Journal of Cell Science

Kir2.1 channel regulates macrophage polarization via the Ca^{2+}/CaMK II/ERK/NF-κB signaling pathway

Kuihao Chen, Qiaoyan Man, Jiaen Miao, Wenjing Xu, Yangchen Zheng, Xiuli Zhou and Zhe Gao
DOI: 10.1242/jcs.259544

Editor: Daniel Billadeau

Review timeline
Original submission: 2 November 2021
Editorial decision: 4 January 2022
First revision received: 3 April 2022
Editorial decision: 13 May 2022
Second revision received: 30 May 2022
Accepted: 6 June 2022

Original submission

First decision letter

MS ID#: JOCES/2021/259544

MS TITLE: Kir2.1 channel regulates macrophage polarization via Ca2+/CaMK II/ERK/NF-κB signaling pathway

AUTHORS: Kuihao Chen, Qiaoyan Man, Jiaen Miao, Wenjing Xu, Yangchen Zheng, Xiuli Zhou, and Zhe Gao
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. Although several comments can be taen care of through appropriate quantitative methods and adding to the Introduction and Materials and Methods section, some key experiments need to be performed to further support the conclusions drawn in the manuscript. Further verification of the M1 and M2 phenotypes using additional markers, as well as more detailed impact of IL-4 and Kir2.1 silencing on macrophage polarization. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then

provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

N/A

Comments for the author

The authors proposed a mechanism underlying the role of Kir2.1 channel in macrophage polarization through a Calcium/CamKII/ERK/NFkb pathway. In general, the paper was very poorly written and needs substantial new data and revision to make the arguments more cohesive and comprehensible.

1. It is important to note that macrophages have a spectrum of polarization states, and M1 and M2 are two extreme ends of the spectrum. Single cell RNA sequencing should be performed to determine the role of Kir2.1 in macrophage polarization spectrum.
2. In the first paragraph of the introduction the authors mention that “many molecules have been found” to participate in macrophage polarization. It would be easier for the readers if the authors could mention some commonly known biophysical and biochemical factors involved in polarization with necessary citations.
3. The authors should expand the second paragraph of the introduction to include recent studies about different ion channels in physiological and pathological conditions. Additionally, the authors need to provide some information about the structure of Kir2.1.
4. The authors could use Density Plot for the flow cytometry data in Fig 3 because the histogram might not be visually conclusive for the readers.
5. The authors need to provide quantification to clearly convey the increase or decrease in the fluorescence intensity in Fig 4F.
6. The authors need to provide quantification for the Western blot analysis for Fig 6, need to specify “cells”, provide all necessary references supporting any statement, and describe the reason behind using LPS for 6 hours.
7. In Fig 7 the authors claimed that the blockade of Kir2.1 promotes the transition from M1 to M2. However, this transition was tested in the presence of IL4 stimulation for 24 h, which is very likely to polarize macrophages to M2 phenotype irrespective of any previous treatment with M1 factors. How do the authors conclude that M1 to M2 transition was only for blockade of Kir2.1 and not due to the presence of prolonged IL4 exposure?
8. In Fig 7, the authors noted a key finding about the role of Kir2.1 in transition of M1 to M2 phenotype based on CD206 and IGF-1 as M2 markers. However, it is important to show other common M2 markers to validate this key claim in the article.
9. In figure 8C and 8D the authors used only two M1 markers TNFα and IL1β to show that blockade of Kir2.1 channel inhibits macrophage M1 polarization in LPS-induced peritonitis model. Additional M1 markers should be shown in support of this key claim.
10. The authors in discussion section mentioned Supp Figure 1 for the Ca2+/CamKII/ERK/NFkb pathway, which should be figure 6 according to the figure panel.

Reviewer 2

Advance summary and potential significance to field

This is an informative study evaluating the role of Kir2.1 in the process of macrophage polarization. It is important to understand the role of potassium channels in macrophage function as a potential therapeutic approach for the treatment of inflammatory disease. This paper is an advancement in our understanding of Kir2.1 in macrophage polarization.

Comments for the author

Reasonable introduction though I would not describe the 2015 Lam and Schlichter paper as recent study.
In the methods, add codes for the all the reagents and antibodies. What type of LPS is used? What is the final dilution of antibodies for flow cytometry and western blot? Add more details of stats and the software used for this statistical analysis. Define cell rate used to analyse the flow cytometry plots in methods.
The authors should comment on the fact they are using a semi-quantitative assessment of protein expression by western blot. For the fluo4 measurements, the authors need to include a max fluo4 response for the ±LPS treatment to ensure the loading of the dye has not changed in the polarized macrophages (e.g., response to a calcium ionophore). What is the impact of ML133 on basal resting calcium in calcium free conditions? Is the ML133 block reversible? Do you see the same effect in the shRNA knockdown macrophages to ensure it is not a non-specific action of the inhibitor?
Add a reference for the Nernst equation.
Measure the expression of toll receptors as an additional control for the changes seen in the signalling pathways during M1 polarization.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Chen et al. describes the role of potassium ion channel Kir2.1 in regulating macrophage polarization. The main idea put forward by the authors is the Kir2.1 supports M1 macrophage polarization.
Inhibition of Kir2.1 genetically with shRNA or pharmacologically with ML133 or high extracellular K+ resulted in reduced pro-inflammatory response during LPS stimulation and enhanced M2 polarization during IL-4 stimulation. The authors further showed that this regulation was due to Ca2+ intake mediated by Kir2.1, and high extracellular Ca2+ was found to be able to restore M1 polarization when Kir2.1 was being inhibited. Overall, these results are novel and provide new insight into the role of Kir2.1 in macrophage effector functions. My main concerns revolve around the activity of Kir2.1 during macrophage activation, and how significant is the contribution of Kir2.1 to the regulation of membrane potential and its downstream Ca2+ entry upon macrophage activation.

Comments for the author

Major points:
1. It is unclear whether LPS or IL-4 stimulation can activate Kir2.1 and so induces Ca2+ entry that leads to the support of NF-kB signaling as proposed by the authors? Figure 1B shows there is no effect of LPS on the current recording, but Figure 4B shows there is an induction of store-operated calcium entry by LPS.
Can the authors explain this? Were these two measurements performed in similar time ranges of stimulation? Alternatively, is it possible that the tonic channel activity is simply required for supporting/potentiating the activation of CRAC or other calcium channels upon LPS stimulation?
2. What is the exact contribution of Kir2.1 to the regulation of the membrane potential in macrophages/RAW cells? Did the authors examine it by using either shRNA or ML133 in the current recording experiment as in Figure 1A?
3. Is LPS-induced calcium influx shown in Figure 4B dependent on Kir2.1? Did the authors examine if Kir2.1 inhibition (e.g., ML133) block the LPS-induced calcium entry?

4. It is known that K+ efflux (as well as Ca2+ influx) due to the activation of ion channel P2X7 by ATP or certain bacterial pore forming toxins can lead to the activation of NLRP3 inflammasome that drives IL-1b secretion in macrophages. Since Kir2.1 can mediates similar ion flux as shown here, did the authors examine the effect of Kir2.1 in inflammasome activation? Alternatively, can the authors discuss about this possibility?

Minor
1. Figure 6: since M2 polarization by IL-4 is enhanced by Kir2.1 inhibition, is IL-4 receptor signaling (e.g., phospho-Stat6) also affected?
2. For general audience, the authors should indicate why using extracellular Ba2+ for the current recording experiment in Figure 1A.
3. The time periods for LPS or IL-4 stimulation or other indicated treatments are missing in the figure legends for Figure 1B, Figure 5, and Figure 6.

First revision

Author response to reviewers' comments

Response to Reviewer #1
(Reviewer's comments appear as in *Italic*)

The authors proposed a mechanism underlying the role of Kir2.1 channel in macrophage polarization through a Calcium/CamKII/ERK/NFkb pathway. In general, the paper was very poorly written and needs substantial new data and revision to make the arguments more cohesive and comprehensible.

It is important to note that macrophages have a spectrum of polarization states, and M1 and M2 are two extreme ends of the spectrum. Single cell RNA sequencing should be performed to determine the role of Kir2.1 in macrophage polarization spectrum.

--**Many thanks for your suggestion. It will be interesting to study the role of Kir2.1 in macrophage polarization spectrum. But, we think it is another story. M1 and M2 indeed are two extreme ends of polarization, meanwhile, they are the predominant and most studied phenotypes in the development of many diseases. In this study, we focused on whether Kir2.1 participates in these two classic polarization states and the possible mechanism. We think our present data can support the hypothesis made in the paper. A new study will be designed to examine the role of Kir2.1 in macrophage polarization spectrum in future.**

In the first paragraph of the introduction the authors mention that "many molecules have been found" to participate in macrophage polarization. It would be easier for the readers if the authors could mention some commonly known biophysical and biochemical factors involved in polarization with necessary citations.

--**Thanks for the suggestion. The related information was added.**

The authors should expand the second paragraph of the introduction to include recent studies about different ion channels in physiological and pathological conditions. Additionally, the authors need to provide some information about the structure of Kir2.1.

--**We added the information in the revised manuscript.**

The authors could use Density Plot for the flow cytometry data in Fig 3 because the histogram might not be visually conclusive for the readers.

--**The Density Plot of flow cytometry data was used in the new figure 3 in the revised manuscript.**

The authors need to provide quantification to clearly convey the increase or decrease in the fluorescence intensity in Fig 4F.

--**In fact, the pictures in Fig 4F correspond some time points of Fig 4E, which displays the quantification changes of fluorescence intensity. The corresponding time points were marked in the revised figure 4E.**

The authors need to provide quantification for the Western blot analysis for Fig 6, need to specify "cells", provide all necessary references supporting any statement, and describe the reason behind using LPS for 6 hours.

--**We added the quantification of the Western blot in the new Fig 6. Specified "cells" and provided all necessary references in the revised manuscript.**

For the reason of using LPS for 6 hours. First, before experiment we read many publications in which macrophages can be activated by LPS in 6 hours; second, our pre-experiments also suggest that 6 hours is enough for LPS to activate macrophages. We described the reason in the revised manuscript.

In Fig 7 the authors claimed that the blockade of Kir2.1 promotes the transition from M1 to M2. However, this transition was tested in the presence of IL4 stimulation for 24 h, which is very likely to polarize macrophages to M2 phenotype irrespective of any previous treatment with M1 factors. How do the authors conclude that M1 to M2 transition was only for blockade of Kir2.1 and not due to the presence of prolonged IL4 exposure?

--**We double-checked the data and found that we made a mistake in statistics, the conclusion "inhibition of Kir2.1 promotes plasticity of polarized macrophages" is not reasonable. So, this figure is deleted in the revised manuscript. We apologize for this mistake very much.**

In Fig 7, the authors noted a key finding about the role of Kir2.1 in transition of M1 to M2 phenotype based on CD206 and IGF-1 as M2 markers. However, it is important to show other common M2 markers to validate this key claim in the article.

--**This figure is deleted in the revised manuscript.**

In figure 8C and 8D the authors used only two M1 markers TNFa and IL1B to show that blockade of Kir2.1 channel inhibits macrophage M1 polarization in LPS-induced peritonitis model. Additional M1 markers should be shown in support of this key claim.

--**Thanks for your suggestion. Other two markers iNOS and IL-6 were detected and the results were displayed in the new figure 7.**

The authors in discussion section mentioned Supp Figure 1 for the Ca2+/CamKII/ERK/NFkb pathway, which should be figure 6 according to the figure panel.

--**The description was corrected in the revised manuscript.**

Response to Reviewer #2
(Reviewer's comments appear as in *Italic*)

This is an informative study evaluating the role of Kir2.1 in the process of macrophage polarization. It is important to understand the role of potassium channels in macrophage function as a potential therapeutic approach for the treatment of inflammatory disease. This paper is an advancement in our understanding of Kir2.1 in macrophage polarization.

--**We appreciate the reviewer #2 for the positive comments and constructive suggestions.**

Reasonable introduction though I would not describe the 2015 Lam and Schlichter paper as recent study.

--**The description was corrected in the revised manuscript.**

In the methods, add codes for the all the reagents and antibodies. What type of LPS is used? What is the final dilution of antibodies for flow cytometry and western blot? Add more details of stats and the software used for this statistical analysis. Define cell rate used to analyse the flow cytometry plots in methods.

--**The information was provided in the revised manuscript as you suggested.**

The authors should comment on the fact they are using a semi-quantitative assessment of protein expression by western blot. For the fluo4 measurements, the authors need to include a max fluo4 response for the ±LPS treatment to ensure the loading of the dye has not changed in the polarized macrophages (e.g., response to a calcium ionophore).

--**Thanks for your suggestion. The recommended concentration of Fluo 4 is 2-5 μM in the manual, we used 5 μM here and make sure the dye is overloaded. Since Fluo 4 enters to cells in a physical manner, it may have nothing to do with cell states. And we referenced many publications, no higher concentration was used. On the other hand, it seems that the Fluo 4 in cells cannot be detected because of very weak fluorescence.**

What is the impact of ML133 on basal resting calcium in calcium free conditions? Is the ML133 block reversible? Do you see the same effect in the shRNA knockdown macrophages to ensure it is not a non-specific action of the inhibitor?

--**Yes, ML133 block is reversible (Wang HR, et al. *ACS Chem Biol*. 2011).**
ML133 doesn't affect basal resting calcium in calcium free conditions (please see panel A in the figure below). The calcium influx was significantly decreased in Kir2.1-shRNA knockdown macrophages (panel B in the figure below).

NOTE: *We have removed unpublished data that had been provided for the referees in confidence.*

Add a reference for the Nernst equation.

--**A related reference was added in the revised manuscript.**

Measure the expression of toll receptors as an additional control for the changes seen in the signalling pathways during M1 polarization.

--**Thanks for your suggestion. TLR4 was detected as a control and displayed in figure 6A.**

Response to Reviewer #3
(Reviewer's comments appear as in *Italic*)

The manuscript by Chen et al. describes the role of potassium ion channel Kir2.1 in regulating macrophage polarization. The main idea put forward by the authors is the Kir2.1 supports M1 macrophage polarization. Inhibition of Kir2.1 genetically with shRNA or pharmacologically with ML133 or high extracellular K+ resulted in reduced pro-inflammatory response during LPS stimulation and enhanced M2 polarization during IL-4 stimulation. The authors further showed that this regulation was due to Ca2+ intake mediated by Kir2.1, and high extracellular Ca2+ was found to be able to restore M1 polarization when Kir2.1 was being inhibited. Overall, these results are novel and provide new insight into the role of Kir2.1 in macrophage effector functions. My main concerns revolve around the activity of Kir2.1 during macrophage activation, and how significant is the contribution of Kir2.1 to the regulation of membrane potential and its downstream Ca2+ entry upon macrophage activation.

-**We appreciate the reviewer #3 for the positive comments and constructive suggestions.**

It is unclear whether LPS or IL-4 stimulation can activate Kir2.1 and so induces Ca2+ entry that leads to the support of NF-kB signaling as proposed by the authors? Figure 1B shows there is no effect of LPS on the current recording, but Figure 4B shows there is an induction of store-operated calcium entry by LPS. Can the authors explain this? Were these two measurements performed in similar time ranges of stimulation? Alternatively, is it possible that the tonic channel activity is simply required for supporting/potentiating the activation of CRAC or other calcium channels upon LPS stimulation?

--**Other group also found that LPS could not activate Kir2.1 (*Lucente Jacopo Di, et al. Glia, 2018*). Although the current and gene/protein expression of Kir2.1 was not affected by LPS or IL-4, the role of Kir2.1 in macrophages could not be ignored. As you said, we also think Kir2.1 channel is simply required for supporting the activation of CRAC upon LPS stimulation by setting up polarized membrane potential.**

What is the exact contribution of Kir2.1 to the regulation of the membrane potential in macrophages/RAW cells? Did the authors examine it by using either shRNA or ML133 in the current recording experiment as in Figure 1A?

--**We recorded the membrane potential of macrophages before and after treated with Ba^{2+} (panel A in the figure below). The inward rectification current was also inhibited by ML133 (panel B in the figure below).**

***NOTE**: We have removed unpublished data that had been provided for the referees in confidence.*

Is LPS-induced calcium influx shown in Figure 4B dependent on Kir2.1? Did the authors examine if Kir2.1 inhibition (e.g., ML133) block the LPS-induced calcium entry?

--**Yes, we think LPS-induced calcium influx is dependent on Kir2.1. Because ML133 can block the LPS-induced calcium entry. Please see the figure below.**

NOTE: *We have removed unpublished data that had been provided for the referees in confidence.*

It is known that K+ efflux (as well as Ca2+ influx) due to the activation of ion channel P2X7 by ATP or certain bacterial pore forming toxins can lead to the activation of NLRP3 inflammasome that drives IL-1b secretion in macrophages. Since Kir2.1 can mediates similar ion flux as shown here, did the authors examine the effect of Kir2.1 in inflammasome activation? Alternatively, can the authors discuss about this possibility?

--**Yes, as you said, we indeed examined the effect of Kir2.1 blocker on NLRP3 inflammasome activation, and found that the gene expressions of NLRP3, ASC, Caspase-1, and IL-18 were significantly decreased in RAW264.7 macrophages treated with ML133 or Ba^{2+}. It is also an interesting story, further study might be performed in future.**

Figure 6: since M2 polarization by IL-4 is enhanced by Kir2.1 inhibition, is IL-4 receptor signaling (e.g., phospho-Stat6) also affected?

--**Yes, p-STAT6 in IL-4 receptor signaling was upregulated by Kir2.1 inhibitor. Please see the figure below.**

NOTE: *We have removed unpublished data that had been provided for the referees in confidence.*

For general audience, the authors should indicate why using extracellular Ba2+ for the current recording experiment in Figure 1A.

--**We added a related reference in the revised manuscript.**

The time periods for LPS or IL-4 stimulation or other indicated treatments are missing in the figure legends for Figure 1B, Figure 5, and Figure 6.

--**Thanks for the suggestion. The information was added in the revised manuscript.**

Second decision letter

MS ID#: JOCES/2021/259544

MS TITLE: Kir2.1 channel regulates macrophage polarization via Ca2+/CaMK II/ERK/NF-κB signaling pathway

AUTHORS: Kuihao Chen, Qiaoyan Man, Jiaen Miao, Wenjing Xu, Yangchen Zheng, Xiuli Zhou, and Zhe Gao
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

I agree with the comments of Reviewer three that the pieces of data presented to the reviewers in the PbP response should be placed into either the main figures or supplmental figures as it is supportive of the main conclusions of the manuscript. I look forward to seeing your revision.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

All my concerns have been addressed.

Comments for the author

All my concerns have been addressed.

Reviewer 3

Advance summary and potential significance to field

In this revised manuscript by Chen et al, the authors have made some minor changes to strengthen the quality of data. However, the main concerns regarding the activity of Kir2.1 and their contribution to Ca2+ entry upon macrophages activation have not yet been addressed in the current version.

Comments for the author

While the authors have provided new data in their response to reviewers, for examples, the Ca2+ influx with Kir2.1-shRNA (response to review #2) and ML133 (review #3), they did not include them in the manuscript.
These data indeed provide a mechanistic link between Kir2.1 and the regulation of Ca2+ influx upon macrophage polarization, which was lacking in the previous version. In addition, their new data for the upregulation of Stat6 phosphorylation by ML133, shown in the response to reviewers, can further support their observations of enhanced M2 polarization (Fig 2 and 3) and should also be included in the main figures if not in supplementary.

Second revision

Author response to reviewers' comments

Response to Reviewer #3
(Reviewer's comments appear as in Italic)

While the authors have provided new data in their response to reviewers, for examples, the Ca2+ influx with Kir2.1-shRNA (response to review #2) and ML133 (review #3), they did not include them in the manuscript. These data indeed provide a mechanistic link between Kir2.1 and the regulation of Ca2+ influx upon macrophage polarization, which was lacking in the previous version. In addition, their new data for the upregulation of Stat6 phosphorylation by ML133, shown in the response to reviewers, can further support their observations of enhanced M2 polarization (Fig 2 and 3) and should also be included in the main figures if not in supplementary.

--Many thanks for your suggestion, the data you mentioned are included in the supplementary figures in the revised manuscript.

Third decision letter

MS ID#: JOCES/2021/259544

MS TITLE: Kir2.1 channel regulates macrophage polarization via Ca2+/CaMK II/ERK/NF-κB signaling pathway

AUTHORS: Kuihao Chen, Qiaoyan Man, Jiaen Miao, Wenjing Xu, Yangchen Zheng, Xiuli Zhou, and Zhe Gao
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.