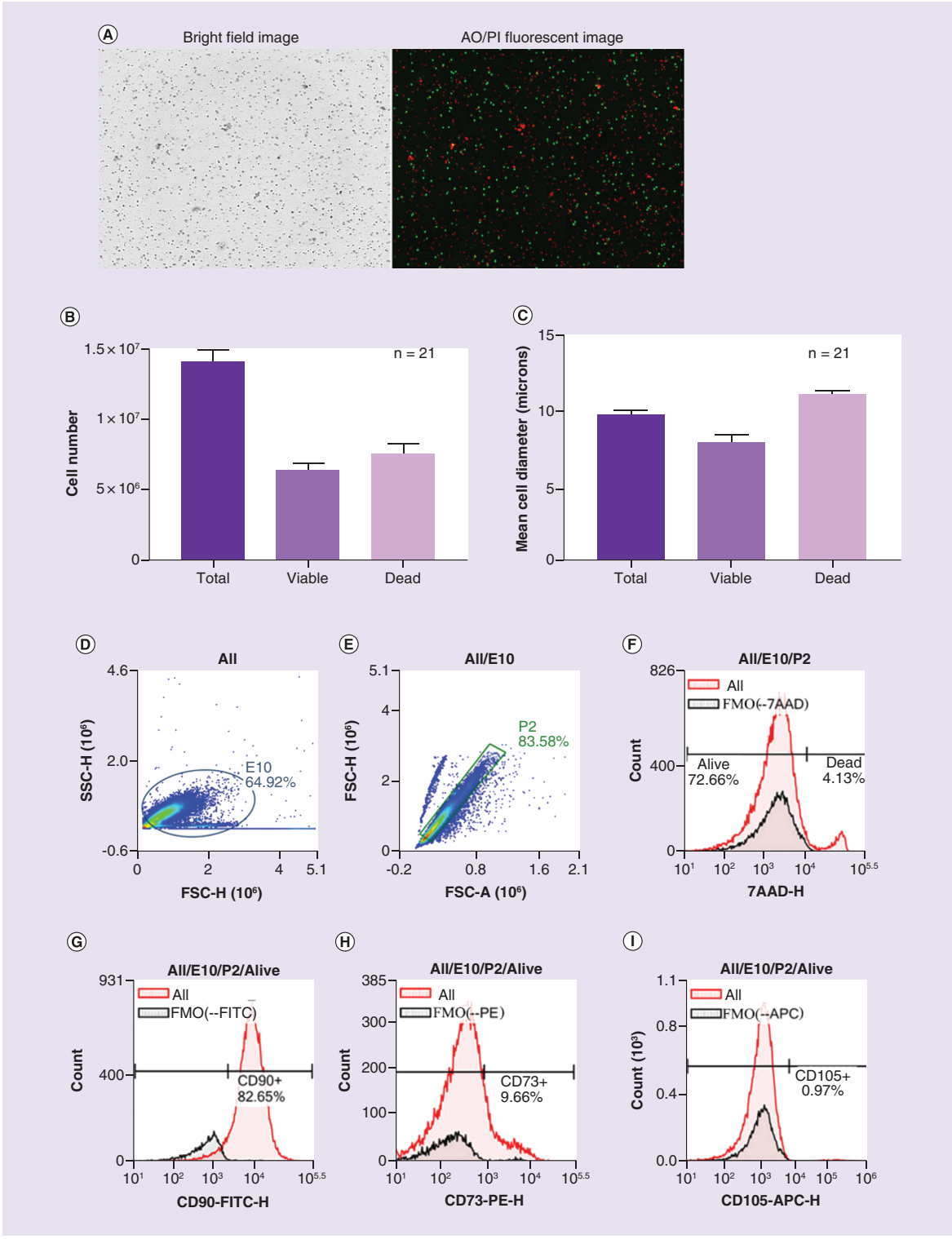



**Figure 1. Characterization of cellular components of umbilical cord blood allograft by cell viability measurement and immunophenotyping using flow cytometry.** UCBr from 21 donors was passively thawed and mixed with AO/PI dye solution and visualized under Nexcelom K2. **(A)** Representative photomicrographs of a random field with bright field (left panel) and fluorescence (right panel) were shown, AO-green/viable and PI-red/dead. **(B)** Cell number and **(C)** cell diameter were quantified. Values are expressed as mean ± standard deviation. **(D–N)** Expression of surface markers of UCBr cells from a representative donor. Cells were appropriately gated **(D, E & K)**. In figures **(F–J)**, 'all' indicates cells stained with CD90-FITC, CD73-PE, CD105-APC, CD45-PerCp-Cy5.5 and 7-AAD monoclonal antibodies and 'fluorophore' indicates cells stained with all the fluorophores minus the one as labeled in respective figures. Viability determined by 7-AAD exclusion **(L)** and stained with CD34 and CD45 monoclonal antibodies to determine $CD34^+$ and $CD45^+$ cells **(M & N)**. **(O)** The average expression percentage of cells stained with individual or combination of antibodies in UCBr.
AO: Acridine orange; PI: Propidium iodide; UCBr: Umbilical cord blood allograft.

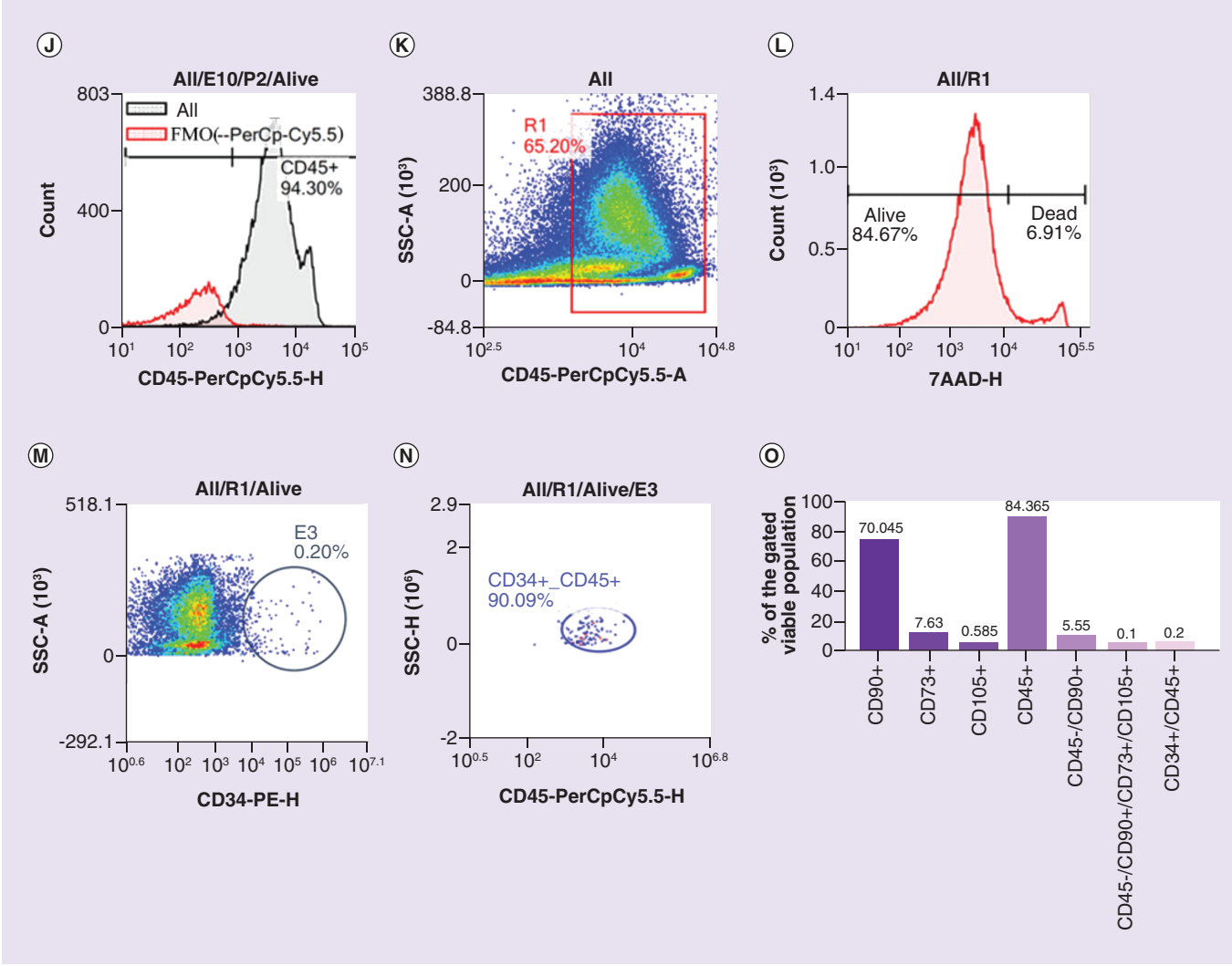



**Figure 1. Characterization of cellular components of umbilical cord blood allograft by cell viability measurement and immunophenotyping using flow cytometry (cont.).** UCBr from 21 donors was passively thawed and mixed with AO/PI dye solution and visualized under Nexcelom K2. **(A)** Representative photomicrographs of a random field with bright field (left panel) and fluorescence (right panel) were shown, AO-green/viable and PI-red/dead. **(B)** Cell number and **(C)** cell diameter were quantified. Values are expressed as mean ± standard deviation. **(D–N)** Expression of surface markers of UCBr cells from a representative donor. Cells were appropriately gated **(D, E & K)**. In figures **(F–J)**, 'all' indicates cells stained with CD90-FITC, CD73-PE, CD105-APC, CD45-PerCp-Cy5.5 and 7-AAD monoclonal antibodies and 'fluorophore' indicates cells stained with all the fluorophores minus the one as labeled in respective figures. Viability determined by 7-AAD exclusion **(L)** and stained with CD34 and CD45 monoclonal antibodies to determine $CD34^+$ and $CD45^+$ cells **(M & N)**. **(O)** The average expression percentage of cells stained with individual or combination of antibodies in UCBr.
AO: Acridine orange; PI: Propidium iodide; UCBr: Umbilical cord blood allograft.

bright field and dual-fluorescence imaging using acridine orange/propidium iodide staining (Figure 1A). The mean total cell count was 14.1 ± 3.4 million and the viability was close to 50%. The mean diameter was 8 ± 1.4 µm for viable and 11.12 ± 0.85 µm for the nonviable cells (Figure 1B & C).

### *Characterization of UCBr cells by flow cytometry*

All studies reporting isolation of MSC from UCB have allowed the cord blood cells to adhere to culture dishes and expand for a few generations before performing flow-cytometry to characterize MSC based on cell surface markers [20–22]. Other methods like fluorescence activated cell sorting (FACS) have been used to enrich and purify the small but significant fraction of stem cells present in UCB [23]. Without such downstream manipulations, the sensitivity of the current assay procedures makes it extremely difficult to identify the stem cell population

directly in the UCB cells [24]. Therefore, instead of identifying a particular subpopulation of cells in UCBr through lineage specific enrichment, we chose to characterize the heterogeneous population by immunophenotyping. Two UCBr samples were thawed, washed and stained with respective antibodies. Dead cells were identified using 7-Aminoactinomycin D (7AAD). Of the viable cells in UCBr, the average expression of CD90, CD73, CD105, CD45 were 70.04 ± 17.8%, 7.63 ± 2.87%, 0.585 ± 0.54%, 84.37 ± 14.1% respectively, indicating a major population of the cells is lymphocytes (Figure 1D–O). CD45 cells that stained positive for CD90 were 5.55 ± 0.48%. As expected in such heterogeneous population of frozen UCB cells, less than 1% of the cells in UCBr stained for all the MSC markers ($CD45^-/CD90^+/CD73^+/CD105^+$). Albeit very low, expression of HSC progenitor marker CD34 was also detected.

## Characterization of the noncellular components of UCBr

### *Mass spectrometric analysis of UCBr*

In addition to cytokines, signaling molecules such as protein kinases and redox enzymes also impact tissue regeneration. To identify such signaling proteins, we performed proteomic analysis of UCBr lysates by mass spectrometry. Only those proteins identified with two or more peptides, an expected p-value of $<0.05$ and a false discovery rate of $\leq 1\%$ were reported. Proteomic analysis of two UCBr samples using Uniprot human protein database identified 1801 and 2335 proteins respectively, including serine threonine kinase, mitogen activated protein kinase, tyrosine kinases, cyclin dependent kinases and von Willebrand factor in addition to growth factors like PDGF, TGFβ1 and FGF2 (data not shown).

We identified 784 proteins that were common between the samples (Figure 2A). The high overlap of proteins between the donors suggests that the UCBr processing technique is consistent and the methods for protein identification are reliable. Bioinformatic analysis of the overlapping proteins using Database for Annotation, Visualization and Integrated Discovery 6.8 and gene ontology annotation indicated that proteins common between the donors were mainly involved in wound healing, cell proliferation and migration, cell cycle, redox metabolism, glucose metabolism and signaling pathways (Figure 2B). Pathways that yielded a Fishers exact p-value $<0.05$ and involved at least eight genes were reported. All the molecular functions and biological processes identified by this bioinformatic analysis are physiologically relevant and clearly established the role of the noncellular component of the UCBr in tissue regeneration.

### *Cytokine profiling of UCBr*

Mass spectrometry analysis identified several signaling molecules including PDGF and VEGF in UCBr that play a critical role in wound healing and tissue repair (data not shown). We have previously reported 44 different cytokines in the cord blood allograft derived CM [9]. In this study, we quantified the cytokines present in the UCBr lysates (Figure 2C). This enabled a better understanding of the molecular composition of the product with a direct clinical relevance. Total 31 cytokines including several growth factors (PDGF and EGF), neurotrophic factors (BDNF), angiogenic factors (VEGF) and interleukins (IL-1, IL-2, IL-5, IL-8, IL-10) were identified in samples prepared from 31–64 donors. The mean concentration of the cytokines ranged from 7.83 ± 0.3 pg/ml (SCF) to 30933 ± 1901.2 pg/ml (IL-1RA). IL-1RA is a major anti-inflammatory cytokine and several clinical trials with IL-1RA have reported pain reduction in patients following acute injury [25]. Presence of important cytokines like EGF (323.16 ± 19.02 pg/ml); PDGF-BB (3256.8 ± 495.9 pg/ml) and VEGF (1600.1 ± 91 pg/ml) in UCBr may be the regulatory factors for inducing cell proliferation, migration and angiogenesis and hence, constitute an important criterion to determine product quality and donor suitability.

## Pre- and postprocessing microbiological safety testing on UCBr

Absence of microbial contamination in the product is critical from clinical safety perspective and must be an integral part of the quality control process. UCBr is manufactured under controlled, clean room conditions with minimal environmental exposure which must be subsequently verified by an array of safety testing to ensure the aseptic nature of the final allograft.

### *Infectious disease testing on maternal peripheral blood samples*

To begin with, a well-defined inclusion–exclusion criterion based on personal lifestyle, medical history, etc. determined donor initial qualification. Serology testing was performed by Clinical Laboratory Improvement Amendment certified lab as per US FDA's guidelines for human cells, tissues, and cellular and tissue-based product (HCT/Ps).

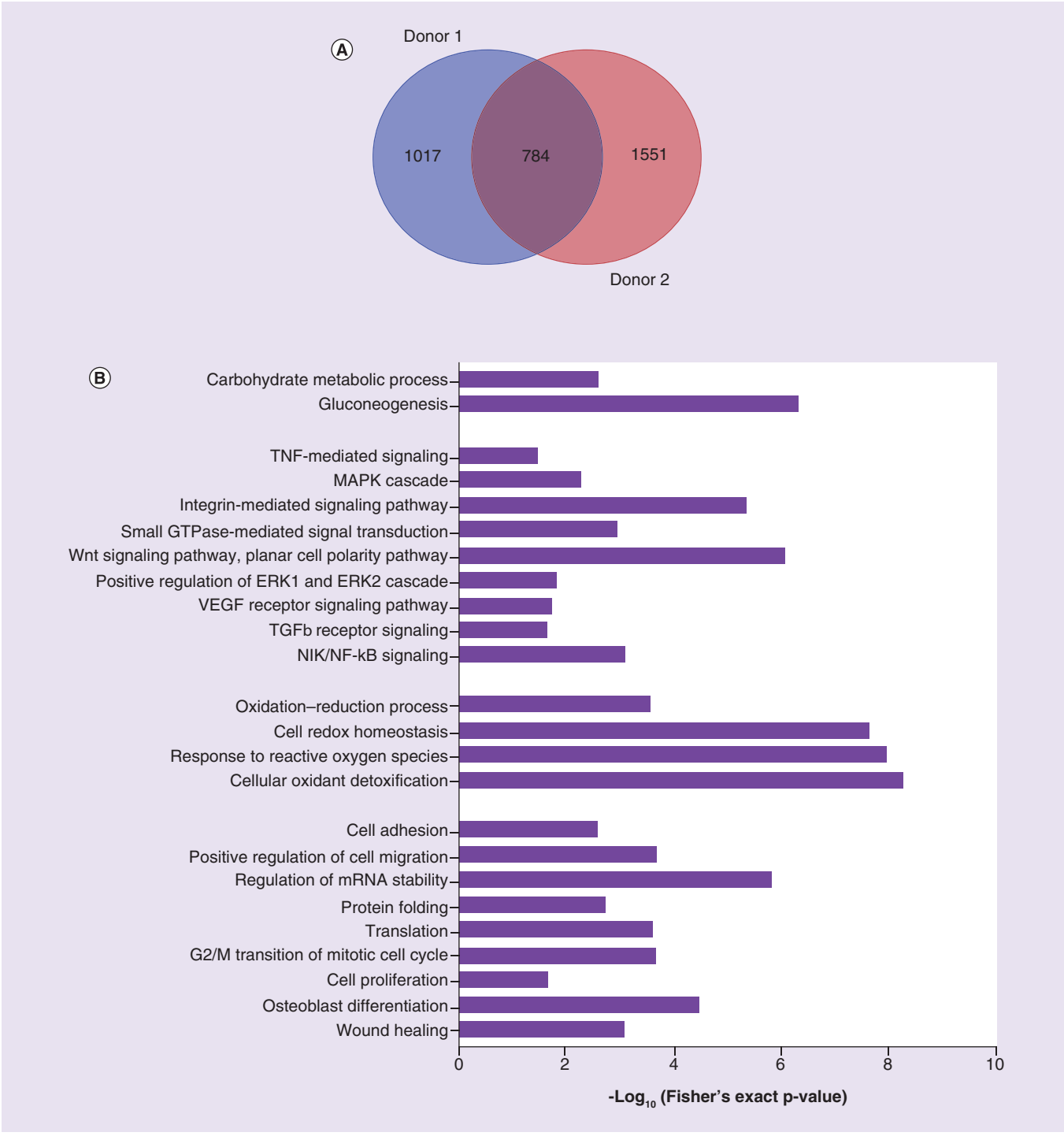



**Figure 2. Characterization of noncellular components of umbilical cord blood allograft by mass spectrometry and protein multiplexing.** **(A)** Venn diagram showing the number of unique and shared proteins in donor samples obtained by mass spectrometry. **(B)** Enrichment analysis of the 784 common proteins in donor samples using DAVID. **(C)** Protein multiplexing analysis for 31 cytokines in UCBr samples. Cytokine concentration was measured by Luminex xMAP technology on MAGPIX™ platform and expressed as mean values in picograms/ml (± standard error of the mean) from 31–64 donors.
UCBr: Umbilical cord blood allograft; DAVID: Database for Annotation, Visualization and Integrated Discovery.

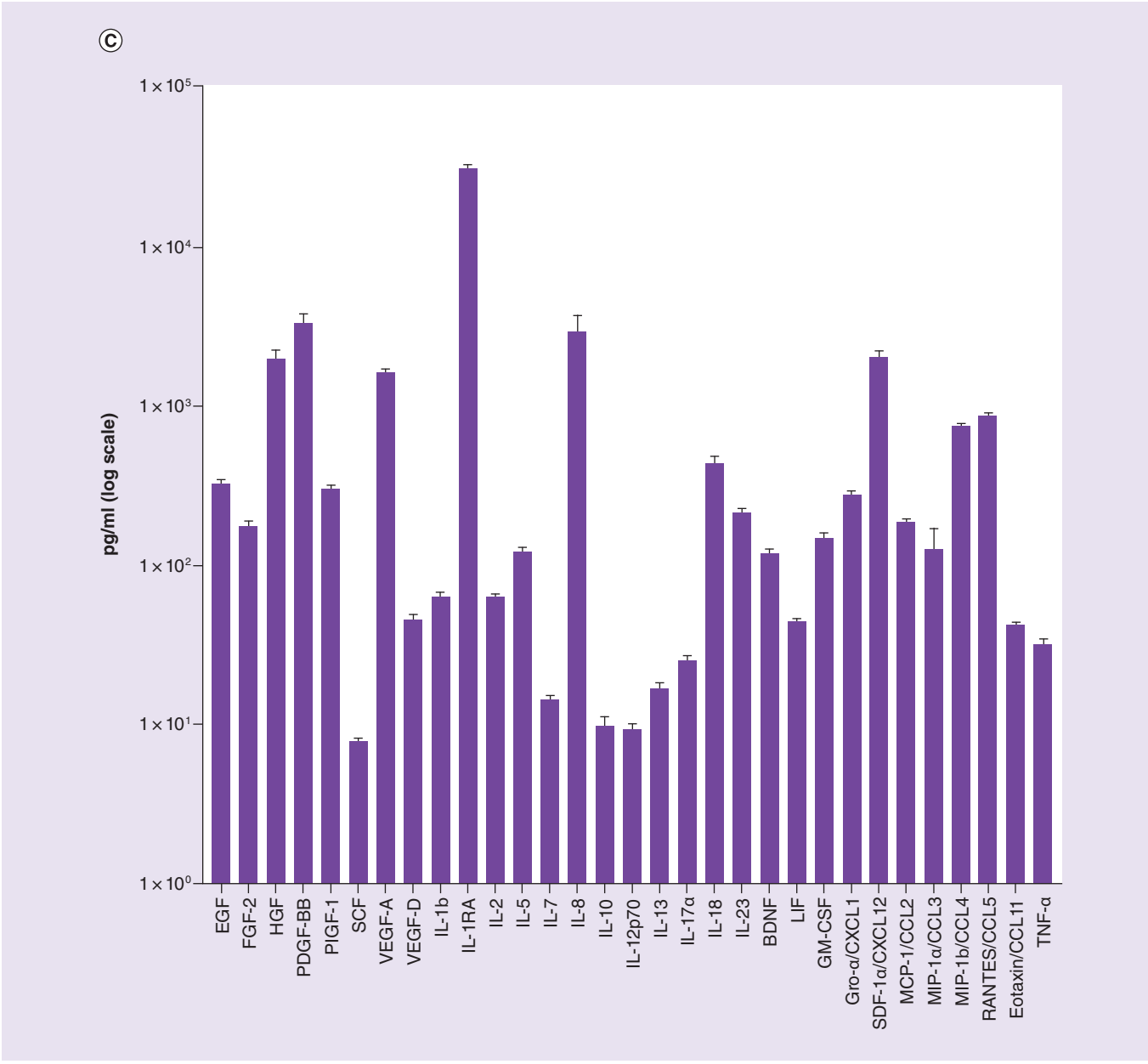



**Figure 2. Characterization of noncellular components of umbilical cord blood allograft by mass spectrometry and protein multiplexing (cont.). (A)** Venn diagram showing the number of unique and shared proteins in donor samples obtained by mass spectrometry. **(B)** Enrichment analysis of the 784 common proteins in donor samples using DAVID. **(C)** Protein multiplexing analysis for 31 cytokines in UCBr samples. Cytokine concentration was measured by Luminex xMAP technology on MAGPIX™ platform and expressed as mean values in picograms/ml (± standard error of the mean) from 31–64 donors.
UCBr: Umbilical cord blood allograft; DAVID: Database for Annotation, Visualization and Integrated Discovery.

Testing included detection of antibodies to HTLV-1 and 2, syphilis and hepatitis B surface antigen. Nucleic acid testing (NAT) was performed for hepatitis B, hepatitis C, HIV and West Nile virus. Peripheral blood samples were also tested for total antibody of CMV, while Zika and other flavivirus were identified by PCR amplification, to eliminate possibility of past or recent exposure to the virus because of traveling to certain geographical zones. In a typical 24 months period, 5.2% of the total donors were rejected due to positive antibody test, 1.2% were rejected due to positive NAT outcomes and 1.0% of the donors were rejected based upon PCR-based identification. Overall, 92.5% donors were negative for any infectious disease serology and the cord blood collected from these donors underwent final processing (Table 1A).

**Table 1. Determination of product safety using pre- and postprocessing microbiological testing.**

| (A) Maternal peripheral blood infectious disease serology testing | | |
|---|---|---|
| **Serology test** | | **Percentage positive** |
| Antibody-based detection | | 5.209 |
| Nucleic acid test | | 1.195 |
| PCR-based identification | | 1.0247 |
| | | 7.42 (Total) |
| **(B) Umbilical cord blood microbial cultures** | | |
| **Incubation temperature** | **Culture media** | **Percentage positive** |
| 22.5°C | Tryptic soy broth | 0 |
| 32.5°C | Fluid thioglycolate broth | 0.028 |
| | Tryptic soy broth | 0.008 |
| | | 0.036 (Total) |
| **(C) Umbilical cord blood product microbiological testing** | | |
| **Incubation temperature** | **Culture media** | **Percentage positive** |
| 22.5°C | Tryptic soy broth | 0 |
| | Sabdex broth | 0.001 |
| | Tryptic soy agar | 0 |
| | Sabdex agar | 0 |
| 32.5°C | Fluid thioglycolate broth | 0.007 |
| | Tryptic soy broth | 0.006 |
| | Tryptic soy agar | 0.007 |
| | | 0.021 (Total) |

**(A)** Percentage of maternal peripheral blood samples that tested positive for the presence of infectious serology using antibody, nucleic acid and PCR based testing in a period of 24 months. **(B)** Percentage of cord blood samples that tested positive in aerobic and/or anerobic microbial cultures over a period of 24 months. **(C)** Percentage of UCBr that were positive in either one or more growth-based microbiological culture tests in 24 months.
UCBr: Umbilical cord blood allograft.

### *Microbial cultures on cord blood*

In order to determine if collection, storage and shipment of cord blood samples introduced any microbial contamination, it is important to test bioburden in cord blood. Each cord blood sample was tested for rapid growth of aerobic and anerobic organisms using tryptic-soy and fluid thioglycolate broth culture methods. In a period of 24 months, less than 0.04% of the total donors were rejected for positive bioburden outcomes (Table 1B).

### *Quarantine & microbiological quality control testing on final product post-thaw*

To ensure that none of the processing step(s) and/or reagent(s) introduced microbial contamination, one vial from each donor lot of the final UCBr product stored at -80°C was thawed and tested. Broth and plate-based cultures were monitored every 3–5 days, up to 2 weeks. Temperature and growth media were suitable to determine presence of fungi and aerobic, anerobic as well as facultative bacteria. In a period of 24 months, approximately 0.02% of the total donor lots tested were found to be positive for one or more types of contamination and summarily discarded (Table 1C).

## Purity of UCBr

### *Endotoxin Assay*

Presence of bacterial endotoxin, 'pyrogens' may induce fever and other adverse reactions caused by inflammatory mediators. Commercially available products marketed as HCT/Ps are regulated solely as section 361 of PHS Act and regulation 21 C.F.R 1271. Such products do not require endotoxin testing under cGTP. Nevertheless, reported use of such allografts often involves different routes of administration which may require information on the endotoxin levels. So, we identified criteria set forth under different regulatory frameworks while determining the allowable limit of endotoxin in UCBr. Therefore, endotoxin testing of UCBr is included in our quality control regimen. Current allowable limits for endotoxin range from 5 EU/kg bodyweight (non-IV drugs) to 0.2 EU/kg bodyweight (intrathecal application). Based on the most stringent criteria, we chose to qualify UCBr at the limit set for intrathecal applications. Considering a subject of 70 kg, 0.2 EU/kg limit allows up to 14 EU in a single application. Endotoxin levels in UCBr were determined using a kinetic chromogenic test in which the reaction

Table 2. **Determination of product purity using endotoxin assay.**

| **(A) Inhibition/enhancement test and detection of MVD** | | | | | | |
|---|---|---|---|---|---|---|
| **Sample dilution** | **S1** | **S2** | **S3** | **S4** | **S5** | **S6** |
| 1:10 | 1298% | 80% | 139% | 81% | 166% | 186% |
| 1:50 | 98% | 81% | 81% | 125% | 110% | 122% |
| 1:100 | 89% | 76% | 98% | 130% | 94% | 127% |
| 1:250 | 76% | 72% | 125% | 115% | 145% | 106% |

| **(B) Endotoxin values of seven representative UCBr by kinetic chromogenic LAL test** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **UCBr** | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
| Dilution | 1:50 | 1:50 | 1:50 | 1:50 | 1:50 | 1:50 | 1:50 |
| Sample reaction time CV | 0% Pass | 0% Pass | 0% Pass | 0% Pass | 0% Pass | 0% Pass | 0% Pass |
| Spike value (recovered) | 0.325 EU/ml | 0.570 EU/ml | 0.460 EU/ml | 0.376 EU/ml | 0.456 EU/ml | 0.786 EU/ml | 0.614 EU/ml |
| Spike reaction time CV | 2.4% Pass | 1.8% Pass | 13.1% Pass | 4.5% Pass | 0.5% Pass | 5.4% Pass | 6.1% Pass |
| Spike recovery | 54% Pass | 95% Pass | 77% Pass | 63% Pass | 76% Pass | 131% Pass | 102% Pass |
| Test suitability | Pass | Pass | Pass | Pass | Pass | Pass | Pass |
| Sample value | <2.5 EU/ml | <2.5 EU/ml | <2.5 EU/ml | <2.5 EU/ml | <2.5 EU/ml | <2.5 EU/ml | <2.5 EU/ml |

**(A)** Percentage spike recovery for UCBr from six donors using inhibition/enhancement test cartridge. Each sample was tested at four different dilution levels (1:10, 1:50, 1:100 and 1:250) with 1:50 dilution showing the most consistent spike recovery across the samples. Dilution of 1:50 was designated as MVD and used in the endotoxin testing. **(B)** Endotoxin testing parameters (sample value, spike recovery, % spike recovery and spike recovery time CV) reported for UCBr from seven random donors using EndoSafe-NexGen-PTS platform.
CV: Coefficient of variation; MVD: Maximum valid dilution; UCBr: Umbilical cord blood allograft.

time of the sample is compared with that of control standard endotoxins (CSE). We tested four dilutions of UCBr ranging from 1:10 to 1:250 (Table 2A) to determine a maximum valid dilution that will not interfere with the biochemical reaction. We concluded that dilution of 1:50 was noninterfering and consistently yielded a spike recovery within 50–200%. The exdotoxin limit for allograft release was set at <2.5 EU/ml based on sensitivity of the cartridge (0.05 EU/ml) and MVD. All UCBr samples tested were <2.5 EU/ml. Representative values of seven samples are provided in Table 2B.

### *Presence of undesirable components*

UCB based allografts are often processed using reagents that may have proteins of nonhuman origin and could potentially end up as residues in final product. Our processing technique for UCBr does not involve any such reagents and thus contaminating molecules are not expected. In order to confirm this, the proteins identified in UCBr samples by mass spectrometry were cross-checked against the Repository of Adventitious Proteins database that identifies contaminating proteins from dust, physical contact or common laboratory reagents like bovine serum albumin. No such contaminants were detected in any of the UCBr samples tested (data not shown).

## Immunogenicity of UCBr

UCB sourced allografts for homologous use are considered non-immunogenic as UCB cells are MHC class I (MHCI) dull and negative for MHC class II (MHCII) [26]. However, as applications of such allografts continue to evolve there is a need for direct measurement of the immunogenicity of the allograft itself as a part of the quality control regimen. Immune responsiveness of each of the three allogeneic human PBMC donors was evaluated in a mixed lymphocyte reaction (MLR) format with 30 UCBr donors. Proliferative response was determined using a BrdU ELISA assay on day 4 (Figure 3). Allogeneic PBMC proliferative responses to all UCBr were negligible, being equal to or below mean baseline PBMC proliferative responses with stimulatory index (SI) <1.2. In contrast, the mean two-way control PBMC allogeneic responses for the three donors had SI values between 3.2 and 28.5. In 4-day MLR reaction, all UCBr samples displayed negligible SI compared with the positive control indicating that these cells were not metabolically active. In summary, UCBr failed to stimulate human PBMCs and were nonimmunogenic.

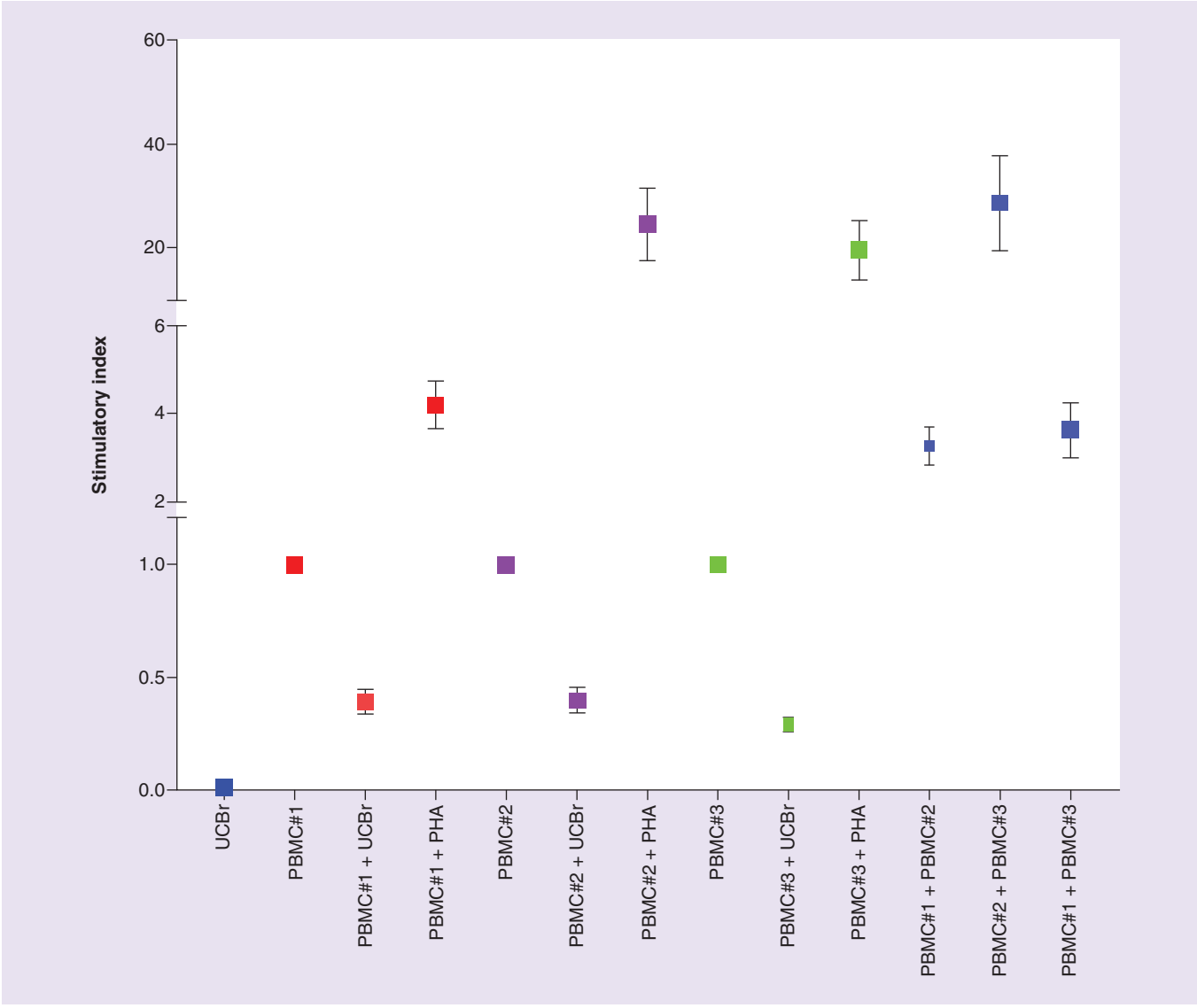



**Figure 3. Immunological characterization of umbilical cord blood allograft *in vitro*. (A)** Evaluation of UCBr immunogenicity in a two-way mixed lymphocyte reaction assay. Stimulatory index of 30 donors and three PBMCs is expressed as mean ± standard deviation. Data were analyzed by Student's t-test and $p \leq 0.05$ was considered statistically significant.
PBMC: Peripheral blood mononuclear cell; UCBr: Umbilical cord blood allograft.

## Determining the biological effects of UCBr

One of the reported applications of UCBr is for articular cartilage injuries. Articular cartilage has inherent limited healing potential, and its damage poses a challenging problem for orthopedic surgeons. The structure and function of this cartilage is dependent on the proliferation, migration and homeostasis of articular chondrocytes that are present in the cartilage. To determine potency, effects of UCBr were investigated on articular chondrocytes isolated from human knee.

### *UCBr induces chondrocyte proliferation*

Articular chondrocytes proliferate and secrete extracellular matrix to maintain and sustain the cartilage, and constitutes a promising strategy for cartilage repair [27]. UCBr lysate at 1:10 and 1:5 dilution induced higher proliferation in HC after 24 h of treatment (Figure 4A) as compared with cells cultured in SFM with no other exogenous factors. HC treated with UCBr at 1:10 dilution showed approximately fourfold increase in proliferation, whereas 1:5 dilution showed about fivefold higher proliferation. Cells that were cultured in the complete growth

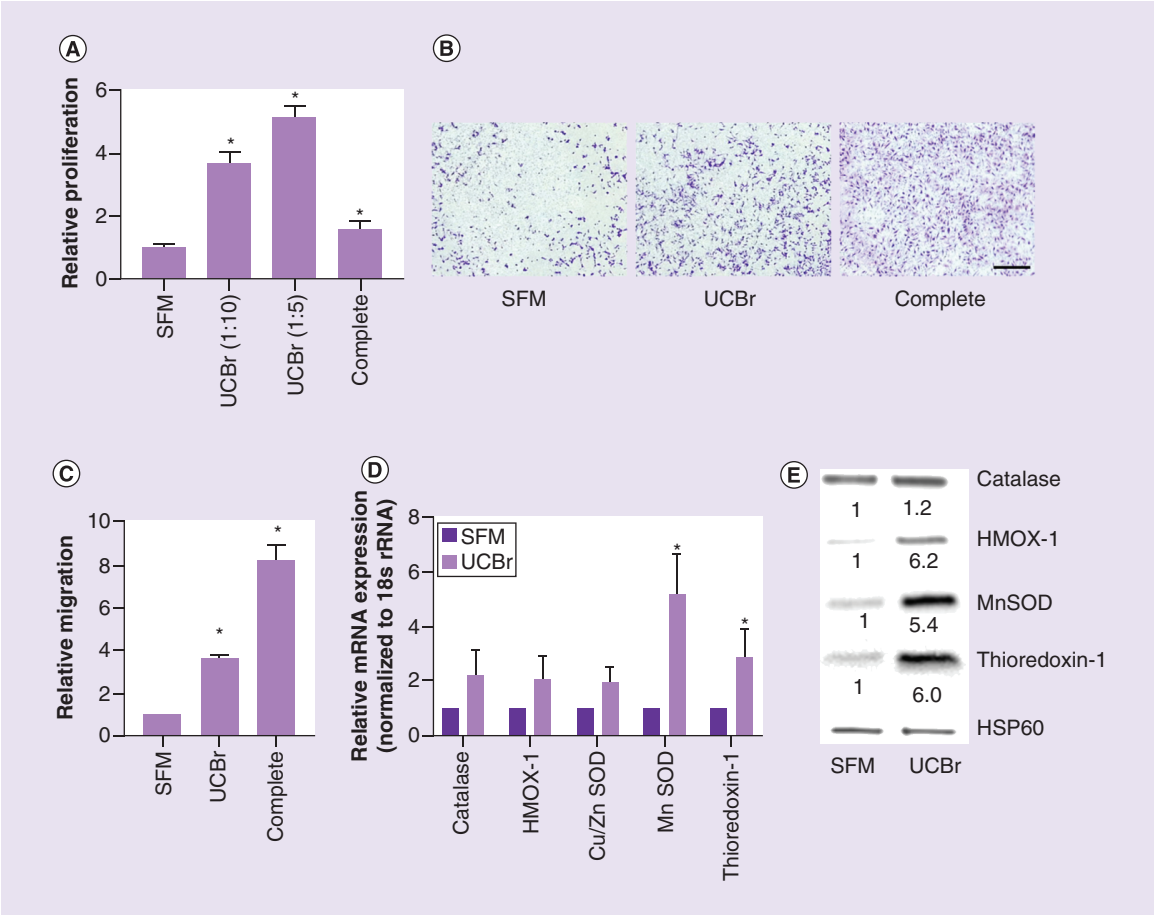



**Figure 4. Biological effects of umbilical cord blood allograft. (A)** Proliferation rate was measured by fluorescence quantification of total cellular DNA in HC treated with either SFM, UCBr lysates or complete media (10% FBS) and data presented as mean ± standard deviation relative to SFM treatment. **(B)** Cell migration of HC was evaluated using transwell system and representative photomicrographs of the migrated cells in response to SFM, UCBr lysates or complete media are provided. **(C)** Cells migrated per visual field were quantified using ImageJ, normalized first to SFM, then to the rate of proliferation and expressed as mean of relative migration ± standard deviation. Experiments were repeated three-times, each time with 3–5 donors pooled together. **(D)** qPCR was performed on RNA extracted from HC treated with SFM or UCBr lysates pooled from at least three donors. The 18S rRNA transcript level was used to normalize the data, which are presented as mean values for fold change ± standard deviation. **(E)** Protein levels of redox markers in HC treated as in **(D)** were determined by western blot. Densitometry was performed taking signals from Hsp60 as the internal control. Values are expressed relative to SFM treated control and indicated in the panels below the immunoblots. Data were analyzed by ANOVA and $p \leq 0.05$ was considered statistically significant.
*$p \leq 0.05$; Scale bar: 50 μm.
FBS: Fetal bovine serum; HC: Human knee articular chondrocytes; Hsp60: Heat Shock Protein Family D; qPCR: Quantitative reverse transcription PCR; Redox: Reduction–oxidation; SFM: Serum-free media; UCBr: Umbilical cord blood allograft.

medium served as an internal reference of the normal cell proliferation rate. The proliferation rate of the UCBr treated cells was higher than the cells cultured in complete medium. Combination of cytokines and growth factors present in UCBr promotes chondrocyte proliferation.

### *UCBr induces chondrocyte migration*

Chondrocyte migration is challenging as the cells need to overcome the density and pressure of the surrounding matrix to migrate to other sites. Research has shown that chondrocyte migration can help in restoring osteochondral defects [28]. The potential of the UCBr as a chemo-attractant was therefore specifically tested by transwell migration assay using complete growth medium (10% FBS), SFM or UCBr lysates (1:10 dilution, Figure 4B). When normalized to their respective proliferation rate, the net migratory index of the HC cells treated with UCBr was

3.6 ± 0.1 ($p < 0.05$) fold higher when compared with that of the control cells (Figure 4C). These data indicate that the UCBr may aid in osteochondral repair by stimulating chondrocyte migration.

### *Impact of UCBr on redox metabolism*

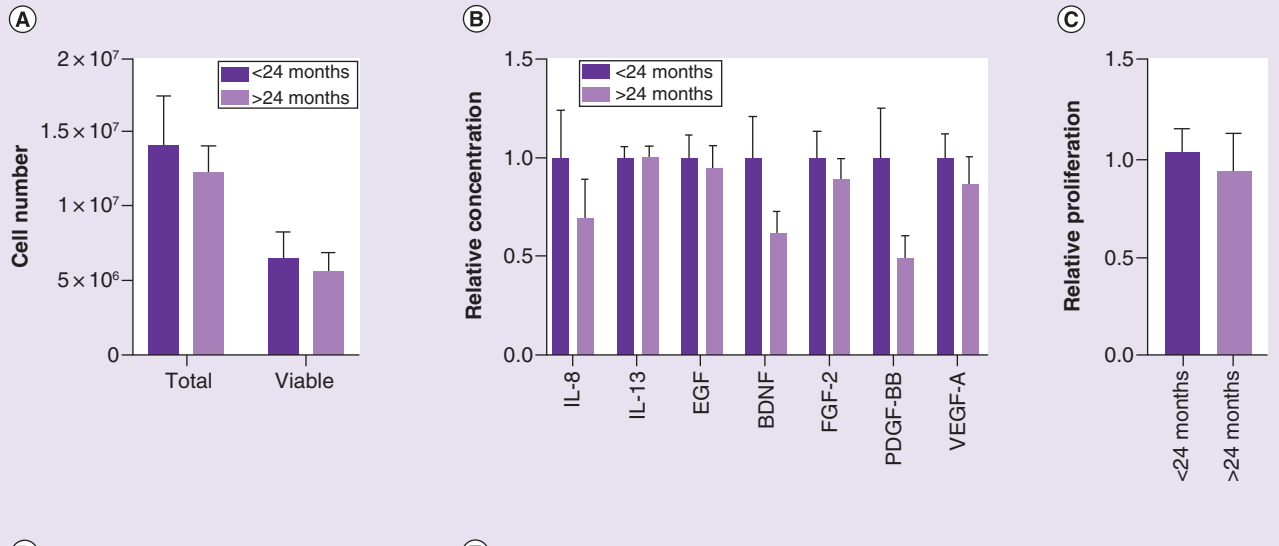
There is ample evidence that cartilage degradation is often due to impaired ROS homeostasis. Strategies to target antioxidant systems may reduce degradation and aid in repair of damaged cartilage [29]. Bioinformatic analysis of the proteins identified in UCBr lysates by mass-spectrometry indicated that several of these proteins are involved in redox metabolism. Therefore, the effect of UCBr on antioxidant enzymes was evaluated both at the transcriptional and translational levels. mRNA expression of several redox marker genes was quantified in chondrocytes treated with UCBr lysates. MnSOD and Thioredoxin 1 were significantly bolstered by 5.2-fold ($p < 0.0001$) and 2.9-fold, respectively ($p < 0.05$), when compared with untreated controls (Figure 4D). Immunoblotting of these markers further confirmed that Thioredoxin 1 and MnSOD were also upregulated at the protein levels (Figure 4E) by similar treatment. Interestingly, mRNA level of *HMOX1* was not significantly upregulated at the transcriptional level but at the protein level HMOX1 expression increased (~6.2-fold) and was similar to Thioredoxin 1 (~6.0-fold) and MnSOD (~5.4-fold). This might indicate a possible post-transcriptional modification of HMOX1 in HC. We were unable to detect Cu/ZnSOD at appreciable levels by immunoblotting of control and treated HC.

## Determining the stability of UCBr

Based on literature, the current expiry period of UCBr was set at 24 months [30]. To establish that this criterion is valid, we compared certain aforesaid quality parameters between samples that were less than 24 months (N = 26, median of 3 months) and those above 24 months (N = 23, median 25 months). No significant difference in cell viability was observed between these groups (Figure 5A). As cytokines in UCBr are the key factors influencing tissue regeneration, concentration of seven cytokines (IL-8, IL-13, EGF, BDNF, FGF-2, PDGF-BB and VEGF-A) was measured in these two groups. compared with the samples grouped under <24 months, there was a slight but not statistically significant drop in the relative cytokine concentration (Figure 5B). The biological potency of the products was tested based on its effect on proliferation of HC. Proliferative rate of the samples less than 24 months and greater than 24 months were similar without any statistically significant difference (Figure 5C). Four additional samples between 24 and 30 months were tested for their aseptic nature and no growth was detected (Figure 5D). The endotoxin values of five representative samples between 24 and 30 months were also tested for their endotoxin limits and were found to be <2.5 EU/ml (Figure 5E) same as the samples with median age of 3 months as shown in Table 2B.

## UCBr increases the yield of MSC derived exosomes

Current dogma in regenerative medicine indicates that the EVs including exosomes released by the MSC have therapeutic benefits. Altering the conditions of culturing MSC have a significant influence not only on the yield but also on the efficacy of exosomes in terms of the proteomic and genomic complexities [14]. Here, we investigated if exosomes derived from MSC treated with UCBr lysates differ in yield and biological functions when compared with the ones derived from untreated MSC. EVs were characterized based on particle concentration and size distribution using fluorescence-based Nanoparticle Tracking Analysis which specifically identifies microparticles with intact membranes (Figure 6A & B). The concentration of the EVs in the samples obtained from UCBr treated MSC was significantly higher (~twofold) compared with the untreated counterpart (Figure 6C). Nano Tracking Analysis also confirmed a more homogeneous population of EVs obtained from untreated MSC (mean 104.4 ± 20.7 nm) compared with samples obtained from treated MSC which ranged from 91 to 698 nm, with a mean of 288.7 ± 4.1 nm (Figure 6A & B). The heterogeneity of the particle size identified in samples derived from treated MSC may be attributed to the yield of a higher ratio of EVs of varying size in response to the UCBr treatment. The proliferative effect of these EVs was further tested on MSC and HC. EVs derived from UCBr lysate treated MSC showed approximately 2.1-fold (MSC) to approximately fivefold (HC) increase in proliferative rate when compared with the respective controls (Figure 6 D & E). Proteomic analysis of MSC-derived EVs has shown that they contain the factors influencing angiogenesis such as FGF, VEGF, HGF, EGF and IL-8 [13]. We measured the concentration of EGF and VEGF present in the exosomes derived from treated or nontreated MSC. In unit volume, EGF was approximately 2.14-fold higher and VEGF was approximately 2.8-fold higher in the exosomes released by the MSC pretreated with UCBr lysate (Figure 6C).




D

| No. of samples >24 months | 4 |
|---|---|
| Growth detected under following conditions | |
| Tryptic soy broth (22.5°C) | 0/4 |
| Sabdex broth (22.5°C) | 0/4 |
| Tryptic soy broth (32.5°C) | 0/4 |
| Fluid thioglycolate (32.5°C) | 0/4 |
| Tryptic soy agar plate (22.5°C) | 0/4 |
| Sabdex agar plate (22.5°C) | 0/4 |
| Tryptic soy agar plate (32.5°C) | 0/4 |

E

| >24 months | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 |
|---|---|---|---|---|---|
| Slope: | -0.402 | -0.402 | -0.402 | -0.371 | -0.371 |
| Intercept: | +2.319 | +2.319 | +2.319 | +2.402 | +2.402 |
| Spike value (recovered): | 0.833 EU/ml | 1.22 EU/ml | 0.863 EU/ml | 0.699 EU/ml | 0.632 EU/ml |
| Spike reaction time CV: | 13.6% pass | 9.7% pass | 21% pass | 5.5% pass | 3.4% pass |
| Spike recovery: | 98% pass | 144% pass | 102% pass | 91% pass | 82% pass |
| Sample value: | <2.5 EU/ml | <2.5 EU/ml | <2.5 EU/ml | <2.5 EU/ml | <2.5 EU/ml |

**Figure 5. Determination of umbilical cord blood allograft stability.** UCBr were divided into two groups based on their manufacturing date, less than 24 months (N = 9–26) or greater (N = 4–23). Cells were stained with AO/PI and counted with Nexcelom K2. Total and viable cell count **(A)** was determined. **(B)** MAGPIX protein multiplexing assay was performed to determine cytokine concentration in UCBr lysates grouped as in **(A)** with N = 20 donors in each group. Cytokine concentration of samples grouped as >24 months was expressed relative to the mean concentration of the UCBr samples grouped as <24 months. **(C)** Proliferation of HC treated with UCBr lysates was determined by CyQUANT assay. Data are normalized to samples grouped as <24 months and represented as mean of relative proliferation rate ± standard deviation. **(D)** Data summarize growth-based microbiological testing to confirm aseptic nature for UCBr from four donors grouped as >24 months. **(E)** Endotoxin levels for UCBr from five donors grouped as >24 months was tested using EndoSafe-NexGen-PTS platform. Data were analyzed by Student's t-test and $p \leq 0.05$ was considered statistically significant.
AO: Acridine orange; HC: Human knee articular chondrocytes; PI: Propidium iodide; UCBr: Umbilical cord blood allograft.

## Discussion

As the clinical application of UCB allografts gains prominence, the mechanistic link between these products and tissue regeneration must be fully explored. Research on such allografts has mainly focused on the stem cell content due to their origin from birth tissues. However, studies in preclinical models make it evident that rather than stem cells, it is the trophic factors that have actual therapeutic impact *in vivo* [8]. We have previously shown that UCB-sourced allografts can induce proliferation, migration and angiogenesis by virtue of the cytokines present in UCB. The knowledge being gained from ongoing research on such allografts has prompted efforts to identify new quality control parameters that may be useful to define suitability release criteria. As the regulatory guidelines continue to evolve, parameters addressing regenerative mechanisms will also be likely included in the developing quality control framework. This is a first-of-its-kind study to put forth a set of quality control attributes which can be used to determine the clinical suitability of UCB-sourced allografts.

We enumerated the cellular component in addition to quantifying cytokines and identifying novel signaling proteins to have a complete picture of the allograft. The total cell count and percentage of cell viability was

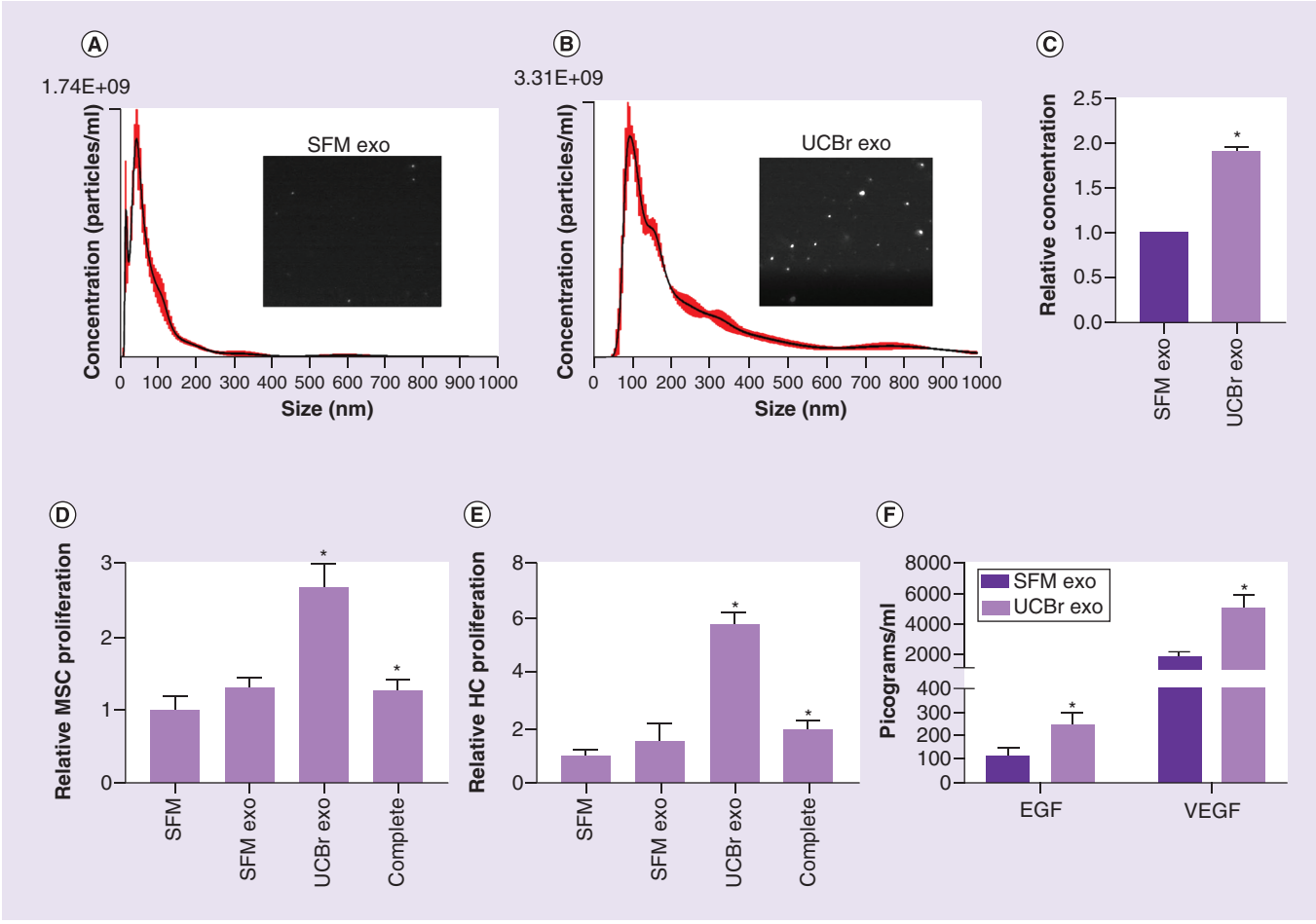



**Figure 6. Effect of umbilical cord blood allograft on mesenchymal stem cell derived exosome.** MSC were treated with SFM or UCBr lysate for 18 h and exosomes were collected from the respective conditioned media using protocol described in materials and method section. **(A & B)** Quality/size distribution and representative screenshots of the isolated exosome as determined by fluorescence based nanoparticle tracking assay. **(C)** Relative yield of the microparticles by MSC treated with SFM or UCBr lysate is provided as mean ± standard deviation. **(D & E)** Proliferation of MSC **(D)** and HC **(E)** treated with either SFM, SFM-Exo, UCBr-Exo or complete media was measured by CyQUANT assay and values are expressed as mean fold change ± standard deviation. **(F)** EGF and VEGF levels were measured using Luminex xMAP assay on MAGPIX™ platform in exosomes isolated from MSC treated with SFM or UCBr lysates.
Data were analyzed by Student's t-test or ANOVA and $p \leq 0.05$ was considered statistically significant.
*$p \leq 0.05$.
Exo/UCBr-Exo: Exosomes derived from MSC treated with SFM/UCBr; HC: Human knee articular chondrocyte; MSC: Mesenchymal stem cell; SFM: Serum free media; UCBr: Umbilical cord blood allograft.

consistent among the samples tested. Although cell viability and its metabolic activity cannot be correlated to the efficacy of the product, it is indicative of the uniformity of the manufacturing process. We identified a significant percentage of viable cells in UCBr; however, a very low fraction stained positive for stem cell markers according to the International Society for Cellular Therapy guidelines [31]. These data do not preclude the possibility of stem cell presence in the cord blood sourced allograft cells. Isolation of stem cells from UCB has been successfully accomplished previously using specific tissue culture approaches [20,32]. It is interesting to see if UCBr cells cultured under such *in vitro* conditions over extended period of time enables better immunostaining of the stem cell surface markers. However, it is beyond the scope of this quality assessment study and may be a focus of future research. While we do not foresee dependence of UCBr on the viable cell population as a function of its clinical benefit, it demonstrates that the DMSO free cryopreservation techniques are gentle but effective to sustain these delicate cells. The two-way mixed lymphocyte reaction indicated that the cells present in UCBr do not proliferate, and the biological effects are independent of the metabolic function of these cells. Proteomic profiling of the samples revealed several proteins that can influence wound healing, ROS metabolism etc. To our knowledge, this is the first

report identifying global proteins associated with UCB allografts. Random sampling for proteomic profiling as a part of routine quality control may identify possible deviation in the manufacturing process. About 31 cytokines that have a direct or indirect role in tissue regeneration were quantified from a large cohort of donors and found to be consistent. While donor variability is inevitable, strict inclusion–exclusion criteria based on quantitative identification of critical trophic factors can limit product variability and be a part of the quality control parameter.

cGTP guidelines were followed for determining the bioburden of the donated cord blood and the aseptic nature of UCBr. Less than 10% of the donated blood samples were rejected due to serology outcomes highlighting the importance of effective donor screening with well-defined donor inclusion–exclusion criteria. Processing in a constantly monitored, controlled environment can drastically reduce microbial contamination. In a span of 24 months, 99.98% processed products were aseptic. Allografts regulated as HCT/Ps are not required to be tested for endotoxin levels or immunogenicity. But based on the analysis of the reported use of these allografts, these tests may bear clinical relevance. Endotoxin levels of all UCBr products tested were <2.5 EU/ml using the most stringent criteria of allowable endotoxin limit for pharmaceuticals. Testing endotoxin levels on each donor lot may be incorporated as a routine quality control measure to ensure clinical safety on certain instances. UCBr was nonimmunogenic as determined by MLR, on the contrary it suppressed the proliferation of the allogeneic PBMC. This can partially be attributed to the anti-inflammatory cytokines like IL-6, -10 and -13. Concentration of another anti-inflammatory cytokine IL-1RA was approximately 30,000 pg/ml, the highest among all the cytokines tested. Clinical trials on inhibition of IL-1 activity with IL-1RA have concluded that it is a safe and efficient way of alleviating GvHD that failed to respond to conventional treatment [33]. In future, it would be interesting to study the effects of UCBr in mitigating GvHD using preclinical models. Assessing the immunogenicity of the product at routine intervals indicates that the minimal processing requirement is effectively met such that the inherent nonimmunogenic property of the cord blood has not been compromised.

Apart from safety, another critical part of controlling product quality is determining consistency and lack of major variability from lot to lot. Reported use of UCB allografts in cartilage and soft tissue repair prompted us to perform experiments using articular chondrocytes as the model system. HC shows a continuous age-related decline in the proliferative response but can be activated by the mitogenic factors and chemokines like PDGF, SDF1α and VEGF [34–36]. Cell free extracts of UCBr induced both proliferation and migration of chondrocytes by several folds. Results observed with a series of individual growth factors and cytokines identified PDGF as the most potent chemotactic factor for human articular chondrocytes [28]. Cytokine profiling of UCBr has confirmed presence of PDGF and several other factors. ROS generation is another age dependent factor leading to cartilage degradation [37,38]. Lack of defense toward the elevated levels of ROS generated from dysfunctional mitochondria has been shown to be mitigated by elevating the expression of antioxidant proteins [39,40]. Proteomic profiling of UCBr identified proteins that demonstrated involvement in ROS homeostasis. Therefore, we evaluated the effect of UCBr on expression of antioxidants. UCBr specifically upregulated MnSOD, Thioredoxin 1 and HMOX-1 but not Catalase, indicating that the antioxidant effect may be specific than indiscriminate. HMOX-1 is known to play a protective role against tissue injury in human cartilage by upregulating MnSOD, and is a therapeutic target in several pathological conditions related to elevated ROS generation [41]. Our data indicate potential involvement of several cytokines, enzymes and signaling molecules of UCBr in activating proliferation and migration of chondrocytes coupled with building antioxidant defense mechanisms to help in cartilage repair.

Long-term stability is another integral aspect of quality assessment. Volume reduced cord blood stored up to 15 years showed no alteration in post thaw quality [30,42,43]. However, there are no established guidelines to determine the maximum storage period for cryopreserved UCB sourced allografts. Initial storage conditions of UCBr at less than -65°C was set for a period of 2 years. All post-thaw quality attributes were met by samples stored between 1 month and 25 months with no significant differences. Given these results, it can be concluded that UCBr is stable up to 2 years, but ongoing analysis as required by regulatory agencies will determine the actual expiration period.

With the advancement of our knowledge on secretome based therapy, we have started to appreciate the role of EVs/exosomes and consider it to be the next quantum leap in regenerative medicine [44–47]. While MSC derived exosomes are known to contain bioactive molecules that can alter the genetic and proteomic landscapes of the target cells, we were particularly interested to know if the yield and function of exosomes can be modulated by application of UCBr. This will be clinically relevant because host MSC activated by UCBr can release exosomes acting as 'enriched capsules' to trigger a more sustained regenerative outcome. Fluorescence-based nanoparticle tracking analysis identified intact EV-specific particle size and concentration. Current EV isolation methods may

include protein aggregates, membrane fractions and other background particles, which have confounding effects. The structural integrity of the prepared EVs needs to be further characterized by cryo-electron microscopy. While most of these ideas are speculative, initial experiments indicate that exosomes derived from UCBr primed MSC have a higher cytokine content and proliferative effect, along with a higher yield. These data argue favorably for a potential liaison among UCBr, host MSC and their derived exosomes to influence tissue regeneration *in vivo*. We are actively pursuing this line of research to elucidate the mechanistic role of EVs in tissue regeneration in greater detail.

## Conclusion

As the field of regenerative medicine continues to evolve, the parameters required to establish its quality must be reviewed periodically and updated accordingly. Here, we put forward a set of experimental approaches to access the identity, safety, purity, potency, and stability of this UCB-based allograft.

## Translational perspective

Procuring birth tissues to manufacturing allografts for regenerative therapy has been one of the most remarkable accomplishments in medical practice. However, leveraging the full benefit of these allografts depend on three major factors. First, there is a need to have a complete understanding of the molecular mechanism of action of the allograft; second, to exercise end-to-end control in the processing of the allograft from the birth tissues and at last to have adequate checks and balances in the quality control regime to maintain the highest standard of clinical safety. As the clinical demand of the allografts increases, the regulatory oversight on their manufacturing, marketing and clinical use is parallelly evolving to ascertain that patient safety and interest are not compromised. Considering these factors currently concerning the clinicians practicing regenerative medicine, we performed an array of experiments to develop a quality control regime suitable to clinically qualify a UCB allograft, based on its molecular/cellular component, purity, safety, efficacy and stability. Lessons from both translational research and ongoing clinical trials have led to a paradigm change in identifying the role of exogenous stem cells in tissue repair with concomitant appreciation of the effects of trophic factors in activating the endogenous host stem cells to execute the regenerative process. Therefore, when accounting for the clinical efficacy of UCB allografts, quality control measures should focus more on its ability to initiate signaling pathways to efficiently activate the host's MSC and other healing cells at the site of injury. In the future more clinical studies are required to explore the role of cytokines and growth factors associated with these allografts to fully harness their healing potential.

### Summary Points

- Umbilical cord blood (UCB) sourced allografts are widely used in clinics for trauma and degenerative conditions. Since many products are registered as human cells, tissues, and cellular and tissue-based products, a defined regulatory framework is still evolving to address quality control attributes for such allografts.
- In this study, we identified tests to address critical quality attributes of UCB-allograft (UCBr) from a safety and potency perspective. In practice, identity, purity and safety tests can be adapted from existing guidelines, but potency assays/end points will vary based on reported application of the product.
- Here, we characterized the 'identity' of UCBr-cellular components, based on cell count, diameter and viability. Flow cytometric analysis of the mixed cell population using stem cell surface markers was used to effectively categorize cellular population in UCBr. Mass spectrometry and protein multiplexing in decellularized UCBr quantified several different proteins, cytokines, growth factors and signaling molecules which constitutes the noncellular portion of this allograft. These tests may be routinely employed to monitor the product and assess the consistency of the manufacturing techniques.
- Endotoxin assay was used to determine 'purity' of the allograft. Aseptic clinical products derived from biological materials have an inherent risk of microbial contamination in the final product. Stringent donor inclusion–exclusion criteria were established based on peripheral blood serology and cord blood microbial testing. Additionally, cryopreserved final product underwent 'safety' testing based on growth-based microbiological cultures.
- Based on the reported application UCBr in cartilage repair and regeneration, biological efficacy of UCBr was tested *in vitro* using human chondrocytes.
- UCBr stored at temperature less than -65°C was stable up to 24 months after production. Stability was established with protein multiplexing (stable chemical composition), endotoxin assay, potency assays (intact biological efficacy) and microbiological testing (maintaining aseptic nature).
- Identifying the tests that will characterize a biologics product is often challenging. To the best of our knowledge, this is the first report enumerating the tests to address identity, purity, safety and potency for a UCB allograft which may be developed into qualified and validated assays.

### Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/rme-2019-0058

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### Financial & competing interests disclosure

MS Sane, H Tang, S Banerjee Mustafi, CD Jones and S Malara are employees of Burst Biologics. N Misra was previously employed at Burst Biologics. Burst Biologics has filed a patent pertaining to the processing method of the UCB sourced allograft described in the manuscript. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

### Ethical conduct of research

UCB was procured from consenting adults and manufactured in a US FDA-registered facility following all necessary regulatory guidelines as per the FDA's current Good Tissue Practice.

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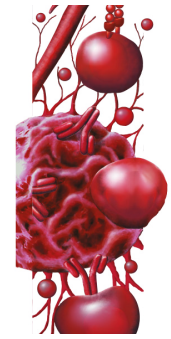

# Extramedullary leukemia relapse after allogeneic stem cell transplantation: a novel mechanism of immune escape?

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**Background:** Relapse is a significant cause of treatment failure after allogeneic stem cell transplantation. In many cases relapse occurs when leukemic cells escape from immune surveillance. **Methods & results:** In the setting of haploidentical transplantation, immune escape is usually the result of the loss of the mismatched haplotype from leukemic cells, while downregulation of HLA-expression has been postulated as a significant cause of immune escape after transplantation with the use of HLA-matched donors. We observed that patients with acute leukemia who relapse at the time of active graft-versus-host-disease, usually develop extramedullary leukemia while they remain free of leukemia in peripheral blood and bone marrow. **Conclusion:** Our observation points toward a novel mechanism of immune escape which is microenvironment-specific.

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**Keywords:** allogeneic stem cell transplantation • extramedullary relapse • graft-versus-host-disease • immune escape • leukemia • tumor microenvironment

Relapse occurs in a significant percentage of patients with hematological malignancies after allogeneic stem cell transplantation (allo-SCT). Although relapse may occur as a result of refractoriness of leukemic cells to conditioning chemotherapy, in many cases relapse is due to the inability of donor immune system to eradicate residual leukemia cells that survived conditioning. Understanding the mechanisms of relapse would be of fundamental importance for the development of novel therapeutic and or prophylactic strategies.

New mutations are acquired by the leukemic cells over time and contribute to leukemia relapse. However, in the post-transplant setting the contribution of novel mutations as a mechanism responsible for relapse is largely unknown due to limited number of studies performed with the use of modern sequencing techniques. In an elegant study conducted by Christopher *et al.*, enhanced exome sequencing was performed in samples taken from patients with acute myeloid leukemia (AML) at baseline and at the time of post-allo-SCT relapse. Interestingly, they observed that the mutations found at the time of post-transplant relapse were similar to the mutations found at the time of initial presentation [1].

Clearance of leukemia cells after allo-SCT is for the most part mediated through the activation of donor immune system against alloantigens expressed by leukemic cells. Given the importance of the so-called graft-versus-leukemia (GVL) effect, it is reasonable to speculate that AML re-emergence after transplantation is due to escape of leukemic cells from the immune surveillance mediated by alloreactive donor T cells.

In the setting of haploidentical stem cell transplant, immune escape has been attributed to the loss of the mismatched HLA haplotype [2]. In a haploidentical stem cell transplant study, genomic loss of the patient-specific HLA haplotype in leukemic cells was observed in 23 out of 69 (33%) cases of relapse [3]. On the contrary, none of the three patients who underwent a mismatched unrelated donor (MMUD) allo-SCT had detectable deletions or mutations in mismatched *HLA* genes [1].

In the setting of allo-SCT from HLA-matched related or unrelated donors, an important mechanism of immune escape is the downregulation of HLA expression by the leukemic cells. In a previous study, RNA expression analysis was performed on AML samples obtained at the time of initial presentation and at the time of post-allo-SCT relapse. An important finding was the decreased expression of class-II but not class-I *HLA* genes. The downregulation of *HLA* class-II gene was also confirmed at the protein level by using flow cytometry analysis on purified leukemic cells [1].

Relapse after allo-SCT might be explained by T-cell exhaustion induced by the continuous state of activation of alloreactive donor T cells. In a recent study, authors examined the expression of immune inhibitory receptors on donor T cells. Alloreactive $CD8^+$ T cells with specificity against minor histocompatibility antigens displayed increased expression of PD-1, TIM-3 and TIGIT compared with CMV-specific $CD8^+$ T cells from healthy donors [4].

In this paper, we describe a pattern of relapse observed in patients with acute leukemia who relapse after allo-SCT.

## Cases of patients

From 2013 to 2018, 127 patients with acute AML, high-risk myelodysplastic syndrome (MDS) and acute lymphoblastic leukemia (ALL) underwent allo-SCT in our department. Diagnosis and staging of acute and chronic graft-versus-host-disease (GVHD) was performed according to Mount Sinai acute GVHD International Consortium (MAGIC) and NIH consensus criteria, respectively [5,6].

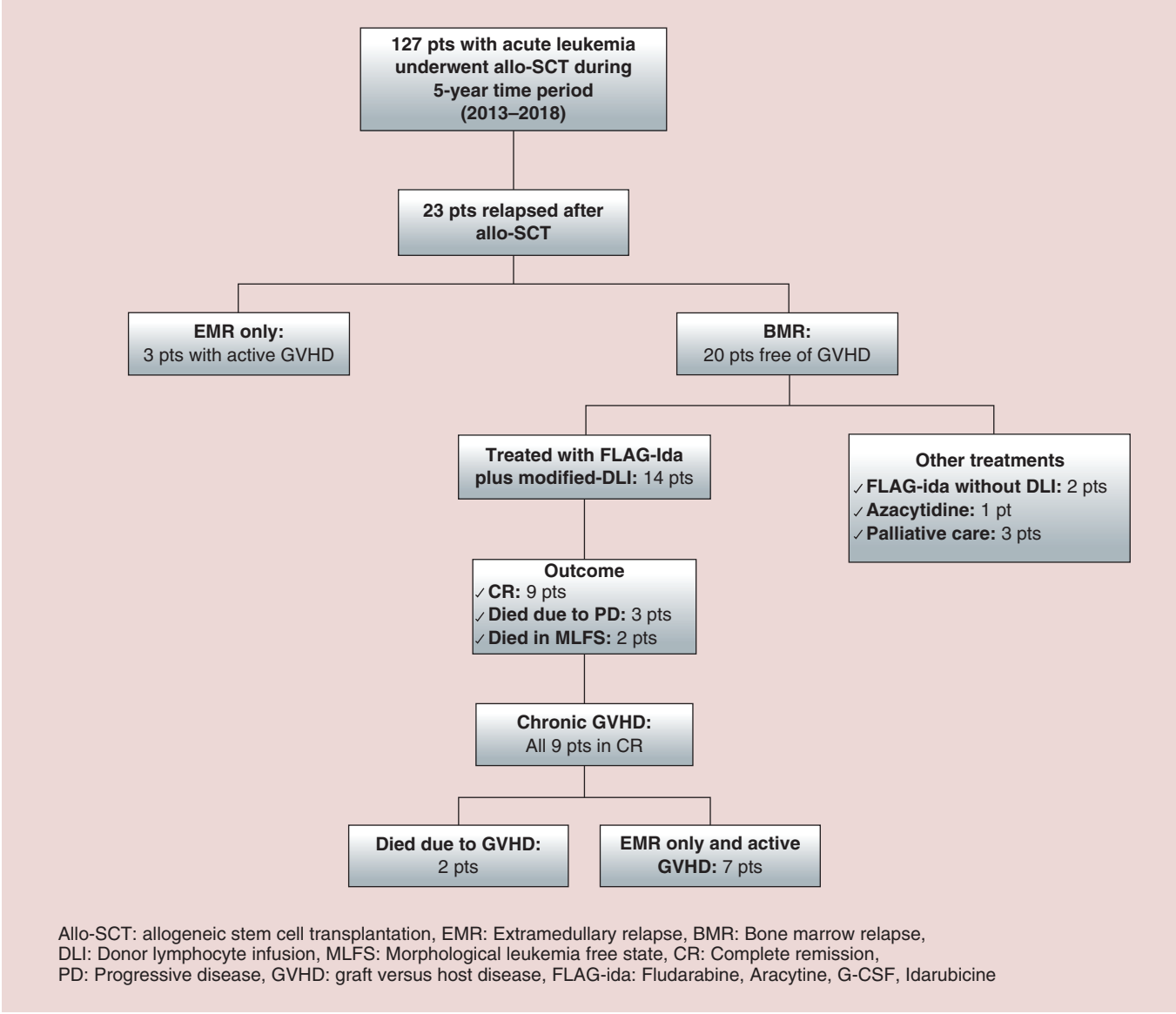
Our standard approach, for patients with acute leukemia or high-risk myelodysplastic syndrome who relapse after allo-SCT and while free of active GVHD, is salvage chemotherapy followed by modified-donor lymphocyte infusion (DLI) infusion and a short course of low dose cyclosporine (CyA). In more detail, salvage chemotherapy consisted of FLAG-Ida regimen (fludarabine, cytarabine, idarubicin, G-CSF priming). Modified-DLI is collected after previous G-CSF stimulation. G-CSF at a dose of 5–10 mcg/kg is administered to original donor for 4 days before peripheral blood mononuclear cell collection. In many cases, a previously frozen portion of the original graft was used as modified-DLI. For those cases without back-up frozen graft available, a second peripheral blood stem cell (PBSC) mobilization with G-CSF stimulation of the original donor was performed. The median CD34(+) and CD3(+) content of modified-DLI was $1.5 \times 10^6$ and $5 \times 10^7$ per kg of recipient body weight (BW). CyA was administered at a dose of 1 mg/kg for 30 days and then abruptly discontinued. Written informed consent was obtained by all patients included in this study. In our department, all patients referred for allo-SCT asked to give their permission for future anonymous use of data regarding their disease, treatment, etc. The study was approved by the bioethics committee and Institutional Review Board (IRB).

23 out of 127 patients had disease relapse and, among them, three patients relapsed while they had active chronic-GVHD. All three patients with active chronic-GVHD at the time of relapse had isolated extramedullary relapse (EMR) without peripheral blood (PB) and/or bone marrow (BM) involvement, while all 20 patients who relapsed while they were free of active GVHD had BM relapse (BMR). Type of relapse and treatment after relapse are shown schematically in Figure 1. Characteristics of patients with EMR after allo-SCT are shown in Table 1.

Fourteen out of 20 patients who relapsed while free of active GVHD received salvage chemotherapy with the Ida-FLAG regimen followed by modified-DLI (CD3 cell dose: $5–10 \times 10^7$/kg) 5 days after the end of chemotherapy. All patients received low dose CyA for 30 days in order to prevent severe acute GVHD. Three patients received only palliative treatment, two patients received Ida-FLAG without DLI administration due to unavailability and one patient received treatment with azacytidine only.

Nine out of 14 patients treated with our standard approach achieved complete morphological remission, two patients died during aplasia but in a morphologically leukemia-free state and three patients died from progressive disease without any clinical or laboratory finding of GVHD. Six out of nine patients who developed acute GVHD were treated successfully with steroids. All nine patients developed moderate to severe chronic GVHD. Two out of nine patients died of severe steroid-refractory chronic GVHD. Seven patients had isolated EMR after salvage treatment while they were in a state of active chronic GVHD. Characteristics of the seven patients with EMR only are shown in Table 1.

Interestingly all ten patients who relapsed while they were in a state of active chronic GVHD had isolated extramedullary involvement. Seven of these patients survived for at least 6-months after relapse and surprisingly in none of them PB and/or BM involvement was detected during follow-up. The treatment used, as well as the outcome of all patients with extramedullary relapse are shown in Table 2.




Allo-SCT: allogeneic stem cell transplantation, EMR: Extramedullary relapse, BMR: Bone marrow relapse, DLI: Donor lymphocyte infusion, MLFS: Morphological leukemia free state, CR: Complete remission, PD: Progressive disease, GVHD: graft versus host disease, FLAG-ida: Fludarabine, Aracytine, G-CSF, Idarubicine

**Figure 1. Treatment schema.**

## Discussion

Previous studies have shown that EMR after allo-SCT occurs in a significant proportion of patients with AML. EMR tends to occur at later time points and is associated with better overall survival in comparison with BMR [7].

Overall, our data showed that relapses occurring in the presence of active GVHD are usually of extramedullary origin with leukemia-free BM. Twenty patients with BMR were GVHD-free at the time of relapse, while all ten patients with only EMR had active chronic-GVHD at the time of relapse. Interestingly, these ten patients developed extramedullary disease in multiple sites while they remained leukemia-free in the BM for a median time of 9 months (range, 3–18 months).

These findings point toward a novel mechanism of immune escape after allo-SCT resulting in leukemia relapse. The absence of leukemia relapse in PB and BM in a patient who shows progression in multiple extramedullary sites is suggestive of a microenvironment-dependent GVL effect.

None of the proposed immune escape mechanisms described above, such as loss of the mismatched haplotype after haploidentical allo-SCT, reduced HLA-class II expression after matched unrelated allo-SCT and/or T-cell exhaustion after protracted stimulation, can explain the type of relapses observed in our study.

**Table 1. Patients characteristics.**

| Relapse after allo-SCT at the time of active chronic GVHD | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Patient (No)** | **Sex** | **Age** | **Disease** | **Disease risk according to ELN-2017** | **Disease status at allo-SCT** | **Lines of treatment before allo-SCT** | **Extramedullary involvement at diagnosis** |
| 1 | F | 53 | AML | High (complex-monosomic karyotype) | CR1 | 1 | Yes |
| 2 | M | 32 | AML | High (*FLT3-ITD* high mutant burden) | CR2 | 3 | No |
| 3 | M | 64 | AML | High (complex-monosomic karyotype) | Refractory disease | 2 | No |
| **Relapse after salvage at the time of active chronic GVHD (All patients relapsed after allo-SCT, treated with Ida-FLAG plus modified-DLI and short course cyclosporine)** | | | | | | | |
| 4 | F | 41 | ALL | High due to t (4;11) | CR1 | 1 | No |
| 5 | F | 32 | AML | Intermediate (normal karyotype) | CR1 | 1 | No |
| 6 | F | 34 | AML | Intermediate (normal karyotype) | CR2 | 2 | No |
| 7 | M | 54 | AML | High-risk (complex karyotype) | Refractory disease | 2 | No |
| 8 | M | 29 | AML | High-risk (*MLL*-rearrangement[†]) | CR1 | 1 | No |
| 9 | M | 39 | AML | Intermediate risk (normal karyotype) | Primary refractory[‡] | 2 | No |
| 10 | M | 35 | AML | Intermediate risk (normal karyotype) | CR1 | 1 | No |

[†]Detected by FISH – partner gene was not identified but PCR for t (9;11) was negative.
[‡]Not in CR after double induction, he underwent allo-SCT while in morphological CR after salvage with FLAG-Ida.
ALL: Acute lymphoblastic leukemia; Allo-SCT: Allogeneic stem cell transplantation; AML: Acute myeloid leukemia; DLI: Donor lymphocyte infusion; FLAG-Ida: Fludarabine, cytarabine, idarubicin, G-CSF priming; GVHD: Graft-versus-host-disease.

**Table 2. Treatment after relapse and outcome.**

| Relapse after allo-SCT at the time of active chronic GVHD | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Patient (No)** | **Type of conditioning** | **Donor** | **Source of graft** | **Time of relapse after allo-SCT** | **Treatment of relapse** | **Death** | **Duration of survival after relapse** | **Sites of involvement after initial relapse** | **PB and or BM involvement after initial relapse** |
| 1 | MAC | MSD | PBSC | 12 m | Azacytidine | Yes | 18 m | Breast, intestine, subcutaneous nodules | No |
| 2 | MAC | MSD | PBSC | 15 m | Azacytidine, Irradiation | Yes | 14 m | Spinal cord | No |
| 3 | RIC | MUD | PBSC | 18 m | Azacytidine | No | + 9 m | Intestine | No |
| **Relapse after salvage at the time of active chronic GVHD (All patients relapsed after allo-SCT, treated with Ida-FLAG plus modified-DLI and short course cyclosporine)** | | | | | | | | | |
| **Patient (No)** | **Type of conditioning** | **Donor** | **Source of graft** | **Time of relapse after salvage plus DLI** | **Treatment of relapse** | **Death** | **Duration of survival after relapse** | **Sites of involvement after initial relapse** | **PB and or BM involvement after initial relapse** |
| 4 | MAC | MSD | PBSC | 3 m | No | Yes | 4 m | Heart | No |
| 5 | MAC | MSD | PBSC | 9 m | Irradiation | No | + 12 m | CNS | No |
| 6 | MAC | MSD | PBSC | 12 m | No | Yes | 3 m | CNS | No |
| 7 | RIC | MSD | PBSC | 6 m | Yes | Yes | 6 m | Subcutaneous nodules | No |
| 8 | MAC | MSD | PBSC | 5 m | Chemotherapy Nivolumab | Yes | 9 m | Subcutaneous nodules, jaw, stomach, liver | No |
| 9 | MAC | MSD | PBSC | 3 m | No | Yes | 4 m | CNS | No |
| 10 | MAC | MSD | PBSC | 6 m | Sorafenib, irradiation, chemotherapy | Yes | 16 m | Subcutaneous nodules, liver, heart, lymph nodes, jaw | No |

Allo-SCT: Allogeneic stem cell transplantation; BM: Bone marrow; DLI: Donor lymphocyte infusion; FLAG-Ida: Fludarabine, cytarabine, idarubicin, G-CSF priming; GVHD: Graft-versus-host-disease; MAC: Myeloablative conditioning; MSD: Matched sibling donor; MUD: Matched-unrelated donor; PB: Peripheral blood; PBSC: Peripheral blood stem cell; RIC: Reduced intensity conditioning.

An important question that needs to be answered is: what is the difference between bone marrow microenvironment and that of other tissues? Why are leukemic cells susceptible to GVL effect in the BM microenvironment while the same immune effect is not active within extramedullary tissues?

Possible tumor microenvironment-dependent immune escape mechanisms have been proposed such as excess production of anti-inflammatory cytokines or of immunosuppression-inducing enzymes in leukemic microenvironment.

High concentration of anti-inflammatory cytokines present in tumor microenvironment can neutralize the cytotoxic potential of invading donor T cells. Indeed, previous studies have shown increased production of anti-inflammatory cytokines by leukemic cells in a significant proportion of patients. Chronic myeloid leukemia (CML) cells have shown capability of producing IL-4, TGF-β, while chronic lymphocytic leukemia (CLL) cells produce increased amounts of IL-10. IL-10 and IL-4 production has been shown in different types of leukemia cells [8,9].

Leukemic cells produce metabolically active molecules such as indoleamine 2,3-dioxygenase-1 (IDO1), arginase, CD39 and CD73. IDO is an intracellular enzyme that catalyzes tryptophan degradation resulting in the production of kynurenine. Kynurenin has an inhibitory effect on T-cell function while it is also an inducer of Treg production. Consistent with these data is the observation of poor prognosis in childhood AML with high IDO1 expression [10].

Arginase is an enzyme that leads to depletion of the amino acid arginin which is an important regulator of T-cell function. Arginin depletion results in inhibition of T-cell proliferation and polarization of macrophages toward a suppressive M2 phenotype. AML cells display arginase activity and create a state of immunosuppression in their microenvironment [11].

## Conclusion & future area of research

A possible explanation for this microenvironment specific immune escape is that certain tissue-environments induce the expression of anti-inflammatory cytokines or of immunosuppression-inducing enzymes. As a result, a site-specific 'tumor-shield' is formed that protects neoplastic cells from immune attack.

Our team is in the process of collecting data from other bone marrow transplantation (BMT) centers. Patients with isolated EMR of acute leukemia after allo-SCT will be included in the study. Disease and detailed clinical characteristics such as the presence of active chronic GVHD at the time of relapse will be recorded. The immune microenvironment of leukemia biopsies from sites of EMRs will be examined by using tissue MicroArrays in paraffin sections. The functional status of tumor infiltrating lymphocytes will be analyzed by the use of multiplexed immunofluorescence. Bone marrow biopsies from the same patients at the time of initial diagnosis, as well as from patients with BMR after allo-SCT will serve as the control group.

In conclusion, we propose that EMR of acute leukemia observed during the strong immune pressure of active GVHD represents a novel mechanism of immune escape from alloreactive response. Examination of tumor microenvironment from biopsies taken during relapses from extramedullary sites is required for the elucidation of this novel immune escape mechanism.

### Summary points

- Relapse is a significant cause of treatment failure after allogeneic stem cell transplantation (allo-SCT) and is usually due to escape of leukemic cells from the immune surveillance.
- In the setting of haploidentical donor, immune escape has been attributed to the loss of the mismatched HLA haplotype, while downregulation of HLA-class II has been observed in many cases of relapse after allo-SCT with matched donors. Immune escape after allo-SCT might also be explained by T-cell exhaustion induced by the continuous state of activation of alloreactive donor T cells.
- In our study, we observed that all patients with acute leukemia who relapsed at a time of active graft-versus-host-disease developed extramedullary leukemia only while they remained free of leukemia in peripheral blood and bone marrow (BM). On the contrary all cases with BM relapse occurred in patients free of active graft-versus-host-disease.
- None of the proposed immune escape mechanisms described above can explain the type of relapses observed in our study. The absence of leukemia relapse in peripheral blood and BM in a patient who shows progression in multiple extramedullary sites is suggestive of a microenvironment-dependent graft-versus-leukemia effect.
- Our observation points toward a novel mechanism of immune escape that is microenvironment-specific. Understanding the mechanisms of relapse would be of fundamental importance for the generation of novel therapeutic and or prophylactic strategies.

### Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

### Ethical conduct of research

Our study is a retrospective observational study. All patients gave written informed consent before allogeneic stem cell transplantation and before treatment of relapse. All patients gave written informed consent that their data will be sent to international registries such as EBMT and CIBMTR. All patients gave written informed consent that their data will be used anonymously in scientific publications. This study has been approved by the Institutional Review Board and the Bioethics Committee of ATTIKO University Hospital.

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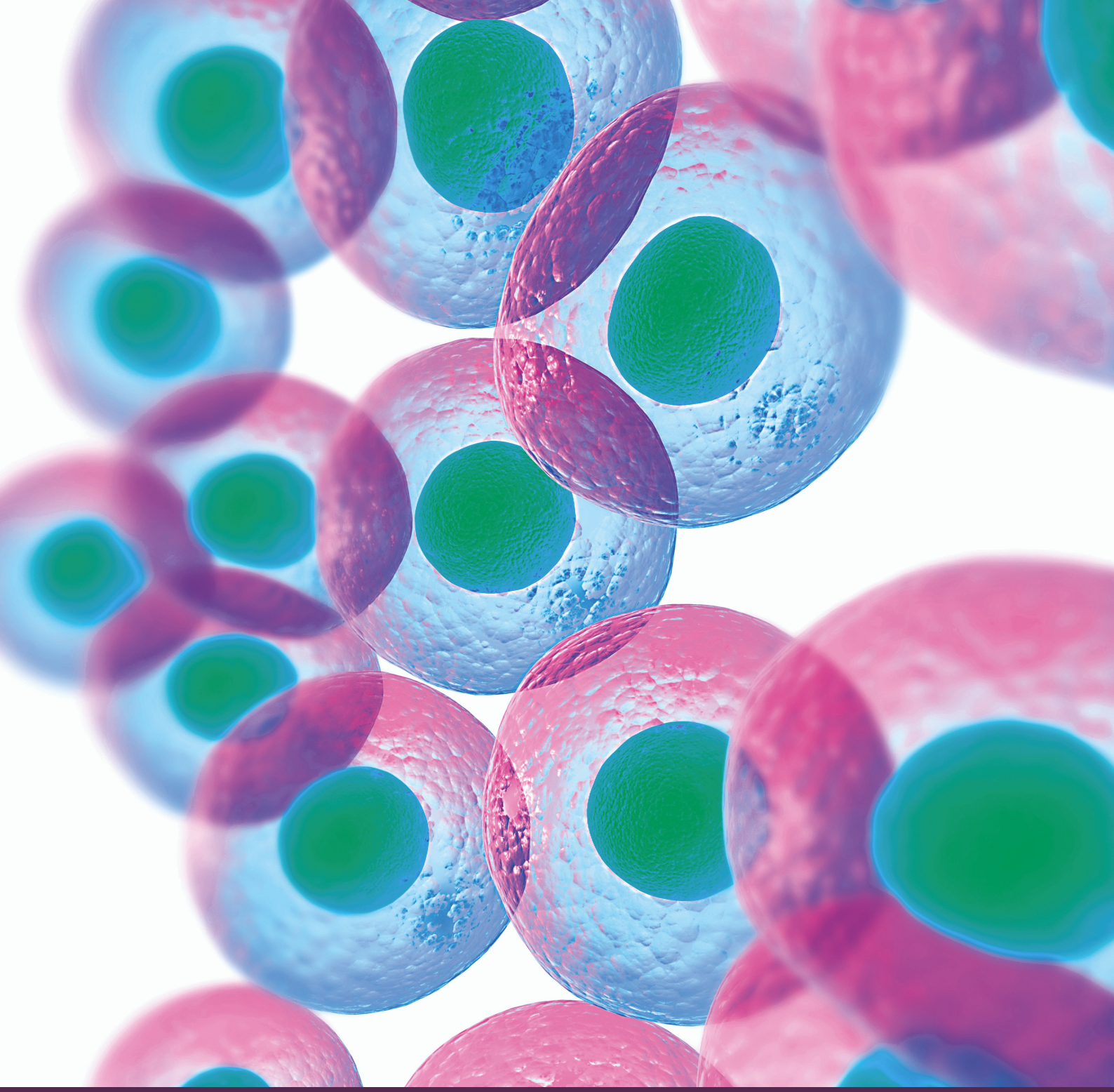

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