Peer Review File
Article information: https://dx.doi.org/10.21037/aob-23-42

Reviewer A

Comment 1

"How do the authors know that this combination is truly 'representative' of IVIG? Activation of ADCC is well known to be selective to the antigen/effector pair. Not all antigens can induce ADCC. Can the authors choose a different antigen or CD16 expressing cell line?"

Response 1:

Thank you for the valuable suggestion. Not all antigens activate ADCC actually. We have tried other target cells, however, due to the materials of IVIG are made from a number of healthy human plasma containing polyclonal antibodies, it is hardly possible to select an antigen that is unique present in healthy people. Since the selected IVIG was produced in China and the plasma source was Chinese population, we considered the background of hepatitis B vaccination in Chinese population and selected HBsAg as the antigen. What we choose as the effect cells are the transgenic reporter cells Jurkat-NFAT-Luc-CD16 cells, which are widely used in the detection of ADCC activity of monoclonal antibodies and are the most stable reporter cells expressing CD16 receptor so far. We also have considered primary cells as effector cells, but the primary cells from donors were unstable, the cell extraction progress was complicated and resulted in different immune response. In our designed system, HBsAg expressed on the surface of target cells only after binds with the Fab fragment of IVIG and then Fc fragment of IVIG will bind to FcγRIIIA on the surface of Jurkat-NFAT-Luc-CD16 cells.

Comment 2

"Can HBsAg monoclonal antibodies be used as a control for ADCC assessment? What if this phenomenon is selective to this antigen and is not general to IVIG?"

Response 2:

HBsAg monoclonal antibodies can be used as a control for ADCC assessment. Due to the

materials of IVIG are made from a number of healthy human plasma containing polyclonal antibodies, it is hardly possible to select an antigen that is unique present in healthy people. Since the selected IVIG was produced in China and the plasma source was Chinese population, we considered the background of hepatitis B vaccination in Chinese population and selected HBsAg as the antigen.

Comment 3a

"What is the glycosylation pattern on the HBsAg subset of IgGs in the IVIG? Are they selectively afucosylated?

a. Can the author enrich for the HBsAg subset of IgGs using antigen immunoprecipitation to better characterize the specific antibody of interest? This appears to have been performed in the literature (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3314627/)"

Response 3a:

Thank you for your serviceable suggestion. we even don't conclude the issue the glycosylation pattern on the HBsAg subset of IgGs in the IVIG and their selectively afucosylation. We will conclude your constructive suggestion into consideration and verify this factor by using COIP.

Comment 3b

"b. How about the use of the FcgRIIIA chromatography column to separate the glycosylation variants to determine high affinity binders?"

Response 3b

Thank you for your valuable and constructive comments. The purpose of our study is to detect the binding ability of IVIG and FcγRIIIA which is focusing on all components in IVIG. As to the high-binding IgG sialylation variant in IVIG will be purified according to your suggestion for further study.

Comment 4

"Enrichment of the small subset of HBsAg for further characterization would be highly desirable."

Response 4

Thank you for your comments. Theoretically, increasing the ability of antigen to bind to IVIG

would further activate more IVIG Fc fragments. However, cell density is limited. Meanwhile, HBsAg on the cell line PLC/PRF/5 can't be purity and it's not necessary.

Reviewer B

Comment 1

"I understand that English is not the first language, this paper needs major overhaul in terms of the English, there are several instances where sentences are incomplete. Basic grammar checks and spell checks were not performed.

Abstract:

Line 27- that sentence is incomplete.

Line 30- spelling correction for "Unknow" is unknown

No conclusions are provided in the abstract, the abstract ends abruptly at results and jumps straight into key findings.

No mention of how many individuals blood samples these tests were done in, and how many times the experiments were replicated.

Results line 39- it should be combined and not "combine" as indicated

No conclusions

Key findings:

Inconsistent use of FCGR3A and FcγRIIIA"

Response 1

We agree with and accept the reviewer's comments. These questions must be reviewed after your caring reading and we have revised in the article.

Comment 2

"Introduction:

No where in the Introduction is it stated how FcγRIIB regulates signals negatively- is this an inhibitory receptor? The authors need to clarify this."

Response 2

Thank you for your questions. In the original article, we did have rarely described the inhibitory

receptor FcγRIIB. When FcγRIIB binds to immune complexes (IgG binding to antigens), it can bind to activator receptors on the cell surface, such as FcγRI or FcγRIII. This connection causes the inhibitory signal with the activation signal, thus weakening cell activation. The intracellular segment of FcγRIIB contains a typical immunoreceptor tyrosine inhibitory motif (ITIM). When FcγRIIB binds to the immune complex, ITIM is phosphorylated and then recruits phosphatases such as SHIP (Src homologous region tyrosine kinase suppressor protein). The activation of these enzymes inhibits the downstream activation signaling pathway, realizes the inhibition of B cell receptor (BCR) or FcγR mediated signaling, reduces the proliferation of B cells and antibody production. As well as regulating phagocytosis and antigen presentation of macrophages and dendritic cells, we have added relevant descriptions in the article. Reference：1.Differential modulation of stimulatory and inhibitory Fc gamma receptors on human monocytes by Th1 and Th2 cytokines. 2.Fcγ receptors and immunomodulatory antibodies in cancer.

Comment 3

"Materials and Methods

No mention of how many participants this in vitro test was done on"

Response 3

Thank you for your questions. Our study collected blood samples from three healthy people for the experiment and we have added relevant descriptions as follows: Donor1, female, B blood type, Donor2, male, O blood type, Donor3, male, O blood type.

Comment 4

"BADTA abbreviation on line 74 has not been defined."

Response 4

Thank you for your questions. *BADTA* is not abbreviated in the specification, We have added it's application the article.

Comment 5

"Line 78- 100ul of fresh whole blood cells- did they remove complement- how did they isolate the cells if they did- this is not clear?"

Response 5

Thank you for your questions. We collected 100μL fresh anticoagulant from whole blood for the experiment without complement inactivation treatment. We have considered inactivated the complement by heating specimen in a water bath at 56℃ for 30 minutes, however, activity of leukocytes is also affected. Meanwhile, Jacob have conclude a similar study and did not inactivate the complement as well. Reference: Exposure of NK cells to intravenous immunoglobulin induces IFN gamma release and degranulation but inhibits their cytotoxic activity.

Comment 6

"Line 90- rephrase "Every concentration was repeated 3 tests"- this is not correct"

Response 6

Thank you for your questions. What we want to express is that different samples concentrations have been repeatedly detected for three times and we have modified the syntax to make it more accurate.

Comment 7

"Line 92-94- Sentence does not make sense"

Response 7

Thanks for your suggestion. We have modified the statement in the article.

Comment 8

"What housekeeping gene was used for the RT PCR? No mention of this in the text."

Response 8

Thanks for your suggestion, we use the following housekeeping gene,

GAPDH-F CGGAGTCAACGGATTTGGTC

GAPDH-R CGGTGCCATGGAATTTGCCA

We have already added it in the article.

Comment 9

"Results

Critique

Overall, I think that this article is more suited for a methodological journal as a short report more so than a full science article. The data that is provided is very one dimensional, not really many results and as I have said that they did not distinguish the sub-population contribution of FcγRIIIa

The FcγRIIIa and FcγRIIIb share 96% sequence homology with FcγRIIIB anchored to the cell membrane. For the expression analyses, how did the authors differentiate between the FcγRIIIa and FcγRIIIb mRNA expression. Could both have been upregulated? I am not sure that both are equal, one is membrane bound? This is important since the FcγRIIIa is expressed on mast cells, macrophages, and natural killer cells as a transmembrane receptor, FcγRIIIb is only expressed on neutrophils- the authors used whole blood so they cannot distinguish between the two FcγRIII based just on mRNA expression?"

Response 9

Thank you for your questions. The structures of FcγRIIIA and FcγRIIIB are different. The transgenic reporter cells we used expressed FcγRIIIA on the cell surface and IVIG bound to theFcγRIIIA on the cell surface. We searched the NCBI database and found the gene which ID number was NM_001386450.1. The gene name is "Homo sapiens Fc gamma receptor IIIa (FCGR3A), transcript variant 8, mRNA" and the primer designed based on this gene is different from the mRNA of FcγRIIIB.

Comment 10

"The authors also spend paragraphs expounding how the ADCC activity can help in cancer etc, but do not themselves test this concept in the experiments. The experiments are few and the results are few- this is not a full science paper but can be a short report."

Response 10

Thank you for your comments. In 2.1, IVIG can kill K562 cells through ADCC, which indicate that IVIG could mediate ADCC to kill tumor cells. Our experimental results are not abundant but we can discuss them in the future research according to the opinions of experts. It should be noted

that as an important antibody drug, the biological activity of the Fc fragment of IgG in IVIG has not been queried as a quality evaluation of IVIG. In addition, there are relatively few reports on the study of FcγRIIIA and its potential activation mechanism of the Fc fragment of IVIG remains to be explored. Therefore, it is necessary to actively promote IVIG ADCC research, which is crucial to evaluate the quality of IVIG.

Comment 11

"Caveat to the methods: The authors used whole blood cells- and not purified cells- this means that complement present in the whole blood may have been responsible for the increased or enhanced FcγRIIIa activity as they conclude- this is fundamentally flawed."

Response 11

As you said, we used fresh anticoagulant whole blood for the experiment without complement inactivation treatment. We have considered the effect of the complement, by heating 56℃ in a water bath for 30 minutes to inactivate the complement, but it will affect the activity of leukocytes. In addition, we chose whole blood cells instead of purified culture of NK cells due to the pH of IVIG may affect the activity of cells. The fresh whole blood represent a more real environment in vivo which can provide stable and reliable results. The NK cells take a small proportion in peripheral blood and IL-2 stimulation is required for in vitro culture to maintain biological activity of NK cells. To some extent, it does not represent the true activity of NK cells.

Comment 12

"The authors also offer no limitations in the discussion.

Throughout the discussion there is inconsistent use of FCGR3A and FcγRIIIA- but this is cosmetic and can be fixed."

Response 12

Thank you for your questions. We will consider and modify the use of FCGR3A and FcγRIIIA in the article uniformly.

Reviewer C

The authors try to show that IVIG can induce ADCC and up-regulate the FcγRIIIA receptor gene and protein expression. There are many problems with this manuscript.

Comment 1

"The authors' English is not good, both grammar and spelling. I suggest that the authors find someone who is good with the English language to go over their manuscript and "fix" the English so that it is acceptable before any resubmission."

Response 1

Thanks for your suggestion, we have revised the syntax and sentences.

Comment 2

"The manuscript itself tries to describe the authors' approach to their question but, to me, it is convoluted and very confusing as to what they did. The Methods section is especially confusing. What is the "whole blood addition" for, what are the authors doing here? How does IVIG lyse the K562 cells"

Response 2

Thank you for your questions. "Whole blood addition" means that 100μL of whole blood is extracted from fresh anticoagulant whole blood and added to the system. Whole blood contains NK cells and activated NK cells will release perforin, granzyme B and other substances. Perforin form "pores" on the cell membrane allowing water and electrolyte to enter the cell rapidly, resulting in cell lysis. Granzyme B enter the cell through "pores" formed by interferon on the cell membrane, activating the apoptosation-related enzyme system and leading to apoptosis of target cells. We have added the principle of the method and detailed steps in the article. IVIG can activate the ADCC biological effect of NK cells through FcγRIIIA and lyse K562 cells. It should be pointed out that the reason why we chose whole blood instead of purified culture NK cells is that the pH of IVIG (4.0) may affect the activity of cells. The cells in fresh peripheral blood represent authentic environment in vivo and can provide stable and reliable results. In vitro culture, IL-2 stimulation is required to maintain biological activity, which can't represent the activity of cells to a certain extent.

Comment 3

"The authors need to show that the IVIG binds to K562 via the Fab before they can suggest that IVIG can lyse K562 cells. The results suggest that IVIG has antibodies that are directed to some epitope(s) on K562 that can result in ADCC. The authors need to prove that this is true."

Response 3

The principle of IVIG lysis of K562 cells is that the Fab fragment of IVIG binds to the antigen epitopes of K562 cells and the Fc fragment binds to the Fc receptors on the surface of effector cells in the whole blood (mainly FcγRIIIA). In effector cells in the whole blood, NK cells will release perforin, granzyme B and other substances, and perforin can form "pores" on the cell membrane. The rapid entry of water and electrolyte into the cell leads to cell lysis and granzyme B can enter the cell through the "pore" formed by interferon on the cell membrane, activating the apoptosis-related enzyme system leading to apoptosis of the target cell. The reaction process in the system can't be directly observed and we have not yet proved the way of combining IVIG and K562 cells, which we will continue to explore in future studies.

Comment 4

"IVIG has isoagglutinins that may bind to RBCs in whole blood. What was the blood group of the whole blood donor?"

Response 4

Thank you for your questions. According to the Pharmacopoeia of the People′ s Republic of China, the agglutinin in IVIG should be lower than 1:64 and the agglutinin should not affect the experiment. At the same time, the index we observed was the lysis of K562 cells which may not be affected by lectins. As for the blood type of the donor, we did not take into account the blood type of the donor in the collection as long as the donor meets the criteria for blood donation. And the previous research people did not raise this issue. The donor information is as follows and we can explore the influence of blood type on IVIG-mediated ADCC in the future. Donor1, female, O blood type, Donor2, male, O blood type, Donor3, male, O blood type. donor information we have added in the text. Reference, 1. Chinese Pharmacopoeia Commission. Pharmacopoeia of the People' s Republic of China（Vol III）. 2. Exposure of NK cells to intravenous immunoglobulin induces IFN gamma release and degranulation but inhibits their cytotoxic activity.