The IgG3 Subclass of β1-adrenergic receptor autoantibody is an endogenous biaser of β1AR signaling

Maradumane Mohan, Yuji Nagatomo, Prasenjit Saha, Sromona Mukherjee, Timothy Engelman, Rommel Morales, Stanley Hazen, W.H. Wilson Tang, and Sathyamangla Naga Prasad

Corresponding author(s): Sathyamangla Naga Prasad, Cleveland Clinic

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|---------------------------|------------|
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
Dear Dr. Naga Prasad,

Your paper "The IgG3 subclass of β1-adrenergic receptor autoantibody is an endogenous biaser of β1AR signaling" has now been evaluated by two experts in the field. As you will see from the enclosed reports, Reviewer 1 raised some points of criticism, but was overall positive. On the other hand, Reviewer 2 felt that your study suffers from major methodological flaws and recommended against acceptance of your paper. Given the different views of the reviewers, I have studied your paper carefully myself. I am afraid that I have to concur with the more critical reviewer, and therefore have no other choice than to reject your paper.

I regret that we could not reach a more positive outcome this time, but would anyhow like to thank you for giving us the opportunity to evaluate your work.

Sincerely yours,

Carl-Henrik Heldin

Thank you for the opportunity to examine this work. We hope that as your studies progress you will consider submitting future manuscripts to Molecular Biology of the Cell (MBoC).

If you have any questions regarding the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

Sincerely,

Carl-Henrik Heldin
Monitoring Editor
Molecular Biology of the Cell

Reviewer #1 (Remarks to the Author):

In this study, Mohan et al. describe the molecular impact of β1AR autoantibodies of the IgG3 class on β1AR ligand-mediated signaling. Using adenylyl cyclase activity and cAMP generation as readouts of G protein-coupling and 2nd messenger generation, as well as phosphorylated ERK1/2 as a readout of G protein-independent signaling, the authors report that the IgG3 class of β1AR autoantibody acts to block β1AR agonist (dobutamine) G protein-dependent effects while preserving G protein-independent effects, whereas it enhances β-blocker (metoprolol) G protein-dependent effects. These results suggest that this class of β1AR autoantibody acts as a modulator of β1AR ligand bias toward particular signaling pathways. Overall this mechanistic pilot study reveals an exciting potentially therapeutic and valuable concept, but could benefit from more clarity in data presentation.

Suggestions:

1. Separation of serum versus purified antibody results. Since there are some differences between the human serum data and purified antibody data, it would be helpful thematically to separate the two into separate figures so the reader isn't jumping back and forth between parts of Figure 2 and SFig 1. The human serum data establishes the paradigm, but it's the purified antibody data that's more important for clarity of effects. To this end, what concentration of purified antibodies are used in the experiments and how does this compare to the concentration of the antibody in the serum?

2. Presentation of data by experiment type to allow insertion of all required controls for proper comparison. Although important to show the model cell line works as expected, some of the data in Fig. 1 would be beneficial to include for comparison in other Figures, potentially made easier by presenting each data output in its own Figure. For instance it's difficult to fully interpret the impact of the antibodies on vehicle control, dobutamine or metoprolol effects on cAMP generation, adenylyl cyclase activity or P-ERK1/2 response without directly comparing to their effects in the absence of the antibodies within the same experiment.

3. G protein-independent effects. Related to the point above, when the P-ERK1/2 data is compared between Fig. 1, where dobutamine caused an increase to ~800%, and Fig 4, where dobutamine increased the response to ~400% in the presence of the antibodies, it's unclear that there's a G protein-independent effect. It seems like both IgG3 (−) and (+) purified antibodies may attenuate the P-ERK1/2 response, which could represent a degree of receptor blockade. Analysis of all conditions in one
experiment (i.e. vehicle control, dobutamine and metoprolol effects on P-ERK1/2 in the absence or presence of IgG3(+), with accompanying histogram comparison) would clarify. Is the antibody-sensitive component of the P-ERK1/2 response β-arrestin-dependent as the authors suggest?

4. Mechanism of potential autoantibody-mediated effects on receptor signal bias. The most interesting and important aspect of the study is the potential impact of the IgG3 class of β1AR antibody on modulating proximal ligand-mediated receptor signaling responses. Based on the growing literature focused on alterations in GPCR structure in response to different ligand types, can the authors postulate how the autoantibody may work to modulate ligand-mediated signal bias?

Reviewer #2 (Remarks to the Author):

The present manuscript by Mohan et al. has investigated the effects of a specific subclass of human anti-beta1 adrenoceptor autoantibodies on the in vitro signaling by this receptor. I have the following major concerns:

1) The use of a physiologically irrelevant cell system (HEK293) expressing supra-physiological levels of the beta1AR, which itself is recombinantly expressed (Flag-tagged, i.e. not the native receptor), dramatically diminishes the physiological relevance and significance of the reported findings.

2) The compositions of the IgG3(-) and IgG3(+) sera used in the present study are not outlined at all by the authors and thus, are completely unclear to this reviewer. For instance, is either serum agonistic (stimulatory) or antagonistic (inhibitory) at the human beta1AR? Also, how can the authors be sure that the receptor binding properties of these sera/antibodies are preserved at the heterologously expressed Flag-tagged beta1AR of their HEK293 cells?

3) In Fig. 1 (specifically panel C), metoprolol appears to act as an inverse agonist. Is that true or an artifact of the heterologous cell system?

4) The observed effects of the autoantibodies on cAMP & AC activity in Fig. 2 could easily be due to beta1AR desensitization induced by the Dob pre-treatment. They do not necessarily reflect altered or biased receptor signaling.

5) In the same vein, the results of Fig. 3 could easily reflect the effect(s) of some other IgG subclass autoantibody(ies) present in the sera and do not necessarily signal beta1AR signaling alterations by IgG3(-) or IgG3(+). In fact, the complete absence of ERK activation under Control or Meto conditions in Fig. 3D vs. Fig. 3A is a clear indication that the IgG3(-) & IgG3(+) sera contain growth factors that most likely drive baseline/background ERK activation.

6) In Fig. 4, the beta2AR appears incapable of activating ERK at all. Shouldn’t Iso induce substantial (if not robust) ERK activation in its own right here?
Editor:

We would like to thank the editor for giving us the opportunity to address the concerns outlined by the reviewers. We have now addressed all the concerns of the reviewers by performing new additional experiments using non-failing human cardiac patient tissue to reflect the physiological system and have provide an in depth rationale for using them. The consistent findings observed in the cell system as well as in the donor human hearts reflects that the unique modulation observed by the human IgG3(+) β1AR autoantibody elucidates an underappreciated and understudied role of autoantibodies in biasing downstream signals. We hope that our comprehensive response to the reviewers assuages their concerns and the manuscript would now be acceptable for publication.

Reviewer #1 (Remarks to the Author):

In this study, Mohan et al. describe the molecular impact of β1AR autoantibodies of the IgG3 class on β1AR ligand-mediated signaling. Using adenylyl cyclase activity and cAMP generation as readouts of G protein-coupling and 2nd messenger generation, as well as phosphorylated ERK1/2 as a readout of G protein-independent signaling, the authors report that the IgG3 class of β1AR autoantibody acts to block β1AR agonist (dobutamine) G protein-dependent effects while preserving G protein-independent effects, whereas it enhances β-blocker (metoprolol) G protein-dependent effects. These results suggest that this class of β1AR autoantibody acts as a modulator of β1AR ligand bias toward particular signaling pathways. Overall, this mechanistic pilot study reveals an exciting potentially therapeutic and valuable concept, but could benefit from more clarity in data presentation.

We would like to thank the reviewer for a very insightful and comprehensive analysis of the manuscript and critical suggestions.

Suggestions:

1. Separation of serum versus purified antibody results. Since there are some differences between the human serum data and purified antibody data, it would be helpful thematically to separate the two into separate figures so the reader isn't jumping back and forth between parts of Figure 2 and SFig 1. The human serum data establishes the paradigm, but it's the purified antibody data that's more important for clarity of effects. To this end, what concentration of purified antibodies are used in the experiments and how does this compare to the concentration of the antibody in the serum?

We thank the reviewer for this excellent suggestion. We have now separated the human serum data [Fig. 2 & Fig. S1] and purified antibody data [Fig. 3 & Fig. S2]. These
changes can now be found in the new results section (serum data and purified autoantibody data) of the revised manuscript which now allows for streamlined reading.

The presence or absence of IgG3 subclass of autoantibody targeting β1AR was determined by ELISA wherein the ELISA plate was coated with fusion protein for the extracellular loop of human β1AR followed by use of IgG3 subclass as secondary antibody which was described previously (Nagatomo et al, 2009, J. Card Fail; Nagatomo et al, 2016, J Card Fail, 22(6):417-422). The positivity for the IgG3 subclass was defined as an optical reading > 2.5 times the background density. The antibodies were purified using the MabTrap kit (detailed in the methods section) and following purification, the autoantibodies were diluted to a final concentration of 0.5 μg/μL. In depth information is now incorporated into the methods section of the revised manuscript.

2. Presentation of data by experiment type to allow insertion of all required controls for proper comparison. Although important to show the model cell line works as expected, some of the data in Fig. 1 would be beneficial to include for comparison in other Figures, potentially made easier by presenting each data output in its own Figure. For instance it’s difficult to fully interpret the impact of the antibodies on vehicle control, dobutamine or metoprolol effects on cAMP generation, adenylyl cyclase activity or P-ERK1/2 response without directly comparing to their effects in the absence of the antibodies within the same experiment.

We would like to thank the reviewer for asking us to streamline the data. This realignment has now allowed us present the data in manner that provides appreciation to the changes that the autoantibodies mediate. We have now separated out serum data from purified autoantibodies and provided data on control (Ctrl) allowing for comparison. We have presented the G-protein coupling adenylyl cyclase activity as a percent change over respective controls giving an appreciation on the unique role the IgG3(+) β1AR autoantibodies play in biasing the β-blocker signal. Furthermore, there were no differences in ERK activation at baseline following treatment of cells with either IgG3(-) or IgG3(+) serum. Similarly, pre-treatment of cells with IgG3(-) or IgG3(+) serum impairs Dob-mediated ERK phosphorylation indicating that there may be components in the serum that may inhibit this pathway. In this context, we did not observe any differences in Dob-mediated ERK phosphorylation following pre-treatment with either IgG(-) or IgG(+) purified β1AR autoantibodies. Also, we have now included the cells with no IgG [Fig. 4D] as control which shows all the differences in ERK phosphorylation in presence or absence of β1AR autoantibodies. However, Dob-mediates significant phosphorylation of ERK following pre-treatment with both IgG3(-) or IgG3(+) β1AR autoantibodies. This is the key contrasting observation as IgG3(+) β1AR autoantibodies selectively abrogates G-protein coupling with Dob but mediates robust ERK activation. This suggests a unique modulatory role for IgG3(+) autoantibody that biases the agonist signaling towards G-protein independent pathway while impairing G-protein coupling.
3. G protein-independent effects. Related to the point above, when the P-ERK1/2 data is compared between Fig. 1, where dobutamine caused an increase to ~800%, and Fig 4, where dobutamine increased the response to ~400% in the presence of the antibodies, it's unclear that there's a G protein-independent effect. It seems like both IgG3 (-) and (+) purified antibodies may attenuate the P-ERK1/2 response, which could represent a degree of receptor blockade. Analysis of all conditions in one experiment (i.e. vehicle control, dobutamine and metoprolol effects on P-ERK1/2 in the absence or presence of IgG3(+), with accompanying histogram comparison) would clarify. Is the antibody-sensitive component of the P-ERK1/2 response $\beta$-arrestin-dependent as the authors suggest?

We would like to thank the reviewer for this thoughtful comment. We would like to clarify that the data presented are not percent changes over control but are arbitrary densitometry units. Furthermore, as suggested by the reviewer we have now run the sample in presence and absence of IgG3(+) autoantibodies in one experimental set as a representative. This shows similar levels of ERK activation in presence or absence of IgG3 autoantibodies suggesting that IgG3(+) pre-treatment does not attenuate ERK activation. Furthermore, as G-protein coupling is impaired with IgG3(+) autoantibodies, we believe that this ERK activation could primarily be through G-protein independent pathways. We believe that this component of ERK activation could be through G-protein $\beta$-arrestin dependent pathway as G-protein coupling is impaired by IgG3(+) autoantibodies. These studies are currently ongoing and is complicated by the availability of the patient samples.

4. Mechanism of potential autoantibody-mediated effects on receptor signal bias. The most interesting and important aspect of the study is the potential impact of the IgG3 class of $\beta$1AR antibody on modulating proximal ligand-mediated receptor signaling responses. Based on the growing literature focused on alterations in GPCR structure in response to different ligand types, can the authors postulate how the autoantibody may work to modulate ligand-mediated signal bias?

We thank the reviewer for this insightful question. We too were initially surprised by our observations. However, given the reproducibility of these observations from seven different patients harboring IgG3(+) $\beta$1AR autoantibodies suggested the presence of yet to understood role of IgG3(+) autoantibodies in uniquely modulating human $\beta$1AR function and signaling. Based on the recent advances made in understanding the GPCR structure, particularly the knowledge gained on allosteric modulation of the receptor function, we believe that IgG3(+) autoantibodies probably acts as Positive Allosteric Modulator (PAM)-antagonist with respect to Dobutamine (elucidated in Fig.7). PAM-antagonist increases the affinity of the receptor for the agonist but concomitantly...
decreases agonist efficacy when co-bound. These kind of antagonism can be useful in correcting inappropriate pathological signaling (Kenakin and Strachan, 2018, Trends Pharmacol Sci 39(8): 748-765). However within the constructs of the IgG3(+) antibody modulation, presence of antibody uniquely impairs agonist mediated G-protein coupling while G-protein independent ERK activation is intact. More importantly, IgG3(+) autoantibodies may allosterically modulate Meto binding to receptor in such a way that it now allows for G-protein activation. We believe that IgG3(+) β1AR autoantibodies in presence of high levels of circulating β-blocker would allow for moderate coupling to G-proteins while, with higher levels of circulating catecholamines it would impair G-protein coupling but importantly allow for G-protein independent signaling. Such opposing yet balanced signaling mediated by IgG3(+) antibodies may underlie the clinical benefits observed in patients with dilated cardiomyopathy and heart failure.

Reviewer #2 (Remarks to the Author):

The present manuscript by Mohan et al. has investigated the effects of a specific subclass of human anti-beta1 adrenoceptor autoantibodies on the in vitro signaling by this receptor. I have the following major concerns:

1) The use of a physiologically irrelevant cell system (HEK293) expressing supra-physiological levels of the beta1AR, which itself is recombinantly expressed (Flag-tagged, i.e. not the native receptor), dramatically diminishes the physiological relevance and significance of the reported findings.

We thank the reviewer for critical evaluation of the manuscript. We agree with the criticism of not using physiologically relevant cell system but we believe that the reviewer would have realized the inherent limitation of this question had he/she suggested specific experiments to address this key concern. On the same note, it is also clear to us that we did not do good job in making a clear case on the limitation of using endogenous primary cell/organ systems to assess the role of IgG3(+) autoantibodies.

Since the auto antibodies are very specific to the human β1AR, it becomes key limitation in terms of using primary human cells for our study as majority of the human cells/organs have little or no endogenous expression of β1AR. In depth analysis on the protein expression profile of β1AR in human tissues using the GeneCards showed that it is primarily expressed in the heart and to a certain extent in adipose tissue and kidney. In recognition of the difficulty to culture these primary cells, we are left with two options: a) to use human non-failing donor heart samples to test for the ability of these auto-antibody to bias the agonist signal using in vitro adenyl cyclase assay or b) to use induced human pluripotent stem cells to test for the ability of the auto antibody to bias agonist signals. Despite number of studies on differentiated cardiomyocytes from human pluripotent stem cells showing contractile response to β-agonist, nothing is known about the expression levels of β1- and/or β2-AR in these cells. Therefore, we
used the differentiated cardiomyocytes from induced human pluripotent stems cells to determine the levels of $\beta_1$- and/or $\beta_2$-AR in these cells by performing radio-ligand binding. Assessment of $\beta_1$- versus $\beta_2$-AR levels in these cells showed that majority of the $\beta$ARs are the $\beta_2$AR compared to $\beta_1$ARs [See Figure 1]. Consistent with this observation an earlier studies showed selective engagement of $\beta_2$AR signaling in differentiated human pluripotent stem cells (Borchert et al, 2017, J Am Col Card, 70(8):975-991). Together, these studies indicates that differentiated human cardiomyocytes may not be a good endogenous system to assess the role of human IgG3(+) autoantibodies. Given this unexpected limitation, we used non-failing human donor hearts to assess for the role of IgG3(+) autoantibodies in modulating adenylyl cyclase activity and are now included as new Figure 5 in the revised manuscript.

Although we do agree on the issue of a heterologous system but due to the key limitations (as outlined above), we believe that the heterologous system used by us provides a valuable alternative to understand the unexpected beneficial clinical outcomes in patients harboring the IgG3(+) sub-class of autoantibodies. The currently used heterologous system to a great extent would reflect that endogenous responses of the receptor given the expression of human $\beta_1$AR in human cells. This is supported by the similar results obtained on modulation of G-protein coupling by IgG3(+) $\beta_1$AR autoantibodies from HEK 293 cells expressing human $\beta_1$ARs and non-failing human hearts.

2) The compositions of the IgG3(-) and IgG3(+) sera used in the present study are not outlined at all by the authors and thus, are completely unclear to this reviewer. For instance, is either serum agonistic (stimulatory) or antagonistic (inhibitory) at the human beta1AR? Also, how can the authors be sure that the receptor binding properties of these sera/antibodies are preserved at the heterologously expressed Flag-tagged beta1AR of their HEK293 cells?

We apologize for not presenting the information on the patients samples and how the IgG3(+) sera was identified. We have now provided this information in the methods section detailing the ELISA and IgG purification from plasma. The compositions and characteristics of the sera used in the study have been published and is part of the comprehensive IMAC (Intervention in Myocarditis and Acute Cardiomyopathy)-2 clinical study (Nagatomo Y et al., JACC, 2017). The aim of our study was to understand the mechanisms underlying this unexpected clinical benefits with $\beta$-blocker. We further believe that the modulation of $\beta_1$AR signaling by autoantibodies are preserved in the heterologous system given the similar results obtained on modulation of G-protein coupling by IgG3(+) $\beta_1$AR autoantibodies in the human heart samples.
3) In Fig. 1 (specifically panel C), metoprolol appears to act as an inverse agonist. Is that true or an artifact of the heterologous cell system?

Treatment of Metoprolol blocks receptor activity which is reflected in the reduction in cAMP. Although it is true that there is significant reduction in cAMP levels, we do not believe that is an inverse agonist as cAMP levels are measured are a snap shot of its accumulation over time. A more direct measure of cAMP generation following β1AR modulation is through real-time measurement of G-protein coupling and adenylyl cyclase activity which is shown in Fig 1 panel D where pre-treatment with metoprolol does not significantly reduce cAMP generation compared to control vehicle treatment. This observation is further supported by the studies in human cardiac plasma membranes.

4) The observed effects of the autoantibodies on cAMP & AC activity in Fig. 2 could easily be due to beta1AR desensitization induced by the Dob pre-treatment. They do not necessarily reflect altered or biased receptor signaling.

We apologize if the explanations were not clear in our methods and/or results section. In all these experiments, there is no Dobutamine pretreatment. We have performed all the studies following pre-treatment with autoantibodies. It is unlikely that autoantibodies can cause receptor desensitization as we did not observe significant changes in cAMP response following treatment with just the purified autoantibodies [Fig. S2].

5) In the same vein, the results of Fig. 3 could easily reflect the effect(s) of some other IgG subclass autoantibody(ies) present in the sera and do not necessarily signal beta1AR signaling alterations by IgG3(-) or IgG3(+). In fact, the complete absence of ERK activation under Control or Meto conditions in Fig. 3D vs. Fig. 3A is a clear indication that the IgG3(-) & IgG3(+) sera contain growth factors that most likely drive baseline/background ERK activation.

We do agree that the serum may contain factors that may potentially contribute to the ERK phosphorylation given multiple inputs for ERK activation. Therefore, to overcome these concerns, we have used purified IgG3(-) or IgG3(+) autoantibodies. The data presented in old Fig. 3D (now new Fig. 4D in the revised manuscript) represents ERK phosphorylation following pre-treatment with purified autoantibodies which would reflect changes only mediated by the purified autoantibody. Our studies show that while IgG3(+) autoantibodies are able to impair Dob-mediated G-protein coupling/adenylyl cyclase activity they are able to maintain robust ERK activation following Dob. This suggests a unique yet to be understood modulation of human β1AR by IgG3(+) subclass of autoantibodies.
6) In Fig. 4, the beta2AR appears incapable of activating ERK at all. Shouldn`t Iso induce substantial (if not robust) ERK activation in its own right here?

We do agree that ISO should mediate robust ERK activation. In our hands strength of ERK activation seems to determine the time span of serum starvation. Use of serum starvation for time longer 6 hours or even overnight mediates very robust ERK activation to ISO [see Figure 2]. Given that all our studies on HEK-β1AR cells were performed at 4 hours of serum starvation, we use the same time window with the HEK-β2AR expressing cells. More importantly, the reason for performing this experiment is to show that the IgG3(+) autoantibodies are specific toward β1ARs. Thus to determine the specificity, we have used both the assessment of receptor phosphorylation as well as ERK activation. We observe that both receptor phosphorylation as well as ERK activation patterns are no different in presence or absence of antibody. Although the ERK activation is not that robust yet, is significantly activated independent of the autoantibody. Since β2AR phosphorylation is a more proximal event, taken together these data consistently shows that these autoantibodies do alter β2AR responses to ISO.
Dear Dr. Prasad:

The revised version of your manuscript "The IgG3 subclass of β1-adrenergic receptor autoantibody is an endogenous biaser of β1AR signaling" has now been evaluated by the two original reviewers. Whereas Reviewer 1 was satisfied with your revision, Reviewer 2 felt that additional information about the direct effect of the autoantibodies on G protein signaling is needed. I concur with the latter conclusion and am therefore unable to accept the present version of your paper. However, if you can provide the data requested by Reviewer 2, I would like to invite you to resubmit a suitable revised version of your paper.

Sincerely yours,

Carl-Henrik Heldin

Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Naga Prasad,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

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Reviewer #1 (Remarks to the Author):
The authors have adequately addressed my previous concerns.

Reviewer #2 (Remarks to the Author):
I applaud the authors for their efforts to address my concerns but, unfortunately, the main findings of the study are still not sufficiently documented, in my opinion. Since the authors claim that IgG3 is a biase of beta1AR signaling, there should have been an exhaustive investigation and documentation of the effects of the autoantibodies on G protein signaling/activity (e.g. via the GTPgammaS binding assay) and on beta-arrestin binding/activation (e.g. use of the DiscoveRx assay system or BRET or other types of protein-protein interaction assays). The reported effects of dobutamine and metoprolol on beta1AR-induced cAMP accumulation in the presence of IgG3 serum, although intriguing and provocative, could be simply due to the receptor switching signaling to Gi (or some other G protein type) or, quite simply, due to differences in expression levels of adenylyl cyclase or in functional beta1AR membrane densities among the various cell/tissue types used in the study (transfected HEK293 cell clones expressing recombinant receptor, human cardiac tissues, etc.).
We would like to thank the handling editor and the reviewers for their insightful review and constructive suggestions to strengthen the manuscript. In the revised manuscript, we have addressed the concerns of the reviewer by performing additional experiments including the use of TANGO β-arrestin recruitment assay to support our observations on the β-arrestin bias promoted by the IgG3(+) β1AR autoantibodies. Please find below point-by-point response to the concerns raised by the reviewers.

Reviewer #1

_The authors have adequately addressed my previous concerns_

We would like to thank the reviewer for stating that we have addressed the reviewer concerns.

Reviewer #2

_I applaud the authors for their efforts to address my concerns but, unfortunately, the main findings of the study are still not sufficiently documented, in my opinion. Since the authors claim that IgG3 is a biaser of beta1AR signaling, there should have been an exhaustive investigation and documentation of the effects of the autoantibodies on G protein signaling/activity (e.g. via the GTPgammaS binding assay) and on beta-arrestin binding/activation (e.g. use of the DiscoveRx assay system or BRET or other types of protein-protein interaction assays). The reported effects of dobutamine and metoprolol on beta1AR-induced cAMP accumulation in the presence of IgG3 serum, although intriguing and provocative, could be simply due to the receptor switching signaling to Gi (or some other G protein type) or, quite simply, due to differences in expression levels of adenylyl cyclase or in functional beta1AR membrane densities among the various cell/tissue types used in the study (transfected HEK293 cell clones expressing recombinant receptor, human cardiac tissues, etc.)._

We would like to thank the reviewer for the insightful comments and suggestions to address the main findings of our study.

As suggested by the reviewer, we have now performed in depth studies to determine the β-arrestin bias of IgG3(+) β1AR autoantibodies by using the PRESTO-Tango assay and β-arrestin recruitment assay by confocal microscopy. These studies further support our previous observations showing that IgG3(+) autoantibodies biases β1AR signaling towards β-arrestin in response to β1AR agonist Dob. These set of comprehensive studies show that IgG3(-) β1AR autoantibodies do not appreciably modulate Dob mediated signaling compared to IgG3(+) β1AR autoantibodies that promote arrestin signaling as assessed by TANGO assay (Fig. 4) and supported by enhanced β-arrestin recruitment (Fig. 5) in presence of IgG3(+) autoantibodies. These data are now incorporated in new figures 4 and 5 and changes in the manuscript can be found in introduction, results, discussion, methods and figure legends in the revised manuscript.

Given our in depth studies, we believe that the unique β-arrestin bias observed in presence of IgG3(+) β1AR autoantibodies are not due to changes in expression levels of adenylyl cyclase or functional β1ARs as the cyclase experiments were performed on plasma membrane fractions from the same set of cells that were pre-treated with autoantibodies for a very short period of time. Finally, the reviewer suggests that the IgG3(+) autoantibodies could potentially allow for Gi coupling that may underlie the bias
towards β-arrestin. This is an interesting question but out-of-the scope of the current study as it involves a complete set of detailed studies on the G-protein switching responses to agonist and antagonist in the presence or absence of IgG3 (+) or (-) β1AR autoantibodies. We believe our current findings sets the stage for future studies in determining the ability of IgG3(+) autoantibodies to switch the G-proteins in response to agonist or antagonist promoting β-arrestin bias that may underlie the observed beneficial clinical outcomes (Nagatomo et al., 2017). In recognition, we have now included this interesting idea in the discussion section of the revised manuscript given the recent findings that Gi coupling is important in β-arrestin bias (Wang et al., 2017). Another key aspect that we hope the reviewer appreciates is the fact that these autoantibodies have to be collected from patients and is the most limiting factor in our study. Given this limitation, we have used our available autoantibody resources to optimally address the key concerns of the reviewer. We hope our in depth additional studies assuages the concerns of the reviewer and the revised manuscript is now acceptable for publication.

References
Nagatomo, Y., McNamara, D.M., Alexis, J.D., Cooper, L.T., Dec, G.W., Pauly, D.F., Sheppard, R., Starling, R.C., Tang, W.H., and Investigators, I.-. (2017). Myocardial Recovery in Patients With Systolic Heart Failure and Autoantibodies Against beta1-Adrenergic Receptors. Journal of the American College of Cardiology 69, 968-977.
Wang, J., Hanada, K., Staus, D.P., Makara, M.A., Dahal, G.R., Chen, Q., Ahles, A., Engelhardt, S., and Rockman, H.A. (2017). Galphai is required for carvedilol-induced beta1 adrenergic receptor beta-arrestin biased signaling. Nature communications 8, 1706.
3rd Editorial Decision

January 22, 2021

RE: Manuscript #E20-06-0394RR
TITLE: “The IgG3 Subclass of β1-adrenergic receptor autoantibody is an endogenous biaser of β1AR signaling”

Dear Dr. Naga Prasad:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Carl-Henrik Heldin
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Naga Prasad:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,
Eric Baker
Journal Production Manager
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