nature portfolio

Peer Review File

Carbohydrate-Mediated Interactions Between Chloroviruses and the Immune System

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this paper, the authors demonstrate glycan-mediated immune responses against giant viruses. This review article hold significance not only for glycobiologists but also for virologists as well as immunologists. However, the current data are not sufficient for acceptance in this journal, as there is a lack of evidence showing that viral glycans themselves directly elicit immune responses.

The authors should clarify why they focused on two systems, such as human monocyte-derived dendritic cells and mouse macrophages.

The authors have highlighted DC-SIGN and Langerin as C-type lectins and Interleukins 6 and 10 as cytokines. This reviewer suggests considering other molecules that may be involved in the immune response to the giant virus. The authors should explain the rationale behind their selection and consider conducting additional experiments.

In Fig. 2, the authors illustrate the binding of viral glycoproteins to human and plant lectins. Demonstrating the direct interaction of glycan chains with lectins would be beneficial for readers. The inhibitory effect could potentially be confirmed by utilizing monosaccharides such as rhamnose or glycan-deficient mutant viruses.

In Figs. 3 and 4, the authors demonstrated IL-6 and IL-10 secretion from human monocyte-derived dendritic cells and mouse macrophages stimulated with viral glycoproteins, respectively. If the authors assume an immune response mediated by C-type lectins to the viral glycoproteins, it is suggested to verify that knockdown or knockout of these C-type lectins will not elicit a response.

In Fig. 3, please include a notation indicating the number of samples.

In the methods section: Change "g" into "g (italic)."

Reviewer #2

(Remarks to the Author)
1. Brief summary of the manuscript
The article by Speciale et al. focuses on the presence of large and giant viruses in the human virome, and the immune response and modulation that results. Giant viruses are unique among viruses in their ability to make some proteins and glycosylation enzymes themselves, and the major capsid proteins (MCP) from chloroviruses express a unique N-glycan structure. To link the viral-encoded glycan structures to the host immune system, they worked with three chlorovirus classes, and the working hypothesis was that the MCP could be recognized by the human innate system.
They focused first on interactions between the viral MCPs and a set of defined lectins. Second, the effects of the MCPs on the induction of cytokines IL-6 and IL-10 were monitored in human monocytes-derived dendritic cells (moDC) and mouse macrophage cell line RAW264.7. Finally, human sera from healthy donors were screened for the presence of IgG capable of recognizing the glycosylated MCPs, which would indicate the MCPs stimulated the human adaptive immune system.
They conclude that the data established that glycosylated molecular patterns on chloroviruses are recognized by the human

innate and adaptive immune systems.

2. Overall impression of the work
My impression is that the work is novel and of interest to those in the virology, immunology, and glycobiology fields, and the experiments are sound and convincing. I have made some suggestions to provide additional information or data to strengthen the conclusions.

3. Specific comments, with recommendations for addressing each comment
A. Fig. 2- Included controls are sound, but could consider inhibition assays with free sugars; 2b- why not show all 6 VGP that are discussed throughout the paper instead of just 4?

B. Fig. 3- Can you provide rationale for why 20 ug/ml viral glycoproteins and 20 hours stimulation were chosen? The data would be strengthened by testing 2 or 3 concentrations of viral glycoproteins to show concentration dependence, especially given the large error bars. Were any statistical calculations performed on the data for significance?

C. Fig. 4- Can you provide rationale for why 20 ug/ml viral glycoproteins and 20 hours stimulation were chosen? The data would be strengthened by testing 2 or 3 concentrations of viral glycoproteins. Were any statistical calculations performed on the data for significance?

D. Discussion- Any further discussion on why or what could be the advantage for the stimulation of both a pro- and an anti-inflammatory cytokine simultaneously? Are there other pathogens/viruses that are capable of this?

E. Methods- It is not clear if either of the anti-IgG detection antibodies are specific for only IgG, or could cross react with IgM or other antibody types? Please provide clarification.

F. General- I do not see a 'data availability' statement in the paper itself, and the Reporting summary says 'n/a' for antibodies and cell lines, however both are used in the paper.

Reviewer #3

(Remarks to the Author)
The paper entitled "Carbohydrate-Mediated Interactions Between Chloroviruses and the Immune System" submitted by Chiodo and de Castro explores how the unique N-glycosylation patterns of chloroviruses is recognized by the innate and adaptive host immune systems.
Chloroviruses in contrast to other viruses such as HIV and SARS-Cov2, express unique N-glycosylation pattern that do not resemble the host glycosylation. In initial ELISA experiments, they showed differential interaction of C type lectins evidencing the interaction of chloroviruses with these innate receptors. Experiments with antigen presenting cells from human and mouse origin are employed to study the IL-6 and IL 10 secretion and finally, IgG profiling in healthy individuals is studied.
The experiments are well performed, and the paper is clear and well written with discussion/conclusions supported by the experimental data, therefore I suggest the publication after minor corrections.

Minor comments/corrections for the authors:
In line 58, please complete/correct the sentence: These responses are typical for viral infection of, or interaction with macrophage cells.

In line 192, please include a more extensive discussion of the interaction with plant lectins.
The authors only see these experiments to confirm the immobilization of the glycoproteins on the plates but I think that these ELISA like experiments with plant lectins could provide new information about the binding specificity of these reagents widely employed in glycobiology research as in general, their binding profiles are described with mammalian like N-glycosylation e. g. CFG glycan microarray data. A more complete study of the chloroviral glycoproteins interactions with different plant lectins (out of the scope of this paper) may also allow identification of plant lectins with potential for the isolation/identification/characterization of these viruses.
In the assay, only galactose/galactosamine recognizing lectins are included, is there a reason for that?

In Figure 3 and 4, it would be nice to include the statistical significance of the data (as it is included in Figure 5)

In the experimental part, in lectin assays, please include how plant lectins are detected biotin-Streptavidin HRP?¿?¿

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The author has sincerely addressed this reviewer's comments and has responded appropriately given the limited time

available. This reviewer considers the manuscript acceptable for publication in Communications Biology.

Reviewer #2

(Remarks to the Author)
The revised manuscript has addressed all of my questions and I agree that it should be published.

Reviewer #3

(Remarks to the Author)

The authors of the article entitled “Carbohydrate-Mediated Interactions Between Chloroviruses and the Immune System” have effectively included the requested data and experiments from the previous revision.

Therefore, I recommend the publication of the article in the present form.

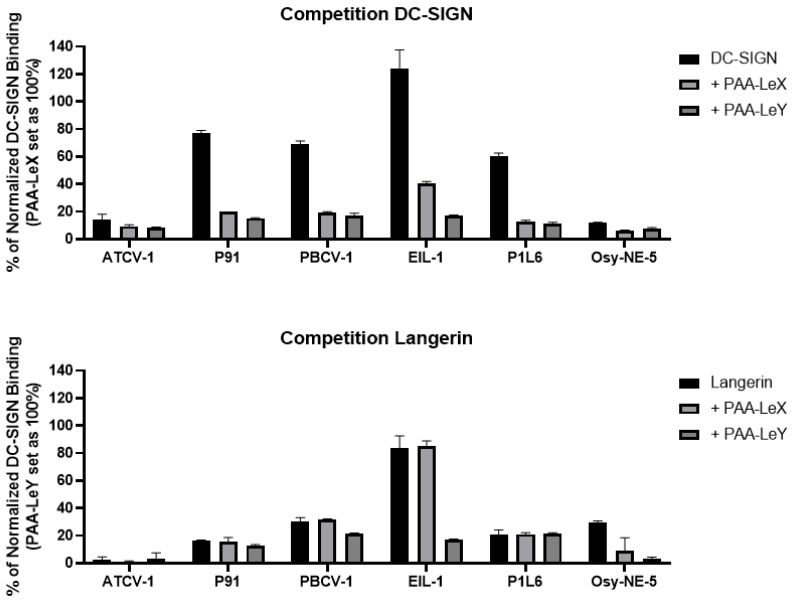
Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this paper, the authors demonstrate glycan-mediated immune responses against giant viruses. This review article hold significance not only for glycobiologists but also for virologists as well as immunologists. However, the current data are not sufficient for acceptance in this journal, as there is a lack of evidence showing that viral glycans themselves directly elicit immune responses.

We thank the reviewer for the analysis and to highlight the importance of our study. The reviewer comments that we have no sufficient data at this stage because there is a "lack of evidence showing that viral glycans themselves directly elicit immune responses". Here we list the evidences we had and the new ones we have added showing and confirming that the viral glycans are responsible for the *in vitro* immune responses we have studied:

1. **As mentioned in the manuscript, the PBCV-1 major capsid protein is decorated with a *N*-glycan that is unusual in terms of structure. To prove the direct effects of different glycans in our different experiments we have tested three mutants of PBCV-1 (P1L6, P91 and EIL-1) expressing the same major capsid protein differing only from the different (truncated) glycans attached on the capsid protein (see Fig. 1). As reported in all our experiments we do see several differences between these glycoproteins and because they only differ in the glycans structures, we have ~~a~~ solid evidence to claim that these glycans are responsible for the responses we see. For example, in our *in vitro* studies, both human and mice, we see clear and statistically significant differences between the wild type glycoprotein PBCV-1 and its mutants (especially EIL-1 and P1L6).**
2. **To further convince the reviewer that the glycans mediate binding and immune responses, we have performed a new set of experiments, competition ELISA, with the tested lectins (Figure S1 in the supporting information). As reported in Figure S1 we have performed the binding experiments in presence of *bona fide* glycosylated ligands of the tested lectins. The reduced EIL-1 binding (and others) we observe if DC-SIGN is pre-incubated with its glycan ligand, a polyacrylamide polymer (PAA) coated with LeX, as well as reduced Langerin binding after pre-incubation with its glycan ligand PAA-LeY, clearly shows the roles of glycans in driving the mentioned binding to lectins.**



3. **As described later in this rebuttal, we are convinced that the viral glycan structures cause the biological affect, as we have tested a generated reporter cell line system expressing DC-SIGN, to prove not binding to this C-type lectins (ELISA solid-phase results) induces cell signaling *in vitro,* which can be inhibited by specific glycan blocking.**

The authors should clarify why they focused on two systems, such as human monocyte-derived dendritic cells and mouse macrophages.

Human monocyte-derived dendritic cells have been widely used to study carbohydrates-mediated interactions especially because they express the majority of the lectins dealing with host-pathogens interactions, and have been well characterized for their specific glycan interactions through these lectins. Moreover, dendritic cells and macrophages are both antigen-presenting cell types that can be modified to change immunity or tolerance, for instance through production of IL-6 and IL-10. Very importantly, these two cell types express C-type lectin receptors and respond with IL-6 and IL-10 production to C-type lectins. Dendritic cells and macrophages have been used together in previous published research to examine cytokine responses to C-type lectins (ex. Mariano Malamud, Gustavo J Cavallero, Adriana C Casabuono, Bernd Lepenies, María de Los Ángeles Serradell, Alicia S Couto. Immunostimulation by Lactobacillus kefiri S-layer proteins with distinct glycosylation patterns requires different lectin partners. J Biol Chem. 2020; 295(42):14430-14444) So, it is very important to use macrophages to confirm dendritic cells data during the responses to C-type lectins.

The authors have highlighted DC-SIGN and Langerin as C-type lectins and Interleukins 6 and 10 as cytokines. This reviewer suggests considering other molecules that may be involved in the immune response to the giant virus. The authors should explain the rationale behind their selection and consider conducting additional experiments.

The main aim of our study is to show for the first time the ability of the immune system to sense the new viral glycoproteins tested in addition to our serological and lectins binding data. It is beyond our goal to study the complete and possible immunological readouts of each individual viral glycoprotein. In addition, IL-6 and IL-10 are the two interleukins that C-type lectins such as DC-SIGN and Langerin are able to modulate during host-pathogens interactions. Most importantly, these two cytokines represent opposing parts of innate immune responses and inflammation. IL-6 is the classical inflammatory cytokine while IL-10 is the classic anti-inflammatory cytokine. Therefore, it is critical to determine whether these C-type lectins induce inflammation or counteract inflammation responses. It appears that for the most part the response of dendritic cells and macrophages to these viral glycans of Chlorovirus is balanced. We thank the reviewer to for this remark, we have added this sentence in the main manuscript: *these two cytokines have been selected as representative to study these type of glycan-based host-pathogens interactions as previously reported.*

In Fig. 2, the authors illustrate the binding of viral glycoproteins to human and plant lectins. Demonstrating the direct interaction of glycan chains with lectins would be beneficial for readers. The inhibitory effect could potentially be confirmed by utilizing monosaccharides such as rhamnose or glycan-deficient mutant viruses.

We thank the referee for this important suggestion. We have performed, as mentioned before, a competition ELISA with *bona fide* ligands of DC-SIGN and Langerin. We did not use simple monosaccharides, because from our experience and literature we know that in particular multivalent systems such the PAA-polymers we have the strength to be used to interfere in this interaction using competition experiments. As mentioned above, we confirmed the specificity of our binding study showing the reduced binding to the viral glycoproteins when the lectins were pre-incubated with PAA-LeX or PAA-LeY. We have already used glycan-truncated mutant viruses by testing three mutants of PBCV-1 (P1L6, P91 and EIL-1) expressing the same major capsid protein differing only in the different (truncated) glycans attached to the capsid protein.

In Figs. 3 and 4, the authors demonstrated IL-6 and IL-10 secretion from human monocyte-derived dendritic cells and mouse macrophages stimulated with viral glycoproteins, respectively. If the authors assume an immune response mediated by C-type lectins to the viral glycoproteins, it is suggested to verify that knockdown or knockout of these C-type lectins will not elicit a response.

We thank the reviewer for this important comment. As the reviewer mentioned, in our work we "assume" that these responses are modulated by the C-type lectins: we have proved a glycan-depend binding and in addition we have shown the glycoproteins with the "strongest" binding to the C-type lectins also triggered IL-6 and IL-10 in moDC. We did not claim that we showed a direct link between the two phenomena however literature is consistent with similar evidences. Unfortunately, no one (as far as we know) has been able to create moDC KO for C-type lectins, as these are primarily cells and dye quickly. We have performed CRISP/Cas9 to KO these lectins however it seems that these cells are not suitable to "silence" lectins efficiently. To approach the reviewer suggestions, and convince that the interactions of viral glycans to C-type lectins leads to cellular alterations we have created a DC-SIGN reporter cell line to readout that binding leads to signaling of DC-

SIGN (solid phase assays described in Figure 2). As depicted in the attached Figure we clearly showed that EIL-1 was not only able to bind DC-SIGN in solid phase assays but was able to trigger the DC-SIGN signaling in these reporter cell lines. The DC-SIGN negative parental cell line was not able to respond to the viral glycoproteins. We hope to convince the reviewer that glycans and C-type lectins are responsible for the interleukins secretion we measured in moDC. It is beyond the scope of this manuscript to deeply investigate the signaling and we have confidentially added these results to the rebuttal. We have added two authors to the manuscript responsible for these results. A separate manuscript is preparation to describe glycan binding receptor reporter cell lines, their specificities and biological responses as add on to functional characterization of moDC. We hope the reviewer appreciates our efforts trying to link glycans structures, binding to C-type lectins and signaling to C-type lectins with the IL responses described *in vitro*.

[Information redacted]

In Fig. 3, please include a notation indicating the number of samples.

In the caption to Fig 3 this sentence is already there: “Mean values from four different experiments (four different human donors) are reported”

In the methods section: Change "g" into "g (italic)."

We have changed the “g” as requested.

Reviewer #2 (Remarks to the Author):

1. Brief summary of the manuscript
The article by Speciale et al. focuses on the presence of large and giant viruses in the human virome, and the immune response and modulation that results. Giant viruses are unique among viruses in their ability to make some proteins and glycosylation enzymes themselves, and the major capsid proteins (MCP) from chloroviruses express a unique N-glycan structure. To link the viral-encoded glycan structures to the host immune system, they worked with three chlorovirus classes, and the working hypothesis was that the MCP could be recognized by the human innate system.
They focused first on interactions between the viral MCPs and a set of defined lectins. Second, the effects of the MCPs on the induction of cytokines IL-6 and IL-10 were monitored in human monocytes-derived dendritic cells (moDC) and mouse macrophage cell line RAW264.7. Finally, human sera from healthy donors were screened for the presence of IgG capable of recognizing the glycosylated MCPs, which would indicate the MCPs stimulated the human adaptive immune system.
They conclude that the data established that glycosylated molecular patterns on

chloroviruses are recognized by the human innate and adaptive immune systems.

2. Overall impression of the work
My impression is that the work is novel and of interest to those in the virology, immunology, and glycobiology fields, and the experiments are sound and convincing. I have made some suggestions to provide additional information or data to strengthen the conclusions.

3. Specific comments, with recommendations for addressing each comment
A. Fig. 2- Included controls are sound, but could consider inhibition assays with free sugars;

We thank the reviewer for this comment. To combine also other comments from other reviewers and to ameliorate our results, we have performed a competition ELISA with the tested lectins. As reported in the Figure S1 in the supporting information, we have performed the binding experiments in the presence of multivalent *bona fide* glycosylated ligands of the tested lectins. We did not use simple monosaccharides, because in our experience to compare the effects from glycans on a glycoprotein it is better to use multivalent systems such the PAA-polymers we have tested in the competition experiments. The reduced EIL-1 binding (and also other ones) we observe when DC-SIGN is pre-incubated with a polyacrylamide polymer (PAA) coated with LeX, as well as the reduced Langerin binding we measure after a pre-incubation with PAA-LeY clearly shows the roles of glycans in driving the mentioned binding to lectins. We have also added this sentence to the manuscript and added these results in the supporting material as Fig. S1.*: *Solid-phase competition experiments pre-incubating DC-SIGN and Langerin with their bona fide multivalent ligands (Polyacrylamide polymers, PAA, coated with LeX or LeY) was also performed. The pre-incubation of DC-SIGN with PAA-LeX or PAA-LeY clearly showed a decrease in binding confirming the involvement of the viral glycans in this interaction and the carbohydrate-recognition domain of DC-SIGN. The pre-incubation of Langerin with PAA-LeY showed also a clear binding reduction in these experimental conditions (see Fig. S1).*

2b- why not show all 6 VGP that are discussed throughout the paper instead of just 4?
As requested, we have added the complete VGP library to the tested lectins by uploading a new Figure 2.

B. Fig. 3- Can you provide rationale for why 20 ug/ml viral glycoproteins and 20 hours stimulation were chosen? The data would be strengthened by testing 2 or 3 concentrations of viral glycoproteins to show concentration dependence, especially given the large error bars. Were any statistical calculations performed on the data for significance?

We thank the reviewer for these important remarks. The 20 ug/mL and the incubation time have been selected due to previous experiments with similar molecules. We have added in the supporting information a titration of seven different concentration of the VGP (Figure S2). The incubation time was still the same (20h) due to our experience with these APC and their interactions with glycosylated macromolecules. We have also added the requested full statistical analysis by uploading a new Figure 3.

C. Fig. 4- Can you provide rationale for why 20 ug/ml viral glycoproteins and 20 hours stimulation were chosen? The data would be strengthened by testing 2 or 3 concentrations of viral glycoproteins. Were any statistical calculations performed on the data for significance?

We have added the requested full statistical analysis uploading a new Figure 4.

D. Discussion- Any further discussion on why or what could be the advantage for the stimulation of both a pro- and an anti-inflammatory cytokine simultaneously? Are there other pathogens/viruses that are capable of this?

We thank the reviewer for this comment. In the context of several host-pathogens interactions it is not clear and obvious to classify cytokines as pro or anti-inflammatory. In general, for most of the TLR ligands and also other PRR, we first have an "inflammatory" signaling that is immediately balanced with a "non-inflammatory" signaling. This is why, for example, the well-known LPS from several gram-negative bacteria, is able to trigger the simultaneous secretion of IL-6 and IL-10 in different APC. So, yes, there are several bacteria able to do this. In addition, the role of lectins in balancing TLR responses has been described. Therefore, it is very important to determine both IL-6 (inflammation) and IL-10 (non-inflammation) cytokines. Our results show that the viral lectins do not induce an unbalanced dendritic cell/macrophage response, at least not initially. We have discussed this point adding this sentence (and references) in the manuscript: *our results are in line with other studies that demonstrated a balance between different PRRs during host-pathogens interactions.*

E. Methods- It is not clear if either of the anti-IgG detection antibodies are specific for only IgG, or could cross react with IgM or other antibody types? Please provide clarification.

We thank the reviewer for this comment. We have used a specific anti-human IgG antibody in this solid-phase assay, as described in the experimental part, so the binding we see is due to the IgG in plasma. For sure, if we would have tested an anti IgM antibody we would have seen a glycan-dependent binding also for the IgM.

F. General- I do not see a 'data availability' statement in the paper itself, and the Reporting summary says 'n/a' for antibodies and cell lines, however both are used in the paper.

We have added these information uploading a new "data availability" in the editorial system.

Reviewer #3 (Remarks to the Author):

The paper entitled "Carbohydrate-Mediated Interactions Between Chloroviruses and the Immune System" submitted by Chiodo and de Castro explores how the unique N-glycosylation patterns of chloroviruses is recognized by the innate and adaptive host immune systems.
Chloroviruses in contrast to other viruses such as HIV and SARS-Cov2, express unique N-glycosylation pattern that do not resemble the host glycosylation. In initial ELISA

experiments, they showed differential interaction of C type lectins evidencing the interaction of chloroviruses with these innate receptors. Experiments with antigen presenting cells from human and mouse origin are employed to study the IL-6 and IL 10 secretion and finally, IgG profiling in healthy individuals is studied.
The experiments are well performed, and the paper is clear and well written with discussion/conclusions supported by the experimental data, therefore I suggest the publication after minor corrections.

Minor comments/corrections for the authors:
In line 58, please complete/correct the sentence: These responses are typical for viral infection of, or interaction with macrophage cells.

We thank the reviewer. A complete sentence has been added to the manuscript: *These responses are typical for viral infection during the interaction with macrophage cells*

In line 192, please include a more extensive discussion of the interaction with plant lectins.

We thank the referee for this remark, we have added this sentence to better explain the plant lectins experiments: *the selected plant lectins are able to recognize Gal and/or GalNAc-containing glyco-conjugates. In Fig. 2B the accessibility of Galactose for the plant lectins has confirmed not only the effectiveness of the ELISA-coating strategy but also the glycans-accessibility to lectins in these experimental conditions.*

The authors only see these experiments to confirm the immobilization of the glycoproteins on the plates but I think that these ELISA-like experiments with plant lectins could provide new information about the binding specificity of these reagents widely employed in glycobiology research as in general, their binding profiles are described with mammalian like N-glycosylation e. g. CFG glycan microarray data. A more complete study of the chloroviral glycoproteins interactions with different plant lectins (out of the scope of this paper) may also allow identification of plant lectins with potential for the isolation/identification/characterization of these viruses.

We thank the reviewer ~~to~~ for this important and key suggestion. We will soon start to test different plant lectins as suggested, not only for technical reasons but - as mentioned - also to better understand the binding profiles of these rare viral glycoproteins.

In the assay, only galactose/galactosamine recognizing lectins are included, is there a reason for that?

Due to the constant presence of Galactose in a position that allows this monosaccharide to be hindered or not by the other closer monosaccharides (especially in the mutants), we have selected Galactose-binding lectins. We agree with the referee that a more complete array of plant lectins should be done in the future.

In Figure 3 and 4, it would be nice to include the statistical significance of the data (as it is included in Figure 5)

We added the requested analysis

In the experimental part, in lectin assays, please include how plant lectins are detected biotin-Streptavidin HRP?

We thank the reviewer for this remark. We have added the extra information in the experimental section. Yes, the tested plant-lectins were biotinylated and detected with Streptavidin-HRP.