

Multinucleation resets human macrophages for specialized functions at the expense of their identity

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Review
COMMONS

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

****Summary:****

The authors compared the various multinucleated cells, osteoclasts, LCG and FBGC. Overall, the manuscript shows rigor in the analyses, and also very interesting approaches for retrieving mononuclear cells, for instance using DC-STAMP siRNA. This work adds very much to understanding the biological differences, as summarized in figure 6h. A lot of work in osteoclast field with for instance qPCR is hampered because, inevitably, a mix of mononuclear and multinucleated cells is always measured. Here, a solid attempt to separate those mixes with cell sorting and subsequent analysis on the mononuclear and multinucleated isolates, really adds. Choice of figures is good, also the extra info of the supplementary figures is relevant and makes it easy to read.

****Major and minor concerns:****

For osteoclasts, various markers exist for their biological characterization, for instance the ability to resorb bone. What, apart from the arrangement and number of nuclei, were the biological parameters that confirmed that the cells made by addition of IFN or IL-4 were LCG and FBGC? In fig 2c: did the authors perform stainings with isotype control antibodies? In my experience, quite often, antibodies stain mononuclear cells much intenser, since the cytoplasm is much more condense, less spread over a large area.

Resorption assay in 6 is not clear. It is weird that osteoclasts apparently display so limited resorption? Also the traces are not typical for osteoclasts. Please explain.

Provide a better image Supplementary 2A, even at 250% the lettering is vague. What do the colours in 2A mean?

CROSS-CONSULTATION COMMENTS

I have read the comments of the other two reviewers, and together. I absolutely agree with their additions, Indeed, supplementary tables are lacking, as well as there could be a bit more emphasis on the fact that it is all in vitro work. Together, I think the three of us are complementary in our comments, with good overlap as well. Any effort to stain for instance pathology material with the markers that have been found, would be great, especially for the LGC and the FBGC, that are much less studied in the field of MNGs. Having said that ,I can also live without this addition, but then it could be highlighted in the discussion that these are the future avenues that should be considered. Collaborate with Pathology!

2. Significance:

Significance (Required)

This manuscript is particularly interesting to those who are interested in the BIOLOGY of MNCs. In essence, three types of MNCs were cultured and compared, with each of them a specific function.

I am an osteoclast expert (76 publications), and have two publications on FBGCs

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

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Reviewer Publons

Yes

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

****Summary:****

In this manuscript, the authors performed a comparative transcriptome analysis of mononuclear and multinuclear human osteoclasts, LGCs and FBGCs. They found that multinucleation triggers a significant downregulation of macrophage identity in all three types of MGCs. Furthermore, RNA-seq data and in-vitro functional analysis of multinucleated cells showed that macrophage cell-cell fusion and multinucleation enhance phagocytosis and contribute to lysosome-dependent

intracellular iron homeostasis. Furthermore, multinucleation of osteoclasts promoted mitochondrial activity and oxidative phosphorylation, resulting in maximal respiration. This unique and interesting study addresses the fundamental question of how cell-cell fusion and multinucleation contribute to cellular activity and biological homeostasis.

****Major comments:****

1. The authors generated mature multinucleated cells by stimulating human PBMC-derived macrophages with either IFN- γ , IL-4, or RANKL. However, no quantitative data have been presented to determine how effectively IL-4, IFN- γ , and RANKL can induce multinucleated giant cells from mononuclear macrophages. Quantitative data showing induction efficiency would provide a more detailed picture of the overall experiment.
2. The authors mentioned, "The distinct morphological appearance of these three types of MGCs (Figure 1B) suggested cell type-specific functional properties and shared mechanisms underlying macrophage multinucleation". However, there is no discussion or data showing how the nuclear arrangement and intracellular location affect the biological function of multinucleated cells.
3. Based on the results of DC-stamp knockdown experiments, the authors concluded that cell-cell fusion and multinucleation suppress the mononuclear phagocytic gene signature. However, to strengthen this hypothesis, it would be necessary to provide at least data showing that DC-stamp knockdown reduces the number of multinucleated cells.
4. In Figure 4, the authors showed that transcripts in LGCs were enriched for antigen presentation and adaptive immune system pathways. In addition, multinucleation of LGCs increased the surface expression of B7-H3 (CD276) and colocalized with CD3⁺ cells, suggesting that LGC multinucleation potentiates T cell activation. However, the authors did not present enough data to demonstrate the antigen-presenting ability of LGCs or their specific T cell activating capacity.
5. Figure 6 clearly shows that mature multinucleated osteoclasts exhibit increased ATP production and maximal respiration. However, the glycolytic pathway did not differ between mononuclear and multinuclear osteoclasts. No explanation for this observation has been provided. It is easy to understand that osteoclasts acquire ATP through aerobic respiration during multinucleation. But how NADPH, which is essential for its redox reaction, is produced? Is it by acquiring α KG from the glutamine pathway?

****Minor comments:****

1. Supplementary tables 1-6 were not provided.
2. Figure 2D right panel, difficult to see DAPI⁺ nuclei.

2. Significance:

Significance (Required)

Although it is well known that multinucleation of cells constantly occurs, especially in osteoclasts, skeletal muscle, and trophoblasts of the placenta, the biological significance of multinucleation and the intracellular functions of multinucleation are not well understood. In this unique study, three types of multinucleated cells were generated from human peripheral blood to elucidate the genetic and functional differences between mononucleated and multinucleated cells. Furthermore, by demonstrating the possibility that the morphological peculiarity of multinucleation can regulate cell function, this paper provides clues to understanding the underlying biology of multinucleated cells and how they maintain cell function in homeostatic and pathological settings.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

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Reviewer Publons

Yes

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

****Summary:****

The manuscript of Ahmadzadeh and Pereira et al is an interesting study of the fusion process key to the formation of multinucleated giant cells (MGCs). Our current ability to discriminate between different types of MGCs is limited, and there are gaps in our understanding of the

molecular determinants of cell fusion. In this study, the authors isolated different MGC variants - osteoclasts, Langhans giant cells (LGCs) and foreign body giant cells (FBGCs) and identified common, as well as MGC-specific genes and pathways involved in the process of cell fusion. The approach of isolating and comparing different types of MGCs is novel, and the manuscript is well presented and written. However, due to the in vitro nature of the study, the physiological significance of the findings is unclear. I have further minor and major points for the authors to address, as detailed below.

****Minor comments:****

- The approach to isolate the different MGCs using FACS and imaging technique is highly novel. However the difference between MGC subtypes isolated isn't immediately apparent beyond the morphological comparisons. In my opinion some of the results of MGC-specific assays from Figures 4, 5 and 6 can be included in Figure 1, e.g. TRAP staining and hydroxyapatite resorption for osteoclasts, to provide evidence of purity and specificity of each MGC subtype early on in the manuscript. Classical or canonical genes associated with each MGC subtype can also be highlighted in the volcano plots in Figure 1C, e.g ACP5, CTSK, TNFRSF11A for osteoclasts.
- The overall decrease in phagocytic identity of all the MGCs, and the specific upregulation of phagocytic pathways in the FBGCs are conflicting. Are there subsets of phagocytic pathways that were down and upregulated during the formation of FBGCs?
- What are the identities of the mononuclear cells in each of the MGC experiment? They appeared to be quite heterogeneous based on the DEGs identified, beyond the common phagocyte signature. Can the authors comment on the difference between the mononuclear cells and whether this will affect the DEG analysis?
- The authors should also frame/discuss the findings in the context of diagnostic and therapeutic potentials to highlight the clinical significance of this study

****Major comments:****

- As mentioned before, the physiological significance of the findings is unclear. Some form of in vivo data is needed to support some of the key conclusions of the study, e.g validating some of the markers of the pathways identified (common and MGC subtype-specific), and the role of lysosome-mediated iron homeostasis in multinucleation. The authors can make use of the FACS and imaging approaches they developed to look at MGCs in relevant tissues.

2. Significance:

Significance (Required)

****Significance:****

- The approach of isolating and comparing different types of MGCs is novel, and the findings

certainly improved our understanding of the fusion processes of MGCs. However the physiological role of these processes in health and disease that involve MGCs is still unclear due to the lack of in vivo data. The findings were discussed in quite a bit of detail in the context of current literature, though clinical impact was not explored.

- My background is bone biology with a very keen interest in osteoclast biology so arguably my knowledge on other MGCs eg LGCs and FBGCs is limited.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

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Reviewer Publons

Yes

Full Revision



Manuscript number: #RC-2022-01638

Corresponding author(s): Jacques Behmoaras

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1. General Statements [optional]

***[Authors' reply].** We thank all the reviewers for their very constructive comments. We note that our findings were unanimously received as novel and unique. The reviewers highlighted the rigor in data analysis (Reviewer 1) and the improvement of our understanding of the fusion process and the underlying biology (Reviewer 1, 2 and 3) – a characteristic of macrophages among all other immune cells. We have addressed all the points raised by the reviewers and provide a fully revised version of our manuscript.*

2. Point-by-point description of the revisions

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Summary: The authors compared the various multinucleated cells, osteoclasts, LCG and FBGC. Overall, the manuscript shows rigor in the analyses, and also very interesting approaches for retrieving mononuclear cells, for instance using DC-STAMP siRNA. This work adds very much to understanding the biological differences, as summarized in figure 6h. A lot of work in osteoclast field with for instance qPCR is hampered because, inevitably, a mix of mononuclear and multinucleated cells is always measured. Here, a solid attempt to separate those mixes with cell sorting and subsequent analysis on the mononuclear and multinucleated isolates, really adds. Choice of figures is good, also the extra info of the supplementary figures is relevant and makes it easy to read.

Major and minor concerns:

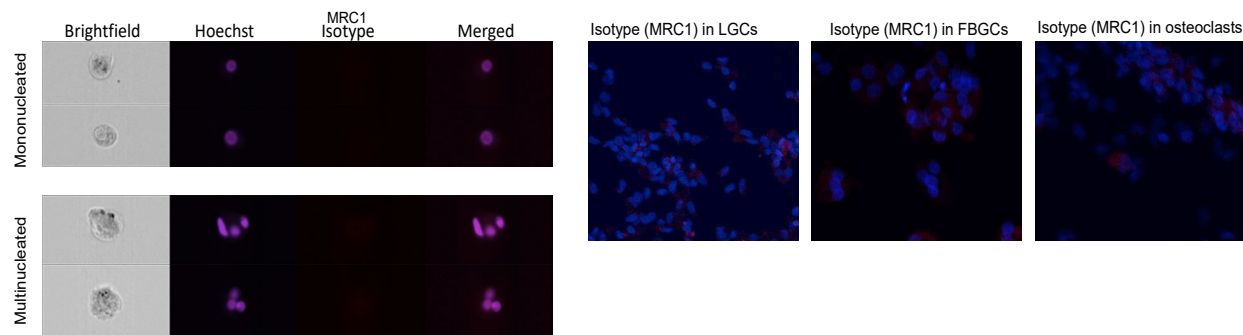
1. For osteoclasts, various markers exist for their biological characterization, for instance the ability to resorb bone. What, apart from the arrangement and number of nuclei, were the biological parameters that confirmed that the cells made by addition of IFN or IL-4 were LCG and FBGC?

***[Authors' reply].** In order to address this point, we focused on gene sets that characterize LCGs and FBGCs. By doing so, we aimed to identify (i) lineage dependent factors and (ii) markers of*

LGCs and FBGCs. (See new Supplementary Figure 1B and C, New Supplementary Table 1 and highlighted text in Results). As expected, and in line with the lineage-determining factors, the transcriptomics comparison between mononucleated/multinucleated IFN- γ and IL-4-differentiated macrophages showed predominance of IFN- γ and IL-4-related pathways, respectively (Supplementary Figure 1B and C and Supplementary Table 1). Among known LGC and FBGC markers, we confirmed up-regulation of CCL7 [1] and CD86 [2], respectively. As per the biological parameters, we indeed confirm that FBGCs show enhanced phagocytosis properties (Figure 5C) while LGCs can form granuloma-like clusters in vitro (Figure 4D and E). Altogether, we characterize LGCs and FBGCs with (i) polykaryon-specific nuclear arrangement, (ii) polykaryon-specific gene expression markers, (iii) previously shown and new phenotypic characteristics such as LGCs' unique ability to form in vitro clusters containing CD3+ cells.

2. In fig 2c: did the authors perform stainings with isotype control antibodies? In my experience, quite often, antibodies stain mononuclear cells much intenser, since the cytoplasm is much more condense, less spread over a large area.

[Authors' reply]. According to the reviewer's suggestion, we provide isotype control staining for MRC1 in IFN- γ -stimulated mononucleated/multinucleated cells by ImageStream (left panel) and immunofluorescence in LGCs, FBGCs and osteoclasts (right panel). There was negligible staining with the isotype control antibody for MRC1 in both settings (see below).



We did not observe a potential artefact of staining in multinucleated cells when compared to mononuclear cells. In fact, some markers of multinucleation such as B7-H3 is augmented in LGCs (Figure 4E).

3. Resorption assay in 6 is not clear. It is weird that osteoclasts apparently display so limited resorption? Also the traces are not typical for osteoclasts. Please explain.

[Authors' reply]. Human osteoclasts are cultured for 2 days on hydroxyapatite-coated plates and the amount of resorption is dependent on the healthy donor the peripheral blood is derived from. In addition to genetic variability, the support (hydroxyapatite) is different from dentine, which is also widely used for measuring osteoclast resorptive activity. The visualization of the human osteoclast resorption is made by transparency (area not coated by hydroxyapatite due to its resorption) on image J.

4. Provide a better image Supplementary 2A, even at 250% the lettering is vague. What do the colours in 2A mean?

***[Authors' reply].** According to the reviewer's suggestion, we now provide the Supplementary Figure 2A with better resolution. In STRING protein-protein interaction analysis, there is no particular meaning of the node color itself.*

CROSS-CONSULTATION COMMENTS

I have read the comments of the other two reviewers, and together. I absolutely agree with their additions, Indeed, supplementary tables are lacking, as well as there could be a bit more emphasis on the fact that it is all in vitro work. Together, I think the three of us are complementary in our comments, with good overlap as well. Any effort to stain for instance pathology material with the markers that have been found, would be great, especially for the LGC and the FBGC, that are much less studied in the field of MNGs. Having said that ,I can also live without this addition, but then it could be highlighted in the discussion that these are the future avenues that should be considered. Collaborate with Pathology!

***[Authors' reply].** We appreciate that the reviewer provides cross-consultation comments which we address in our revised manuscript. As such, we discuss future avenues regarding the translatability of these results to human pathology involving MGCs.*

Reviewer #1 (Significance (Required)):

This manuscript is particularly interesting to those who are interested in the BIOLOGY of MNCs. In essence, three types of MNCs were cultured and compared, with each of them a specific function.

I am an osteoclast expert (76 publications), and have two publications on FBGCs

***[Authors' reply].** We sincerely thank the reviewer for his/her pertinent comments, enthusiasm for our findings and for providing us an overall summary of our findings in view of all other reviewer comments.*

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary:

In this manuscript, the authors performed a comparative transcriptome analysis of mononuclear and multinuclear human osteoclasts, LGCs and FBGCs. They found that multinucleation triggers a significant downregulation of macrophage identity in all three types of MGCs. Furthermore, RNA-seq data and in-vitro functional analysis of multinucleated cells showed that macrophage cell-cell fusion and multinucleation enhance phagocytosis and contribute to lysosome-dependent intracellular iron homeostasis. Furthermore, multinucleation of osteoclasts promoted mitochondrial activity and oxidative phosphorylation, resulting in maximal respiration. This unique

and interesting study addresses the fundamental question of how cell-cell fusion and multinucleation contribute to cellular activity and biological homeostasis.

Major comments

#1 The authors generated mature multinucleated cells by stimulating human PBMC-derived macrophages with either IFN-g, IL-4, or RANKL. However, no quantitative data have been presented to determine how effectively IL-4, IFN-g, and RANKL can induce multinucleated giant cells from mononuclear macrophages. Quantitative data showing induction efficiency would provide a more detailed picture of the overall experiment.

[Authors' reply]. According to the reviewer's suggestion, quantitative data showing the efficiency of these cytokines to induce multinucleation (i.e. fusion index) is now provided as part of the revised Supplementary Figure 1A (right panel).

#2 The authors mentioned, "The distinct morphological appearance of these three types of MGCs (Figure 1B) suggested cell type-specific functional properties and shared mechanisms underlying macrophage multinucleation". However, there is no discussion or data showing how the nuclear arrangement and intracellular location affect the biological function of multinucleated cells.

[Authors' reply]. This is good point and is now discussed in the revised manuscript (see highlighted text in revised manuscript and below).

Whether MGC-specific nuclear arrangements and/or numbers are indicative of specialized function is currently unclear. Intracellular nuclei arrangement is likely to be important for the sealing zone formation in a polarized bone-resorbing osteoclast. Furthermore, whether distinct transcriptional activities are assigned to different nuclei of the MGC also remain to be tested. Recent elegant work performed in multinucleated skeletal myofibers suggest transcriptional heterogeneity among the different nuclei of the polykaryon [3].

#3 Based on the results of DC-stamp knockdown experiments, the authors concluded that cell-cell fusion and multinucleation suppress the mononuclear phagocytic gene signature. However, to strengthen this hypothesis, it would be necessary to provide at least data showing that DC-stamp knockdown reduces the number of multinucleated cells.

[Authors' reply]. According to the reviewer's suggestion, we provide data showing that DCSTAMP knockdown reduces multinucleation in LGCs and FBGCs (see below and new Supplementary Figure 2F). For human osteoclasts, the data was included in our previously published paper ([4] and below).

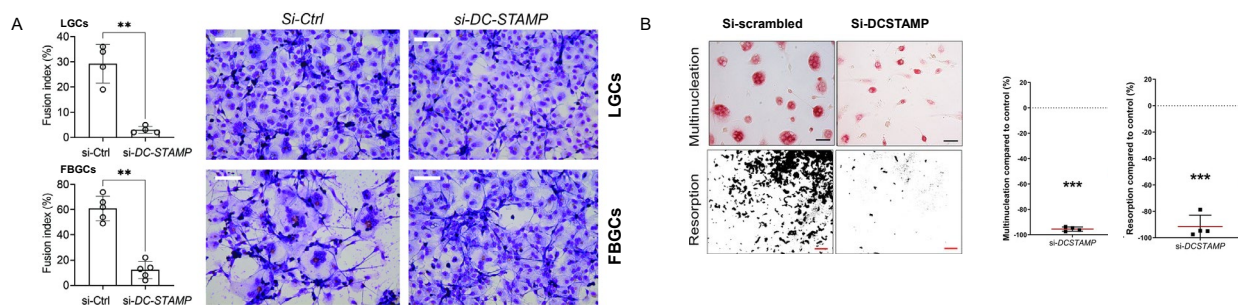
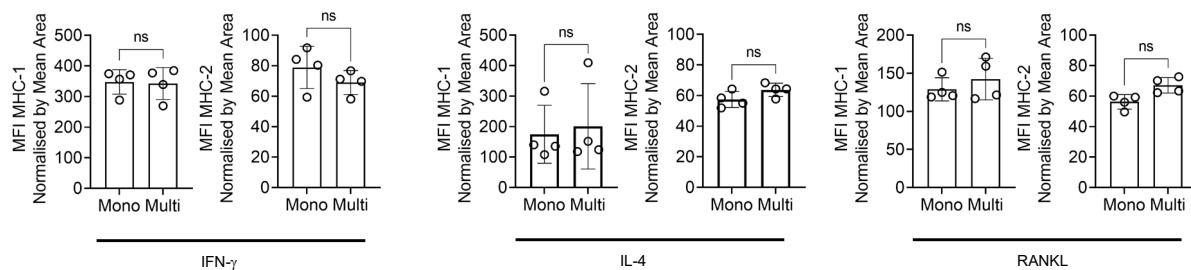


Figure. A. Fusion index following DC-STAMP knockdown in human LGCs (upper panel) and FBGCs (lower panel); n=4 donors; significance tested by paired t-test. Fusion was measured in cells stained with Giemsa. **B.** Human osteoclast multinucleation (upper panel, TRAP staining) and resorption (lower panel, hydroxyapatite) in si-Scrambled and si-DCSTAMP conditions. Quantification is shown on the right panel. Data presented in panel B are from Pereira et al., *Elife* 2020 [PMID:32553114].

#4 In Figure 4, the authors showed that transcripts in LGCs were enriched for antigen presentation and adaptive immune system pathways. In addition, multinucleation of LGCs increased the surface expression of B7-H3 (CD276) and colocalized with CD3⁺ cells, suggesting that LGC multinucleation potentiates T cell activation. However, the authors did not present enough data to demonstrate the antigen-presenting ability of LGCs or their specific T cell activating capacity.

[Authors' reply]. We agree with the reviewer that our data on a potential role of LGCs' on T cell activation is based on increased surface expression of B7-H3 and the unique CD3⁺ cluster forming ability of LGCs. In order to check for further markers of antigen presentation, we have performed MHC-1 and MHC-2 quantification by ImageStream in 3 types of MGCs (see below).



Although there was no difference in MHC-1/MHC-2 between the mononucleated and multinucleated macrophages, the mean fluorescent intensity (MFI) range was the highest in IFN- γ -stimulated macrophages, suggesting that LGCs may be better equipped for antigen presentation than the other 2 types of MGCs. A more comprehensive analysis of antigen presentation requires enzymatic digestion and isolation and phenotyping of LGCs from clusters *in vitro* and human tissues *in vivo*. This is a program of research that we have initiated as part of a separate study, which will focus on the *in vivo* relevance of the current findings such as the unique Ag presentation ability of LGCs in a non-sterile tissue environment.

5 Figure 6 clearly shows that mature multinucleated osteoclasts exhibit increased ATP production and maximal respiration. However, the glycolytic pathway did not differ between mononuclear and multinuclear osteoclasts. No explanation for this observation has been provided. It is easy to understand that osteoclasts acquire ATP through aerobic respiration during multinucleation. But how NADPH, which is essential for its redox reaction, is produced? Is it by acquiring α KG from the glutamine pathway?

[Authors' reply]. This is a point worth expending (see also discussion; highlighted text). Osteoclast multinucleation is characterized by increased mitochondrial gene expression which also translates into increased spare respiratory capacity (SRC or maximal respiration). This metabolic rewiring does not modify glycolysis and basal respiration rate. As the reviewer correctly states, increased SRC may be a way to supply more ATP to the energy-demanding polykaryon.

As per the production of NAD(P)H as an electron source for ETC, it could indeed be through glutamine rather than glucose usage in multinucleated osteoclasts. Furthermore, as iron is an essential cofactor for ETC activity through activity of iron-sulfur clusters, the mitochondrial

concentration of iron is likely to be critical for the mitochondrial activity of multinucleated osteoclasts (see also discussion).

Minor comments:

#6 Supplementary tables 1-6 were not provided.

[Authors' reply]. We apologize for this. The revised versions of supplementary tables are provided as part of the revised manuscript.

#7 Figure 2D right panel, difficult to see DAPI+ nuclei.

[Authors' reply]. Thanks for pointing this out. We have now replaced Figure 2D with a more pronounced DAPI+ nuclei.

Reviewer #2 (Significance (Required)):

Although it is well known that multinucleation of cells constantly occurs, especially in osteoclasts, skeletal muscle, and trophoblasts of the placenta, the biological significance of multinucleation and the intracellular functions of multinucleation are not well understood. In this unique study, three types of multinucleated cells were generated from human peripheral blood to elucidate the genetic and functional differences between mononucleated and multinucleated cells. Furthermore, by demonstrating the possibility that the morphological peculiarity of multinucleation can regulate cell function, this paper provides clues to understanding the underlying biology of multinucleated cells and how they maintain cell function in homeostatic and pathological settings.

[Authors' reply]. We thank the reviewer for finding our study unique and biologically meaningful. We also thank the reviewer for all the suggestions that improved significantly the overall message of the manuscript.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:

The manuscript of Ahmadzadeh and Pereira et al is an interesting study of the fusion process key to the formation of multinucleated giant cells (MGCs). Our current ability to discriminate between different types of MGCs is limited, and there are gaps in our understanding of the molecular determinants of cell fusion. In this study, the authors isolated different MGC variants - osteoclasts, Langhans giant cells (LGCs) and foreign body giant cells (FBGCs) and identified common, as well as MGC-specific genes and pathways involved in the process of cell fusion. The approach of isolating and comparing different types of MGCs is novel, and the manuscript is well presented and written. However, due to the in vitro nature of the study, the physiological significance of the findings is unclear. I have further minor and major points for the authors to address, as detailed below.

Minor comments:

1. The approach to isolate the different MGCs using FACS and imaging technique is highly novel. However the difference between MGC subtypes isolated isn't immediately apparent beyond the morphological comparisons. In my opinion some of the results of MGC-specific assays from Figures 4, 5 and 6 can be included in Figure 1, e.g. TRAP staining and hydroxyapatite resorption for osteoclasts, to provide evidence of purity and specificity of each MGC subtype early on in the manuscript. Classical or canonical genes associated with each MGC subtype can also be highlighted in the volcano plots in Figure 1C, e.g ACP5, CTSK, TNFRSF11A for osteoclasts.

[Authors' reply]. We thank the reviewer for this point and we agree it is important to highlight markers for each polykaryon early in the manuscript. In accordance with this reviewers' comment (and also with Reviewer 1's point), we first verified existence of lineage-dependent factors and markers of LGCs and FBGCs as these cells are relatively less well-defined compared to osteoclasts. (New Supplementary Figure 1B and C and New Supplementary Table 1). As expected, and in line with the lineage-determining effects, the transcriptomics comparison between mononucleated/multinucleated IFN- γ and IL-4-differentiated macrophages showed predominance of IFN- γ and IL-4-related pathways, respectively (New Supplementary Figure 1B and C and New Supplementary Table 1). Among known LGC and FBGC markers, we confirmed up-regulation of CCL7 [1] and CD86 [2], respectively (New Supplementary Table 1). We have added this information in the revised manuscript (see highlighted text). Osteoclast phenotyping is provided by TRAP staining and resorption assay (Figure 6C) and we also confirm that CTSK is indeed significantly up-regulated upon multinucleation (LogFc=1.69; $P=9.2 \times 10E-6$; highlighted in the revised manuscript).

2. The overall decrease in phagocytic identity of all the MGCs, and the specific upregulation of phagocytic pathways in the FBGCs are conflicting. Are there subsets of phagocytic pathways that were down and upregulated during the formation of FBGCs?

[Authors' reply]. This is a very good point. As the reviewer indicates, the results suggest that subsets of phagocytic pathways are changed upon multinucleation. All three types of MGCs show a downregulation of transcripts that belong to Fc receptors and complement C1Q family. However only FBGCs show an up-regulation of S. Aureus bioparticle-mediated phagocytosis. Hence the exact surface receptors responsible for this pathogen clearance remain to be identified. FBGC phagocytosis is a complex process including non-canonical phagocytosis pathways and participation of increased membrane area and endoplasmic reticulum [5, 6]. Whether these pathways are specifically induced in human FBGCs remain to be identified. We now discuss this point in the revised manuscript (see highlighted text in Discussion).

3. What are the identities of the mononuclear cells in each of the MGC experiment? They appeared to be quite heterogeneous based on the DEGs identified, beyond the common phagocyte signature. Can the authors comment on the difference between the mononuclear cells and whether this will affect the DEG analysis?

[Authors' reply]. This is also a very relevant point that we now address in the revised manuscript (New Supplementary Figure 1B and C; New Supplementary Table 1 and highlighted revised text in Results). The reviewer is correct that MGC-specific pathways are in line with the known function of each polykaryon (Figure 4A, 5A and 6A). To what extent lineage-dependent effects (e.g. IFN- γ and IL-4) are conserved between the mononucleated and multinucleated state is yet to be determined. In order to address this point, we compared DEG in IFN- γ and IL-4-differentiated mononucleated macrophages to the ones obtained in multinucleated macrophages (New Supplementary Figure 1B and C; New Supplementary Table 1). The results showed that the multinucleated cell state preserves the majority of the lineage-dependent pathways which are very significantly represented at the mononucleated cell state (e.g. IFN- γ and IL-4-related pathways). Interestingly, although less significant, this analysis also showed pathways that were specific to the mononucleated or multinucleated state in IFN- γ -differentiated macrophages when compared to IL-4-differentiated ones and vice versa. (Supplementary Figure 1B and C). For instance, TRAF3-dependent IRF activation pathway is specific to mononucleated IFN- γ -differentiated macrophages (Supplementary Figure 1B).

4. The authors should also frame/discuss the findings in the context of diagnostic and therapeutic potentials to highlight the clinical significance of this study.

[Authors' reply]. We thank the reviewer for this point and we now discuss our results from a clinical/diagnostic perspective (see highlighted text in the Discussion and below).

From a clinical perspective, since lysosome-regulated intracellular iron homeostasis appears to be a general condition for macrophage multinucleation across different tissues, its blockade may hold therapeutic potential. However, it is still unclear whether granulomatous disease can benefit from targeting LGC fusion. For non-granulomatous inflammatory diseases, inhibiting MGC formation by targeting lysosomes may be a therapeutic avenue. This approach would avoid FBGC-related adverse effects during foreign body reaction or inhibit the formation of MGCs of white adipose tissue during obesity. v-ATPase inhibitors have been previously proposed to inhibit osteoclast activity and bone resorption [7] so their selective targeting in the lysosomal compartment may be generalized to other MGCs such as FBGCs. In addition to potential clinical translation, the results presented in this study require confirmation in tissues originating from human pathology involving MGCs.

Major comments:

- As mentioned before, the physiological significance of the findings is unclear. Some form of in vivo data is needed to support some of the key conclusions of the study, e.g validating some of the markers of the pathways identified (common and MGC subtype-specific), and the role of lysosome-mediated iron homeostasis in multinucleation. The authors can make use of the FACs and imaging approaches they developed to look at MGCs in relevant tissues.

[Authors' reply]. This is an important point that we would like to explore in a comprehensive way. We have initiated a 2-year program to undertake a Multiplexed Immunohistochemistry (mIHC)

using MILAN (Multiple Iterative Labeling by Antibody Neodeposition) <https://www.lpcm.be/multiplex-ihc-milan> approach in human biopsies using >100 antibodies. The current study is pivotal in selecting the gene targets (i.e. common and MGC-specific markers) for prioritization. We foresee to gain critical pathophysiological information about the tissue characteristics of MGCs. The reviewer would acknowledge that these high-throughput and biopsy-based initiatives are lengthy and not the primary scope of our current findings which set the foundation of major cellular events governing multinucleation in macrophages.

Reviewer #3 (Significance (Required)):

Significance:

- The approach of isolating and comparing different types of MGCs is novel, and the findings certainly improved our understanding of the fusion processes of MGCs. However the physiological role of these processes in health and disease that involve MGCs is still unclear due to the lack of in vivo data. The findings were discussed in quite a bit of detail in the context of current literature, though clinical impact was not explored.

[Authors' reply]. We are grateful to Reviewer 3 for raising relevant and constructive points regarding the main findings. His/her review significantly improved the clarity of the overall manuscript.

We recognize our study lacks human clinical association but we highlight the prospective translatability of our findings and the usage of donor-based human macrophages throughout the manuscript. As also recommended by Reviewer 1 in his/her cross-consultation, we discuss the potential clinical impact of our findings in the Discussion of our revised manuscript.

- My background is bone biology with a very keen interest in osteoclast biology so arguably my knowledge on other MGCs eg LGCs and FBGCs is limited.

References

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Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have primary datasets been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section (p16)
Were human clinical and genomic datasets deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are computational models that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective data citations in the reference list.	Not Applicable	