Peer Review File

Article information: http://dx.doi.org/10.21037/atm-20-2788.

Reviewer A

The manuscript by Yue et al examines the effect of regulatory M2b macrophages in a rat model of myocardial ischemia/reperfusion. The authors show that injection of M2b macrophages improves cardiac function and survival and that this is associated with decreased phosphorylation of platelet derived growth factor receptor. The study concludes that administration of M2b macrophages attenuates cardiac remodeling and that reduced activation of PDGF receptors in cardiac fibroblasts represents an important part of the protective effect.

I have the following critique:

1. It appears as though survival is dramatically improved in the group receiving M2b macrophages. In fact, figure 1A shows that survival is 100% despite the fact that deficits remain in cardiac function and there is substantial fibrosis. How do the authors explain these findings? This should be discussed.

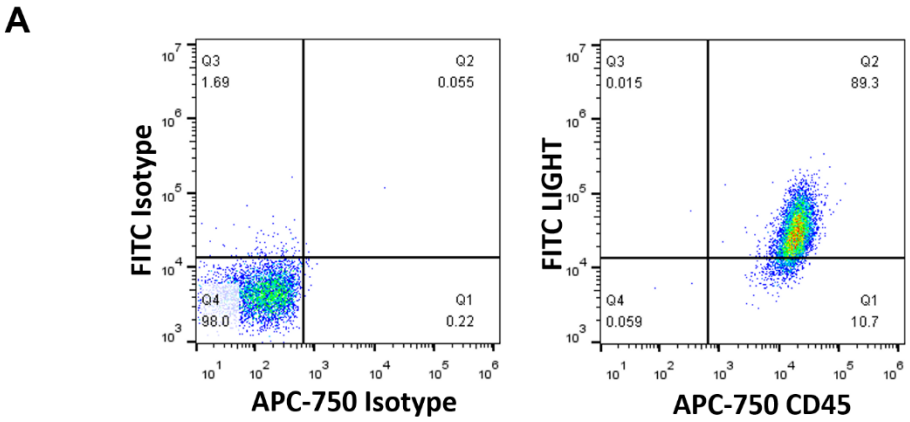
Response: Thank you for your comment. During the surgical procedure of model establishment, we have excluded rats with severe arrhythmia, cardiac arrest, or respiratory failure. And there were 4, 4 and 2 rats that showed these effects in the CK, MT and SO groups, respectively. After excluding these rats with acute serious events, different survival rates were showed among groups. The rats receiving M2b macrophages (MT group) maintained a 100% survival rate. However, the survival rate of rats receiving normal saline (CK group) was 80% (16/20). Heart failure was the most likely cause of death in the CK group, manifested as severe hypoactivity, shortness of breath, and weight loss. Although decreased cardiac function and substantial fibrosis could also be observed in the MT group, the degree was significantly reduced compared with the CK group. The transplantation of M2b macrophages may prevent extremely severe heart failure by protecting cardiac function and reducing ventricular remodeling, thus preventing death in the 2-weeks experiment.

The above information has been added to the discussion section. (see Page 12, line 231)

2. The authors need to provide more evidence that the macrophages are indeed of the M2b phenotype.

Response: We appreciated this very important suggestion by the reviewer. We have performed additional flow cytometry experiments for identification of M2b macrophages and added this data to Figure 1. Quantitative results showed that 89.30% of transplanted cells were M2b macrophages. The results, figure, figure legends and supplementary data subsection have been revised as follows:

Figure 1A



Results

The results of flow cytometry showed that 89.30% of the cells were LIGHT⁺ CD45⁺ (markers of M2b macrophages) after stimulation, indicating that most BMDMs were polarized into M2b macrophages (Fig. 1A).

Figure legends

(A) M2b macrophages were stained to assess LIGHT and CD45 expression and were analyzed by flow cytometry.

Detailed Methods

Identification of M2b macrophages by flow cytometry

Cells were conjugated with LIGHT antibody (Abcam, Cambridge, MA, USA) or isotype control (Abcam) and then with Alexa Fluor 488-conjugated antibody (Invitrogen, Carlsbad, CA, US). After that, the cells were combined with APC A750-rat CD45

(eBioscience, San Diego, CA, USA) or isotype control (eBioscience). Finally, flow cytometry was performed using a CytoFLEX flow cytometer (Beckman Coulter, Miami, FL, USA). The results were analyzed with FlowJo software.

3. Did the authors perform any immunohistochemistry and/or flow cytometry at the end of the experimental period to show that the M2b macrophages were still present? If they are not, how do the authors explain the findings of the study.

Response: We appreciate and agree with the important suggestion from the reviewer, and we have to admit that we failed in detection of M2b macrophages at the end of the experimental period for technical reasons. In this study, BMDMs were induced to differentiate into M2b macrophage subtypes *in vitro* and then transplanted into a rat model of MI/R injury. Although we observed that transplantation of M2b macrophages improved cardiac function and reduced cardiac fibrosis, However, whether and how M2b macrophages were kept activated after transplantation remain to be investigated. One possible explanation is that the cardiac fibroblasts (CFs) awaken quite soon after the cardiac injury, then intimately participate in all critical phases, consisting of inflammation, proliferation of non-myocytes and scar maturation (1). Following the transplantation of M2b macrophages into the injured myocardium, the expression of various cytokines and pro-fibrotic factors is down-regulated in CFs, leading to decreased proliferation of these cells and ultimately, the remission of fibrosis. We have stressed out this explanation to the discussion section. (see Page 13, line 256)

Reference

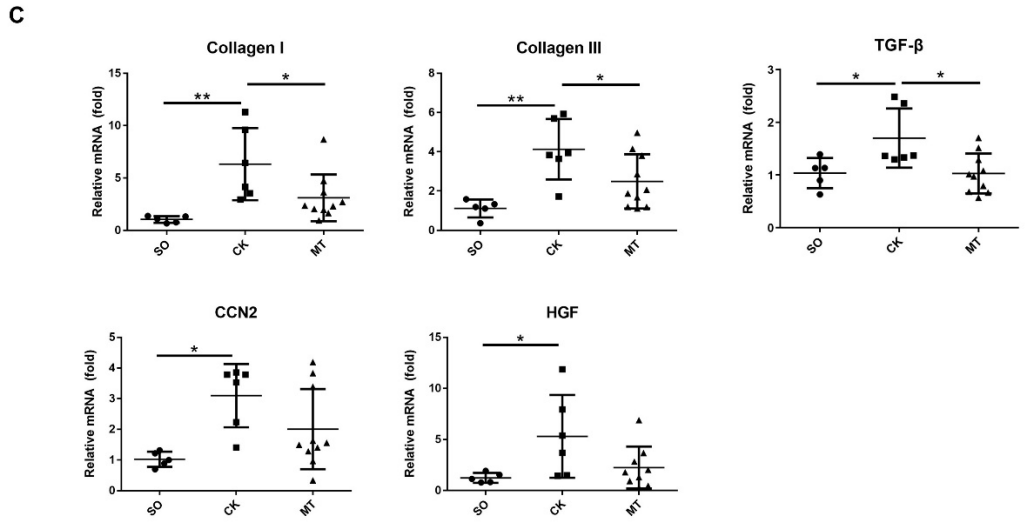
1. Nahrendorf M, Swirski FK. Monocyte and macrophage heterogeneity in the heart. Circ Res. 2013;112(12):1624-33.

4. Does administration of M2b macrophages affect MMP activity? Also, it is surprising that the authors did not examine TGFb levels as this is an important regulator of matrix deposition.

Response: Thank you for your comment. We have performed additional experiments for detection of TGF-β mRNA expression in heart tissue and added this data to Figure

2C. The results, figure, figure legends and supplementary data subsection have been revised as follows:

Figure 2C



Results

In addition, the mRNA levels of the fibrosis-related genes, collagen I, collagen III, TGF-β, connective tissue growth factor (CCN2/CTGF), and HGF are shown in Figure 2C. The I/R injury significantly increased the expression of these genes when compared to that with vehicle treatment, and M2b macrophage transplantation attenuated the increases of collagen I, collagen III and TGF-β compared to those in the CK group.

Figure legends

(C) Myocardial mRNA expression of collagen I, collagen III, TGF-β, CCN2 and HGF in different groups.

Detailed Methods

*TGF-β Fw: ATCGACATGGAGCTGGTGA, Rev: TTGGCATGGTAGCCCTTGG; (*see Supplementary Data Page 4, line 101)

Unfortunately, we could not complete the detection of MMP activities *in vivo* within 3 weeks and we are deeply sorry for that. However, we have studied the MMPs by *in vitro* experiments. Same as the cell experiments in current study, CFs were co-cultured with M2b macrophages, or cultured with M2b macrophage supernatant or the same

volume of culture medium (blank control, NT). Culture supernatants of the 3 groups were collected after 24h, proteins from the supernatants were quantified by Label-free quantitative proteomics. We then compared the contents of proteins from the 3 groups of supernatants. Among these proteins, detected MMPs were separately shown in Table 1 below. Results showed that the concentrations of MMP-12 and MMP-2 were significantly increased after M2b macrophages treatment.

Table 1. Relative quantification of MMPs.

Protein	M2b Co-culture vs. NT		M2b Supernatant vs. NT	
	Ratio	P value	Ratio	P value
MMP-12	190.5924	0.045345	227.0029	0.000224
MMP-9	306.4204	0.197716	250.4505	0.17038
MMP-2	1.697614	0.040369	2.382982	0.008609
MMP-19	0.587391	0.472963	2.762578	0.143544

N=3 in each group

However, a large number of experiments are still required for further verification, and the conclusion is currently incomplete. Therefore, the relevant results cannot yet be published in the manuscript. Follow-up studies will be published in the future. We would appreciate if the reviewer could consider both our explanation and request.

5. Figure 4 clearly shows that treatment of cardiac fibroblasts with M2b supernatant is as effective as co culture with M2b macrophages, indicating that the responsible factor is secreted from M2b macrophages. Can the authors speculate on the identity of this substance or have the authors performed any cytokine/chemokine ELISAs to determine what this might be?

Response: Thank you for your comment. As mentioned by the reviewer, we are also exploring the responsible factors secreted by M2b macrophages. Same as the cell experiments in current study and "*response to comment 4"*, CFs were cultured with M2b macrophage supernatant or the same volume of culture medium (blank control, NT). Culture supernatants of the 2 groups were collected after 24h, proteins from the

supernatants were quantified by Label-free quantitative proteomics. We then compared the contents of proteins. The top 10 higher and lower proteins in conditional-cultured CF supernatant (cultured with M2b macrophage supernatant) than in CF supernatant (NT) are listed respectively in the Table 2 below.

Table 2. Proteins affected by M2b macrophages in CF supernatant.

Conditional-cultured CF supernatant vs. CF supernatant	Protein	Ratio	P value
Top 10 higher-level proteins	MMP-12	227.00	0.00
	LYZ2	110.67	0.00
	C2	86.18	0.01
	TREM2	50.18	0.04
	CTSD	32.30	0.01
	B2M	21.40	0.00
	LCP1	20.60	0.00
	APOE	18.38	0.00
	SERPING1	16.14	0.00
	CCL2	14.20	0.00
Top 10 lower-level proteins	COL11A1	0.10	0.04
	COL3A1	0.11	0.03
	OGN	0.11	0.00
	COL5A1	0.12	0.02
	CLSTN1	0.18	0.04
	FKBP10	0.22	0.05
	S100A6	0.25	0.00
	ME1	0.25	0.03
	CALU	0.26	0.04
	SPTAN1	0.28	0.01

Ratio: relative quantification of proteins in Conditional-cultured CF supernatant vs. relative quantification in proteins of CF supernatant. N=3 in each group

Results showed that M2b macrophage had a significant influence on collagens in CF supernatant, and it was consistent with conclusions in the manuscript. MMP-12, LYZ2, C2, TREM2, CTSD, B2M, LCP1, APOE, SERPING1, CCL2 are the top 10 proteins with significantly higher concentration with M2b macrophage supernatant treatment. However, further experiments of finding the key molecule are still being performed and no exact conclusion have been obtained.

MMP-12 might be a potential signal molecule secreted by M2b macrophage, according to previous studies. MMP-12, also known as macrophage elastase, is the first MMP confirmed to decompose elastin, which is mainly secreted by macrophages. In addition to its substrate elastin, MMP-12 can also widely degrade other extracellular matrix components, including type IV collagen, fibronectin, fibrillin-1, laminin, etc. (2), and even degrade some chemokines and other Non-matrix proteins (such as MBP, Pro-TNFα, etc.) (3). Merry L. L found that the level of MMP-12 continued to increase in the early stage of acute myocardial infarction. And compared with the control group, inhibiting the expression of MMP-12 would lead to the deterioration of cardiac function, showing the protective effect of MMP-12 after myocardial infarction (4). Akihiko Kubota also found that MMP-12 produced by Ly6Clow macrophages can improve the prognosis of myocardial infarction in mice and prolong survival time (5). However, the role of MMP-12 in M2b macrophages still needs further experimental verification. Therefore, the relevant results cannot yet be published in the manuscript. Follow-up studies will be published in the future. We would appreciate if the reviewer could consider both our explanation and request.

References

2. Chen YE. MMP-12, an old enzyme plays a new role in the pathogenesis of rheumatoid arthritis? Am J Pathol. 2004;165(4):1069-70.
3. Marchant DJ, Bellac CL, Moraes TJ, et al. A new transcriptional role for matrix metalloproteinase-12 in antiviral immunity. Nat Med. 2014;20(5):493-502.
4. Iyer RP, Patterson NL, Zouein FA, et al. Early matrix metalloproteinase-12 inhibition worsens post-myocardial infarction cardiac dysfunction by delaying inflammation resolution. Int J Cardiol. 2015;185:198-208.
5. Kubota A, Suto A, Suzuki K, et al. Matrix metalloproteinase-12 produced by Ly6C(low) macrophages prolongs the survival after myocardial infarction by preventing neutrophil influx. J Mol Cell Cardiol. 2019;131:41-52.

6. The authors conclude that M2b macrophages protect against myocardial remodeling after I/R injury and that “although the reduction of acute injury by M2b macrophages may be responsible for the reduced fibrosis, it is conceivable that the direct effects of M2b macrophages on the CFs is more important”. Furthermore, the authors conclude that depressed kinase activation of PDGFR may be part of the mechanism through

limiting CFs activation. Unfortunately, there is little data to support this last statement. Certainly from the western blot data in figure 4C there appears to be a reduction in phosphorylated PDGFRβ in cells either co-cultured with the macrophages or in cells treated with the supernatant from macrophages. These effects are associated with reduced alpha SMA levels but the association does not prove causality.

Response: Thank you for your comment. Our statement in the final conclusion is indeed inappropriate and the conclusion and limitation in the manuscript has been rewritten as follows: (see Page 15, line 303)

Finally, although the activation of fibroblasts has been shown to be inhibited by M2b macrophages and is considered to be the main reason for reducing fibrosis, more explorations are needed to be completed to exclude the confounding factor -- reduction of acute heart injury by M2b macrophages.

In conclusion, this study showed that M2b macrophages protect against myocardial remodeling after I/R injury. And the direct effects of M2b macrophages on the CFs may be responsible for the reduced fibrosis.

Thanks to the reviewers for pointing out our deficiencies in explaining the relationship between PDGFRβ and α-SMA. Our previous *in vitro* studies have demonstrated that the inhibition of PDGFRs significantly reduced the expression of α-SMA in CFs (6). We have added this information to the discussion section. (see Page 13, line 250)

Reference

6. Wang LX, Yang X, Yue Y, et al. Imatinib attenuates cardiac fibrosis by inhibiting platelet-derived growth factor receptors activation in isoproterenol induced model. PLoS One. 2017;12(6):e0178619.