

Peer Review File

Structural reconstruction of mouse acute aortic dissection by intravenously administered human-Muse cells without immunosuppression



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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In the present study, Dr. Takahashi et al. aimed to determine if intravenously administrated human-Muse cells can reconstruct mouse acute aortic dissection and improve survival. The study found that Muse cells had lowered the incidence of aortic rupture compared to the MSC-50K and vehicle group in a mouse model of human type B-AAD. Further, Muse cells more effectively homed to the ADD tissue, differentiated into CD31+ and alpha-SMA+ cells and produced elastic fibers that suppressed diameter expansion. Searching for the mechanisms, they found that higher expression of S1P in the ADD region is important for recruiting Muse cells, while MSC-750K reduced S1P levels resulting in less cells in the ADD region. HLA-G expression in Muse cells was the key to prevent rejection and Muse cells contributed to reinforcement of the aortic wall by producing and reorganizing elastic fibers. This is very interesting and novel study showing the efficacy of Muse cells, but not similar number of MSC, in preventing aortic rupture. Overall, the study design is reasonable, and the data are consistent to support their conclusion. The study will be of interest to the field of stem cell therapy, especially treating aortic dissection. Statistical analysis is appropriate. This reviewer would accept the paper for publication with minor revision. There are a few comments.

Specific comments:

1. Did authors confirm that mice died due to aortic rupture?
2. Figure 1B shows the survival after cell administration. It's unclear when cells were intravenously given following human angiotensin administration. 24 hours after angiotensin or CT scan confirming the ADD? This needs to be clarified.
3. Figure 2 shows that the majority of MSC-750 accumulated in the lung at 1 week, but disappeared at 4 weeks, likely due to rejection. The authors need to confirm if MSC express HLA-G.
4. Figure 3 and figure 6 examined GFP+alpha-SMA+ cells and the impact of non-Muse cells on S1P expression in AAD-aorta. Authors concluded that non-Muse MSC reduced/suppressed S1P expression/production. It would be better to discuss the potential mechanism.
5. Do non-Muse MSC express S1P receptors?

Reviewer #2 (Remarks to the Author):

Takahashi and colleagues conclude that a specialized cell called MUSE cells (SSEA-3 positive) have superiority in aortic repair than do basic MSCs. The study uses an elegant model of aortic dissection in the mouse, generated by using β -aminopropionitrile fumarate and angiotensin-II resulting in reproducible Stanford type B aortic dissection.

The study is interesting and rigorously conducted providing potentially interesting insights, that the subtraction of bone marrow MSCs known as MUSE cells are the ones with the greatest propensity to engraft and repair a damaged aorta in this model. Moreover, this process is HLA-G dependent.

One minor criticism is the over-reliance on statistical significance to draw conclusions. Statistical significance is important but magnitude of effect also is important and should be relayed as effect size with confidence intervals. With regard to statistical significance, has appropriate correction for multiple comparisons been employed? and if not why not?

An additional question relates to sex differences. Is the same effect seen in Male and Female mice?

Can the authors address the impact of various culture expansion techniques with regard to preservation or enhancement of the the MUSE subfraction?

Reviewer #3 (Remarks to the Author):

Yoshikatsu Saiki and colleagues demonstrated that intravenous injection of human muse cells in aortic aneurysm and dissection (AAD) mice mitigated the mortality and diameters enlargement rates effectively. In addition, the authors found that the muse cells are not immunosuppressed and could reside in aneurysmal or dissected aortas dependently on HLA-G and S1P-mediated chemotaxis. Mechanistically, the authors indicated that muse cell reduced B-AAD mortality by differentiating into smooth muscle cells and endothelial cells, and producing elastin. This is overall an interesting study with clinical significance. However, the manuscript showed some flaws in methodological design and lack of some critical evidence to support the conclusions what author proposed.

Major concerns:

1. The reason for investigating Stanford type B-AAD is not sufficient. The surgery is highly invasive and carries the risk of significant mortality and devastating morbidities is same as Stanford type A-AAD, why not the authors investigated it?
2. The authors should clarify the definitions of Stanford type B-AAD and Stanford type A-AAD. Figure 1C: some of aortic tissue are clearly Stanford type A-AAD. The authors should provide all the aortic images and clarify the type of AAD by each mouse.
3. Figure 6A-C: non-muse group decreased S1P in plasma and CD45 positive cell numbers markedly, could non-muse group improve AAD mice mortality and diameter enlargement rates? The experimental groups should be designed as (1) no cells (Vehicle); (2) MSCs-750 k; (3) muse cells; (4) MSCs-non-muse cells.
4. As mentioned in discussion part, muse cells produced many cytokines and other beneficial substances. Were there other things produced by muse cell that improve Stanford B-AAD.
5. The introduction part is too long. Please reorganize it, just state the direct associated content and research rationale in current study.

Minor concern:

1. The experimental design was ambiguous, schematic illustration of the experimental design should be provided.
2. The supplementary figures in manuscript is out of order.
3. Figure 1C: please make sure the size of scale bar.
4. What was the reason for the lower survival rate of MSC-50k than vehicle group?
5. Statistical analysis part: please make sure the method of multiple comparison when there were more than two groups.
6. Line 548-550: please correct the contradictory statement.
7. Figure 4A-F: the number of double positive cells were much less than those in the statistical

figure, please confirm.

8. Figure 5A: what is the reason for no blood in false lumen?

Dear referees,

Thank you for providing us with the referees' comments on our manuscript, "Structural reconstruction of mouse acute aortic dissection by intravenously administered human-Muse cells without immunosuppression (COMMSMED-23-0491-T)". We would also thank the referees for their thoughtful and constructive feedback, which we believe has helped us to greatly improve our manuscript. We appreciate your kind consideration and substantially revised our manuscript to address the issues raised by the referees.

We carefully considered each of the concerns raised by the referees, and our point-by-point responses to each comment are provided below. Thank you once again for your kind consideration of our paper.

Yours sincerely,

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Reviewers' comments:

Reviewer #1:

In the present study, Dr. Takahashi et al. aimed to determine if intravenously administered human-Muse cells can reconstruct mouse acute aortic dissection and improve survival. The study found that Muse cells had lowered the incidence of aortic rupture compared to the MSC-50K and vehicle group in a mouse model of human type B-AAD. Further, Muse cells more effectively homed to the ADD tissue, differentiated

into CD31+ and alpha-SMA+ cells and produced elastic fibers that suppressed diameter expansion. Searching for the mechanisms, they found that higher expression of S1P in the ADD region is important for recruiting Muse cells, while MSC-750K reduced S1P levels resulting in less cells in the ADD region. HLA-G expression in Muse cells was the key to prevent rejection and Muse cells contributed to reinforcement of the aortic wall by producing and reorganizing elastic fibers. This is very interesting and novel study showing the efficacy of Muse cells, but not similar number of MSC, in preventing aortic rupture. Overall, the study design is reasonable, and the data are consistent to support their conclusion. The study will be of interest to the field of stem cell therapy, especially treating aortic dissection. Statistical analysis is appropriate. This reviewer would accept the paper for publication with minor revision. There are a few comments.

Specific comments:

1. Did authors confirm that mice died due to aortic rupture?

→ Yes, we did. The information is newly mentioned in Page 20, 2nd paragraph:

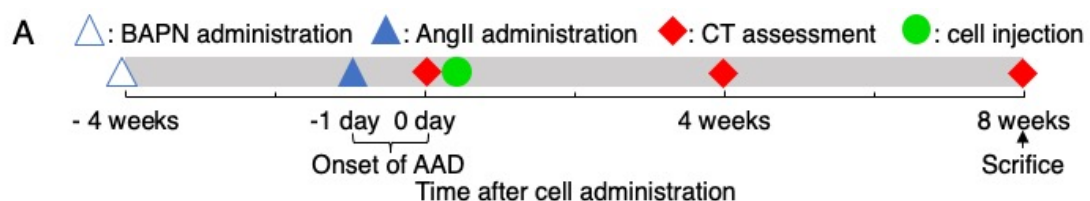
“Approximately 20% of AAD model mice died within 24 h after Ang II administration. The dissection of those mice clarified that almost all of the deaths were due to the rupture of the dissected thoracic aorta.”

2. Figure 1B shows the survival after cell administration. It's unclear when cells were intravenously given following human angiotensin administration. 24 hours after angiotensin or CT scan confirming the ADD? This needs to be clarified.

→ Thank you for the comment. The timing of the cell administration was described in the cell infusion section, page 11, 3rd paragraph:

“Cells were administered intravenously immediately after CT imaging (Figure 1A).”

We also created a new Figure 1A and added the following information in the legend.



Page 40, Figure legend:

“A) The outline of this study. Stanford B-AAD develops within 24 hours of starting Ang II administration. Twenty-four hours after the administration of Ang II, the mice were randomly assigned to the following 4 groups and underwent CT imaging. Cells were administered

intravenously immediately after CT imaging. BAPN: β -aminopropionitrile fumarate salt. Ang II: angiotensin II. AAD: acute aortic dissection.”

3. Figure 2 shows that the majority of MSC-750 accumulated in the lung at 1 week, but disappeared at 4 weeks, likely due to rejection. The authors need to confirm if MSC express HLA-G.

→ Thank you for the comment. According to the previous report, the HLA-G expression in MSCs is considerably lower than in Muse cells¹⁹. MSCs that were accumulated in the lung at 1 week might be due to the capillary trap after intravenous injection rather than selective migration to the lung^{19,38,39}. The disappearance of MSCs from the lung at 4 weeks might be due to immune rejection, partly because of their low HLA-G expression. We newly included these in the main text as follows;

Page 28, the bottom line:

“Furthermore, the expression of HLA-G in MSCs was reported to be significantly lower than that in Muse cells¹⁹, suggesting the lower immune tolerance of MSCs compared to Muse cells.”

Page 29, the last five lines:

“The accumulation of MSCs in the lung at 1 week after intravenous injection might be due to capillary trap rather than selective migration to the lung^{19,38,39}. As reported, the disappearance of MSCs from the lung at 4 weeks might be due to rejection, owing to the low HLA-G expression¹⁹.”

For these parts, we newly cited papers below in the main text;

19. Yamada, Y., *et al.* S1P-S1PR2 Axis Mediates Homing of Muse Cells Into Damaged Heart for Long-Lasting Tissue Repair and Functional Recovery After Acute Myocardial Infarction. *Circ Res* **122**, 1069-1083 (2018).
38. Shono, Y., *et al.* Protection of liver sinusoids by intravenous administration of human Muse cells in a rat extra-small partial liver transplantation model. *Am J Transplant* (2020).
39. Yabuki, H., Wakao, S., Kushida, Y., Dezawa, M. & Okada, Y. Human Multilineage-differentiating Stress-Enduring Cells Exert Pleiotropic Effects to Ameliorate Acute Lung Ischemia-Reperfusion Injury in a Rat Model. *Cell Transplant* **27**, 979-993 (2018).

4. Figure 3 and figure 6 examined GFP+ α -SMA+ cells and the impact of non-Muse cells on S1P expression in AAD-aorta. Authors concluded that non-Muse MSC reduced/suppressed S1P expression/production. It would be better to discuss the

potential mechanism.

→ Thank you for the valuable suggestion. As the referee pointed out, we didn't make it clear enough in the previous version. We revised the main text as follows;

Page 29, 2nd paragraph:

“S1P receptor 2 was reported to be expressed in non-Muse cells with lower level than that in Muse cells^{12,19}. Therefore, the presence of a large number of non-Muse cells may compete with Muse cells for receiving S1P in the MSC group.”

For this part, we cited the following articles:

12. Sato, T., *et al.* A Novel Type of Stem Cells Double-Positive for SSEA-3 and CD45 in Human Peripheral Blood. *Cell Transplant* **29**, 963689720923574 (2020).
19. Yamada, Y., *et al.* S1P-S1PR2 Axis Mediates Homing of Muse Cells Into Damaged Heart for Long-Lasting Tissue Repair and Functional Recovery After Acute Myocardial Infarction. *Circ Res* **122**, 1069-1083 (2018).

5. Do non-Muse MSC express S1P receptors?

→ Thank you for the comment. As mentioned above, non-Muse MSC express S1P receptors as reported previously:

12. Sato, T., *et al.* A Novel Type of Stem Cells Double-Positive for SSEA-3 and CD45 in Human Peripheral Blood. *Cell Transplant* **29**, 963689720923574 (2020).
19. Yamada, Y., *et al.* S1P-S1PR2 Axis Mediates Homing of Muse Cells Into Damaged Heart for Long-Lasting Tissue Repair and Functional Recovery After Acute Myocardial Infarction. *Circ Res* **122**, 1069-1083 (2018).

Reviewer #2:

Takahashi and colleagues conclude that a specialized cell called MUSE cells (SSEA-3 positive) have superiority in aortic repair than do basic MSCs. The study uses an elegant model of aortic dissection in the mouse, generated by using β -aminopropionitrile fumarate and angiotensin-II resulting in reproducible Stanford type B aortic dissection.

The study is interesting and rigorously conducted providing potentially interesting insights, that the subtraction of bone marrow MSCs known as MUSE cells are the ones

with the greatest propensity to engraft and repair a damaged aorta in this model. Moreover, this process is HLA-G dependent.

One minor criticism is the over-reliance on statistical significance to draw conclusions. Statistical significance is important but magnitude of effect also is important and should be relayed as effect size with confidence intervals. With regard to statistical significance, has appropriate correction for multiple comparisons been employed? and if not why not?

→ Thank you for the comment. We consulted our data with statistical expert Dr. Hiroki Yoshida (Data Seed Inc., Tokyo, Japan) to ensure the appropriate conduction of our reanalysis regarding multiple comparisons. The Steel-Dwass test performs non-parametric multiple comparisons of all pairs, and the Tukey-Kramer test makes multiple comparisons of the means of all pairs. Multiplicity was appropriately corrected in the both methods. We used the Tukey-Kramer test which is easier to find statistical significance when it follows a normal distribution in the previous version. However, considering the small sample size of this study, we agree to take into account the importance of performing nonparametric multiple testing rather than focusing on whether the results followed a normal distribution. In the revised version, we reanalyzed all of the multiple comparisons by using the Steel-Dwass test. Although we understand the importance of confidence intervals, we used standard deviations rather than confidence intervals because this study was an exploratory study and was not directly related to the results of nonparametric tests.

We revised the information as follows,

Page 18, line 1:

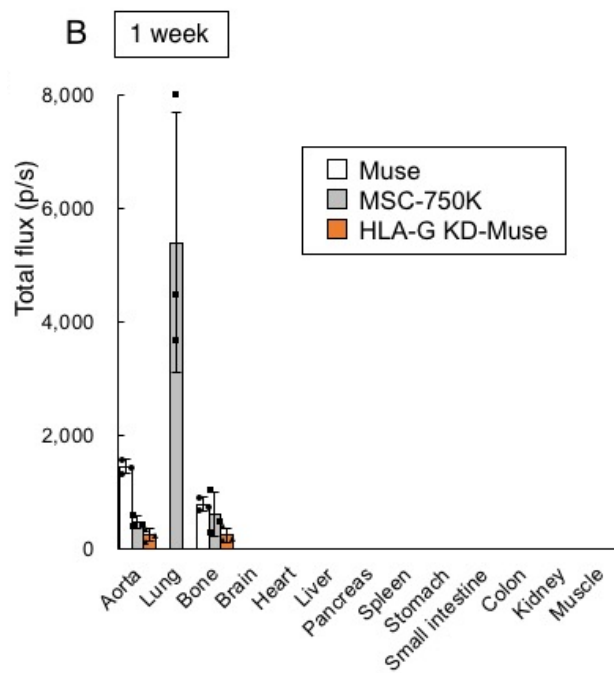
“The comparison of three or more groups was performed by using Steel-Dwass test,”

Page 23, 2nd paragraph:

“Comparison of the total light intensity of Akaluc-GFP Venus in each organ revealed that the signal in the aorta in the Muse group was 2.99 times higher than that in the MSC-750K group ($P=0.19$) and 5.97 times higher than that in the HLA-G KD-Muse group ($P=0.19$) at 1 week (Figure. 2B). The signal in the MSC-750K group was 2.00 times higher than that in the HLA-G KD-Muse group ($P=0.19$) at 1 week (Figure 2B).”

Due to the different statistical analysis, final results were changed as follows:

Figure 2B: No statistically significant difference observed.



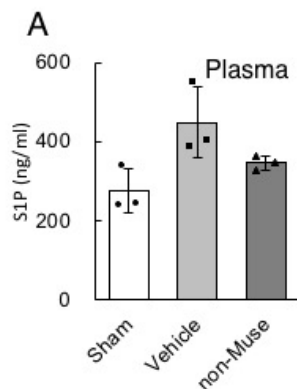
Page 42, Figure legend line 11:

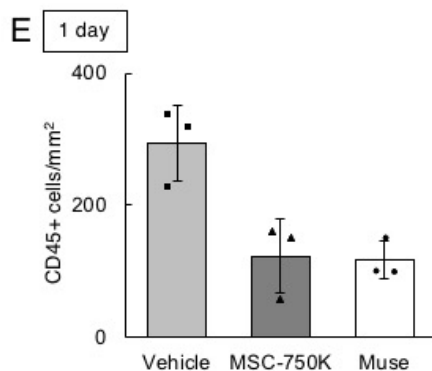
“by Steel-Dwass test (**B**)”

Page 26, the last paragraph:

One day after cell injection, the plasma S1P concentration (ng/ml) in the vehicle group was the highest among the 3 groups **without** significance to the sham group ($P=0.19$).

Figure 6A, 6E: No statistically significant difference observed.





Page 50, Figure legend line 19:

“by Steel-Dwass test (A, B, E)”

Although the significant difference disappeared due to more detailed multiple testing, a trend we mentioned in the previous version was maintained.

We did not mention Dr. Yoshida’s name in the acknowledgement because he told us that we do not need to include his name in the acknowledgement.

An additional question relates to sex differences. Is the same effect seen in Male and Female mice?

→As we used only male mice in this study, the effect on female mice is unknown. We did not use female mice because previous papers used only male mice^{30,31}, probably in order to exclude the effect of estrous cycle on aortic dissection. We mentioned about this in Method section as follows,

Page 10, line 3:

“Male 4-week-old wild-type mice (C57BL/6NCrS1c, Japan SLC, Inc., Hamamatsu, Japan) were used. Stanford B-AAD model mice were created according to the previously described method with minor modifications^{30,31}.”

We cited the following articles:

30. Kurihara, T., *et al.* Neutrophil-derived matrix metalloproteinase 9 triggers acute aortic dissection. *Circulation* **126**, 3070-3080 (2012).
31. Anzai, A., *et al.* Adventitial CXCL1/G-CSF expression in response to acute aortic dissection triggers local neutrophil recruitment and activation leading to aortic rupture. *Circ Res* **116**, 612-623 (2015).

Can the authors address the impact of various culture expansion techniques with regard to preservation or enhancement of the MUSE subfraction?

→ Thank you for the comment. Regarding the culture of bone marrow MSCs and the

sorting method of Muse cells, we believe that the protocol is reproducible because the protocol for adjusting SSEA-3 positive cells has been established²⁹. We mentioned this point in Method section as follows,

Page 8, line 3:

“Human-Muse cells and -MSCs

Human BM-derived MSCs (PT-2501, Lonza, Basel, Switzerland) were used as the source of Muse (SSEA-3(+)) cells and non-Muse MSCs (cells other than Muse cells in MSCs [SSEA-3(-)]), as previously reported^{11,29}. Human BM-MSCs were cultured at 37°C and 5% CO₂ in low glucose Dulbecco's Modified Eagle Medium (10567, Thermo Fisher Scientific, Waltham, MA) with 10% (vol/vol) fetal bovine serum (FBS); HyClone (SH30910.03, GE Healthcare, Chicago, IL) and 0.1 mg/mL kanamycin (15160-054, Thermo Fisher Scientific). For collection of Muse and non-Muse MSCs, eighth-passage MSCs were incubated with rat anti-SSEA antigen-3 (SSEA-3) IgM antibody (1:1000; 330302, BioLegend, San Diego, CA) as the primary antibody (rat IgM isotype control antibody, 1:1000; 400801, BioLegend), fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgM antibody (1:100; 112-095-075, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) as the secondary antibody, and anti-FITC MicroBeads (1:50; Miltenyi Biotec Inc, Auburn, CA) as the tertiary antibody. MSCs were then separated into anti-FITC(+) and anti-FITC(-) cell fractions by magnetic-activated cell sorting (MACS) (autoMACS Pro Separator, Miltenyi Biotec Inc, Auburn, CA) and the FITC(+) cell fraction was used for the Muse cell injection group. Analysis by FACS (BD FACS Aria II, Becton Dickinson, Franklin Lakes, NJ) revealed that human BM-MSCs contained 5.0±0.9% SSEA-3(+)-Muse cells (Figure S1). The FITC(+) cell fraction sorted by MACS contained 76.5±6.0% SSEA-3(+)-Muse cells (Figure S1). Therefore, SSEA-3(+)-Muse cells were enriched from ~5% to ~76% by MACS. The SSEA-3(-) cell fraction was isolated by fluorescence activated cell sorting (FACS) and used as non-Muse MSCs (Figure S1).”

For this part, we cited the following articles:

11. Kuroda, Y., *et al.* Unique multipotent cells in adult human mesenchymal cell populations. *Proc Natl Acad Sci U S A* **107**, 8639-8643 (2010).

We newly cited the article below:

29. Kuroda, Y., *et al.* Isolation, culture and evaluation of multilineage-differentiating stress-enduring (Muse) cells. *Nat Protoc* **8**, 1391-1415 (2013).

In addition, therapeutic effects have been obtained in various models^{17-19,21-23}. We mentioned this point in Introduction as follows,

Page 6, line 8:

“Studies in which Muse cells were systemically administered in animal disease models of acute myocardial infarction, stroke, liver cirrhosis, chronic kidney injury, epidermolysis bullosa, and aortic aneurysm revealed unique features of Muse cells^{17-19, 21-23}”

We cited the following articles:

17. Uchida, N., *et al.* Beneficial Effects of Systemically Administered Human Muse Cells in Adriamycin Nephropathy. *J Am Soc Nephrol* **28**, 2946-2960 (2017).
18. Iseki, M., *et al.* Muse Cells, Nontumorigenic Pluripotent-Like Stem Cells, Have Liver Regeneration Capacity Through Specific Homing and Cell Replacement in a Mouse Model of Liver Fibrosis. *Cell Transplant* **26**, 821-840 (2017).
19. Yamada, Y., *et al.* S1P-S1PR2 Axis Mediates Homing of Muse Cells Into Damaged Heart for Long-Lasting Tissue Repair and Functional Recovery After Acute Myocardial Infarction. *Circ Res* **122**, 1069-1083 (2018).
21. Uchida, H., *et al.* Human Muse Cells Reconstruct Neuronal Circuitry in Subacute Lacunar Stroke Model. *Stroke* **48**, 428-435 (2017).
22. Fujita, Y., *et al.* Intravenous Injection of Muse Cells as a Potential Therapeutic Approach for Epidermolysis Bullosa. *J Invest Dermatol* (2020).
23. Hosoyama, K., *et al.* Intravenously injected human multilineage-differentiating stress-enduring cells selectively engraft into mouse aortic aneurysms and attenuate dilatation by differentiating into multiple cell types. *J Thorac Cardiovasc Surg* **155**, 2301-2313 e2304 (2018).

However, when considering clinical application, we think this should be considered as a future issue. Thank you again for valuable comments.

Reviewer #3:

Yoshikatsu Saiki and colleagues demonstrated that intravenous injection of human muse cells in aortic aneurysm and dissection (AAD) mice mitigated the mortality and diameters enlargement rates effectively. In addition, the authors found that the muse cells are not immunosuppressed and could reside in aneurysmal or dissected aortas dependently on HLA-G and S1P-mediated chemotaxis. Mechanistically, the authors indicated that muse cell reduced B-AAD mortality by differentiating into smooth muscle cells and endothelial cells, and producing elastin. This is overall an interesting study with clinical significance. However, the manuscript showed some flaws in methodological design and lack of some critical evidence to support the conclusions

what author proposed.

Major concerns:

1. The reason for investigating Stanford type B-AAD is not sufficient. The surgery is highly invasive and carries the risk of significant mortality and devastating morbidities is same as Stanford type A-AAD, why not the authors investigated it?

→ Thank you for the comment. As the mortality rate for Stanford type A-AAD is high, emergency surgery is the first choice. In other words, patients with Stanford type A-AAD will die within a few days without emergency surgery. This short time window may not be feasible for stem cell therapy because Stanford type A-AAD patients will die before the therapeutic effect of the administered cells is brought. For these reasons, we considered that cell therapy is not suitable for Stanford type A dissociation.

On the other hand, conservative treatment is selected for Stanford type B-AAD because the mortality rate is low if there are no complications. In other words, surgery is not absolutely required because it is highly invasive and the mortality rate for surgery is the same as that for conservative treatment. However, in the chronic phase, the fragile dissected vessels often enlarge and require surgery. This means that in Stanford B-AAD, unlike Stanford type A-AAD, there is a grace period until surgical treatment is required. Therefore, we focused on Stanford type B-AAD in this study, expecting stem cell treatment in the acute phase to structurally strengthen the dissected blood vessels, enabling us to avoid surgery in the chronic phase.

We newly mentioned this issue as follows,

Page 5, 1st paragraph:

“Acute aortic dissection (AAD) is a common, life-threatening disease that occurs with chest pain and/or back pain, mainly related to high blood pressure. AAD begins with a tear (entry) in the intima of the aortic wall and progresses into the media, forming the original aortic lumen (true lumen) and a newly created wall lumen (false lumen), and is associated with inflammation of the aortic tissue¹⁻⁴. AAD is classified into two types⁵; Stanford type A-AAD (Stanford A-AAD), which involves the ascending aorta, requires emergent surgery to place a prosthetic vascular graft because the prognosis is generally poor; Stanford type B-AAD (Stanford B-AAD) that does not involve the ascending aorta but extends distal to the aortic arch is usually treated conservatively for avoiding surgical risks if there are no complications^{6,7}. However, in Stanford B-AAD, the vulnerable dissected aorta often dilates over time and develops an aneurysmal transformation, increasing the risk of rupture⁸, which ultimately leads to significant mortality. This requires highly invasive surgery with the risk of serious complications such as spinal cord infarction⁹. Thoracic endovascular aortic

repair (TEVAR), a surgical treatment intended to close the entry that connects the true lumen and false lumen, in the acute phase may improve mortality and aneurysmal changes during the chronic phase¹⁰, while this treatment includes risk of complications. In other words, there is no debate that emergency surgery is necessary for Stanford A-AAD, but surgery for the acute phase of Stanford B-AAD remains controversial. We focused on Stanford type B-AAD, expecting to avoid surgery in the chronic phase through structural strengthening of the dissected aorta in the acute phase by stem cell treatment.”

2. The authors should clarify the definitions of Stanford type B-AAD and Stanford type A-AAD. Figure 1C: some of aortic tissue are clearly Stanford type A-AAD. The authors should provide all the aortic images and clarify the type of AAD by each mouse.

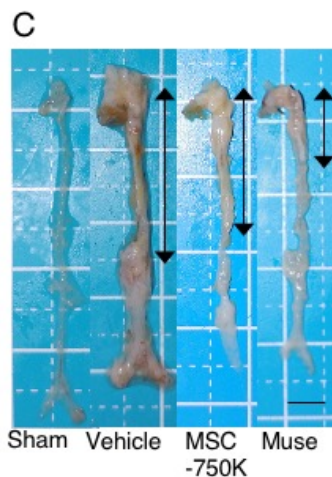
→ Thank you for the comment. Type B dissection is diagnosed by CT examination (Page 11, 2nd paragraph). We newly mentioned how Stanford B-AAD was diagnosed as follows;

Page 11, 2nd paragraph:

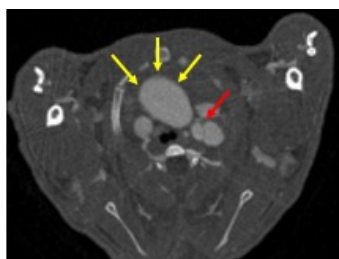
“Stanford B-AAD was diagnosed by confirming the presence of 2 cavities in the aorta other than the ascending aorta on multiple short axis images of contrast-enhanced CT. All CT images used for this study are provided in the supplementary data as follows. Repository URL: <https://doi.org/10.57760/sciencedb.13656>.”

While macroscopic findings in the previous Figure 1C indeed showed an enlargement of the ascending aorta, all the samples were confirmed to be an aneurysm rather than dissection in the ascending aorta by CT imaging. CT images of each sample before the preparation of specimens used in the previous Figure 1C are shown below.

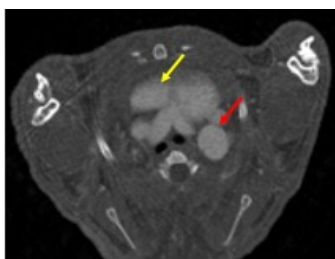
The previous Figure 1C:



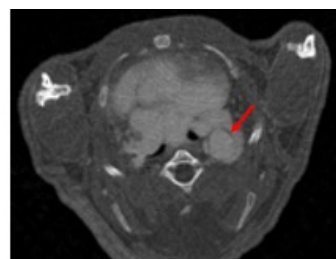
CT images of the vehicle group in the previous Figure 1C is shown below (All these CT images are in repository file: Vehicle diameter No.3-3 (8weeks)). The images showed that an enlargement of the ascending aorta is due to aneurysm, not dissection.



Although there is aneurysm (yellow arrow), there is no dissection in the proximal aortic arch. There is dissection in the distal aortic arch (red arrow).



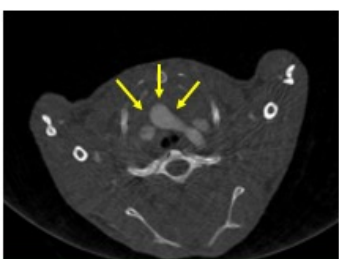
Although there is aneurysm (yellow arrow), there is no dissection in the ascending aorta. There is dissection in the descending aorta (red arrow).



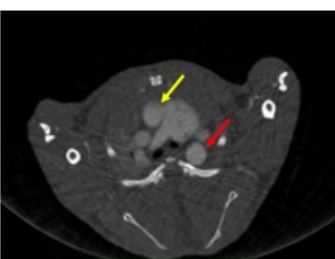
There is dissection in the descending aorta (red arrow).

Other CT images for Fig 1C samples are shown below:

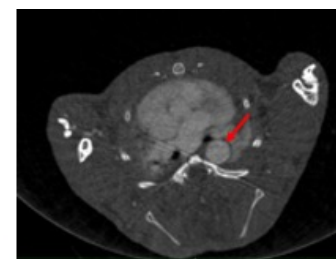
CT images of the MSC-750K group in the previous Figure 1C (All these CT images are in repository file: MSC-750K vascular marker at 8 weeks No.3 (8weeks)).



There is no dissection in the proximal aortic arch (yellow arrow).

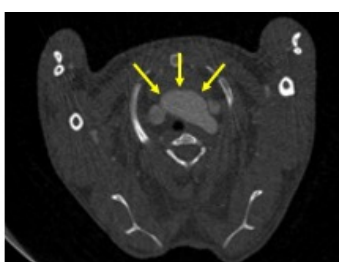


There is no dissection in the ascending aorta (yellow arrow). There is dissection in the descending aorta (red arrow).



There is dissection in the descending aorta (red arrow).

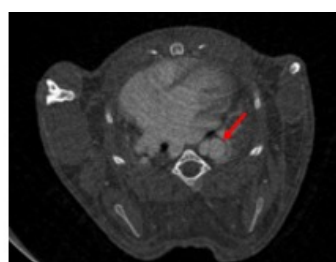
CT images of the Muse group in the previous Figure 1C (All these CT images are in repository file: Muse diameter No.7-3 (8weeks)).



Although there is aneurysm (yellow arrow), there is no dissection in the aortic arch.



There is no dissection in the ascending aorta (yellow arrow). There is dissection in the descending aorta (red arrow).



There is dissection in the descending aorta (red arrow).

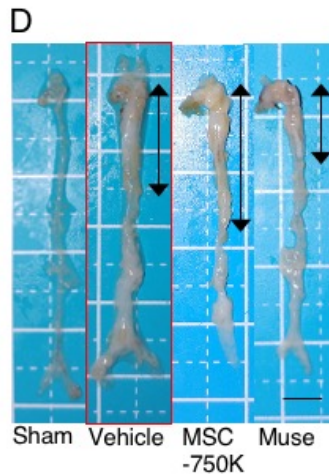
All mice used in this study were confirmed to be Stanford type B-AAD. All the CT images used for diagnosis are provided in the supplementary data as follows.

Repository URL: <https://doi.org/10.57760/sciencedb.13656>.

However, we agree with the referee's comment that the macroscopic image of the vehicle aorta in the previous Figure 1C was confusing and misleading. We therefore

replaced it with a clearer sample in the revised Figure 1D (the previous Figure 1C was changed to 1D because Figure 1A was newly created in the revised version).

The revised Figure 1D:



CT images of the revised vehicle group is as follows (All these CT images are in repository file: Vehicle diameter No.7-3 (8weeks)), confirming that there was no dissection in the ascending aorta. Thank you for the comment.



3. Figure 6A-C: non-muse group decreased S1P in plasma and CD45 positive cell numbers markedly, could non-muse group improve AAD mice mortality and diameter enlargement rates? The experimental groups should be designed as (1) no cells (Vehicle); (2) MSCs-750 k; (3) muse cells; (4) MSCs-non-muse cells.

→ Thank you for the comment. There are several reports comparing the effect of vehicle, MSCs, Muse cells and non-Muse cells, as the referee suggested²³. These reports, including the study of aortic aneurysm (Hosoyama et al., J Thorac Cardiovasc Surg, 2018), demonstrated nearly the same outcomes between the MSCs and non-Muse groups. This is because the composition of MSC and non-Muse cells is very close. Muse cells correspond to one to several percent of total MSCs. In other words,

non-Muse cells occupy 97~99% of MSCs and thus non-Muse cells and MSCs are very close each other. For this reason, we did not set the non-Muse group for evaluating therapeutic effect in this study. For S1P analysis, however, we considered that the separation of Muse and non-Muse cells might be critical, rather than setting MSCs that contain several percent of Muse cells, instead of non-Muse cells. Indeed, the aortic aneurysm study exhibited that the non-Muse group demonstrated very similar results to that of the MSC group²³. For these reasons, the non-Muse group is expected to exhibit very close outcomes in mortality and diameter expansion to those in the MSC group.

23. Hosoyama, K., *et al.* Intravenously injected human multilineage-differentiating stress-enduring cells selectively engraft into mouse aortic aneurysms and attenuate dilatation by differentiating into multiple cell types. *J Thorac Cardiovasc Surg* **155**, 2301-2313 e2304 (2018).

4. As mentioned in discussion part, muse cells produced many cytokines and other beneficial substances. Were there other things produced by muse cell that improve Stanford B-AAD.

→ Thank you for the comment. We did not search new other factors in this study. As previous reports demonstrated that Muse cells produces various factors related to anti-inflammatory and immune regulation such as prostaglandin E2, HGF, IDO and so on, these factors are likely to be effective in this model. The exploration of new cytokines and beneficial substances the future subject to be done. We newly mentioned in the main text as follows,

Page 30, 2nd paragraph:

“Muse cells produce factors relevant to anti-inflammation and immunomodulation, such as prostaglandin E2, interleukin-10, matrix metalloproteinase-2, hepatocyte growth factor, and indoleamine-2,3-dioxygenase^{17,39}. These factors are considered effective for Stanford B-AAD. On the other hand, searching for new cytokines and factors is a challenge for the future.”

17. Uchida, N., *et al.* Beneficial Effects of Systemically Administered Human Muse Cells in Adriamycin Nephropathy. *J Am Soc Nephrol* **28**, 2946-2960 (2017).

39. Yabuki, H., Wakao, S., Kushida, Y., Dezawa, M. & Okada, Y. Human Multilineage-differentiating Stress-Enduring Cells Exert Pleiotropic Effects to Ameliorate Acute Lung Ischemia-Reperfusion Injury in a Rat Model. *Cell Transplant* **27**, 979-993 (2018).

5. The introduction part is too long. Please reorganize it, just state the direct associated content and research rationale in current study.

→ We deleted sentences below to make Introduction short and succinct:

“Therapeutic effects of stem cells such as mesenchymal stem cells (MSCs) were evaluated in aortic aneurysm in which all three layers of the aortic wall were dilated^{11,12,13}, while only 1 study demonstrated statistically meaningful effectiveness of MSCs on AAD¹⁴, mainly because of the low engraftment of cells due to high levels of inflammation and stressful microenvironment induced by the aortic wall tearing¹⁵.

The revised introduction is now as follows:

Page 5, 1st paragraph:

“Acute aortic dissection (AAD) is a common, life-threatening disease that occurs with chest pain and/or back pain, mainly related to high blood pressure. AAD begins with a tear (entry) in the intima of the aortic wall and progresses into the media, forming the original aortic lumen (true lumen) and a newly created wall lumen (false lumen), and is associated with inflammation of the aortic tissue¹⁻⁴. AAD is classified into two types⁵; Stanford type A-AAD (Stanford A-AAD), which involves the ascending aorta, requires emergent surgery to place a prosthetic vascular graft because the prognosis is generally poor; Stanford type B-AAD (Stanford B-AAD) that does not involve the ascending aorta but extends distal to the aortic arch is usually treated conservatively for avoiding surgical risks if there are no complications^{6,7}. However, in Stanford B-AAD, the vulnerable dissected aorta often dilates over time and develops an aneurysmal transformation, increasing the risk of rupture⁸, which ultimately leads to significant mortality. This requires highly invasive surgery with the risk of serious complications such as spinal cord infarction⁹. Thoracic endovascular aortic repair (TEVAR), a surgical treatment intended to close the entry that connects the true lumen and false lumen, in the acute phase may improve mortality and aneurysmal changes during the chronic phase¹⁰, while this treatment includes risk of complications. In other words, there is no debate that emergency surgery is necessary for Stanford A-AAD, but surgery for the acute phase of Stanford B-AAD remains controversial. We focused on Stanford type B-AAD, expecting to avoid surgery in the chronic phase through structural strengthening of the dissected aorta in the acute phase by stem cell treatment.”

We cited the following articles for this paragraph:

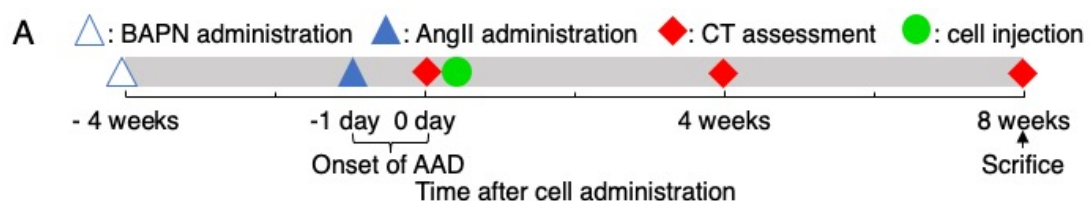
1. Nienaber, C.A. & Eagle, K.A. Aortic dissection: new frontiers in diagnosis and management: Part I: from etiology to diagnostic strategies. *Circulation* **108**, 628-635 (2003).

2. Erbel, R., *et al.* Diagnosis and management of aortic dissection. *Eur Heart J* **22**, 1642-1681 (2001).
3. Nakashima, Y., Kurozumi, T., Sueishi, K. & Tanaka, K. Dissecting aneurysm: A clinicopathologic and histopathologic study of 111 autopsied cases. *Hum Pathol* **21**, 291-296 (1990).
4. Nakashima, Y., Shiokawa, Y. & Sueishi, K. Alterations of elastic architecture in human aortic dissecting aneurysm. *Lab Invest* **62**, 751-760 (1990).
5. Bossone, E., LaBounty, T.M. & Eagle, K.A. Acute aortic syndromes: diagnosis and management, an update. *Eur Heart J* **39**, 739-749d (2018).
6. Kaji, S. Acute medical management of aortic dissection. *Gen Thorac Cardiovasc Surg* **67**, 203-207 (2019).
7. Estrera, A.L., *et al.* Outcomes of medical management of acute type B aortic dissection. *Circulation* **114**, I384-I389 (2006).
8. Schwartz, S.I., *et al.* Predictors of late aortic intervention in patients with medically treated type B aortic dissection. *J Vasc Surg* **67**, 78-84 (2018).
9. Bicknell, C.D., Riga, C.V. & Wolfe, J.H. Prevention of paraplegia during thoracoabdominal aortic aneurysm repair. *Eur J Vasc Endovasc Surg* **37**, 654-660 (2009).
10. Nienaber, C.A., *et al.* Endovascular repair of type B aortic dissection: long-term results of the randomized investigation of stent grafts in aortic dissection trial. *Circ Cardiovasc Interv* **6**, 407-416 (2013).

Minor concern:

1. The experimental design was ambiguous, schematic illustration of the experimental design should be provided.

→ Thank you for the valuable suggestion. We newly made the schema below and incorporated it into the main figure as Figure 1A.



Page 39, newly created Figure 1A and Figure Legend Page 40:

“(A) The outline of this study. Stanford B-AAD develops within 24 hours of starting Ang II administration. Twenty-four hours after the administration of Ang II, the mice were randomly assigned to the following 4 groups and underwent CT imaging. Cells were administered

intravenously immediately after CT imaging. BAPN: β -aminopropionitrile fumarate salt. Ang II: angiotensin II. AAD: acute aortic dissection.”

2. The supplementary figures in manuscript is out of order.

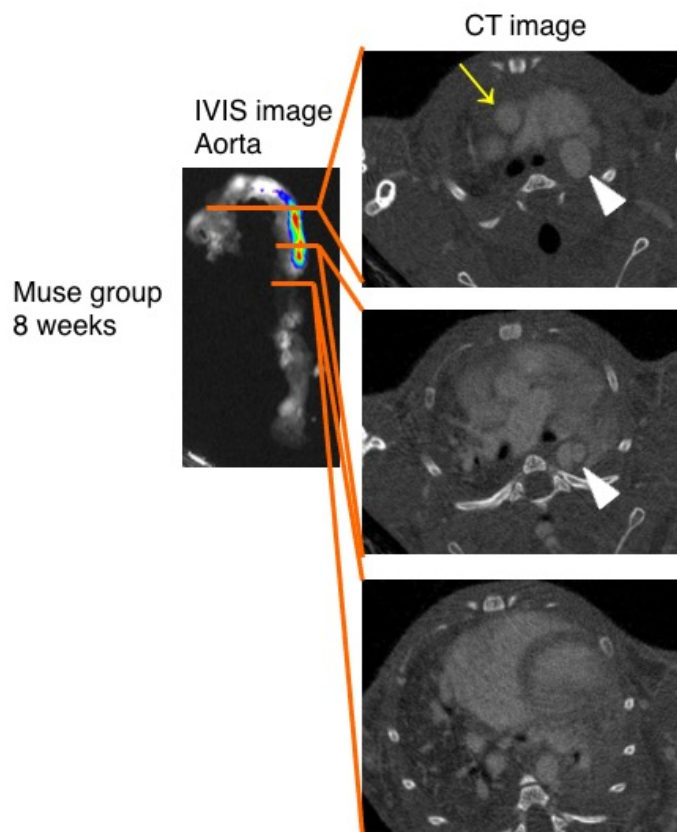
→ We checked the order of supplementary figures in the main text. As many of the supplementary figures are quoted in Method section, the order seems disorganized in Result section but the revised version is now confirmed throughout the main text.

Thank you for the suggestion.

In addition, the CT image in Supplementary Figure S2 was changed to a clearer image (All these CT images are in repository file: Muse IVIS at 8 weeks No.2 (0day).

Repository URL: <https://doi.org/10.57760/sciencedb.13656>), and the legend was also modified to be more accurate as follows;

Supplementary Figure S2:



Supplementary Figure S2 legend:

Correspondence between the IVIS Spectrum CT image and contrast-enhanced CT. CT was performed before IVIS imaging to confirm the site of Stanford **type B** acute aortic dissection (AAD). The distribution of Muse cells was shown to be concentrated on the dissected aorta and not on the intact aorta. Muse group **8 weeks**: **Stanford type B-AAD**

mice at 8 weeks after administered with Venus-Akaluc GFP-labeled 50,000 Muse cells. CT image: This CT images were taken before cell administration (at 0 day after cell administration). Yellow arrow: the ascending aorta without aneurysm. White arrowhead: the dissected and dilated descending thoracic aorta.

3. Figure 1C: please make sure the size of scale bar.

→ Thank you for pointing it out. I apologize for repeating my mistake. The correct value is 5 mm, not 50 mm. We corrected figure legend as follows,

Page 40, line 7 from the bottom:

“Scale bar: 5 mm.”

4. What was the reason for the lower survival rate of MSC-50k than vehicle group?

→ We considered this was due to a small number of animals in the MSC-50K group (n=10). The mortality rate of the MSC-50K group was higher than the MSC-750K group and was rather similar to that of the vehicle group up at the first n=10 experiment. Based on this, we decided to omit the MSC-50K group in the subsequent experiments and set the MSC-750K group as representative of the MSC groups (n=31). Since there was no statistically significant difference in mortality between the MSC-50K group (n=10) and the vehicle group (n=30) ($P=0.20$), we considered that the MSC-50K group was not inferior to the vehicle group.

These were mentioned on Page 21, 2nd paragraph:

“Because the MSC-750K group had better survival than the MSC-50K group and the data of the MSC-50K group was close to that of the vehicle group ($P=0.20$) (Figure 1C), the MSC-750K group was selected as representative of the two MSC groups in subsequent experiments.”

5. Statistical analysis part: please make sure the method of multiple comparison when there were more than two groups.

→ Thank you for the comment. We consulted our data with statistical expert Dr. Hiroki Yoshida (Data Seed Inc., Tokyo, Japan) to ensure the appropriate conduction of our reanalysis regarding multiple comparisons. The Steel-Dwass test performs non-parametric multiple comparisons of all pairs, and the Tukey-Kramer test makes multiple comparisons of the means of all pairs. Multiplicity was appropriately corrected in the both methods. We used the Tukey-Kramer test which is easier to find statistical significance when it follows a normal distribution in the previous version. However, considering the small sample size of this study, we agree to take into account the

importance of performing nonparametric multiple testing rather than focusing on whether the results followed a normal distribution. In the revised version, we reanalyzed all of the multiple comparisons by using the Steel-Dwass test.

We revised the information as follows,

Page 18, line 1:

“The comparison of three or more groups was performed by using Steel-Dwass test,”

Page 23, 2nd paragraph:

“Comparison of the total light intensity of Akaluc-GFP Venus in each organ revealed that the signal in the aorta in the Muse group was 2.99 times higher than that in the MSC-750K group ($P=0.19$) and 5.97 times higher than that in the HLA-G KD-Muse group ($P=0.19$) at 1 week (Figure. 2B). The signal in the MSC-750K group was 2.00 times higher than that in the HLA-G KD-Muse group ($P=0.19$) at 1 week (Figure 2B).”

Due to the different statistical analysis, final results were changed as follows:

Figure 2B: No statistically significant difference observed.

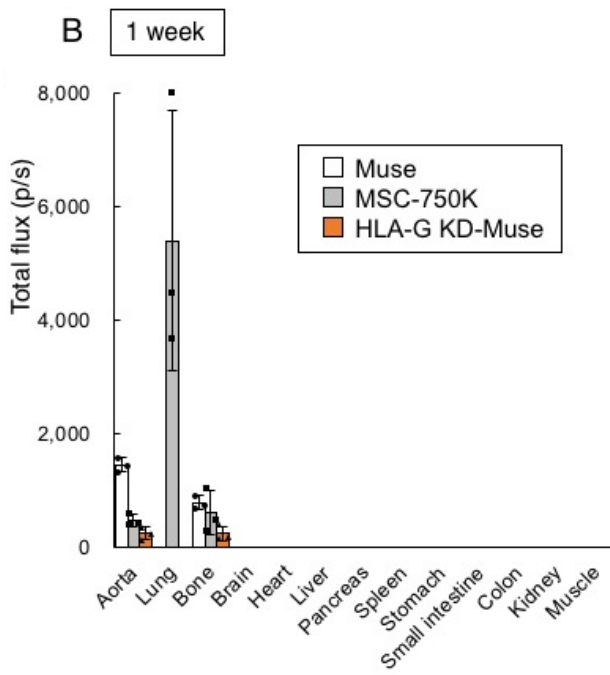


Figure legend line 11:

“by Steel-Dwass test (B)”

Page 26, the last paragraph:

One day after cell injection, the plasma S1P concentration (ng/ml) in the vehicle group was the highest among the 3 groups without significance to the sham group ($P=0.19$).

Figure 6A, 6E: No statistically significant difference observed.

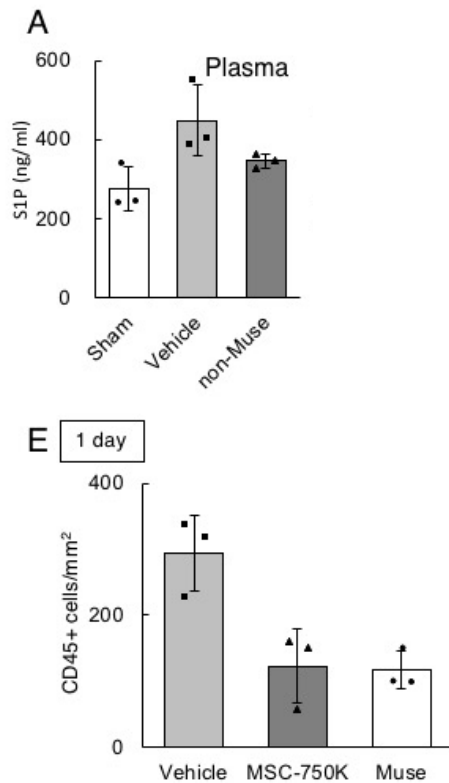


Figure 6, the figure legend:

“by Steel-Dwass test (A, B, E)”

Although the significant difference disappeared due to more detailed multiple testing, a trend we mentioned in the previous version was maintained.

We did not mention Dr. Yoshida’s name in the acknowledgement because he told us that we do not need to include his name in the acknowledgement.

6. Line 548-550: please correct the contradictory statement.

→ We revised the sentence as follows,

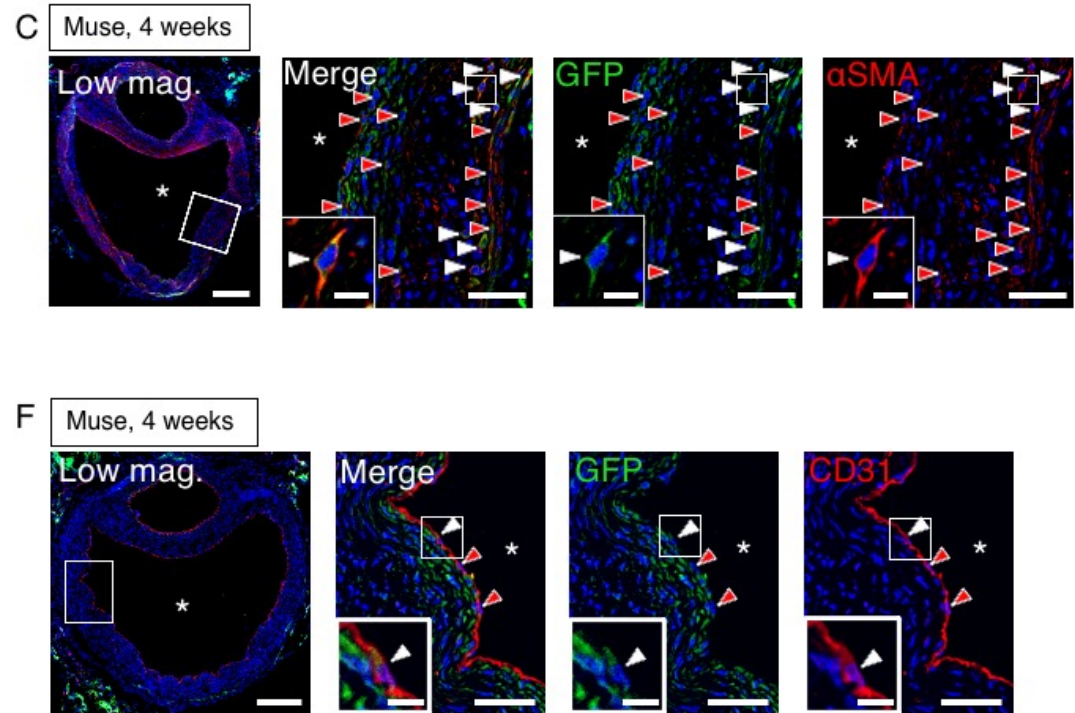
Page 23, 1st paragraph:

“The signal in the aorta of the HLA-G KD-Muse group was lower than that of the Muse group but was rather comparable to that of the bones of the same group (Figure 2A).”

7. Figure 4A-F: the number of double positive cells were much less than those in the statistical figure, please confirm.

→We think the referee might be referring to Figures 3A-F. Thank you for pointing this out. I apologize for my mistake. The graphs (Figure 3A, 3B, 3D, 3E) were correct, but the images (Figure 3C, 3F) were not appropriately organized. Some of the cells

counted as double positive cells were not marked with arrowheads. We carefully modified Figures 3C and 3F as follows;



Red arrowheads indicate double positive cells that were not incorporated in the previous version.

Additionally, although unrelated to the images, there were some simple mistakes in the values in the Supplementary dataset. Therefore, we modified it as follows;

The previous Supplementary Data Figure 3:

Figure 3					
Vascular marker expression in Muse cells and MSCs in the AAD-tissue					
mouse No.	Time after cell administration	GFP+ α SMA+ /mm ²	GFP+ α SMA+ /GFP	GFP+CD31+ /mm ²	GFP+CD31+ /GFP
GFP-Muse 1	2 weeks	123	0.46	16.8	0.092
GFP-Muse 2	2 weeks	89	0.61	7.6	0.056
GFP-Muse 3	2 weeks	53	0.54	7	0.06
GFP-Muse 4	4 weeks	135	0.509	21.4	0.064
GFP-Muse 5	4 weeks	111	0.429	11.9	0.053
GFP-Muse 6	4 weeks	101	0.333	25.3	0.083
GFP-Muse 7	8 weeks	124	0.548	13.6	0.087
GFP-Muse 8	8 weeks	144	0.38	28.7	0.091
GFP-Muse 9	8 weeks	123	0.463	16.8	0.092
GFP-MSC-750K 1	2 weeks	21.6	0.121	1.7	0.01
GFP-MSC-750K 2	2 weeks	12.8	0.083	1	0.016
GFP-MSC-750K 3	2 weeks	15.2	0.1	1.9	0.04
GFP-MSC-750K 4	4 weeks	25.9	0.189	3.7	0.038
GFP-MSC-750K 5	4 weeks	23.4	0.269	3.4	0.043
GFP-MSC-750K 6	4 weeks	11.4	0.227	4.6	0.025
GFP-MSC-750K 7	8 weeks	9.8	0.259	2.8	0.067
GFP-MSC-750K 8	8 weeks	19.4	0.185	3.9	0.032
GFP-MSC-750K 9	8 weeks	15.2	0.222	1.9	0.04

The revised Supplementary Data Figure 3:

Figure 3					
Vascular marker expression in Muse cells and MSCs in the AAD-tissue					
mouse No.	Time after cell administration	GFP+ α SMA+/mm ²	GFP+ α SMA+/GFP	GFP+CD31+/mm ²	GFP+CD31+/GFP
GFP-Muse 1	2 weeks	123	0.463	16.8	0.092
GFP-Muse 2	2 weeks	89	0.606	7.6	0.056
GFP-Muse 3	2 weeks	53	0.543	7	0.06
GFP-Muse 4	4 weeks	135	0.509	21.4	0.064
GFP-Muse 5	4 weeks	111	0.429	11.9	0.053
GFP-Muse 6	4 weeks	101	0.333	25.3	0.083
GFP-Muse 7	8 weeks	124	0.548	13.6	0.087
GFP-Muse 8	8 weeks	144	0.379	28.7	0.091
GFP-Muse 9	8 weeks	155	0.493	22.3	0.113
GFP-MSC-750K 1	2 weeks	21.6	0.121	1.7	0.01
GFP-MSC-750K 2	2 weeks	12.8	0.083	1	0.016
GFP-MSC-750K 3	2 weeks	15.2	0.1	1.9	0.04
GFP-MSC-750K 4	4 weeks	25.9	0.099	3.7	0.038
GFP-MSC-750K 5	4 weeks	23.4	0.127	3.4	0.043
GFP-MSC-750K 6	4 weeks	11.4	0.114	4.6	0.025
GFP-MSC-750K 7	8 weeks	15.5	0.064	3.4	0.02
GFP-MSC-750K 8	8 weeks	19.4	0.185	3.9	0.032
GFP-MSC-750K 9	8 weeks	25.7	0.101	5.6	0.033

The values in the graphs (Figure 3A, B, D, E) were accurate.

8. Figure 5A: what is the reason for no blood in false lumen?

→ This was written on page 13, 3rd paragraph, "Histologic Assessment" in Methods :

"Mice were killed under deep isoflurane anesthesia and perfused transcardially (right ventricle) with 4% paraformaldehyde in phosphate buffered saline (PBS)."

When sacrificing, the aorta was fixed by sufficiently perfusing 4% paraformaldehyde solution from the heart into the aorta. Therefore, blood is washed out and no blood remains in the open false lumen.

Thank you for all the valuable comments.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

In the revised manuscript, the authors have addressed all questions from reviewers. Overall, this study is well designed and rigorously conducted providing interesting data to the field of stem cell therapy. This reviewer would recommend the revised paper for publication.

Reviewer #2 (Remarks to the Author):

Generally, the authors adequately addressed my comments, including those related to statistics. However, in the Results section, the comparison of the total light intensity, where $P=0.19$ consistently over the first 2 weeks (Fig. 2B). Since they were not significantly different, the authors should refrain from stating that some values are higher than others. Similarly, the plasma S1P concentrations were not significantly different ($P=0.19$) and, again, should not be referred to as higher or lower.

Reviewer #3 (Remarks to the Author):

The authors well addressed my comments. No more concerns exist.

“Generally, the authors adequately addressed my comments, including those related to statistics. However, in the Results section, the comparison of the total light intensity, where $P=0.19$ consistently over the first 2 weeks (Fig. 2B). Since they were not significantly different, the authors should refrain from stating that some values are higher than others. Similarly, the plasma S1P concentrations were not significantly different ($P=0.19$) and, again, should not be referred to as higher or lower.”

→ The referee is right. According to the advice, we deleted all the descriptions regarding comparisons between the groups without statistically significant differences. The sentences rewrote in Results section were as follows:

Page 24, 1st paragraph:

“In the Muse group, the signal of the aorta (1461 ± 128 p/s) was higher than that of the bone (793 ± 121 p/s), whereas, in the HLA-G KD-Muse group, those of the aorta (245 ± 111 p/s) and bone (257 ± 124 p/s) were close to each other and were both lower than that of the aorta of the Muse group (Figure 2a, 2b). However, there was no statistically significant difference in the aorta signals among the Muse, MSC-750K, and HLA-G KD-Muse groups or between the signals of the aorta and bone in both the Muse and HLA-G KD-Muse groups, respectively (Figure 2a, 2b).” Page 28, 1st paragraph: “To address this question, we created 3 groups (sham, vehicle, and non-Muse [receiving 712,000 SSEA-3(-) non-Muse MSCs]) and examined how non-Muse MSCs affected the plasma S1P level after intravenous injection. One day after cell injection, there were no statistically significant differences in the plasma S1P levels and S1P concentrations (ng/mg protein) of the AAD-aorta homogenate among the 3 groups (Figure 6a and 6b)”. In accordance with the above changes, we revised the description in Discussion section was revised as follows: Page 29, 5th paragraph:

“HLA-G is associated with immune tolerance in the placenta¹⁹. When HLA-G was knocked down, the effect on the specific homing of Muse cells to the AAD-tissue and survival of the cells in the body was insignificant at one week (Figure 2a and 2b). Since the HLA-G gene expression was decreased by 44.3% in HLA-G KD-Muse cells compared to naïve Muse cells in ddPCR, as mentioned in the Method section, whether HLA-G is involved in protecting Muse cells from immunologic attack at the acute phase and whether HLA-G is enabling Muse cells to safely home to the damaged tissue after intravenous injection is still open to question. How Muse cells maintain their immune privilege after differentiation in the host tissue for an extended period of time without immunosuppressant remains to be clarified. A possible explanation for the consistently lower therapeutic effect in the MSC-750K group than the Muse group, despite both groups contained the same number of Muse cells, might be the presence of non-Muse MSCs that occupy ~98% of the total MSC-750K cells. Since S1P receptor 2 was reported to be expressed in non-Muse cells with lower level than that in Muse cells^{12,19}, the presence of a large number of non-Muse cells may compete with Muse cells for receiving S1P in the MSC group. In addition, the presence of non-Muse MSCs substantially reduced S1P production in the AAD-aortic tissue as shown in Figure 6c. Therefore, the presence of non-Muse MSCs in the MSC-750K group might lead to the inefficient engraftment of Muse cells, thereby preventing adequate replenishment of vascular cells and production of elastic fibers in the AAD tissue.”