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The $CD45^{low}CD271^{high}$ cell prevalence in bone marrow samples may provide a useful measurement of the bone marrow quality for cartilage and bone regenerative therapy

Abstract

Background

Bone marrow (BM) **aspirates/concentrates are increasingly used for musculoskeletal regenerative therapies providing bone and cartilage** progenitors. **However, the quality of these BM samples remains imprecise within the clinical settings. As** there is an urgent need **for the development of these therapies, a method to count CD45^{low}CD271^{high} cells was optimised and tested as an indicator of BM sample quality.**

Methods

BM aspirates were collected from 54 donors (28 males and 26 females, median age: 48). The reagent concentrations were optimized for a fast staining **and Attune** flow-cytometer **was used** enabling **an** automated **CD45^{low}CD271^{high} cell** counting **in** BM aspirates, BM concentrates and those loaded onto **a collagen** scaffold. The **CD45^{low}CD271^{high} cell** numbers were compared to those obtained using another flow-cytometry (LSRII)-based method and to connective tissue progenitor (CTP) numbers counted using the colony forming unit-fibroblast (CFU-F) assay.

Results

The optimised **method enabled the** count**ing of** CD45^{low}CD271^{high} cells within only 15 minutes. The quantified cell numbers (median: 1,520, range: 96-20,992 cells/ml of BM) **were positively** correlated with the CTP counts ($p<0.0001$**, r=0.7237**). **In agreement with CFU-F and LSRII-based assays, the CD45^{low}CD271^{high} cell numbers counted using the Attune-based method were evidently decreasing with age in females but not males (p=0.0015 and p=0.3877 respectively). A significant increase of CD45^{low}CD271^{high} cell numbers was detected**

following BM concentration (mean: 5-fold, CI: 3.6-7.2). Additionally, the $CD45^{low}CD271^{high}$ cell numbers attached to the scaffold were positively correlated with progenitor cell numbers survived on the scaffold after 2-week culture (p=0.0348).

Conclusions

An assay counting $CD45^{low}CD271^{high}$ cells may provide a useful measurement of the BM quality. While the specificity of this measurement for CTPs remains low in our experimental conditions, $CD45^{low}CD271^{high}$ counts are positively and modestly correlated with the prevalence of CTPs.

Clinical Relevance

A fast and automated assessment of the BM aspirate/concentrate **quality using $CD45^{low}CD271^{high}$ cell counting can be** a **useful** tool for the **regenerative therapy improvement**.

Introduction

The field of regenerative medicine is constantly evolving with new approaches for cartilage and bone healing dominating both clinical and research activities. Targeting **the environment of non-united fractured bone or degenerative** joint with biological modifiers such as progenitor cells and/or growth factors represent promising **therapeutic** strategies (1-4). The rationale **behind these strategies** is that repopulation of cartilage and bone defects is possible, as long as **the progenitor** cells are present. **For example**, the **potential** efficacy of the micro fracture technique **for cartilage repair in osteoarthritis (OA) could be related to the effect of the subchondral bone** progenitors **that produce** growth factors and tissue matrix helping cartilage repair (5). Furthermore, the use of BM progenitors **with or without platelet rich plasma** has been demonstrated to aid bone repair **in pre-clinical and clinical studies of the osteochondral defects, metaphyseal bone defects and** femoral head avascular necrosis (AVN) (6-10).

Previous research has proven the clinical value of BM aspirates/concentrates showing a positive correlation between the numbers of applied BM progenitors and favourable clinical outcomes in tibia fracture non-union (11), hip osteonecrosis (12), OA (13) and AVN therapy (14-16). Despite the advantages of **using** BM aspirates or concentrates, the **quality of these samples** remains difficult to assess and **is poorly** control**led**. Furthermore, the numbers of **progenitor cells** in BM aspirates are widely variable depending on the aspiration site, volume and **surgical** technique (17, 18), as well as donor-related factors such as age and gender (19). Determination of the quality of BM **samples is crucial in order** to optimize clinical outcomes, cost and time associated with cell-based therapies. The colony forming unit-fibroblast

(CFU-F) assay **facilitates the** counting **of connective tissue progenitors (CTPs)** and is commonly used as an indicator for **BM sample quality** (20, 21), however it usually takes several days to be informative. **CTPs represent the progenitors in native tissues that are able to form colonies in vitro. However, the CTP concentration and prevalence can be influenced by BM processing methods. The efficiency of colony formation (the likelihood that a viable CTP will form a colony when placed into CFU-F assay) is also dependent on culture conditions** (18, 22).

The aim of the current study was to introduce a fast and automated **method** with minimum sample processing that helps to **indicate the quality of** BM aspirates and concentrates. BM cells isolated based on the $CD45^{low}CD271^{high}$ phenotype are known to express CD73, CD90 and CD105, but not hematopoietic lineage markers and generate cultures of multipotential stromal cells fully consistent with the international society **for cellular** therapy (ISCT) criteria (23-25). **Importantly, several groups have reported that no colony-forming cells are present in CD271-negative fraction of BM cells and the detected** BM colony-forming activity **was completely** confined to the $CD45^{low}CD271^{high}$ cells (25-31). Therefore, we chose to quantify in a flow-cytometry based assay, the numbers of $CD45^{low}CD271^{high}$ cells to assess the **quality of** BM aspirates and concentrates. **We hope that the work carried out will contribute to the standardization of therapies setting thresholds between 'success' and 'failure' of the** musculoskeletal regenerative therapies.

Materials and Methods

Bone marrow aspirates

BM samples from 54 donors were used for this study under ethical approval, ***Blinded by JBJS***. The donors were admitted at ***Blinded by JBJS*** for orthopaedic surgery, but did not have any systemic illness, cancer or metabolic diseases. The donors were 28 males and 26 females with an age range, 22-80 years and median of 48 years. **Two groups of patient samples were used as described in Table 1**. All BM aspirates were consistently harvested from the **same location (zone 6) of** posterior iliac crest as previously described (17, 26, 32). **Each sample analysis was carried out on one BM sample harvested from one individual at one-time point.**

Using flow-cytometry for **counting $CD45^{low}CD271^{high}$ cells**

A 100µl volume of whole BM, was stained using a three-marker panel containing Vybrant® DyeCycle™ Ruby 2.5mM solution in DMSO (Thermo Fisher Scientific, Waltham, MA, USA), a DNA-selective dye that only labels the nucleated cells enabling gating out of RBCs and platelets. Additionally, the panel contained anti-CD45 antibody (V450, clone: HI30, mouse IgG1κ, 100µg/ml, BD Biosciences) and anti-CD271 antibody (PE, clone: ME20.4-1.H4, mouse IgG1, concentration: 0.75µg/ml, Miltenyi Biotec Ltd, Surrey, UK). The phenotype **indicating BM progenitor cells** ($CD45^{low}CD271^{high}$ cells) was applied as shown previously (18). The **manufacturer recommendation** for CD45 and CD271 antibodies was 15 minutes at room temperature (RT) **and for Vybrant DyeCycle Ruby dye, at 37°C for 15 minutes**. However, the antibody/dye staining was optimised to count $CD45^{low}CD271^{high}$ cells within the shortest time (described in the results). An acoustic focusing flow-cytometer, Attune® (Thermo Fisher Scientific) was used **allowing** an automated cell counting. For **some experiments**, the $CD45^{low}CD271^{high}$ cell numbers were **counted** using our previously published flow-cytometry

based method (18). Briefly, this method involved BM sample staining (using CD90, CD271 and CD45), red blood cell (RBC) lysis then adding CountBright™ absolute counting beads (Thermo Fisher Scientific). The data acquisition was performed using LSRII (BD biosciences).

Colony Forming Unit-Fibroblasts assay

The colony forming unit-fibroblast (CFU-F) assay was employed as described previously (18) to count CTPs whereby the BM samples were added to StemMACS MSC expansion media (Miltenyi Biotec) then cultured for 14 days. **The colonies** were visualised using methylene blue and counted manually. Each colony was defined as having at least 50 cells (33).

BM concentration

BM samples **(n=15)** were concentrated based on the gradient centrifugation using the BioCUE™ device (Zimmer Biomet, Warsaw, USA). **The** BM aspirates **were** collected into syringes washed with anticoagulant acetate citrate dextrose (ACD) and loaded into the BioCUE device. From both pre- and post-concentration fractions, aliquots were analysed for $CD45^{low}CD271^{high}$ cell and CTP **counts** using the **Attune-based method** and CFU-F assays respectively. Counting of platelets was performed for some samples (n=10) using an automated haematopoietic cell counter, Sysmex (Sysmex Ltd, Milton Keynes, UK).

Loading of BM samples on a collagen scaffold

The BM aspirates were used to load a collagen scaffold, Bio-Gide® (Geistlich Sons Limited, Manchester, UK). The pre-loading and the remaining post-loading parts of the BM samples were processed to count $CD45^{low}CD271^{high}$ cells. Additionally, the BM-loaded scaffolds were cultured

for 2 weeks and subsequently processed to quantify **BM** progenitors that survived on the scaffolds as previously described (22). Briefly, the scaffolds were digested using 0.25% collagenase (Stem Cell Technologies, Grenoble, France). As the surface expression of CD271 can be reduced on cultured cells (25), the extracted cells were **stained** using CD45, CD90 (BioLegend, CA, USA) and CD73 (Miltenyi Biotec) antibodies and counted using the counting beads.

Statistical analysis

The statistical analysis and graph preparation were performed using GraphPad Prism software version 7.0a. The normal distribution of the data was assessed using the Shapiro-Wilk normality test and the appropriate test for **the data** analysis was applied accordingly. The statistical significance was considered when p value < 0.05.

Source of Funding

Blinded by JBJS. The funding sources did not have any role in the study design, sample collection, data analysis or interpretation.

Results

1. Optimisation of marker concentration

The **fast** staining of BM samples was initially optimised. For anti-CD45 antibody, the $CD45^{low}CD271^{high}$ cells counted using 10µl of **this** antibody was higher **compared to** 5µl

(p=0.0486), **but with no** difference between **using** 10**µl** and 20µl (p=0.3969), (Figure 1, A). Regards **anti-**CD271 **antibody**, the $CD45^{low}CD271^{high}$ cell numbers quantified using 20µl of the antibody was significantly higher **than using** 10µl (p=0.0450), but similar to 40µl (p=0.5443), (Figure 1, B). Using three different volumes of the Vybrant DyeCycle Ruby dye, the $CD45^{low}CD271^{high}$ cell numbers were similar (p=0.1901 and p=0.1140 for 3µl versus 5µl and 10µl versus 5µl respectively), (Figure 1, C).

We next **tested the use of** CD45 and CD271 **antibodies** followed by Vybrant DyeCycle Ruby (two-step staining) versus the addition of all markers in one step. The cell numbers were not significantly different **between one-** or two-step staining **methods** (p=0.6581), (Figure 1, D). **The** $CD45^{low}CD271^{high}$ cell numbers were **also** similar **using different staining temperatures** (p=0.7237, p=0.1261, p=0.3558 for 4°C versus RT, 37°C versus RT and 4°C versus 37°C respectively), (Figure 1, E). **Also,** the $CD45^{low}CD271^{high}$ cell numbers were not significantly different comparing **5-minute staining** versus 10, 5 versus 15 or 10 versus 15 minutes (p=0.1981, p=0.5028, p=0.7870 respectively), (Figure 1, F).

For each BM sample, the acquisition **time** on Attune **was completed within 10 minutes. An** internal control (counting beads) **for automated counting** was used and both automated and bead-dependent quantification were comparable (p=0.3750, Figure 1, G). The data also showed **similar** cell numbers quantified when BM samples were 5-time or 10-time diluted compared to undiluted **ones** (Figure 1, H). Collectively, we **optimised an automated and simple** assay of $CD45^{low}CD271^{high}$ cells within only 15 minutes.

2. Comparison of the **Attune-based** assay versus another flow-cytometry and CFU-F assays

Using the Attune-based method, the median percentage of $CD45^{low}CD271^{high}$ cells per total BM cells was 0.016% (95% Confidence Interval (CI): 0.009-0.032%). The absolute counts of $CD45^{low}CD271^{high}$ cells had a median of 1,520 cells/ml of BM (CI: 1,056-6,112, range: 96-20,992 cells/ml of BM).

The results **obtained by Attune, LSRII and CFU-F assays** were consistent **indicating for example,** high or low **quality BM samples** (Figure 2, A and B). The **$CD45^{low}CD271^{high}$ cells** numbers obtained **using Attune** were close to those **counted using** LSRII (median 1,311 and CI: 900-5,533, range: 87-20,471 cells/ml of BM). **However**, the $CD45^{low}CD271^{high}$ cell numbers obtained by **Attune** were higher than CTPs (median 60, CI: 45-190, range 3-900 CTPs/ml of BM). **Interestingly**, the $CD45^{low}CD271^{high}$ cell numbers measured using **Attune** were positively, correlated with the data of LSRII ($p<0.0001$, $r=0.9801$), (Figure 2, C) and CTPs ($p<0.0001$, $r=0.7237$), (Figure 2, D).

When the **data** were analysed in relation to the age and gender of the donors, a clear pattern of a negative correlation between the $CD45^{low}CD271^{high}$ cell numbers with **donor** ageing was observed in females ($p=0.0015$, $r=-0.6900$), (Figure 3, A, left), but not in the males ($p=0.3877$, $r=-0.2102$), (Figure 3, B, left). This was consistently detected using the LSRII-based method (females: $p=0.0070$, $r=-0.6563$, Figure 3, A, middle and males: $p=0.3708$, $r=-0.2577$, Figure 3, B, middle) and CFU-F assays (females: $p=0.0055$, $r=-0.6904$, Figure 3, A, right and males: $p=0.1461$, $r=-0.4093$, Figure 3, B, right). Altogether, **the numbers of $CD45^{low}CD271^{high}$ cells were comparable between Attune and LSRII and** positively correlated with **the** CTP counts.

3. <u>The **assessment** of $CD45^{low}CD271^{high}$ cells in BM concentrates</u>

Our **optimisation** results showed that the quantified $CD45^{low}CD271^{high}$ cell numbers **in BM concentrates** were generally higher after 10-time dilution compared to 5-time dilution and non-dilution (Figure 4, A) **thus** 10-time dilution **of BM concentrates** is needed to ensure accurate **estimation**. The $CD45^{low}CD271^{high}$ cell numbers were increased significantly **after BM** concentration ($p<0.0001$), (Figure 4, B). The fold increase of the $CD45^{low}CD271^{high}$ cell numbers **(mean: 5-fold, CI: 3.6-7.2) and that of CTPs (mean: 4.6-fold, CI: 3.1-6) were comparable** ($p=0.1894$, Figure 4, C). The Sysmex results showed an increase of the platelet numbers in BM concentrates ($p=0.6255$) with a mean increase of 4.5-fold (CI: 3-6), (Figure 4, D). **In summary, we have shown a fast assessment** of increased $CD45^{low}CD271^{high}$ cell numbers in the BM concentrates.

4. <u>The assessment of $CD45^{low}CD271^{high}$ cells attached to a collagen scaffold</u>

We used BM aspirates to load Bio-Gide scaffold then the number of attached $CD45^{low}CD271^{high}$ cells was calculated by **counting these cells** in the pre- **and post-**loading samples (Figure 5, A). The numbers of $CD45^{low}CD271^{high}$ cells attached to Bio-Gide were variable between samples, but consistently dependent on the pre-loading cell quantities (Figure 5, B). Furthermore, the numbers of attached $CD45^{low}CD271^{high}$ cells strongly correlated with those survived on Bio-Gide ($p=0.0348$, $r=0.8434$), (Figure 5, C). **The** $CD45^{low}CD271^{high}$ cell **assessment** helped to detect **the donor-related** differences **in cell attachment onto scaffolds.**

Discussion

Bone marrow samples contain CTPs that are potentially useful in treating degenerative musculoskeletal diseases and non-united bone fractures. The processing of BM samples helps to concentrate these CTPs. However, the concentration and prevalence of CTPs vary widely between individuals and according to different aspiration locations and techniques (17-19). The gold standard CFU-F assay requires at least 6 days (19) thus clinicians currently have no way of knowing at the time of the procedure, the quality of the BM sample utilised. It would be desirable, therefore to have a rapid measurement that could provide an insight into BM quality on the day of procedure. Here, **we introduced a** fast and automated **assessment** of $CD45^{low}CD271^{high}$ cells **in BM preparations that may be used to judge the quality of BM samples. This assay was compared to another more time-consuming flow-cytometry assay using LSRII (18) and provided a similar range of $CD45^{low}CD271^{high}$ cells. Both assays confirmed an age-related decline in $CD45^{low}CD271^{high}$ cells in females but not males as previously reported for CTPs (19).**

The specificity of the $CD45^{low}CD271^{high}$ cell measurement compared to CTP numbers was low (0.05 on average) i.e. 20 times more $CD45^{low}CD271^{high}$ cells than CTPs measured by CFU-F. This finding agrees with previous studies (18, 27). However, the $CD45^{low}CD271^{high}$ counts were positively and modestly correlated with the prevalence of CTPs (r=0.7237). This low specificity does not prevent the use of this assay for estimating of aspirate quality, however it is clear that it does not enable exact measurement of CTP numbers. This might be related to the senescence of some $CD45^{low}CD271^{high}$ cells in culture during CFU-F assay as a result of plating at very low clonal densities. Another possible explanation for this disparity is that CTPs represent only a subset of the $CD45^{low}CD271^{high}$ population as suggested recently (34). It is possible that with the addition of more markers,

this subpopulation could be defined allowing increased specificity of the assay. In one study, CD146 marker was tested, but no further enrichment in CTP numbers was detected in the $CD146^{+}CD271^{+}$ fraction compared to $CD146^{-}CD271^{+}$ fraction (27). Subsequently, the same group showed that the majority of CTPs resided in the $CD140a^{-}CD271^{+}$ fraction (29) however, our group did not find such a clear subpopulation (35) and we are investigating this further. Others have not yet devised additional, more selective markers while all agree on the value of CD271 (36).

We believe the assessment of $CD45^{low}CD271^{high}$ cells has a very high sensitivity (close to 100%) as other studies have shown that all BM colony-forming activity is confined to $CD45^{low}CD271^{high}$ cells and CD271-negative cells did not have any colony forming ability (25-31). The implications of this assay with high sensitivity and relatively low specificity is that no CTPs are missed out while some progenitor cells with potentially lower colony forming capacity than detected in our experimental conditions can be counted.

The CFU-F assay data can be varied depending on the patient age and BM aspiration site and volume (18, 22). This could explain that CTPs numbers in this study showed some variability from previous work (37). Using various BM processing methods could have an additional effect on variability of CTP counts e.g. using lymphoprep causes CTP loss (38). We have ensured optimal and consistent culture conditions by using complete and batch-tested media for CFU-F assays. Thus, the possibility of underestimation of CTPs is small but still exists.

The BM aspirates or concentrates loaded on scaffolds have been demonstrated to enhance cartilage repair in OA knee or hip (39-41), focal condylar lesions of knee articular cartilage and talar osteochondral injuries with promising outcomes (10, 42). The results **presented here** have

277 demonstrated that the $CD45^{low}CD271^{high}$ cell number**s were increased** 5-fold after BM
278 concentration. We also reported that platelets were concentrated 4.5-fold showing an additional
279 value **of un-fractioned BM concentrates via providing** growth factors (43). Compared to our
280 data, Dawson et al showed 4-fold increase of CTPs in BM concentrates (44). Another recent
281 study has shown that two different concentrator devices produced significantly different numbers
282 of CTPs and dissimilar levels of growth factors (45). Our data also showed that the numbers of
283 attached $CD45^{low}CD271^{high}$ cells onto Bio-Gide were variable depending on the initial cell
284 counts in the BM samples. Collectively, this further emphasises the **potential** value of
285 **$CD45^{low}CD271^{high}$ cell count assessment** to indicate the **quality of BM samples after**
286 **concentration or when loaded** on**to** scaffolds.
287 In conclusion, **our aim was to report on a method that can help to indicate the**
288 **'potency/quality' of the BM sample applied in clinical settings**. **The quantitative assessment**
289 of $CD45^{low}CD271^{high}$ cells in BM aspirates can be performed rapidly and the numbers of
290 $CD45^{low}CD271^{high}$ cells are **positively** correlated with the numbers of CTPs. **While the**
291 **specificity of $CD45^{low}CD271^{high}$ cell assessment is low compared to CFU-F assay, the**
292 **sensitivity of this method is very high**. **Since the CFU-F data cannot be immediately**
293 **available on the day of surgery, these findings support the view that an assay measuring**
294 **$CD45^{low}CD271^{high}$ cells could be useful as a surrogate measure of BM quality on the day of**
295 **surgery, if that information were available**. **Future studies on the rapid measure of CTP**
296 **prevalence in BM samples with inclusion of other specific markers are desirable to further**
297 **enhance the method described in this study**.

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Figure legends

Figure 1: Optimisation of the staining and **counting** of $CD45^{low}CD271^{high}$ cells on Attune flow-cytometer

The $CD45^{low}CD271^{high}$ cell numbers quantified using 3 different volumes of anti-CD45 and CD271 antibodies and Vybrant DyeCycle Ruby (VDR) dye, were compared (Student's paired t-test, n=10 samples), (A, B, C). The comparison was performed between $CD45^{low}CD271^{high}$ cell numbers counted using the one-step versus two-step staining (Student's paired t-test, n=6 samples), (D), at different staining temperatures (Student's paired t-test, n=7 samples), (E) and after 5-, 10- and 15-minute staining (Student's paired t-test, n=10 samples), (F). The comparison of $CD45^{low}CD271^{high}$ cell numbers enumerated on Attune using automated counting versus counting beads (Wilcoxon matched-pairs signed rank test, n=9 samples), (G). The $CD45^{low}CD271^{high}$ cell numbers were counted in BM aspirates that were stained either undiluted, 5-time or 10-time diluted (n=5 samples, S: sample), (H).

Figure 2: The $CD45^{low}CD271^{high}$ cell numbers detected by the **Attune** versus LSRII-based method and CTP numbers.

The $CD45^{low}CD271^{high}$ cell numbers counted using Attune were compared to those obtained by another flow-cytometry method using LSRII and CTP numbers. Examples of high-quantity (A) and low-quantity (B) BM progenitor samples were shown. The correlation was analysed between $CD45^{low}CD271^{high}$ cell numbers quantified on Attune versus LSRII-based method (n=33 samples), (C) or CTPs using CFU-F assay (n=33 samples), (D). Spearman r test was used for the correlation analysis.

Figure 3: The numbers of $CD45^{low}CD271^{high}$ cells and CTPs in the BM samples of different age and gender donors.

The correlation between the numbers of $CD45^{low}CD271^{high}$ cells and ageing in females (A) and males (B) are shown. The results of the two methods for **counting** of $CD45^{low}CD271^{high}$ cells (using Attune and LSRII-based) and CTP numbers using CFU-F assay were compared. For females (A), 18, 16 and 15 samples were included respectively. For males (B), 19, 14 and 14 samples were used respectively. Spearman r test was used for the correlation analysis.

Figure 4: The **assessment** of $CD45^{low}CD271^{high}$ cells in BM concentrates.

The samples of BM concentrates were either undiluted, 5-time or 10-time diluted then was used to count $CD45^{low}CD271^{high}$ cells (n=8 samples, S: sample), (A). The numbers of $CD45^{low}CD271^{high}$ cells were compared between pre- and post-concentration (Conc) samples (Wilcoxon matched-pairs signed rank test, n=15 samples), (B). The fold increase of $CD45^{low}CD271^{high}$ cell numbers **was compared** versus CTPs **after BM concentration** (Student's paired t-test, n=13 samples), (C). The fold increase of $CD45^{low}CD271^{high}$ cells (calculated using the **Attune-based method**) and platelets (calculated using Sysmex) was compared **after BM concentration**. The figure showed the mean with 95% CI (Unpaired t-test, n=15 sample for $CD45^{low}CD271^{high}$ cells and n=10 samples for platelets), (D).

Figure 5: The **assessment** of $CD45^{low}CD271^{high}$ cells attached to Bio-Gide scaffold.

The number of $CD45^{low}CD271^{high}$ cells attached to Bio-Gide was calculated **by** count**ing** these cells in pre- and post-loading (remaining) BM samples (A). The numbers of pre-loading $CD45^{low}CD271^{high}$ cells and those attached to Bio-Gide were shown (n=6 samples, S: sample),

(B). The correlation was analysed between the number of $CD45^{low}CD271^{high}$ cells attached to Bio-Gide and **the progenitor cells** ($CD45^{-}CD90^{+}CD73^{+}$) survived on Bio-Gide after 2-week culture (Pearson r test, n=6 samples), (C).