nature portfolio

Peer Review File

Kynurenine promotes neonatal heart regeneration by stimulating cardiomyocyte proliferation and cardiac angiogenesis

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The IDO1 enzyme and the metabolites produced along the kynurenine pathway represent an important mechanism of catabolism of the essential amino acid tryptophan (Trp) but, perhaps most importantly, also a mean to control both inflammation and adaptive immune responses. Among the produced kynurenines, the most studied is l-kynurenine (Kyn), an agonist of the Aryl hydrocarbon Receptor (AhR). AhR is indeed a ubiquitous, ligand-activated transcription factor that can be activated by several compounds, of both endogenous and external origin (i.e., the so-called xenobiotics), with discrete (sometimes unknown and unpredictable) consequences or effects.

In the present study by Zhang and colleagues, Kyn, produced by upregulated IDO1, was found to induce potent proliferative and thus regenerative effects in cardiomyocytes of neonatal mice subjected to apical resection (AR). Kyn, a secreted compound, induced such effect by acting on AhR expressed by cardiomyocytes and also nearby endothelial cells, thus promoting a pro-angiogenetic effect that favored the regeneration of cardiomyocytes themselves.

Although proven only in neonatal mice and in a restricted temporal space, this is a very exciting and original work that opens new perspectives for the treatment of cardiac diseases such cardiac insufficiency. Moreover, it increases the range of effects triggered by Kyn and AhR.

Nevertheless, I have some minor concerns that preclude the publication of the manuscript in its present form. In particular, there are inaccuracies in the Figure legends, as detailed below. In addition, because TDO can also produce Kyn, the Authors should also evaluate TDO expression and its possible modulation at the beginning of the Results (Figure 1A). In general, the duration of treatments with Kyn and also 1-MT is not always clear (up to P28 or shorter?).

Minor concerns:

Figure 1: The fold change relative to sham (and not the absolute concentration levels of Trp and Kyn) are shown in panel B. Which is the biologic replicate?

Figure 2: Panel I, why Kyn contents are different in sham- and AR-treated samples? When the ex vivo analysis was performed?

Figure 6: Some quantitative data of immunofluorescence are lacking. Panels B-D, which is the meaning of Rel Kyn? Panel E, the 20 μm specification is missing. In the same panel, the term Rel IDO1 intensity and so on should also be clarified. In panels F-L, bar units should be μm and not μM.

Fig. S8 should be moved into the main figures.

Reviewer #2:
Remarks to the Author:
In this manuscript, Zhang et al. studied the role of Trp-Kyn metabolism during neonatal heart regeneration. They found that IDO1, the enzyme that catalyzed the Trp-Kyn metabolism, is activated after apical resection in the P1 regenerative heart. Deletion of Ido1 in cardiomyocytes prevented heart regeneration and inhibited angiogenesis. This regeneration defect of Ido1 KO hearts can be rescued by exogenous supplement of Kyn. The authors also did a series of mechanistical studies to show that Trp-Kyn regulates regeneration by promoting the YAP/ERK pathway in cardiomyocytes and by direct transcriptional regulation of angiogenic genes via AHR, which is the cytoplasmic receptor of Kyn. Taken together, the authors showed that the Trp-Kyn metabolic pathway, which is a well-known immunosuppressive mechanism, regulates neonatal heart regeneration via a previously unrecognized mechanism acting on the non-immune cells. Overall, the findings of this study are novel and the characterization of cell types where Trp-Kyn pathway is acting on was well performed. However, the authors are required to address the

following concerns:

1. As authors stated, the Trp-Kyn pathway suppresses immune responses by inhibiting T cell activation. Even though IdoI was deleted in cardiomyocytes, its effect on Kyn can be cell type non-specific, given that Kyn can be extracellular. Does the reduced Kyn level caused by IdoI CM deletion affect the degree of inflammation and fibroblast activation?
2. The Trichrome Masson staining done in this study appears to be unsuccessful as the fibrotic region was not labeled clearly in blue. This can affect data interpretation and quantification. The authors are required to repeat all the Trichrome Masson staining experiments and the related quantification.
3. On page 8, "Similarly, Ido1-deletion CMs also showed reduced levels of m-MYC and CCNB1…". Please provide the supporting data.
4. Figs 3B, 4B and S1, please provide better images as the colocalization of staining signals is not clear.
5. Fig 3C, YAP is not detected in the nucleus which contradicts the authors' model in which Trp-Kyn pathway is proposed to activate YAP/ERK pathway to regulate cardiomyocyte proliferation.
6. Fig 4D, the authors are trying to show that the extracellular Kyn activates AHR in endothelial cells and directly regulates transcription of angiogenic genes. To show that, AHR ChIP should be performed specifically in endothelial cells instead of the whole heart, because AHR is localized to multiple cell types in the heart.
7. In discussion, the authors mentioned that Kyn generation through IDO is also induced after adult MI. Why this induction is not sufficient to activate regeneration as seen in the neonatal heart? This needs to be discussed.

Response to previous review:

We thank the editors and the reviewers for their careful readings. We are delighted to know that ***"this is a very exciting and original work that opens new perspectives for the treatment of cardiac diseases such cardiac insufficiency. Moreover, it increases the range of effects triggered by Kyn and AhR."*** **(Reviewer #1), "*Overall, the findings of this study are novel and the characterization of cell types where Trp-Kyn pathway is acting on was well performed"* (Reviewer #2)**. Thanks to these highly positive comments, we are extremely encouraged to revise the manuscript. To address the reviewers' comments, we have also performed several new experiments. We also found the reviewers' comments very helpful, which guided our revisions resulting in this much-improved paper. Based on the reviewers' comments and our newly added data, we have made limited changes to this paper. The changes in text are marked with yellow highlights.

Below is our point-by-point response as the reviewers' comments: Please note the texts in *Italic* are the original comments of the reviewers.

Response to Reviewer #1

(Main comments) *"The IDO1 enzyme and the metabolites produced along the kynurenine pathway represent an important mechanism of catabolism of the essential amino acid tryptophan (Trp) but, perhaps most importantly, also a mean to control both inflammation and adaptive immune responses. Among the produced kynurenines, the most studied is l-kynurenine (Kyn), an agonist of the Aryl hydrocarbon Receptor (AhR). AhR is indeed a ubiquitous, ligand-activated transcription factor that can be activated by several compounds, of both endogenous and external origin (i.e., the so-called xenobiotics), with discrete (sometimes unknown and unpredictable) consequences or effects.*

In the present study by Zhang and colleagues, Kyn, produced by upregulated IDO1, was found to induce potent proliferative and thus regenerative effects in cardiomyocytes of neonatal mice subjected to apical resection (AR). Kyn, a secreted compound, induced such effect by acting on AhR expressed by cardiomyocytes and also nearby endothelial cells, thus promoting a pro-angiogenetic effect that favored the regeneration of cardiomyocytes themselves.

Although proven only in neonatal mice and in a restricted temporal space, this is a very exciting and original work that opens new perspectives for the treatment of cardiac diseases such cardiac insufficiency. Moreover, it increases the range of effects triggered by Kyn and AhR."

Response: We thank you very much for your highly positive comments. We greatly appreciate your time and efforts.

"Nevertheless, I have some minor concerns that preclude the publication of the manuscript in its present form. In particular, there are inaccuracies in the Figure legends, as detailed below. In addition, because TDO can also produce Kyn, the Authors should also evaluate TDO expression and its possible modulation at the beginning of the Results (Figure 1A). In general, the duration of treatments with Kyn and also 1-MT is not always clear (up to P28 or shorter?)."

Responses: This is a very valid concern. Both IDO and TDO (also known as TDO2) catalyze the rate-limiting step of tryptophan catabolism along the kynurenine pathway [1, 2]. IDO was extensively demonstrated in various organs, while the expression of TDO2 is tissue specific in adult human (The Human Protein Atlas). To investigate the expression profile of TDO2 in neonatal mice, we collected the major organs, including brain, lung, liver, kidney, heart and regenerating heart from the mice at 3rd postnatal day and detected the TDO2 protein expression by Western blot assay. As displayed in left **Figure**, TDO2 is mainly expressed in lung tissue and was undetectable in brain, heart and regenerating heart. Thus considering undetectable levels of TDO2 in hearts, we conclude that TDO2 very unlikely contribute to cardiac regeneration.

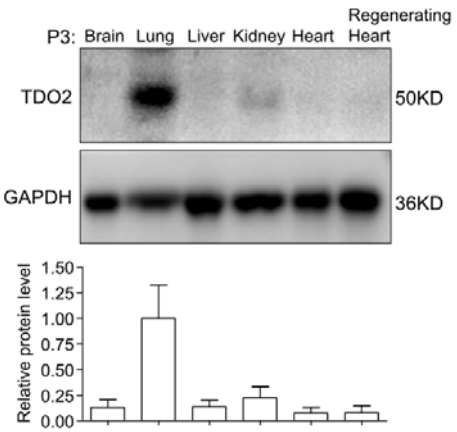

Figure. Representative western blot image and quantitative data of TDO2 protein quantification in neonatal mice (postnatal 3 days). n=4 for each organ. TDO2 antibody obtained from Novus (H00006999-B01P, 1:1000 dilution).

For the second part of your questions on the duration of treatments with Kyn and also 1-MT, we apologize for the confusions. The mice had intraperitoneal injection of Vehicle or Kyn (100 mg/kg) every other day from P1 to P28. We have revised this section accordingly (page 18).

Minor comments:

"Figure 1: The fold change relative to sham (and not the absolute concentration levels of Trp and Kyn) are shown in panel B. Which is the biologic replicate?"

Response: This is a valid concern. In the revised manuscript, we have clarified the data as follow. Postnatal 1 day (P1) of wild-type mice underwent heart apical resection (AR) or sham surgery. About half of apical regenerating hearts were collected at P3 (n=8), P7 (n=8) and P21 (n=6) for HPLC (Figure 1b) assay. Since the regenerating hearts were very small, two or three hearts mixed into one pooled sample for HPLC assay. Thus, there were three quantitation data for each stage in total.

"Figure 2: Panel I, why Kyn contents are different in sham- and AR-treated samples? When the ex vivo analysis was performed?"

Response: Thanks for this query**.** We found a significant elevation of IDO1 protein levels and a higher level of Kyn (**Figure 1a, b**), on day 28 after apical resection surgery compared with Sham group. From these data (**Figure 2i**), we could confirm that, higher level of Ido1 was associated with higher level of Kyn concentration in AR-treated samples compared with Sham group.

"Figure 6: Some quantitative data of immunofluorescence are lacking. Panels B-D, which is the meaning of Rel Kyn? Panel E, the 20 µm specification is missing. In the same panel, the term Rel IDO1 intensity and so on should also be clarified. In panels F-L, bar units should be µm and not µM."

Response: Thank you very much for your careful reading and we apologize for this error. The revision was done as per your kind suggestion. Please see the revised **Figure 6** legend in page 20. The scale bars were also corrected. See below:

"Relative (Rel.) Kyn concentrations was set as 1 in 0 hour group in panel **b**, and in 24 hours group in panel **c** and **d**. Rel. IDO1 immunostaining intensity was set as 1 in Control (Ctrl) group. Bar=50 μm in **f**, 500 μm in **i,** and 200μm in **l"**.

"Fig. S8 should be moved into the main figures."

Response: We agree. **Fig. S8** has been integrated into the main figure as **Fig.7**.

Responses to Reviewer #2

General comments *(In this manuscript, Zhang et al. studied the role of Trp-Kyn metabolism during neonatal heart regeneration. They found that IDO1, the enzyme that catalyzed the Trp-Kyn metabolism, is activated after apical resection in the P1 regenerative heart. Deletion of Ido1 in cardiomyocytes prevented heart regeneration and inhibited angiogenesis. This regeneration defect of Ido1 KO hearts can be rescued by exogenous supplement of Kyn. The authors also did a series of mechanistical studies to show that Trp-Kyn regulates regeneration by promoting the YAP/ERK pathway in cardiomyocytes and by direct transcriptional regulation of angiogenic genes via AHR, which is the cytoplasmic receptor of Kyn. Taken together, the authors showed that the Trp-Kyn metabolic pathway, which is a well-known immunosuppressive mechanism, regulates neonatal heart regeneration via a previously unrecognized mechanism acting on the non-immune cells. Overall, the findings of this study are novel and the characterization of cell types where Trp-Kyn pathway is acting on was well performed. However, the authors are required to address the following concerns:"*

Responses: *We appreciate your expert comments and we are very delighted to know our data are novel and important.*

Specific comments:

Query 1: "*As authors stated, the Trp-Kyn pathway suppresses immune responses by inhibiting T cell activation. Even though Idol was deleted in cardiomyocytes, its effect on Kyn can be cell type non-specific, given that Kyn can be extracellular. Does the reduced Kyn level caused by Idol CM deletion affect the degree of inflammation and fibroblast activation?*"

Response: We appreciate the Reviewer's careful reading and asking for this interesting question.

Emerging data are indicating that there is a complex feedback and feedforward inflammation signaling network among IDO1, KYN, and the ligand-activated transcription factor AhR[1, 3]. Recent data also shows that AhR activation by IDO1 product KYN leads to both hyper- and anti-inflammatory effects depends on the cell types [4]. Type I IFN produced by DCs induces IDO1, which serves as a cell-autonomous control of type I IFN and antagonizes its downstream effects [3, 5]. AhR activation was shown to negatively regulate the type I IFN response by promoting the expression of the TCDD-inducible poly (ADP-ribose) polymerase (TiPARP), which further disturbs the innate immune responses to support the tumor growth [6]. Similar mechanism was also found in our current heart regeneration model. The actual inflammation in neonatal heart after apical resection activated the IDO1-Kyn-AhR signaling. Interestingly, *Ido1* deleted cardiomyocytes have less active IDO1-Kyn-AhR signaling, resulting into the excessive inflammatory action and exiting of cell cycle which lead to the DNA damage during cardiac regeneration (**Figure 3a** and **S6**). However, the activation and quantification of inflammatory cells such as dendritic cells, natural killer cells, macrophages, primary murine T cells and regulatory T cells still need to be done for further investigation.

Fibroblasts are homeostatic and producing extracellular matrix (ECM) during normal heart physiology. The excessive activation of fibroblasts have been implicated as major determinants of maladaptive repair in a range of mammalian contexts [7]. Due to myocardial infarction, cardiac fibroblast response would be activate along the time continuum. During the events of injury, fibroblasts convert into pro-inflammatory cells, and polarize into the proliferative and pro-angiogenic stage in where they promote ECM synthesis. This forms the infarct scar and inhibits angiogenic signaling; finally return to a state of neo-homeostasis, once again supporting the normal turnover of ECM to support the new infarct environment [8, 9]. We found that *Ido1* deleted heart showed a series of fibroblast activations, for example the robust induction of pro-

inflammatory signaling (**Figure 3a**, **S6**), reduction of alpha-smooth muscle actin (αSMA) positive cells within the lesion (**Figure 4a**), anti-angiogenesis and fibrosis formation (**Figure 4b-e, 1d**). Furthermore, some key matrix metalloproteinase (MMP) enzymes, including MMP2, MMP3, MMP9 and MMP13 mRNA were upregulated by Ido1 deletion (**Following figure**). These represent highly conserved and specific markers for fibroblasts activation associated with the promotion of collagen synthesis and fibrosis[10] was directly or indirectly changed by *Ido1* deletion during neonatal heart regeneration.

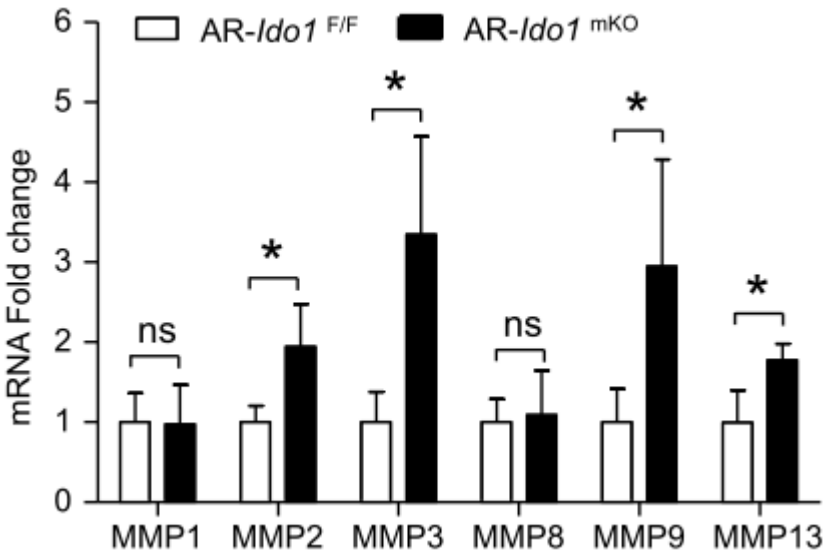

Figure. Quantification of matrix metalloproteinase (MMP) enzymes mRNA expression in apical resected (AR) neonatal hearts with Ido1 deletion at cardiomyocyte (Ido1 ^{mKO}). Ido1 floxed (Ido1 $^{F/F}$) mice were used as control. Total RNA was isolated from postnatal 7 days after AR surgery performed at postnatal 1 day, and analyzed by qRT-PCR assay. n=4 for each group. Data are mean±SD. *p<0.05 by Student t-test. ns, non-significance.

Query 2: *"The Trichrome Masson staining done in this study appears to be unsuccessful as the fibrotic region was not labeled clearly in blue. This can affect data interpretation and quantification. The authors are required to repeat all the Trichrome Masson staining experiments and the related quantification."*

Response: We appreciate and agree with the Reviewer. The Masson trichrome staining assay was performed again and quantification was done. The revised data is included in the current form of the manuscript.

Query 3: "*On page 8, "Similarly, Ido1-deletion CMs also showed reduced levels of m-MYC and CCNB1…". Please provide the supporting data."*

Response: Thanks for your query**.** The supporting data can be found in **Figure 3c, d**. *Ido1*-deleted CMs showed reduced levels of MYC and CCNB1 in nucleus (Western blot and quantification data).

Query 4: "*Figs 3B, 4B and S1, please provide better images as the co-localization of staining signals is not clear."*

Response: As per the Reviewer's recommendation, the indicated staining was repeated to clearly indicate the co-localization. Better and representative images are included in the revised manuscript.

Query 5: "*Fig 3C, YAP is not detected in the nucleus, which contradicts the authors' model in which Trp-Kyn pathway is proposed to activate YAP/ERK pathway to regulate cardiomyocyte proliferation*."

Response: We thank Reviewer for this critical question. As shown in **Figure 3c**, the bands of YAP in nucleus looks weaker, comparing to the $Ido1^{mKO}$, the level of YAP protein in $Ido1^{F/F}$ is higher. This instigates an inevitable role of nuclei localized YAP protein in determining the cardiac regeneration in our experimental setup. Furthermore, previous studies have demonstrated a weak expression of YAP in nucleus comparing with in cytoplasm in regenerating hearts [11, 12].

Query 6: "*Fig 4D, the authors are trying to show that the extracellular Kyn activates AHR in endothelial cells and directly regulates transcription of angiogenic genes. To show that, AHR ChIP should be performed specifically in endothelial cells instead of the whole heart, because AHR is localized to multiple cell types in the heart."*

Response: Thanks for the Reviewer's suggestion. As Reviewer indicated both the transcriptional expression and the AHR enrichment of angiogenic genes should be performed specifically in endothelial cells. We isolated endothelial cells (method section) from experimental animals (Sham and Apical resected heart (P7) with *Ido1* knockout and control). Total RNA was extracted from ECs and qRT-PCR was re-performed (revised **Figure 4c**). AHR-ChIP assay was also re-performed specifically in endothelial cells and represented in the revised **Figure 4d**.

Query 7: *In discussion, the authors mentioned that Kyn generation through IDO is also induced after adult MI. Why this induction is not sufficient to activate regeneration as seen in the neonatal heart? This needs to be discussed.*

Response: We highly appreciate the Reviewer's insightful and helpful comments on our manuscript. The discussion focus on this comment was added in page 14 of the revised manuscript.

Following injury in the adult human heart, almost all of the cardiomyocytes (CMs) exit from the cell cycle, which is the major reason of adult human heart why it fails to regenerate [13]. Likewise, the cell division related genes and pathways were disappeared in adult heart compared with neonatal heart. The data from our lab (data no shown) and others' study show that Kyn generation through IDO is also induced after adult myocardial infarction [14], but not sufficient to activate regeneration. Because the IDO-Kyn target genes such as the Myc, Yap, Notch and GATA, as well as Vegfa (which are well established cell cycle regulators) and their gene expression become very weak after myocardial infarction [15, 16, 17]. However, the other targets of IDO-Kyn signaling including such as, NF-□B and IFN regulation become much stronger, which would damage adult heart regeneration. Conversely, loss of function of IDO1 as well as pharmacological inhibition by 1MT (1-methyl tryptophan) attenuated cardiac deleterious remodeling as well as cardiac dysfunction after adult myocardial infarction[14]. In reverse, EC-derived IDO1 promotes the cardiac injury and cardiac dysfunction after myocardial infarction in adult heart. It might be related to the switching of expression and location of IDO1, the micro-environment of inflammation and the cardiac regeneration activity between neonatal and adult under injury stimulation. Thus, appropriate inflammatory response control may provide an important therapeutic strategy to repair the damaged heart.

Reference:

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17. Braile M*, et al.* VEGF-A in Cardiomyocytes and Heart Diseases. *Int J Mol Sci* **21**, (2020).

Reviewers' Comments:

Reviewer #2:
Remarks to the Author:
The authors have addressed all my previous comments; therefore, I would recommend for acceptance.

Reviewer #3:
Remarks to the Author:
Basically, I share the previous Reviewers' criticisms and I find that they have been adequately addressed by the revision. The manuscript is innovative and though not exactly exhaustive mechanistically, it is ground-breaking, and so to be considered for publication as it stands. Use of the language is suboptimal.

Point-by-point responses:

We appreciate the Editor's favorable decision in principle, to publish a suitably revised version in Nature Communications [NCOMMS-21-41724A]. We were happy to receive the two reviewers' highly positive comments and agreement to be considered for publication since we have addressed all their previous comments.

Below is our point-by-point response as the reviewers' comments:

Reviewer #2 (Remarks to the Author):

The authors have addressed all my previous comments; therefore, I would recommend for acceptance.

Response: We thanks to this positive comment about acceptance our manuscript and appreciate the Reviewer's time and efforts to evaluate our manuscript.

Reviewer #3 (Remarks to the Author):

Basically, I share the previous Reviewers' criticisms and I find that they have been adequately addressed by the revision. The manuscript is innovative and though not exactly exhaustive mechanistically, it is ground-breaking, and so to be considered for publication as it stands. Use of the language is suboptimal.

Response: We appreciate the Reviewer's encouraging comments and careful reading. We have improved our language by a native English speaker.