nature portfolio

Peer Review File

The Cryo-EM structure of human CD163 bound to haptoglobin-hemoglobin reveals molecular mechanisms of hemoglobin scavenging.

Corresponding Author: Professor Christian Andersen

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 the manuscript “The structure of human scavenger receptor CD163 in complex with haptoglobin-hemoglobin discloses the molecular mechanism for hemoglobin scavenging.”, the authors explore the interaction between CD163 receptor and the haptoglobin:hemoglobin (HpHb) complex. They combine structural (cryo-EM SPA), biochemical (SEC-MALS and SPR) and biomolecular (flow cytometry) techniques to characterize the key determinants and the role of a never reported ternary complex composed of HpHb and a trimer of the CD163 receptor. The biological role of the receptor has been thoroughly studied over the years (Van Gorp H et al, doi:10.1016/j.molimm.2010.02.008), however the results presented in this study aim at filling the gap in our understating of the fine structural details on how the machinery works. Indeed, the obtained cryo-EM maps, which have an acceptable resolution, allow to clearly fit the structure of CD163 receptor in complex with HpHb. Interestingly, the authors presented a model where receptor trimerization, which is regulated by the presence of calcium, is a reversible key step that initially modulates HpHb scavenging and internalization, and then its release within microsomes, allowing for the receptor regeneration. The authors were able to identify calcium ions within some of the expected binding sites and reveal how calcium mediates CD163 oligomerization and HpHb recognition. They selected key residues involved in CD163 receptor oligomerization and produced variants where these residues were mutated to alanine with the aim of confirming their role. These variants were tested both in vitro and in cellulo and mostly supported the authors’ conclusions. The results reported in the paper are original and novel; therefore, this reviewer believes that the structural and mechanistic information provided offers important insights on the process of HpHb internalization by CD163 that, importantly, might be extended to other members of the SRCR protein superfamily, thus making the manuscript attractive for a more general audience. However, before this work can be considered acceptable for publication, the authors need to (i) improve the description of the methods used, (ii) revise the existing figures and add new ones, and (iii) further discuss the differences and roles attributed to the two different oligomeric forms of the CD163 receptor that they have identified.

Major points:

Role of calcium. One of the main findings of the paper is the disclosure of the structural basis for the well-assessed dependence of the CD163 function on calcium. To well promote this finding the authors should clarify some aspects:

How does the calcium concentration used in the experiments (5 mM) compare to the physiological one?

Lines 164-177: whether the mutagenesis is hampering oligomerization due to the loss of (i) calcium coordination or (ii) electrostatic interactions remains elusive, mainly because the mutated residues could also directly form stabilizing salt bridges with the other subunit. The authors are suggesting that the Lys substitution by Ala interfere with the correct formation of a calcium binding site, but without never explicitly stating it. Also, it is not clear from Experimental Procedures section whether the SEC-MALS experiment in Figure 3D was performed in the presence or in the absence of calcium. The reviewer suggests testing both these conditions to assess the relevance of calcium in oligomer stabilization in this mutant. Moreover, a dependence of oligomerization of this variant on calcium concentration might be helpful to understand if the effect of the ion is completely abrogated or only reduced.

Lines 108-111: it would be helpful if the authors might provide an explanation of why the calcium ions are missing in some of the expected binding sites.

Cryo-EM. The structures presented have a decent resolution, albeit not as high as one would expect based on the MW of the complexes examined. Some technical insight might be helpful for the reader to better appreciate the limits and the assets of the work

The authors should explain why they utilized a 1:2 stoichiometry to prepare the CD163 SRCR 1-9 complex with HpHb.

The authors identified a mixture of dimers and trimers of the receptor on the grids. Based on SEC-MALS analysis in figure 6A did they expect such heterogeneity? Have they tried higher calcium concentrations to better stabilize the trimer?

A flow chart of SPA is missing and it would really help the reader in understanding how the analysis has been performed. Authors should add the number of (i) recorded micrographs, (ii) picked particles and (iii) particles used for each map.

It is not clear why the complex where CD163 is a dimer (smaller complex) has higher resolution compared to the one where CD163 is a trimer (bigger complex). Indeed, bigger specimens are expected to reach higher resolution in cryo-EM. It is because of the number of pcs in each map? Is the complex with CD163 in the dimer form more stable? Authors should better explain this point.

With reference to the map at higher resolution, the reviewer would like to be reassured on the feasibility of defining residues' side chains position based on the map density. The authors never show zoomed views of the fitted model inside the map. This would be helpful for supporting:

Lines 127-129: the authors refer to a disulphide bond forming between two Cys residues of SCRC6-7 domains which stabilizes a flexible segment. Is this information from the literature, in this case the reference is missing, or is the map describing this bond?

All the "CD163-HpHb interaction" chapter would benefit from a more detailed depiction of residues' side chains and their fitting into the cryo-EM map.

Why did the authors impose C2 symmetry while refining their best map? A figure showing the symmetry axes in model where the refinement was performed without symmetry might help the reader to understand the rationale of this choice. At the same time, the authors need to further discuss line 160-162.

Lines 121-124: the authors report an interesting "rigid two-domain architecture" that might have relevance in the understanding of the mechanistic behaviour of CD163 binding. However, this evidence needs further discussion and a figure to support the finding.

Lines 129-131: a figure in support of the statement "A recent crystal structure of porcine CD163 SRCR5-9 (RCSB ID 8H7J), corroborates the configuration of CD163 SRCR5-9 observed in the cryo-EM structure" might be helpful

The chapter "Structure of human Hp" should be moved to supplementary information to improve readability, since the structure of isolated Hp has been determined only with the purpose of assessing a potential Hb-induced conformational change that eventually was not observed.

SEC-MALS. The reviewer suggests moving the presentation of the SEC-MALS results before the section dedicated to cryo-EM. This would create a smoother logical flow, where (i) the activity of calcium in receptor oligomerization is demonstrated using a standard biochemical technique, and (ii) the high-resolution investigation of the ternary complex between the CD163 receptor and HpHb follows. Moreover, the SEC-MALS analysis could benefit from revising the following parts:

Fig 3D: the reviewer would like to point out that SEC and SEC-MALS experiments are not directly comparable. The authors should better explain the experimental procedure (FPLC system, SEC column, size of the loop in the Experimental Procedures section).

Fig 6A needs further discussion to explain the increasing trend of estimated MW of CD163 receptor in the presence of calcium. Are the authors expecting heterogeneity under this condition? Did they consider the possibility that the measurement was performed at a calcium concentration close to the dissociation constant of the observed oligomer? Did they try a concentration series to check if the MW estimation was changing?

Lines 252-254: the authors should better clarify the meaning of "with a molecular weight average of the two components individually" later stating that "the components co-elute but do not form complexes". Which is the expected average MW? The molar mass (and the elution volume) looks quite similar to the one calculated for the second peak in the top-left panel of figure 6B. However, the authors refer to this latter peak as "it indicates the presence of smaller complexes". Also, the authors should comment a bit more on the results obtained with CD163 SRCR1-5 in complex with HpHb.

Cellular up-take of HpHb and Hb (lines 269-271): the authors explain the residual ability of cells expressing the trimer-destabilizing substitutions to internalize Hb by the ability of monomeric CD163 to perform Hb uptake. Why did the authors

discard the simpler hypothesis that the small residual fraction of dimeric/trimeric CD163 (Figure 3D) is responsible for the uptake? SPR experiments assessing affinity for either HpHb or Hb alone of CD163 SRCR1-5 (which is expected to be monomeric) would help understanding how oligomerization of the receptor is improving affinity for its targets and if internalization by a monomeric receptor is indeed possible.

The chapter “Quantification of the CD163 affinity for HpHb and Hb” did not convince the reviewer that the CD163 trimer is the relevant species over the dimer. Could the authors further discuss their statement?

Minor points

Lines 46-48: please clarify to what extent the Hp genotype (either Hp2-2 or Hp1-1) does not affect the efficiency of Hb scavenging. Dealing with this point: why have the authors chosen the Hp1-1 phenotype?

Using two different colors for αHb and βHb would improve the clarity of all the figures in the manuscript.

Abstract should not have references. Please remove ref.1

Change colors in figure 7: orange and red are too similar and difficult to discriminate

Line 141: Supplementary Fig. 3 is missing in the submitted files. In the following sentence, it is likely that the cited Figure is 2B and not 2D.

Line 157, authors are most likely mentioning Figure 2A and not 3A

Line 181, the text is describing Figure 4A+B+C and not 4A+B. Moreover, the reviewer suggests changing “AB region” with “AB loop” because it is more adherent to the classical nomenclature of Hb.

Line 213, the statement “data not shown” is against the Nature Communication editorial policies. Moreover, the SPR experiment with CD163 and Hp would be of interest for the discussion in the paper, and it should be added as supplemental material.

Lines 218-219, it is not clear if the absence of CD163 binding by Hp is known from literature or experimentally determined in the present work.

Sentence in lines 321-322 is not clear and needs more details.

Figure 7: please specify how many replicates were averaged and the meaning of error bars (standard deviation? Standard error?)

As per Nature Portfolio policy, “Official validation reports from the wwPDB are required for peer review” and “Accessibility in repositories must be designated for immediate release on publication.". The reviewer suggests adding the accession numbers in the Data availability session.

A scheme of the Hp1-1 structure would be beneficial, especially because 3D reconstructions of the ternary complex show only the Hp domain involved in Hb binding (HpSP), without resolution of the rest of the structure (HpCCP).

Reviewer #2

(Remarks to the Author)
Etzerodt A et al. The structure of human scavenger receptor CD163 in complex with haptoglobin-hemoglobin discloses the molecular mechanism for hemoglobin scavenging.

The present study offers new insight into the structural features of CD163-HbHp complex formation based on cryo-EM, in-silico modeling, and experimental analysis that includes mutant construct receptor component expression, SEC-MALLS, binding studies using SPR and flow cytometry. Their study provides a strong balance of techniques to reach a plausible conclusion for Hp1-1 binding to CD163. The binding of calcium ions to CD163 is crucial not only for ligand binding but also for receptor oligomerization. The authors have also made an important observation regarding the role of Ca2+ ions in the process of binding. Overall, the manuscript results support the authors conclusions, and the work advances an understanding of the Hb, Hp, CD163 binding paradigm.

Comments:

1. Do glycosylation differences in Hp1-1, 2-1 and 2-2 affect CD163 binding in this model? Some further insight into the differences in Hp2 phenotypes and their interactions with CD163 could be of interest.
2. The absence of the CD163 SRCR1 domain in the authors models may be an important limitation. The authors should better explain how the model is affected by this omission.
3. The authors discuss the binding of Hb in the absence of Hp and the potential relevance of Hb dimerization. It would be useful to provide modeling data on the concentration dependent binding of Hb tetramer and dimer to CD163 in the absence of Hp. This data will help with understanding the low-level clearance of Hb in absence of Hp during disease and the Hp null

genotype.
4. The discussion becomes speculative in areas. For example, “The present structural data may also provide insight into the uncoupling of CD163 and HpHb in the endosomes, where calcium is gradually pumped. This loss of Ca2+ may promote the release of HpHb from CD163 by a combined process of distinct mechanisms.”

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The reviewer acknowledges the considerable effort made by the authors in addressing the reviewers' suggestions, as well as their care in rewriting the manuscript and preparing more effective and clearer figures. These efforts have resulted in an improved manuscript that addresses many of the concerns previously raised by the reviewer. However, there are still some major issues that prevent the paper from being considered acceptable in its current form. Additionally, some results could benefit from a more thorough discussion to enhance their clarity and draw more substantiated conclusions. The reviewer’s concerns involve mainly the physiological relevance of the trimeric assembly for the receptor’s function and the role of calcium, plus different scattered issues/observations.
One of the main findings of the work is the identification of a calcium-dependent oligomerization of CD163 in both the absence and presence of ligands that should have relevance in ligand binding and internalization by this receptor. The oligomers formed are likely a mixture of dimers and trimers in equilibrium with the monomeric species as clearly indicated by SEC-MALS experiments and by the heterogeneity of the particles in cryo-EM, where both dimeric and trimeric CD163 is seen bound to Hp/Hb. These findings might be suggestive of the existence of this assembly on the cell’s membrane, however there is not direct proof of this, and the authors seem to neglect the possibility that the dimeric species is as efficient as the trimer and might be relevant for Hb acquisition. The authors state that the trimeric assembly is responsible for the high-avidity binding of Hp/Hb to the receptor, although the cryo-EM structure only allows to spot stable interactions between one subunit of CD163 and Hp/Hb, while interactions with the two remaining subunits are openly defined by the authors as weaker (“suggesting that these domains are not or only loosely associated with HpHb”). The determination of the binding affinity for the trimer vs monomer is performed by SPR on an engineered receptor where the trimer is stabilized by the addition of a coiled coil that might, in principle, lead to an overestimation of the affinity by reducing the conformational dynamics of the protein. Considering all the above-mentioned points, the authors should avoid overly stress that i. the physiological assembly on the cell membrane of the receptor is trimeric, and ii. trimeric CD163 is more relevant for Hb/Hp internalization due to an avidity effect of the interactions established by the three subunits with the ligand. The authors should clearly state the limits of their findings, mentioning alternative interpretations of their data to warrant the readers’ critical view of this system.
Moving to the second major point (e.g. the role of calcium in oligomerization), the main issue is related to the lack of any structural direct evidence of the molecular mechanism by which calcium is responsible of favouring CD163 quaternary assembly and its interaction with Hp/Hb. In many instances the authors seem to suggest a mechanism that involves a lysine residue, but they never come to a clear conclusion. The reviewer suggestion is to make the presentation of the data more schematic and to clearly state which are the hypothesis about the molecular mechanism. More specific suggestions are detailed below. The reviewer advises limiting speculative discussions (e.g. lines 367-369 and 377-378) about the oligomerization of proteins sharing similar tertiary structures, as the calcium-dependent oligomerization mechanism for CD163 has yet to be fully explained.
Point-by-point observations:
1) Line 22: only the trimer is mentioned in the abstract. Reference to the existence of dimeric species should be added
2) Lines 46-48: to reviewer’s knowledge the Hp isoform influences the efficiency of Hb removal from the plasma, with the Hp2-2 phenotype being responsible for a less efficient scavenging and thus to a phenotype more prone to Hb-related oxidative stress. Please clarify this point. See also lines 267-270 in this manuscript.
3) Lines 65-71: the paragraph requires revision for more clarity
4) Lines 79-80: data presented do not allow to discard the possibility that, using the appropriate stoichiometric ratio, a Hp/Hb dimer bound to two CD163 trimers might be observed. This sentence should be removed from the introductory paragraph or modified.
5) Section “Analysis of CD163 oligomerization using multi-angle light scattering”: supplementary Figure 1 demonstrates that Ca2+ concentrations higher than 2 mM do not further affect oligomerization. However, the MALS results indicate a maximum MW of 200 kDa, which is substantially lower than the one shown in Figure 1A. The authors should provide a clear explanation for this discrepancy. As a minor point the reviewer suggests modifying Figure 1 and to overlay the chromatograms in the absence and presence of calcium on a single figure. This would allow to better appreciate the effect of calcium on the oligomerization/complex formation.
6) Lines 98-100: SEC-MALS analysis (Figure 1C) does not allow for a definitive determination of the oligomeric state and the stoichiometry of the ternary complex between Hb, Hp, and the CD163 receptor. Indeed, could the estimated MW also

correspond to two CD163 dimers bound to both sides of HpHb complex? To definitively clarify how the CD163 receptor binds HpHb, it is necessary to run a sample without stoichiometric excesses, where a single sharp peak is present in the chromatogram. Also, the use of the right stoichiometry might have allowed the stabilization of the complex and, possibly, a reduction of the conformational flexibility ultimately leading to an increase in the structure resolution and to the determination of the missing part of the Hp/Hb structure. Furthermore, as stated at lines 268-270, the ability of a Hp/Hb dimer to interact with more than one CD163 might have physiological relevance. More in general, the reviewer suggests adding a table (or a scheme) where the expected/theoretical MWs are indicated for each potential assembly of the single proteins and of the complex. This would facilitate the critical reading of the manuscript and the assessment of the correctness of the conclusions drawn from SEC-MALS experiments.

7) Lines 105-106: please specify the meaning of “1:1 stoichiometry”. The MW estimated by MALS is below 200 kDa while the theoretical MW should be 270 kDa, if one CD163 binds to 1 Hp/Hb (not a Hp/Hb dimer).
8) 108-110: please add a reference for this statement
9) Section “Cryo-EM structure determination of CD163 SRCR1-9-HpHb”: Supplementary Figure 2. It is necessary to add (i) an image of a micrograph and (ii) a panel of the selected 2D classes to proceed with the 3D analysis. The addition of these two images will allow an expert reader of the technique to dispel any doubt regarding the presence or absence of the entire deposited complex. Indeed, all the refined maps lack a significant region of the complex (the two alpha chains and one beta chain of Hp, and a dimer of Hb).
10) Line 135: please provide a detailed explanation on how the AlphaFold model was fitted into the density. Was the monomer used or a generated trimeric assembly? Since AlphaFold3 has improved functions, like prediction of quaternary structures and of metal binding, authors might consider using a newly generated model to fit cryo-EM data.
11) Line 155: SRCR 1-4 should be changed in SRCR 2-4 since domain 1 is not visible.
12) Line 162: specify that domain 3 is involved in ligand binding
13) Paragraph lines 168-175: the part on the disulfide bridge is not clear. How was the bridge identified: was it in the density of the 3.8 Å map or the AlphaFold prediction?
14) Paragraph lines 177-187: the order in which data are presented and discussed generates confusion. A suggestion is to start with the identification of the potential calcium binding sites based on homology, state that resolution does not allow to solve the sites in domains 6, 7 and 8, state that calcium is defined in domains 2 and 3 in two out of three sites. After this introduction, the sites and interaction with the ion can be described.
15) Lines 191-192: add reference to figure S4
16) Role of calcium (Section line 198, section line 275, and discussion): the authors have only partially addressed the reviewer's concerns. The SEC-MALS analysis of the receptor variant in the presence and absence of Ca^{2+} was performed as requested; however, the experimental design does not allow for a reasonably solid conclusion to be drawn. The reviewer believes that a greater effort is needed in analysing the role of the identified Lys residues and the effect of their substitution with alanine, as a significant portion of the subsequent experiments and discussion is based on these points. What do the authors believe is the cause of the abrogation of oligomerization of the variant used? Mutated lysines are forming electrostatic interaction with residues forming a predicted calcium-binding site; however, the oligomerization of the K811A/K1021A mutant appears to be independent from calcium (Supplementary Figure 5). The authors should clarify whether they believe that the lack of oligomerization is due to (1) a reduced affinity between protomers caused by the loss of the electrostatic interactions mentioned in the text, in which case they should clarify the role of calcium accordingly, or (2) a reduced affinity for calcium itself. Also consider that calcium coordination requires the formation of eight bonds (https://doi.org/10.1016/j.bbamem.2012.11.025), a property that questions how this is possible if the identified lysines form electrostatic interactions with the Asp and Glu residues involved in metal coordination. A similar pattern of interactions seems to happen between site 2 on SRCR2 and a Lys residue of Hp (Fig. 5A, lines 299-300 and lines 310-311 of the discussion), thus the authors should include an explanation for this behaviour as well.
17) Lines 213-214: The sentence “This pattern of interaction in repeating in the three subunits, resulting in the formation of the triangular base.” should be revised to improve the clarity.
18) Lines 216-219 and Figure 4C: the text states that K811 and K1021 form electrostatic interactions with D746 and D956, respectively. However, Figure 4C shows interactions also with E812 and E1022. The authors should solve this inconsistency.
19) Lines 222-223: in figure 4 no oligomeric species are visible in the SEC-MALS analysis.
20) Section “CD163-HpHb interaction”: how the trimeric assembly should stabilize Hb/Hp binding is not convincingly stated. The interactions with subunits 2 and 3 are defined as loose and identified interactions involve structure with an inadequate resolution. Maybe a comparison between the dimeric and trimeric assembly with respect to Hp/Hb binding could be useful.
21) Line 257: do the authors have an explanation on why the engineered protein has a MW higher than those observed with the wt trimeric assembly?
22) SPR: please add errors for the Kd values
23) Lines 265-267: please add a comment on whether the loose interactions detected between subunit 2/subunit 3 of CD163 and Hp/Hb are compatible with a 70-fold increase in ligand binding affinity of the trimer with respect to the monomer
24) Section “Cellular uptake of HpHb and Hb”: which are the concentrations of Hp/Hb used for this experiment? How do they compare with the physiological ones? Should we discard the hypothesis that the more efficient uptake mediated by the trimer is observed in the presence of non-physiological ligand concentrations? In the same vein: do the authors discard the hypothesis that the dimeric form contributes to efficient internalization? Did they try to express the engineered CD163-CC receptor in the cell line used for internalization experiments?
25) Lines 358-359 require revision for more clarity
26) The final discussion (lines 410-418) would benefit from an introductory section on the already known role of the soluble form of the CD163 receptor, as well as a revision of the speculations made. This is relevant because the work presented in the current manuscript does not determine affinity constants for receptor oligomerization, neither in its soluble form nor in its membrane-bound form.

Reviewer #2

(Remarks to the Author)
The authors have addressed my comments

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised version of the manuscript “The structure of human CD163 bound to haptoglobin-hemoglobin discloses the molecular mechanism for hemoglobin scavenging” by Andersen and colleagues has now improved to the extent that it might be suitable for publication in Nature Communications.
I still have some suggestions, especially aimed at enhancing the reading experience also for non-expert readers.

Major points:
- The role of calcium in mediating receptor trimerization and ligand binding is strongly emphasized by the authors, who take for granted that most of the readers are aware of the structural role played by calcium in this type of interactions. In my opinion, the authors should stress at the very beginning what they believe is the mechanism by which a positively charged ion might bridge the ion interaction between a positively (i.e. Lys/Arg) and negatively (Asp/Glu) residues. They postpone this information to the discussion section (lines 315-319), making difficult for the general reader to understand the results (section “CD163 1-9 oligomerizes via SRCR7 and SRCR9 interaction“).
- In the discussion the narrative flow might be improved, with special focus on the role of calcium on receptor oligomerization and in ligand binding. In the present version the authors mix comments on either one aspect or the other, hindering an easier, more direct interpretation of data. I suggest the authors to start the discussion, after the introductory paragraph, with a section on the oligomerization aspects and then move to the role of calcium in ligand recognition. For example, the sentence about the role of lysines, that is deeply intertwined with the role of calcium is postponed to line 386-394, while it would be more logical if moved when the mechanism of calcium-mediated Asp-Lys interaction is explained.
- In general, the discussion is very long. Once the authors have rearranged the sections, they may want to move the text that refers to figures 8 and 9 in the supplemental material

Minor points:
- The reviewer suggests moving the text in lines 173-180 ("CD163 SRCR5-9 are organized into …") to an earlier section, specifically where the lack of density between SRCR6 and SRCR7 is discussed (line 143). This change would clarify that cryo-EM does not directly resolve this region and that the integration of AlphaFold2 predictions and existing literature addresses the issue.
- Supplementary Figure 7 will be more informative if CD163 protomer are colored differently.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In the manuscript "The structure of human scavenger receptor CD163 in complex with haptoglobin-hemoglobin discloses the molecular mechanism for hemoglobin scavenging.", the authors explore the interaction between CD163 receptor and the haptoglobin:hemoglobin (HpHb) complex. They combine structural (cryo-EM SPA), biochemical (SEC-MALS and SPR) and biomolecular (flow cytometry) techniques to characterize the key determinants and the role of a never reported ternary complex composed of HpHb and a trimer of the CD163 receptor. The biological role of the receptor has been thoroughly studied over the years (Van Gorp H et al, doi:10.1016/j.molimm.2010.02.008), however the results presented in this study aim at filling the gap in our understating of the fine structural details on how the machinery works. Indeed, the obtained cryo-EM maps, which have an acceptable resolution, allow to clearly fit the structure of CD163 receptor in complex with HpHb. Interestingly, the authors presented a model where receptor trimerization, which is regulated by the presence of calcium, is a reversible key step that initially modulates HpHb scavenging and internalization, and then its release within microsomes, allowing for the receptor regeneration. The authors were able to identify calcium ions within some of the expected binding sites and reveal how calcium mediates CD163 oligomerization and HpHb recognition. They selected key residues involved in CD163 receptor oligomerization and produced variants where these residues were mutated to alanine with the aim of confirming their role. These variants were tested both in vitro and in cellulo and mostly supported the authors' conclusions. The results reported in the paper are original and novel; therefore, this reviewer believes that the structural and mechanistic information provided offers important insights on the process of HpHb internalization by CD163 that, importantly, might be extended to other members of the SRCR protein superfamily, thus making the manuscript attractive for a more general audience. However, before this work can be considered acceptable for publication, the authors need to (i) improve the description of the methods used, (ii) revise the existing figures and add new ones, and (iii) further discuss the differences and roles attributed to the two different oligomeric forms of the CD163 receptor that they have identified.

We thank the reviewer for comments and suggestions. Below is a point-by-point reply to the points raised.

Major points:

Role of calcium. One of the main findings of the paper is the disclosure of the structural basis for the well-assessed dependence of the CD163 function on calcium. To well promote this finding the authors should clarify some aspects:

How does the calcium concentration used in the experiments (5 mM) compare to the physiological one?

The physiological calcium concentration is 1-2 mM and 2 mM Ca^{2+} is sufficient for full binding to HpHb (reference 18). This is clarified in the revised manuscript on line 108-109. We chose a slightly hyperphysiological concentration at 5 mM Ca^{2+} and we have now included an additional experiment quantifying CD163 oligomerization at different calcium concentrations (Supplementary figure 1) showing that an even higher Ca^{2+} concentration do not further affect oligomerization.

Lines 164-177: whether the mutagenesis is hampering oligomerization due to the loss of (i) calcium coordination or (ii) electrostatic interactions remains elusive, mainly because the mutated residues could also directly form stabilizing salt bridges with the other subunit. The authors are suggesting that the Lys substitution by Ala interfere with the correct formation of a calcium binding site, but without never explicitly stating it. Also, it is not clear from Experimental Procedures section whether the SEC-MALS experiment in Figure 3D was performed in the presence or in the absence of calcium. The reviewer suggests testing both these conditions to assess the relevance of calcium in oligomer stabilization in this mutant. Moreover, a dependence of oligomerization of this variant on calcium concentration might be helpful to understand if the effect of the ion is completely abrogated or only reduced.

As suggested by the reviewer we have now analysed CD163 K811A/K1021A in the pressence of Ca^{2+} and in the pressence of EDTA. This showed that the observed high-molecular weight peak observed in the original manuscript was not calcium dependent and it therefore likely stems from an unspecific aggregation of CD163. Futher purification revealed that the introduction of K811A and K1021 mutations completely abolishes CD163 oligomerization (Figure 4d). Addition of EDTA does not appear to affect the stability of CD163 K811A/K1021A in size exclusion (Supplementary figure).

Lines 108-111: it would be helpful if the authors might provide an explanation of why the calcium ions are missing in some of the expected binding sites.

Positions of potentially bound calcium ions at CD163 SRCR5-9 cannot be unambiguous determined due to the low resolution of the cryo-EM maps covering this region. This is described on line 304-306 in the revised manuscript.

However, the lack of density at site 1 in both SRCR2 and SRCR3 was completely unexpected. In the revised manuscript, we have now included a table of SRCR structures in the Protein Data Bank and listed calcium site occupance and the content of calcium and magnesium in the crystallization conditions. This table shows that the intact canonical sites are usually occupied when calcium is present in the crystallization conditions. The discussion of calcium site occupancy has been elaborated at line 304-311 in the revised mannuscript.

Cryo-EM. The structures presented have a decent resolution, albeit not as high as one would expect based on the MW of the complexes examined. Some technical insight might be helpful for the reader to better appreciate the limits and the assets of the work

We have added a supplementary figure showing the cryoEM data processing (Supplementary Figure 2) and descibe potential reasons for the limited resolution of the reconstructions (line 127-133 in the revised manuscript).

The authors should explain why they utilized a 1:2 stoichiometry to prepare the CD163 SRCR 1-9 complex with HpHb.

HpHb was added in excess for saturation of the complex as free HpHb could be separated from CD163-HpHb using size-exclusion chromtography. This is now explained in line 525 in the revised manuscript.

The authors identified a mixture of dimers and trimers of the receptor on the grids. Based on SEC-MALS analysis in figure 6A did they expect such heterogeneity? Have they tried higher calcium concentrations to better stabilize the trimer?

The heterogeneity of the sample was expected based on the SEC-MALS analysis. We have added an experiment showing the oligomerization at different calcium concentrations (Supplementary figure 1). Here, already from 2 mM calcium we observe maximal formation of the CD163 oligomer. Hence, increasing the calcium concentration beyond 5 mM will most likely not result in stabilization of the trimer. This experiment is described in line 111-114 in the revised manuscript.

A flow chart of SPA is missing and it would really help the reader in understanding how the analysis has been performed. Authors should add the number of (i) recorded micrographs, (ii) picked particles and (iii) particles used for each map.

As suggested by the reviewer, a flow chart of the SPA has been added to the revised manuscript (Supplementary Figure 2).

It is not clear why the complex where CD163 is a dimer (smaller complex) has higher resolution compared to the one where CD163 is a trimer (bigger complex). Indeed, bigger specimens are expected to reach higher resolution in cryo-EM. It is because of the number of pcs in each map? Is the complex with CD163 in the dimer form more stable? Authors should better explain this point.

It is not evident from the data why the dimeric complex refines to a higher resolution than the trimeric complex. A possible explanation is that CD163 is highly flexible, while the ligand (HpHb) is stable. Consequently, the more subunits of CD163 is included in the refinement the lower the resolution. This is now explained in line 130-133 in the revised manuscript.

With reference to the map at higher resolution, the reviewer would like to be reassured on the feasibility of defining residues' side chains position based on the map density. The authors never show zoomed views of the fitted model inside the map. This would be helpful for supporting:

Lines 127-129: the authors refer to a disulphide bond forming between two Cys residues of SCRC6-7 domains which stabilizes a flexible segment. Is this information from the literature, in this case the reference is missing, or is the map describing this bond?

The disulfide bridge was identified in the crystal structure of porcine CD163 SRCR5-9 (RSCB ID 8H7J) and is also formed in Alphafold predictions of human CD163. We have added a figure (Figure 3c) showing a superimposition of human CD163 SRCR5-9 in the crystal structure of porcine CD163 SRCR5-9.

All the "CD163-HpHb interaction" chapter would benefit from a more detailed depiction of residues' side chains and their fitting into the cryo-EM map.

The resolution of the cryoEM maps are 3.8 Å, 4.5 Å and 5.2 Å. Therefore, the position of side chains should be interpreted with caution. An improved Figure 5 showing the densities has been included in the revised manuscript. Furthermore, the resolution of the individualy maps are shown in the figure for clearity. Also, this limitation is described on line 240-242 in the revised manuscript.

Why did the authors impose C2 symmetry while refining their best map? A figure showing the symmetry axes in model where the refinement was performed without symmetry might help the reader to understand the rationale of this choice. At the same time, the authors need to further discuss line 160-162.

We are unsure how the reviewer concluded that C2 symmetry was applied during the refinement. The map was not refined using C2 or C3 symmetry. A extended discussion of the lack of symmertry of CD163 homotrimer has been included on line 202-209 in the revised manuscript.

Lines 121-124: the authors report an interesting "rigid two-domain architecture" that might have relevance in the understanding of the mechanistic behaviour of CD163 binding. However, this evidence needs further discussion and a figure to support the finding.

A figure showing a close up of structure of CD163 SRCR3-4 has been added in the revised manuscript (Figure 3b). A further discussion of this phenomenon has also been added in line 162-166 in the revised manuscipt.

Lines 129-131: a figure in support of the statement "A recent crystal structure of porcine CD163 SRCR5-9 (RCSB ID 8H7J), corroborates the configuration of CD163 SRCR5-9 observed in the cryo-EM structure" might be helpful

As described above, a figure (Figure 3c) showing a superimposition of human CD163 SRCR5-9 on the crystal structure of porcine CD163 SRCR5-9 (8H7J) has been included in the revised manuscript.

The chapter “Structure of human Hp” should be moved to supplementary information to improve readability, since the structure of isolated Hp has been determined only with the purpose of assessing a potential Hb-induced conformational change that eventually was not observed.

We agree with the reviewer and have moved the figure showing the structure of Hp to the supplement and have moved this section to the Discussion in the revised manuscript (line 400-408).

SEC-MALS. The reviewer suggests moving the presentation of the SEC-MALS results before the section dedicated to cryo-EM. This would create a smoother logical flow, where (i) the activity of calcium in receptor oligomerization is demonstrated using a standard biochemical technique, and (ii) the high-resolution investigation of the ternary complex between the CD163 receptor and HpHb follows. Moreover, the SEC-MALS analysis could benefit from revising the following parts:

We agree with the reviewer and the section describing the SEC-MALS has been moved in the revised manuscript.

Fig 3D: the reviewer would like to point out that SEC and SEC-MALS experiments are not directly comparable. The authors should better explain the experimental procedure (FPLC system, SEC column, size of the loop in the Experimental Procedures section).

The technical details on SEC-MALS have been added to the methods section as suggested by the reviewer.

Fig 6A needs further discussion to explain the increasing trend of estimated MW of CD163 receptor in the presence of calcium. Are the authors expecting heterogeneity under this condition? Did they consider the possibility that the measurement was performed at a calcium concentration close to the dissociation constant of the observed oligomer? Did they try a concentration series to check if the MW estimation was changing?

As described above, we have included an experiment showing the CD163 oligomerization at different calcium concentrations. Here 2 mM Ca^{2+} is sufficient for oligomerization and further addition of Ca^{2+} did not increase oligomerization. This experiment is descibed on line 111-114 in the revised manuscript.

Lines 252-254: the authors should better clarify the meaning of “with a molecular weight average of the two components individually” later stating that “the components co-elute but do not form complexes”. Which is the expected average MW? The molar mass (and the elution volume) looks quite similar to the one calculated for the second peak in the top-left panel of figure 6B. However, the authors refer to this latter peak as “it indicates the presence of smaller complexes". Also, the authors should comment a bit more on the results obtained with CD163 SRCR1-5 in complex with HpHb.

The expected MW and the measured MW has now been included in the manuscript. Labels have been added to the figure to specify the component of the individual peaks (Figure 1c+d in the revised manuscript) and the text has been improved for clarification (line 102-105 in the revised manuscript).

Cellular up-take of HpHb and Hb (lines 269-271): the authors explain the residual ability of cells expressing the trimer-destabilizing substitutions to internalize Hb by the ability of monomeric CD163 to perform Hb uptake. Why did the authors discard the simpler hypothesis that the small residual fraction of dimeric/trimeric CD163 (Figure 3D) is responsible for the uptake? SPR experiments assessing affinity for either HpHb or Hb alone of CD163 SRCR1-5 (which is expected to be monomeric) would help understanding how oligomerization of the receptor is improving affinity for its targets and if internalization by a monomeric receptor is indeed possible.

We have perfomed and included the suggested experiments meassuring the affinity of CD163 SRCR1-5 to both HpHb and Hb (Figure 6b in the revised manuscript). This shows an 70 to 90 fold decrease in ligand affinity of monomeric vs. trimeric receptor. This experiment in now described on line 408-412 in the revised manuscript.

The chapter “Quantification of the CD163 affinity for HpHb and Hb” did not convince the reviewer that the CD163 trimer is the relevant species over the dimer. Could the authors further discuss their statement?

In order to confirm that CD163-CC is on a trimeric form, we have analysed the sample using SEC-MALS, which is included as Suplementary Figure 6 in the revised manuscript. Furthermore we have added SPR experiments of HpHb and Hb binding to monomeric CD163 for comparison (Figure 6b in the revised manuscript). A section disussing the CD163 trimer in a physiological context is in line 264-267 in the revised manuscript.

Minor points

Lines 46-48: please clarify to what extent the Hp genotype (either Hp2-2 or Hp1-1) does not affect the efficiency of Hb scavenging. Dealing with this point: why have the authors chosen the Hp1-1 phenotype?

The choice of the Hp1-1 phenotype was based on its homogeneity compaed to the other more complext phenotypes. Both Hp2-2 and Hp2-1 phenotypes result in a mix of Hp multimers ranging from small dimers to large multimeric forms. A section describing the relationship between efficiency and Hp phenotype has been added in line 267-273 in the revised manuscript.

Using two different colors for αHb and βHb would improve the clarity of all the figures in the manuscript.

The color scheme for Hb has been updated in the revised manuscript.

Abstract should not have references. Please remove ref.1

The reference has been removed in the revidsed manuscript

Change colors in figure 7: orange and red are too similar and difficult to discriminate

The colors in figure 7 has been changed in the revised manuscript.

Line 141: Supplementary Fig. 3 is missing in the submitted files. In the following sentence, it is likely that the cited Figure is 2B and not 2D.

The supplementary figures and citations have been updated in the revised manuscript.

Line 157, authors are most likely mentioning Figure 2A and not 3A

The figures and citations have been updated in the revised manuscript.

Line 181, the text is describing Figure 4A+B+C and not 4A+B. Moreover, the reviewer suggests changing “AB region” with “AB loop” because it is more adherent to the classical nomenclature of Hb.

The AB region has been replaced with AB loop in the revised manuscript.

Line 213, the statement “data not shown” is against the Nature Communication editorial policies. Moreover, the SPR experiment with CD163 and Hp would be of interest for the discussion in the paper, and it should be added as supplemental material.

A figure showing the data has been included in figure 6a.

Lines 218-219, it is not clear if the absence of CD163 binding by Hp is known from literature or experimentally determined in the present work.

References to the litterature have been included in the revised manuscript (on line 261-262) and SRP binding curves of Hp binding to CD163 included in figure 6a.

Sentence in lines 321-322 is not clear and needs more details.

The sentence has been rephrased in the revised manuscript.

Figure 7: please specify how many replicates were averaged and the meaning of error bars (standard deviation? Standard error?)

The number of replicates has been added to the legend and methods section in the revised manuscript.

As per Nature Portfolio policy, “Official validation reports from the wwPDB are required for peer review” and “Accessibility in repositories must be designated for immediate release on publication.". The reviewer suggests adding the accession numbers in the Data availability session.

The accession numbers have been added to the data availability section in the revised manuscript.

A scheme of the Hp1-1 structure would be beneficial, especially because 3D reconstructions of the ternary complex show only the Hp domain involved in Hb binding (HpSP), without resolution of the rest of the structure (HpCCP).

In the revised manuscript, the entire structure of Hp1-1 is depicted in Figure 4a (in gray) for visualization of Hp1-1-Hb docking into the central cavity of the CD163 trimer. Furthermore, in order to visualize the expected binding of Hp2-2-Hb to CD163, we have added a model of trimeric Hp2-2 in complex with Hb binding to CD163 (Figure 10).

Reviewer #2 (Remarks to the Author):

Etzerodt A et al. The structure of human scavenger receptor CD163 in complex with haptoglobin-hemoglobin discloses the molecular mechanism for hemoglobin scavenging.

The present study offers new insight into the structural features of CD163-HbHp complex formation based on cryo-EM, in-silico modeling, and experimental analysis that includes mutant construct receptor component expression, SEC-MALLS, binding studies using SPR and flow cytometry. Their study provides a strong balance of techniques to reach a plausible conclusion for Hp1-1 binding to CD163. The binding of calcium ions to CD163 is crucial not only for ligand binding but also for receptor oligomerization. The authors have also made an important observation regarding the role of Ca2+ ions in the process of binding. Overall, the manuscript results support the authors conclusions, and the work advances an understanding of the Hb, Hp, CD163 binding paradigm.

We thank the reviewer for comments and suggestions. Below is a point-by-point reply to the points raised.

Comments:

1. Do glycosylation differences in Hp1-1, 2-1 and 2-2 affect CD163 binding in this model? Some further insight into the differences in Hp2 phenotypes and their interactions with CD163 could be of interest.

To our knowledge the glycosylation patterns are basically identical in the different Hp phenotypes. To verify that Hp2 phenotypes also bind trimeric CD163, we have

quantified the binding of Hp2-2 to trimeric CD163. This is now included as figure 6c in the revised manuscript and the results elaborated in line 267-273.

2. The absence of the CD163 SRCR1 domain in the authors models may be an important limitation. The authors should better explain how the model is affected by this omission.

We observe density for CD163 SRCR1 in our initial low-resolution reconstructions. However, at higher resolutions the domain no longer shows interpretable density, most likely because it is in a flexible position with respect to SRCR2-4. Therefore, we hypothezise that SRCR1 is not involved in binding to HpHb. This has been elaborated on line 141-146 in the revised manuscript.

3. The authors discuss the binding of Hb in the absence of Hp and the potential relevance of Hb dimerization. It would be useful to provide modeling data on the concentration dependent binding of Hb tetramer and dimer to CD163 in the absence of Hp. This data will help with understanding the low-level clearance of Hb in absence of Hp during disease and the Hp null genotype.

We have attempted to predict the complex between CD163 and tetrameric Hb using Alphafold. However, the results obtained does not make sense according to our insights from the CD163-HpHb structure and has therefore not been included in the present manuscript.

4. The discussion becomes speculative in areas. For example, “The present structural data may also provide insight into the uncoupling of CD163 and HpHb in the endosomes, where calcium is gradually pumped. This loss of Ca2+ may promote the release of HpHb from CD163 by a combined process of distinct mechanisms.”

The crucial role of Ca^{2+} in ligand binding and release in endocytic recptors is a well-decribed phenomenon. To clearify this further, we have now added a sentence describing this (including references) in the beginning of the paragraph at line 330 in the revised manuscript.

The reviewer acknowledges the considerable effort made by the authors in addressing the reviewers' suggestions, as well as their care in rewriting the manuscript and preparing more effective and clearer figures. These efforts have resulted in an improved manuscript that addresses many of the concerns previously raised by the reviewer. However, there are still some major issues that prevent the paper from being considered acceptable in its current form. Additionally, some results could benefit from a more thorough discussion to enhance their clarity and draw more substantiated conclusions. The reviewer's concerns involve mainly the physiological relevance of the trimeric assembly for the receptor's function and the role of calcium, plus different scattered issues/observations.

We thank the reviewer for thorough and critical evaluation of the manuscript. Below, we provide a point-by-point response addressing the reviewer's comments.

One of the main findings of the work is the identification of a calcium-dependent oligomerization of CD163 in both the absence and presence of ligands that should have relevance in ligand binding and internalization by this receptor. The oligomers formed are likely a mixture of dimers and trimers in equilibrium with the monomeric species as clearly indicated by SEC-MALS experiments and by the heterogeneity of the particles in cryo-EM, where both dimeric and trimeric CD163 is seen bound to Hp/Hb. These findings might be suggestive of the existence of this assembly on the cell's membrane, however there is not direct proof of this, and the authors seem to neglect the possibility that the dimeric species is as efficient as the trimer and might be relevant for Hb acquisition. The authors state that the trimeric assembly is responsible for the high-avidity binding of Hp/Hb to the receptor, although the cryo-EM structure only allows to spot stable interactions between one subunit of CD163 and Hp/Hb, while interactions with the two remaining subunits are openly defined by the authors as weaker ("suggesting that these domains are not or only loosely associated with HpHb"). The determination of the binding affinity for the trimer vs monomer is performed by SPR on an engineered receptor where the trimer is stabilized by the addition of a coiled coil that might, in principle, lead to an overestimation of the affinity by reducing the conformational dynamics of the protein. Considering all the above-mentioned points, the authors should avoid overly stress that i. the physiological assembly on the cell membrane of the receptor is trimeric, and ii. trimeric CD163 is more relevant for Hb/Hp internalization due to an avidity effect of the interactions established by the three subunits with the ligand. The authors should clearly state the limits of their findings, mentioning alternative interpretations of their data to warrant the readers' critical view of this system.

We agree with the reviewer that CD163 dimers and even monomers may be present on the surface of cells and that these forms could be functionally relevant in the clearence of HpHb and Hb. In order to acknowledge the reviewers concerns we have made several modifications throughout the revised manuscript and added a section descibing that monomeric CD163 could be functional on the surface of cells (line 353-359).

Moving to the second major point (e.g. the role of calcium in oligomerization), the main issue is related to the lack of any structural direct evidence of the molecular

mechanism by which calcium is responsible of favouring CD163 quaternary assembly and its interaction with Hp/Hb. In many instances the authors seem to suggest a mechanism that involves a lysine residue, but they never come to a clear conclusion. The reviewer suggestion is to make the presentation of the data more schematic and to clearly state which are the hypothesis about the molecular mechanism. More specific suggestions are detailed below. The reviewer advises limiting speculative discussions (e.g. lines 367-369 and 377-378) about the oligomerization of proteins sharing similar tertiary structures, as the calcium-dependent oligomerization mechanism for CD163 has yet to be fully explained.

Our structural data strongly suggest that the electrostatic interactions between Lys811/Lys1021 and Asp956/Asp746 are critical for CD163 oligomerization. This is further supported by mutagenesis, where the K811A/K1021A mutation completely abolishes oligomerization (Figure 4d). Additionally, we observe a nearly identical interface in the CD6 structure, a protein known to dimerize. These findings, we belive, justifies the proposal that other proteins containing SRCR domains with lysine residues in analogous positions may engage in similar interactions. Importantly, our proposition are made solely from observed structural interactions, and we refrain from speculating on the calcium dependence of these interactions.

In order to limit speculative discussion, we have removed lines 377-378 in the revised manuscript. Furthermore we have added a section in the beginning of the discussion, that descibes our hypotesis based on previous structural data (line 315-327)

Point-by-point observations:
1) Line 22: only the trimer is mentioned in the abstract. Reference to the existence of dimeric species should be added

The existence of dimers has been added to the abstract in the revised manuscript.

2) Lines 46-48: to reviewer's knowledge the Hp isoform influences the efficiency of Hb removal from the plasma, with the Hp2-2 phenotype being responsible for a less efficient scavenging and thus to a phenotype more prone to Hb-related oxidative stress. Please clarify this point. See also lines 267-270 in this manuscript.

A possible explanation for the phenomenon is that the plasma concentration of Hp in the Hp2-2 phenotype is lower that in the Hp1-Hp1 phenotype. Also, as stated on line 284-285 in the revised manuscript increased efficiency in uptake is likely lost in Hp2-2 if not fully saturated with Hb.

In the revised manuscript, we now describe that the Hp plama concentration is lower in individuals with the Hp2-2 phenotype (line 51-53).

3) Lines 65-71: the paragraph requires revision for more clarity

The paragraph has been revised for clarity in the revised manuscript.

4) Lines 79-80: data presented do not allow to discard the possibility that, using the appropriate stoichiometric ratio, a Hp/Hb dimer bound to two CD163 trimers might be observed. This sentence should be removed from the introductory paragraph or modified.

The sentence has been modified in the revised manuscript.

5) Section “Analysis of CD163 oligomerization using multi-angle light scattering”: supplementary Figure 1 demonstrates that Ca2+ concentrations higher than 2 mM do not further affect oligomerization. However, the MALS results indicate a maximum MW of 200 kDa, which is substantially lower than the one shown in Figure 1A. The authors should provide a clear explanation for this discrepancy.

The experiment in Supplementary Figure 1 was performed using a 24 ml Superdex 200 column, whereas the experiment in Figure 1A used a 15 ml Wyatt SEC column. Due to the larger column size, the sample is more diluted on the 24 ml Superdex 200 column, resulting in a lower sample concentrations. At lower concentrations, the equilibrium of CD163 oligomers shifts slightly toward monomers, which accounts for the lower observed molecular mass. This explanation has now been included in the legend of Supplementary Figure 1.

As a minor point the reviewer suggests modifying Figure 1 and to overlay the chromatograms in the absence and presence of calcium on a single figure. This would allow to better appreciate the effect of calcium on the oligomerization/complex formation.

As recommended by the reviewer, the figures have been overlaid in the revised manuscript to facilitate direct comparison and enhance clarity.

6) Lines 98-100: SEC-MALS analysis (Figure 1C) does not allow for a definitive determination of the oligomeric state and the stoichiometry of the ternary complex between Hb, Hp, and the CD163 receptor. Indeed, could the estimated MW also correspond to two CD163 dimers bound to both sides of HpHb complex? To definitively clarify how the CD163 receptor binds HpHb, it is necessary to run a sample without stoichiometric excesses, where a single sharp peak is present in the chromatogram. Also, the use of the right stoichiometry might have allowed the stabilization of the complex and, possibly, a reduction of the conformational flexibility ultimately leading to an increase in the structure resolution and to the determination of the missing part of the Hp/Hb structure.

Analyzing the stoichiometry of a complex formed by multiple low-affinity binding sites is challenging. Altering the ratio of mixed samples does not produce a single sharp peak but instead results in a shift towards lower molecular weight complexes and free HpHb. In our optimized protocol, we use an excess of HpHb to fully saturate CD163, followed by size exclusion chromatography to remove any unbound HpHb. Additionally, we ensure that the sample is prepared at a concentration where the top fraction can be used directly for grid preparation, avoiding any dilution that might cause dissociation of

the formed complexes. This approach yields multiple types of complexes while eliminating free HpHb that could interfere with data processing.

While SEC-MALS can be used to determine the molecular weights of complexes, it is only accurate if the complexes can be separated by the column. Otherwise, the molecular weight obtained will be an average of all the complexes present. Thus, this method cannot provide a definitive determination of the oligomeric state or stoichiometry of individual complexes. In the manuscript, we propose that the top fraction corresponds to a CD163 trimer bound to HpHb. This is based not only on SEC-MALS data but also on observations from Cryo-EM.

We have made numerous attempts to identify complexes of HpHb with CD163 (dimer or trimer) bound to both ends in our Cryo-EM micrographs, however this has not been possible. We are aware that this does not rule our their existence and we have added a section describing this on line 287-290 in the discussion.

Furthermore, as stated at lines 268-270, the ability of a Hp/Hb dimer to interact with more than one CD163 might have physiological relevance. More in general, the reviewer suggests adding a table (or a scheme) where the expected/theoretical MWs are indicated for each potential assembly of the single proteins and of the complex. This would facilitate the critical reading of the manuscript and the assessment of the correctness of the conclusions drawn from SEC-MALS experiments.

As noted earlier, SEC-MALS analysis cannot determine the molecular weights of individual CD163-HpHb complexes but provides an average molecular weight for the co-eluting complexes. Therefore, our conclusions about the composition of the complexes are based not only on SEC-MALS data but also on observations from Cryo-EM. In the revised manuscript, we have included the expected molecular weights of the individual components in the main text to help the reader more easily assess the validity of our conclusions.

7) Lines 105-106: please specify the meaning of “1:1 stoichiometry”. The MW estimated by MALS is below 200 kDa while the theoretical MW should be 270 kDa, if one CD163 binds to 1 Hp/Hb (not a Hp/Hb dimer).

The affinity of CD163 SRCR 1-5 for HpHb is relatively low, estimated at 766 nM in SPR, which could lead to partial dissociation of the complex during column separation and may explain the observed discrepancy. Additionally, the overlap of this peak with the peak corresponding to free HpHb could further impact the estimated molecular weight. In the revised manuscript, we have rewritten this section for clarity (line 109-110).

8) 108-110: please add a reference for this statement

A reference has been added in the revised manuscript

9) Section “Cryo-EM structure determination of CD163 SRCR1-9-HpHb”: Supplementary Figure 2. It is necessary to add (i) an image of a micrograph and (ii) a

panel of the selected 2D classes to proceed with the 3D analysis. The addition of these two images will allow an expert reader of the technique to dispel any doubt regarding the presence or absence of the entire deposited complex. Indeed, all the refined maps lack a significant region of the complex (the two alpha chains and one beta chain of Hp, and a dimer of Hb).

Supplementary figure 2 has been modified as suggested by the reviewer.

10) Line 135: please provide a detailed explanation on how the AlphaFold model was fitted into the density. Was the monomer used or a generated trimeric assembly? Since AlphaFold3 has improved functions, like prediction of quaternary structures and of metal binding, authors might consider using a newly generated model to fit cryo-EM data.

Individual SRCR domains of the AlphaFold2 prediction of human CD163 was manually docked into the density followed by automated molecular dynamics flexible fitting and real-space refinement using Namdinator pipe-line tool. This has now been specified on line 579-582 in the revised manuscript.

We have submitted a range of different jobs to the AlphaFold3 server. However, similar to AlphaFold2, AlphaFold3 is unable to predict a reliable CD163 trimer or accurately model its interaction with the HpHb complex.

11) Line 155: SRCR 1-4 should be changed in SRCR 2-4 since domain 1 is not visible.

Done

12) Line 162: specify that domain 3 is involved in ligand binding

Done

13) Paragraph lines 168-175: the part on the disulfide bridge is not clear. How was the bridge identified: was it in the density of the 3.8 Å map or the AlphaFold prediction?

The conformation of the extended linker, including the disulfide bond formation, was revealed in a recent crystal structure of porcine CD163 SRCR 5-9 (RCSB ID 8H7J). Notably, the disulfide bond is also predicted in the AlphaFold model. This is described on line 178-180 in the revised manuscript.

14) Paragraph lines 177-187: the order in which data are presented and discussed generates confusion. A suggestion is to start with the identification of the potential calcium binding sites based on homology, state that resolution does not allow to solve the sites in domains 6, 7 and 8, state that calcium is defined in domains 2 and 3 in two out of three sites. After this introduction, the sites and interaction with the ion can be described.

In response to the reviewer's suggestion, we have reorganized this section in the revised manuscript to improve clarity and logical flow.

15) Lines 191-192: add reference to figure S4

Done

16) Role of calcium (Section line 198, section line 275, and discussion): the authors have only partially addressed the reviewer's concerns. The SEC-MALS analysis of the receptor variant in the presence and absence of Ca2+ was performed as requested; however, the experimental design does not allow for a reasonably solid conclusion to be drawn.

The reviewer believes that a greater effort is needed in analysing the role of the identified Lys residues and the effect of their substitution with alanine, as a significant portion of the subsequent experiments and discussion is based on these points. What do the authors believe is the cause of the abrogation of oligomerization of the variant used? Mutated lysines are forming electrostatic interaction with residues forming a predicted calcium-binding site; however, the oligomerization of the K811A/K1021A mutant appears to be independent from calcium (Supplementary Figure 5).

The authors should clarify whether they believe that the lack of oligomerization is due to (1) a reduced affinity between protomers caused by the loss of the electrostatic interactions mentioned in the text, in which case they should clarify the role of calcium accordingly, or (2) a reduced affinity for calcium itself.

Also consider that calcium coordination requires the formation of eight bonds (https://doi.org/10.1016/j.bbamem.2012.11.025), a property that questions how this is possible if the identified lysines form electrostatic interactions with the Asp and Glu residues involved in metal coordination. A similar pattern of interactions seems to happen between site 2 on SRCR2 and a Lys residue of Hp (Fig. 5A, lines 299-300 and lines 310-311 of the discussion), thus the authors should include an explanation for this behaviour as well.

Regarding the K811A/K1021A mutant: In our recent revision, we generated a new batch of CD163 SRCR 1-9 K811A/K1021A and employed an improved purification protocol. This confirmed that oligomerization of the mutant was completely abrogated. The previously observed high-molecular-weight forms were likely due to nonspecific aggregation or contaminants. This section has been updated accordingly in the revised manuscript (Lines 225-229).

To further investigate the interaction between Lys811/Lys1021 and Asp956/Asp746, we have included an additional experiment in the revised manuscript (Supplementary Figure 5) demonstrating the effect of the D746A/D956A mutation. Similar to the K811A/K1021A mutation, the D746A/D956A mutation completely abolished oligomerization, confirming that the electrostatic interaction between Lys811/Lys1021

and Asp956/Asp746 is critical for CD163 oligomerization. This is described on line 225-229 in the revised manuscript.

The coordination of acidic residues by calcium ions to facilitate interaction with positively charged amino acids from a ligand is a well-established phenomenon and has been demonstrated in various structures (reviewed in DOI:10.1016/j.tibs.2013.12.003, ref. 33 in the revised manuscript). We have revised our hypothesis on how calcium mediates CD163 oligomerization for greater clarity (lines 315-327 in the revised manuscript).

17) Lines 213-214: The sentence "This pattern of interaction in repeating in the three subunits, resulting in the formation of the triangular base." should be revised to improve the clarity.

The sentence has been modified for clarity in the revised manuscript.

18) Lines 216-219 and Figure 4C: the text states that K811 and K1021 form electrostatic interactions with D746 and D956, respectively. However, Figure 4C shows interactions also with E812 and E1022. The authors should solve this inconsistency.

The section has been corrected to include interaction with E812 and E1022.

19) Lines 222-223: in figure 4 no oligomeric species are visible in the SEC-MALS analysis.

The text has been modified according to the new data presented in figure 4d.

20) Section "CD163-HpHb interaction": how the trimeric assembly should stabilize Hb/Hp binding is not convincingly stated. The interactions with subunits 2 and 3 are defined as loose and identified interactions involve structure with an inadequate resolution. Maybe a comparison between the dimeric and trimeric assembly with respect to Hp/Hb binding could be useful.

We agree with the reviewer and have added a paragraph at the end of the section (line 256-262), explaining that there seems to be an uneven contribution from the individual CD163 subunits. Specifically, we highlight that $CD163_C$ has dissociated in the dimeric assembly. Also, in figure 5, we have highlighted the interactions observed in the assembly of dimeric CD163 with HpHb.

21) Line 257: do the authors have an explanation on why the engineered protein has a MW higher than those observed with the wt trimeric assembly?

The SEC-MALS data indicate that wild-type CD163 SRCR1-9 exists in solution as an equilibrium of monomers, dimers, and trimers. Since these oligomeric forms cannot be fully separated by size-exclusion chromatography, the measured MWs represent averages of the different species present. As a result, the MW of the larger oligomers is slightly underestimated. In contrast, the engineered trimeric CD163 remains intact on

the column, allowing for a more accurate determination of its MW. This is now described in the legend for Supplementary Figure 6.

22) SPR: please add errors for the Kd values

Errors are now added to Figure 6

23) Lines 265-267: please add a comment on whether the loose interactions detected between subunit 2/subunit 3 of CD163 and Hp/Hb are compatible with a 70-fold increase in ligand binding affinity of the trimer with respect to the monomer

Several studies have highlighted the significant avidity effects of multivalent interactions. For example, one study reported a 500-fold increase in ligand affinity for a divalent IgG antibody compared to its single-chain fragment (DOI: 10.4049/jimmunol.179.5.2815, ref. 29 in the revised manuscript). In this context, the observed 70-90-fold increase in affinity of trimeric CD163 SRCR 1-9 compared to monomeric CD163 SRCR 1-5 is consistent with multiple low-affinity interactions working together to significantly increase the functional affinity. We have modified the text and included a reference to the study in the revised manuscript (line 279-281).

24) Section “Cellular uptake of HpHb and Hb”: which are the concentrations of Hp/Hb used for this experiment? How do they compare with the physiological ones? Should we discard the hypothesis that the more efficient uptake mediated by the trimer is observed in the presence of non-physiological ligand concentrations? In the same vein: do the authors discard the hypothesis that the dimeric form contributes to efficient internalization? Did they try to express the engineered CD163-CC receptor in the cell line used for internalization experiments?

The concentration of Haptoglobin in serum is in the range 0.3-3 mg/ml. However, the physiological concentration of HpHb and Hb is dependent on the degree of hemolysis and is expected to be much lower due to its rapid clearance by macrophages. For the experiments we have chosen a concentration af 10 µg/ml in order to get a sufficient signal for Hp uptake. At this concentration we observe a clear effect of the mutations, indicating that CD163 oligomerization does affect HpHb and Hb uptake by the cells. We agree with the reviewer that this experiment does not exactly compare with a physiologgical setting, since we do not know the physiological range of HpHb and Hb concentrations. However, we do show that in this experimental setting CD163 oligomerization on the cell membrane plays a functional role. In the revised manuscript, the sections covering the uptake studies (both in the results (line 293-306) and dissusion (line 353-366)) have been modified. We have not performed internalization experiment on CD163-CC.

25) Lines 358-359 require revision for more clarity

The lines have been removed in the revised manuscript.

26) The final discussion (lines 410-418) would benefit from an introductory section on the already known role of the soluble form of the CD163 receptor, as well as a revision of the speculations made. This is relevant because the work presented in the current manuscript does not determine affinity constants for receptor oligomerization, neither in its soluble form nor in its membrane-bound form.

We agree with the reviewer that, without an affinity constant for receptor oligomerization, we cannot definitively determine whether the oligomers will dissociate once cleaved from the membrane. In the revised manuscript, we have modified this section accordingly to reflect this uncertainty.

Reviewer #1 (Remarks to the Author):

The revised version of the manuscript “The structure of human CD163 bound to haptoglobin-hemoglobin discloses the molecular mechanism for hemoglobin scavenging” by Andersen and colleagues has now improved to the extent that it might be suitable for publication in Nature Communications.
I still have some suggestions, especially aimed at enhancing the reading experience also for non-expert readers.

Major points:
- The role of calcium in mediating receptor trimerization and ligand binding is strongly emphasized by the authors, who take for granted that most of the readers are aware of the structural role played by calcium in this type of interactions. In my opinion, the authors should stress at the very beginning what they believe is the mechanism by which a positively charged ion might bridge the ion interaction between a positively (i.e. Lys/Arg) and negatively (Asp/Glu) residues. They postpone this information to the discussion section (lines 315-319), making difficult for the general reader to understand the results (section “CD163 1-9 oligomerizes via SRCR7 and SRCR9 interaction“).

We agree with the reviewer and have moved at section to the introduction as suggested.

- In the discussion the narrative flow might be improved, with special focus on the role of calcium on receptor oligomerization and in ligand binding. In the present version the authors mix comments on either one aspect or the other, hindering an easier, more direct interpretation of data. I suggest the authors to start the discussion, after the introductory paragraph, with a section on the oligomerization aspects and then move to the role of calcium in ligand recognition. For example, the sentence about the role of lysines, that is deeply intertwined with the role of calcium is postponed to line 386-394, while it would be more logical if moved when the mechanism of calcium-mediated Asp-Lys interaction is explained.

We agree with the reviewer and have rearranged the discussion as suggested.

- In general, the discussion is very long. Once the authors have rearranged the sections, they may want to move the text that refers to figures 8 and 9 in the supplemental material

We believe that the text referring to Figures 8 is highly important for the broader scope of the results presented in the manuscript and would prefer to keep it in the main text if possible. To address the reviewers' suggestion, we have removed Figure 9 from the revised manuscript.

Minor points:
- The reviewer suggests moving the text in lines 173-180 ("CD163 SRCR5-9 are organized into ...") to an earlier section, specifically where the lack of density between SRCR6 and SRCR7 is discussed (line 143). This change would clarify that cryo-EM does not directly

resolve this region and that the integration of AlphaFold2 predictions and existing literature addresses the issue.

We respectfully acknowledge the reviewer's perspective; however, we believe the two sections address distinct contexts. The first focuses on the docking of models into the electron density of the cryo-EM reconstructions, while the second discusses the structural composition of SRCR5-9 in relation to prior structural studies and AlphaFold predictions.

- Supplementary Figure 7 will be more informative if CD163 protomer are colored differently.

We agree with the reviewer and have changed the colors in Supplementary Figre 7 accordingly.