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Memory IgM protects endogenous insulin from autoimmune destruction

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1st Editorial Decision 17th Feb 2021

Dear Hassan,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees find the analysis insightful and interesting. They raise a number of different concerns that I would like to ask you address in a revised version. I think it would be good to discuss the raised issues further and I am happy to so via phone or video. Let me know what works best for you.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Thank you for the opportunity to consider your work for publication. I look forward to discussing the revisions further.

Yours sincerely,

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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Referee #1:

The manuscript of Amendt and Jumaa examines the cellular mechanisms of antibody mediated autoimmunity. They use a very simple wild type mouse model, immunizing either soluble or multimerized whole insulin or insulin peptides in the presence of CpG. The multivalent complexes generate an early IgM and IgG response, that shifts towards IgG with time and causes a diabetic phenotype. Interestingly, adding excess monovalent insulin peptide to the multivalent complexes favour IgM over IgG and dampens to autoimmune symptoms. The authors suggest that the monovalent peptides are inducing autoreactive affinity matured memory IgM responses that is distinct from the primary IgM that is polyreactive and possibly B1 cell derived. How exactly the monoreactive IgM protects the endogenous insulin from the pathogenic IgG remains unclear.

Overall this is an interesting study. The experiments are appropriate, and the data presented in a clear fashion. Although in the discussion the authors focus mostly on fundamental tolerance mechanisms, I feel that the model and the results may be most pertinent for the understanding of so-called "inhibitors" of injected therapeutic proteins. The authors mention insulin antibody syndrome, but another very similar issue is anti-factor VIII Abs in hemophilia, which are significant clinical problems.

Specific comments
1. Beyond a couple of very basic FACS plots in figure 2 and S2 there is little characterisation of the immune response elicited to the insulin complexes + CpG. Some more work in characterising the model would be helpful for the interpretation. Are germinal centers formed? What is the IgM and IgG status of the GC and memory B cells? Are the IgM and IgG clonally related? The authors provide very limited evidence for affinity maturation for IgM (fig 6d) and none for IgG. Are the affinity changes and improvements the same for each isotype?
2. The authors build up a narrative that no B cell response to an endogenous protein such as insulin would be expected due to highly efficient tolerance mechanisms during B cell development. Yet at least superficially, there are several other similar models that elicit Ab responses after immunization with endogenous antigens +adjuvant (examples include EAE, arthritis models), so this is not

unprecedented.
3. The polyvalent insulin immunization differs from the monovalent versions in that they also bring in large protein carriers with foreign T cell epitopes. I realise that this may be difficult to experimentally address, so could the authors at least discuss, the impact of the changes in the antigen beyond the insulin valency. For example, what would be the response to polyvalent insulin linked to an endogenous protein as a carrier.
4. In light of the autoantibody responses that can be elicited by endogenous proteins in EAE or arthritis models (often using CFA), is the IgM memory effect seen here adjuvant specific? Similarly, is the CpG used in the recall immunizations.
5. The labelling in figure 3 legend is incorrect (going to panel "e').

Referee #2:

In this Manuscript, Amendt and Jumaa present a set of experiments based on the injection of peptides into mice as means to develop a new model of studying B cell autoreactivity in the context of Diabetes. The manuscript is proposed to offer insight into the fundamental mechanisms of how B central cell tolerance can shape the BCR repertoire in terms of self-recognition to endogenously produced antigens. The experiments are well performed and presented in a clear manner. This model would indeed be useful, and the authors are correct in cautioning about the use of transgenic models. There is consistent use of hyperbolic phrases in the text that gives the impression of a more dramatic phenotype than is actually presented. In particular, the claims of severe autoimmunity do not appear based on pathological or physiological parameters.

Can the authors provide histology of the pancreas? Does the increased immune infiltration lead to organ damage? Can the authors provide OGTT following injection of IgG immune complexes? Can the increase in blood glucose following anti-insulin IgG injection be reversed by also injecting Insulin?

As far as this reviewer is aware, injection of any peptide with a suitable adjuvant will produce self-reactive B cell clones, as the GC reaction will allow for mutation of recruited germline alleles towards self. Are the anti-insulin responses after extended periods reflective of germline specificity? If not, I'm not sure why this paper challenges the notion that central tolerance effectively removes a large amount of self-reactivity, or will indeed lead to the 'paradigm-shift' claimed in the discussion.

There are a few technical points that also need addressing:

The use of infinite in the opening line of the manuscript is incorrect.

Lines 169-174 are difficult to read and I would recommend re-writing as I do not completely follow the logic.

The injected Mice were aged 8 to 30 weeks. This is a big difference, not least for studying autoreactivity. The MZ is also not fully formed at 8 weeks in all mice. No gender information is provided, again a critical aspect in autoimmunity.

The statistical analysis is often incorrect. Repeated measure ANOVA needs to be used when sampling on the same mice is done multiple times, and one-way ANOVA in groups of more than one. There seems to be no checks done on the normality of the data and the need for non-parametric approaches.

Referee #3:

The manuscript by Amendt and Jumaa describes the induction of protective regulatory IgM by immunization with of a combination of monovalent and polyvalent antigen. Using insulin as target antigen and the induction of signs of diabetes, they first demonstrate that immunization with complexed (polyvalent) insulin C-peptide induces anti-insulin IgG, hyperglycemia, glucosuria and pancreas inflammation. IgG purified from immunized mice showed up to 40% reactivity with is insulin and induced hyperglycemia. Compared to this immunization protocol, combined immunization with soluble monovalent and polyvalent C-peptide induced higher levels of specific IgM and lower levels of specific IgG, also after further challenge with the antigen. Similar data were obtained when insulin alpha chain peptides were used as immunogens with positive effects of hyperglycemia and glucosuria with mixed (monovalent and polyvalent) antigens, resulting in a decreased insulin-specific IgG:IgM ratio. Furthermore, the authors could show that these "protective regulatory IgM" (PR-IgM) persist for a long time and can be boosted. Characterization of the IgM responses identified differences between specific d7 IgM and d85 IgM (PR-IgM), with only d7 IgM inducing hyperglycemia following infusion. Similarly, administration of PR-IgM with polyvalent InsA immunization or anti-insulin IgG prevented induction of diabetes signs. The authors suggest a higher affinity and lower polyreactivity of PR-IgM vs d7 IgM characterization both serum IgM and affinity purified IgM.

This is a very interesting and important study documenting differential antibody responses against an autoantigen depending on the preparation of the immunogen. This builds on previous work by the authors, but detailed mechanistic studies in this context are not provided. They further demonstrate different functional activities of early and late (affinity matured) IgM responses with respect to the induction of immunity, which is the main finding of this manuscript. Insights into the mechanisms of these effects require additional experiments that may shed more light on this. There are also a number of other issues that the authors need to clarify.

Major:
1) What is the mechanism of the differential antibody response when monovalent antigens are added? This needs to be discussed with respect to reference 14.
2)The authors suggest that also endogenous insulin could modulate this. Would immunization of hyperinsulinemic vs normoinsulinemic mice with polyvalent antigens also result in differential response? This could be tested.
3) Regarding the characterization of the IgM responses (Fig 6d and 7d-g), the authors need to provide more data on affinity and specificity, including baseline levels (before immunization), raw data, competition assays using different soluble antigens. Also, please provide serum dilutions and IgM concentrations for Fig 7d-g.
4) What is the mechanism for the different activities for 7d IgM vs PR-IgM? Shouldn't PR-IgM better neutralize insulin? One may suspect that these are different (polyclonal vs oligoclonal) specificities. Epitope mapping would identify these differences that may result in different avidities or interference with insulin function. Half-lifes of both IgM preparation following injection need to be compared.
5) The authors need to be careful in presenting the effects causing hyperglycemia, as these are (in case of IgM) caused by neutralization of insulin rather than destruction of pancreatic beta cells, which is the relevant autoimmune pathology.

Minor points:
1) Flow cytometric characterization of insulin-specific IgG bound to macrophages. How can the authors exclude the possibility that they are not looking at expression of insulin receptors?
2) Suppl Fig 4a: Please, also show IgM titers.
3) In general, the authors need to clarify in the figure legends the antigens used in the ELISA.
4) The authors claim that 40% of IgG in immunized mice is specific for insulin. This seems a lot and they should re-evaluate that.
5) Figure annotation on page 8, line 306-308 seems incorrect. Please, correct.
6) The authors mention IgM+ insulin reactive B cells in the discussion, but the data are not provided.

We thank all referees for their comments that were addressed as discussed below.

Referee#1 *describes our study as "an interesting study" and that "the experiments are appropriate, and the data presented in a clear fashion.". Further, she/he is highlighting our results in relevance of "so-called "inhibitors" of injected therapeutic proteins."*
We thank the referee for her/his comments that we addressed below. Changes in the manuscript text are highlighted in green.

Specific comments:

1. Beyond a couple of very basic FACS plots in figure 2 and S2 there is little characterisation of the immune response elicited to the insulin complexes + CpG. Some more work in characterising the model would be helpful for the interpretation. Are germinal centers formed? What is the IgM and IgG status of the GC and memory B cells? Are the IgM and IgG clonally related? The authors provide very limited evidence for affinity maturation for IgM (fig 6d) and none for IgG. Are the affinity changes and improvements the same for each isotype?

For a more detailed characterization of the immune response, especially of the **germinal center** response, we performed immunization experiments by injecting mice with cInsulin or control immunization (CI, i.e. CpG only). We found that germinal centers (GC) are formed (see Fig. 1 for Ref#1) as shown by immunohistochemistry of spleens analyzed on day 5 after boost. Moreover, FACS analysis of splenocytes revealed highly increased numbers of GC B cells of cInsulin immunized mice compared to CI mice (Fig. 1i). Interestingly, these GC B cells showed a significant portion of cells reactive to native insulin (Fig. 1i). We integrated these FACS data into figure 1 of the revised version of the manuscript as we think that these data significantly improve the characterization of the autoimmune response (Fig. 1i).

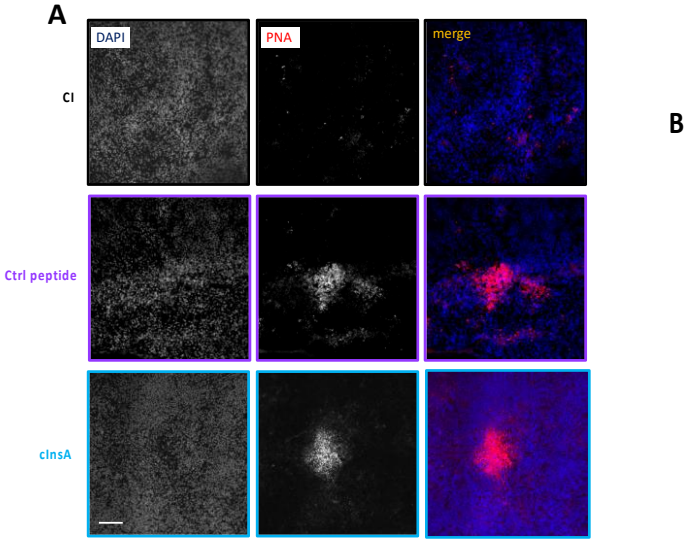

Figure 1 for Ref#1. Germinal center formation in mice immunized with cInsulin.
A: Immunohistochemistry of spleen sections of cInsulin, control peptide and control (CpG only) immunized mice on day 5 post boost. Germinal center B cells are stained with Peanut-Agglutinin (PNA) in red and nuclei with DAPI in blue. This figure is not shown in the revised version as we already included the FACS staining for GC B cells in Fig. 1i.

To further confirm poly-specificity for primary IgM and mono-specificity for PR-IgM, we performed a **direct competition assay** by ELISA. Here, insulin-binding of IgM was assessed after pre-incubation with BSA (untreated control, UT) or soluble dsDNA (+ DNA) as competitors. Effects of competitors on insulin binding are shown relative to insulin binding of the UT samples. As expected, dsDNA reduced the insulin binding of primary IgM drastically, whereas binding of PR-IgM to insulin was only slightly affected (Suppl. Fig. 14). These data support the notion of affinity-matured mono-specific PR-IgM compared to polyreactive and poly-specific primary IgM and are included in the new supplementary figure 14 in the revised version of the manuscript.
Initially, we addressed **IgM affinities** by common ELISA assays[1,2] using biotinylated peptide antigen bound to mono-valent or polyvalent streptavidin to generate different antigen densities. To further confirm our findings, we performed bio-layer interferometric assays[3]. Here, we loaded streptavidin probes with native biotinylated insulin and applied purified insulin-reactive IgM from wild-type mice previously immunized with InsA-KLH. Anti-insulin primary IgM refers to antibodies isolated from serum of mice analyzed at day 7 post immunization, whereas anti-insulin PR-IgM refers to serum of mice analyzed at day 85 (boost on day 78; IgG was depleted prior to IgM pulldown). As recommended by the manufacturer, all antibodies were used at concentrations of 0.5 µg/mL. Interestingly, PR-IgM shows a clear shift towards high-affinity compared to primary IgM regarding insulin binding (Fig. 6f). The presented results suggest a significant affinity-improvement for the memory-derived anti-insulin PR-IgM. We incorporated these new data into figure 6 in the revised version of the manuscript (Fig. 6f).
If the insulin-specific IgM and IgG antibodies are clonally related could not be tested, as this requires single cell sequence analysis which is not established in our group and is out of the scope of this study.

2. The authors build up a narrative that no B cell response to an endogenous protein such as insulin would be expected due to highly efficient tolerance mechanisms during B cell development. Yet at least superficially, there are several other similar models that elicit Ab responses after immunization with endogenous antigens +adjuvant (examples include EAE, arthritis models), so this is not unprecedented.

The referee mentions other established autoimmunity mouse models such as the experimental autoimmune encephalomyelitis model (EAE)[4–6] that vastly relies on the transfer of activated myelin-specific T cells or induction of autoreactive T cells[4–6]. Further, rheumatoid arthritis induced by chicken collagen (CIA) and complete Freud adjuvant (CFA)[7,8] relies heavily on a complex mix of different highly potent adjuvants activating various pathways at the same time. It is not exactly clear, what the underlying mechanism is for the induction of experimental arthritis in these mice.
We believe that, by using insulin as a well-defined and important autoantigen, our approach provides an antigen-specific system for inducing B cell-mediated autoimmunity in wild-type animals. To underline that, we updated the methods section (Mice) explaining in detail how we performed immunization experiments.

Since we only used the CpG adjuvant in the first injection (d0) and since the mice showed repeated signs of autoimmune pathology after boosting without CpG (d21, d42; Fig. 1 – 3) we believe that the effect of the adjuvant is neglectable. Additionally, we included data showing that immunization by injecting only insulin-derived antigen without any CpG results in the generation of insulin-specific antibodies (see point#4 below).

3. The polyvalent insulin immunization differs from the monovalent versions in that they also bring in large protein carriers with foreign T cell epitopes. I realise that this may be difficult to experimentally address, so could the authors at least discuss, the impact of the changes in the antigen beyond the insulin valency. For example, what would be the response to polyvalent insulin linked to an endogenous protein as a carrier.

To address this issue, we performed immunization experiments with native Insulin coupled to a monomeric streptavidin (SAvPhire, Sigmal-Aldrich, SAE0094). This way we generated monovalent Insulin complexes carried by the same exogenous protein as the polyvalent Insulin-SAV complexes. Injections of wild-type mice with the monovalent Insulin-SAV complex did not elicit a significant anti-insulin IgM or IgG response at day 7 and day 14. In full agreement, no changes in blood glucose levels were observed at day 7 post immunization. The presented results underline the necessity of autoantigens to be present in a polyvalent form in order to elicit immune responses. Moreover, this experiment suggests that the foreign protein carrier is most likely not important for autoantibody responses against insulin. We think that these data are important to support the concept presented in the manuscript and included this experiment in the new supplementary figure 3 and discussed it in the revised version of the manuscript.
A polyvalent insulin complex coupled to an endogenous carrier is interesting but needs to be established by comparing different potential carriers. This will be addressed in future studies.

4. In light of the autoantibody responses that can be elicited by endogenous proteins in EAE or arthritis models (often using CFA), is the IgM memory effect seen here adjuvant specific? Similarly, is the CpG used in the recall immunizations.

In this study we used CpG for initial immunizations and did not add the adjuvant to the recall immunizations (compare Fig. 1d, 3c, 4a, 5). To clarify this, we updated the methods section explaining this in detail. We also updated the scheme in figure 1 showing the usage of CpG (Fig. 1c) in the revised version of the manuscript.
Furthermore, to definitely exclude that CpG and TLR-9 signaling is responsible for provoking robust autoantibody responses against insulin, we injected mice with InsA-KLH without any adjuvant. Wild-type mice immunized with InsA-KLH showed detectable insulin-specific IgM and a significant blood glucose increase at day 7. These results clearly demonstrate that the adjuvant (CpG) that we used for initial

immunizations does not break B cell tolerance, and is not required to trigger autoimmune responses.
We integrated these new data into the new supplementary figure 2 in the revised version of the manuscript.

5. The labelling in figure 3 legend is incorrect (going to panel "e').

We apologize for this labelling mistake. Accordingly, we corrected the figure legend.

Referee #2 *describes the performed experiments as "well performed and presented in a clear manner.". Further, she/he refers to the used wild-type autoimmunity mouse model that "this model would indeed be useful, and the authors are correct in cautioning about the use of transgenic models.". We thank the referee for her/his comments that we addressed below. Changes in the manuscript text are marked in pink.*

Specific comments:
1. Can the authors provide histology of the pancreas? Does the increased immune infiltration lead to organ damage? Can the authors provide OGTT following injection of IgG immune complexes? Can the increase in blood glucose following anti-insulin IgG injection be reversed by also injecting Insulin?

Increased immune cell infiltration into the pancreas is a strong indicator of acute pancreas inflammation as suggested in the manuscript. To further examine this, we took supernatants of pancreas cell suspensions of wild-type mice immunized with cInsulin or control immunization (CI) at day 26 and performed a cytometric inflammatory cytokine bead array (BD Biosciences CBA, Cat.: 552364). We observed a significant increase of pro-inflammatory cytokines such as TNF-α and IL-12 in the pancreas supernatant of cInsulin immunized mice (Suppl. Fig. 4). The source of the secreted TNF-α and IL-12 are most likely infiltrating macrophages[9,10] described in the manuscript (Fig. 2e). Further, it is tempting to speculate that the subsequently recruited neutrophils (Fig. 2e) induce transient organ damage[11] leading to temporal acute pancreas inflammation, but not to permanent cell death as the mice become resistant to disease (Fig. 5). We included these data in the new supplementary figure 4 and discussed it in the revised version of the manuscript.
Additionally, to examine if transient pancreas damage occurred in cInsulin immunized mice, we monitored the presence of pancreatic lipase (PL) in serum. Usually, serum PL is used as a biomarker for pancreas damage due to its absence in the serum of healthy individuals. We found that mice immunized with cInsulin show highly increased serum PL titers at day 26 p.i. compared to days 49, 80 or the control immunization. These results indicate that the reported insulin-reactive IgGs (Fig. 1g, h) and the infiltration of immune cells into the pancreas (Fig. 2e) lead to transient organ damage. We think that these data are important to show the extend of autoimmune pancreatitis induced by cInsulin injections and integrated them into figure 2 (Fig. 2f) in the revised version of the manuscript.

Unfortunately, we cannot provide oral glucose tolerance tests (OGTT) following injections of IgG immune complexes since these experiments are not included in the animal permission license used for this study. Similarly, it is technically extremely difficult to calculate insulin concentrations to counteract the anti-insulin-IgG for intravenous injections. The risk of hypoglycemia leading to death of the animals when injecting soluble insulin after i.v. injection of anti-insulin-IgG is too high. Likewise, setting up a suitable facility to produce pancreas sections acceptable for immuno-histochemistry is complicated by the fact that the pancreas is rapidly damaged by its own proteases in vitro after isolation. We will apply for OGTT and work on pancreas histology for future studies.

2. As far as this referee is aware, injection of any peptide with a suitable adjuvant will produce self-reactive B cell clones, as the GC reaction will allow for mutation of recruited germline alleles towards self. Are the anti-insulin responses after extended periods reflective of germline specificity? If not, I'm not sure why this paper challenges the notion that central tolerance effectively removes a large amount of self-reactivity, or will indeed lead to the 'paradigm-shift' claimed in the discussion.

The initial anti-Insulin-IgM responses described in the manuscript show no affinity maturation as compared to memory responses at days 28 and 78 (Fig. 6d). We assume that day 7 IgM responses refer to primary specificity since stable germinal center formation which is required for affinity maturation takes at least 6 – 8 days[12,13]. Efficient plasma cell output from germinal centers is most likely to be present after 8 – 10 days[12–14]. Later IgM responses at day 78 showing the same affinity for Insulin (day 28) clearly refer to memory responses (Fig. 6d).

<u>There are a few technical points that also need addressing:</u>
The use of infinite in the opening line of the manuscript is incorrect.
We changed “infinite” to “enormous” in the summary.

Lines 169-174 are difficult to read and I would recommend re-writing as I do not completely follow the logic.
In the revised version of the manuscript we re-wrote the mentioned passage to ensure a clear structure and good readability.

The injected Mice were aged 8 to 30 weeks. This is a big difference, not least for studying autoreactivity. The MZ is also not fully formed at 8 weeks in all mice. No gender information is provided, again a critical aspect in autoimmunity.

Immunization experiments were exclusively performed with mice aged **8 – 12 weeks**. However, due to monitoring of long-term immune responses in these mice (compare Fig. 5 – 7), we stated an age of mice until 30 weeks. To clarify this, we re-wrote the *Mice* section in the methods part explaining this in detail. In addition, recent studies suggest that marginal zones of mice aged 8 weeks are already fully formed[15–17]. We also included gender information in the methods section, all mice presented were female animals. However, comparable results were also obtained by immunizing

male animals. The usage of only one gender was selected to ensure optimal comparability between groups and control mice were always age-matched.

The statistical analysis is often incorrect. Repeated measure ANOVA needs to be used when sampling on the same mice is done multiple times, and one-way ANOVA in groups of more than one. There seems to be no checks done on the normality of the data and the need for non-parametric approaches.

We updated the methods section *Statistics* and added the strategy we took for statistical analysis. Further, we added the used statistical tests to the figure legends in the revised version of the manuscript.

Referee #3 *describes our study as "a very interesting and important study documenting differential antibody responses against an autoantigen depending on the preparation of the immunogen.". However, she/he states that "insights into the mechanism require additional experiments". We thank the referee for her/his comments that we addressed below. Changes in the manuscript text are marked in blue.*

Major:
1) What is the mechanism of the differential antibody response when monovalent antigens are added? This needs to be discussed with respect to reference 14.

To address this issue, we discussed the potential mechanism with regard to the IgD function described in reference 14.
Moreover, we are investigating the role of IgD-class BCR in the generation of anti-insulin auto-antibodies. Using IgD-deficient mice, we are preparing an independent manuscript, in which we show how monovalent antigen through IgD-BCR modulates immune responses *in vivo*. For this study we include NP-hapten, either soluble or coupled to KLH, as foreign antigen for immunization and test it along with insulin in wild-type as compared with IgD-deficient mice. These resulting data will be submitted soon for publication.

2)The authors suggest that also endogenous insulin could modulate this. Would immunization of hyperinsulinemic vs normoinsulinemic mice with polyvalent antigens also result in differential response? This could be tested.

It would be interesting to immunize hyperinsulinemic mice. We would then expect the hyperinsulinemic mice to show a more drastic shift towards anti-insulin IgM. Further, we expect these mice to become resistant to the disease more quickly due to faster generation of PR-IgM and higher titers. We tried to simulate this scenario by immunizing wild-type mice with ratios of soluble InsA to complex InsA of 100:1 as shown in Figure 4 and Figure 5. In particular, these mice were already protected by day 28 and also resistant to further disease inductions by additional boosts. Treating wild-type mice with increased concentration of insulin to mimic the hyperinsulinemic condition is problematic as it requires an independent animal experimenting license.

Unfortunately, we do not have access to hyperinsulinemic mice, nor do we have such experiments permitted in the animal experimenting license used for this study and therefore cannot conduct the proposed experiments. Nevertheless, we will apply for these experiments in the future.

3) Regarding the characterization of the IgM responses (Fig 6d and 7d-g), the authors need to provide more data on affinity and specificity, including baseline levels (before immunization), raw data, competition assays using different soluble antigens. Also, please provide serum dilutions and IgM concentrations for Fig 7d-g.

To further confirm poly-specificity for primary IgM and mono-specificity for PR-IgM, we performed a **direct competition assay** via ELISA. Here, insulin-binding of IgM was assessed after pre-incubation with BSA (untreated control, UT) or soluble dsDNA (+ DNA) as competitors. Effects of competitors on insulin binding are shown relative to insulin binding of the UT samples. As expected, dsDNA reduced the insulin binding of primary IgM (red) drastically, whereas binding of PR-IgM to insulin was only slightly affected (blue). These data support the notion of affinity-matured mono-specific PR-IgM compared to polyreactive and poly-specific primary IgM. We included these data into the new supplementary figure 14 and discussed it in the revised version of the manuscript as it supports the concept of specificity increase of protective IgM.
For **IgM affinity** see also response to point#1 of Referee#1 and the new data we integrated in Fig. 6f of the revised version of the manuscript.
Concerning **antibody specificity**, we initially addressed this by using HEp2 slides that bear several nuclear molecules. This method is routinely used in the clinic for the characterization of autoreactive antibodies[21]. Binding of IgM to HEp2 cells suggested polyreactivity as observed for early primary IgM but not for late PR-IgM (Fig. 7e, g). Here, we used either serum (Fig. 7e) diluted to **500 ng/mL insulin-specific IgM** (1:20 – 1:50) final concentration, or insulin-specific IgM (Fig. 7g) diluted to **500 ng/mL insulin-specific IgM** (1:200 – 1:600) final concentration. Respectively, we updated the methods section regarding final concentrations used. Detailed serum dilutions and IgM concentrations are now provided in the figure legends (Fig. 6d and 7d – g) and methods section.
At the indicated serum dilution levels, no insulin-specific IgM was detected in naïve wild-type mice prior to immunization. Similarly, the CpG only controls did not show detectable titers of insulin-reactive IgM at the used dilutions.

4) What is the mechanism for the different activities for 7d IgM vs PR-IgM? Shouldn't PR-IgM better neutralize insulin? One may suspect that these are different (polyclonal vs oligoclonal) specificities. Epitope mapping would identify these differences that may result in different avidities or interference with insulin function. Half-lifes of both IgM preparation following injection need to be compared.

Insulin-specific primary and PR-IgM differ clearly in their biochemical properties such as specificity, affinity and therefore avidity (Fig. 6, 7). Importantly, we showed that primary IgM is polyreactive and of low affinity, whereas PR-IgM is monospecific and

of higher affinity (Fig. 7d – g, see response to comment#3). Furthermore, intravenous transfer experiments suggest that insulin-binding by primary IgM leads to neutralization while insulin-binding by PR-IgM leads to protection of insulin (Fig. 7i). Although mice were immunized with an eleven amino acid long insulin-derived peptide and usually epitopes range from 5 to 11 amino acids (Rubinstein et al. 2008), we cannot exclude overlapping epitopes for the different antibodies. Determining the exact epitope of each of the polyclonal antibody is beyond the scope of this study since it requires multiple complicated ELISA set-ups with various overlapping peptide fragments. However, to further support our model of different IgM functions, we included data showing that primary IgM is able to form large immune complexes in the presence of insulin and other antigens such as dsDNA, whereas PR-IgM is unable to do so (Fig. 7h). To test that complex formation is associated with insulin degradation, we added insulin together with primary IgM or PR-IgM to in vitro cultured macrophages. We found that roughly 80% of the insulin can be recovered after 4 h incubation with macrophages in the presence of PR-IgM, while only 10% was recovered when primary IgM was used. These data are not shown, as the direct evidence, i.e. immune fluorescence, for insulin uptake by macrophages is not yet established. However, the proposed mechanism has been explained in the discussion section.

5) The authors need to be careful in presenting the effects causing hyperglycemia, as these are (in case of IgM) caused by neutralization of insulin rather than destruction of pancreatic beta cells, which is the relevant autoimmune pathology.

In the manuscript text, we refer to observed pathologies (Fig. 2, 4 – 7) caused by insulin-neutralizing antibodies as shown by intravenous transfer experiments for instance (Fig. 2h, 6f, 7i). The FACS data that indicate pancreas inflammation (Fig. 2e, f, Suppl. Fig. 4) demonstrate that high titers of insulin-specific IgG are able to initiate pancreatitis and transient pancreas damage that is resolved on day 49 (Suppl. Fig. 11). Since the mice immunized with cInsulin or cInsA recover with time and become resistant to disease induction via anti-insulin PR-IgM, destruction of beta-cells is more likely a transient event and not the main cause of dysglycemia. Thus, neutralization of insulin by primary IgM or IgG is most likely responsible for the presented pathology.

<u>Minor points:</u>
1) Flow cytometric characterization of insulin-specific IgG bound to macrophages. How can the authors exclude the possibility that they are not looking at expression of insulin receptors?

Pancreatic macrophages isolated from mice immunized with cInsulin + CpG or CpG alone were immediately stained for FACS analysis and are most likely already saturated with insulin on insulin-receptors (Fig. 2e). Furthermore, to exclude measuring insulin binding to insulin receptors, we directly compared identically handled pancreatic macrophages from cInsulin and control mice. Since cInsulin

immunized mice show a clear shift compared to the control macrophages for insulin-binding, binding of insulin to the insulin receptor can be excluded.

2) Suppl Fig 4a: Please, also show IgM titers.

In the revised version of the manuscript we included ELISA experiments showing anti-KLH-IgM serum titers of WT mice immunized with different InsA-peptide valence ratios. (now Suppl. Fig. 7a: anti-KLH-IgM, Suppl. Fig. 7b: anti-KLH-IgG). The presented results indicate that the protein carrier has no effect on regulatory mechanisms triggered by soluble InsA.

3) In general, the authors need to clarify in the figure legends the antigens used in the ELISA.

We addressed this problem by updating the figure legends of ELISA experiments clearly stating the antigen used by “(coating: antigen)”.

4) The authors claim that 40% of IgG in immunized mice is specific for insulin. This seems a lot and they should re-evaluate that.

We were also surprised to see such a high amount of IgG binding to insulin after immunization. Yet, we cannot explain this phenomenon. However, to avoid overstatement we edited this passage in the revised version of the manuscript.

5) Figure annotation on page 8, line 306-308 seems incorrect. Please, correct.

We corrected the wrong annotation in the text.

6) The authors mention IgM+ insulin reactive B cells in the discussion, but the data are not provided.

This point is addressed in the new Fig. 1i of the revised version of the manuscript showing IgM^+ GC B cells binding insulin.

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1st Revision - Editorial Decision 10th May 2021

Dear Hassan,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by two of the original referees and their comments are provided below. As you can see the referees appreciate that added data and text changes and support publication here.

I am therefore pleased to let you know that I will accept the manuscript for publication here. Before sending you the formal acceptance letter there are a few editorial points to sort out in a last round of revision.

- You are missing a data availability section. This is the place to enter accession numbers etc. As far as I can see no data is generated that needs to be deposited in a database. If this is correct please state: This study includes no data deposited in external repositories. Please place it after the Materials and methods and before Acknowledgements

- We need a Conflict-of-Interest statement

- The reference section needs to be properly formatted.

- We don't allow data not shown pgs 3 + 13. Please either add the data or re-phrase

- The figures need to be uploaded as single figure files.

- Please double check callouts to Suppl. Fig. panels - I think that they are missing

- The appendix needs a ToC. Please also remove synopsis image and bullet points are from the appendix and uploaded as separate files.

- "Methods" needs correcting to Materials and Methods and should follow the Discussion section.

- If a figure only has one panel it doesn't need a panel label.

- We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

- I have asked our publisher to do their checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

Please submit a point-by-point response to the editorial points when you submit the revised version.

That should be all - Congratulations on a nice paper!

Let me know if we need to discuss anything further

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 8th Aug 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

the authors have done an adequate job of revision and I am now in favour of publication as is

Referee #3:

The authors have responded to all my questions.
In particular the characterisation of the IgM affinity and specificity are valuable new data. I also understand that the use of endogenously hyperinsulinemic mice may be tricky and as straight forward as one would think. I would certainly hope to see data along these lines in the future.
While epitope mapping was not done, the immune complex formation adds some additional insights.
The changes in the text are ok.
Altogether, this is a nice and well conducted study that sheds light on a poorly understood aspect of humoral immunity.

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The EMBO Journal

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Date: 21st May 2021

Dear Karin,

Thank you for handling of our manuscript. We are pleased to be able to publish it with you.
Below you will find our point-by-point response to the editorial points:

- You are missing a data availability section. This is the place to enter accession numbers etc. As far as I can see no data is generated that needs to be deposited in a database. If this is correct please state: This study includes no data deposited in external repositories. Please place it after the Materials and methods and before Acknowledgements
We included a data availability section as suggested.

- We need a Conflict-of-Interest statement
We included a conflict of interest statement in the revised version of the manuscript.

- The reference section needs to be properly formatted.
We formatted the reference section according to the EMBOJ standards.

- We don't allow data not shown pgs 3 + 13. Please either add the data or re-phrase
We re-phrased these sentences on pages 3 and 13.

- The figures need to be uploaded as single figure files.
We uploaded all figures in separate single figure PDF files.

- Please double check callouts to Suppl. Fig. panels - I think that they are missing
We checked the callouts and corrected them properly. All Suppl. Fig. panels are now mentioned within the text.

- The appendix needs a ToC. Please also remove synopsis image and bullet points are from the appendix and uploaded as separate files.
We removed the synopsis image and bullet points from the appendix and uploaded it as separate PDF files. Further, we included a table of contents in the appendix as suggested.

- "Methods" needs correcting to Materials and Methods and should follow the

Discussion section.
We corrected the methods section to Materials and Methods.

- If a figure only has one panel it doesn't need a panel label.
We removed panel labelling “a” from figures with only one panel.

- We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.
We included single PDF files with source data of gels used in the figures.

- I have asked our publisher to do their checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.
We updated the figure legends as suggested by the publisher team.

Sincerely,

Hassan Jumaa

2nd Revision - Editorial Decision 28th May 2021

Dear Hassan,

Thank you for submitting your manuscript to The EMBO Journal. I have now had a chance to take a careful look at everything and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://emboj.embopress.org/about#Transparent_Process

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Corresponding Author Name: Prof. Hassan Jumaa
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2020-107621

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Three or more biological replicates are routinely used and accepted standard in the fied. The sample sizes were dependent on the availability of mice specified by the responsible animal permission council and number calculated by biostatisticians. The exact number of individual mice (n) is stated in the figure legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Age- and sex-matched mice were randomly immunized with stated antigens.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was used.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data sets were analyzed by D'Agostino & Pearson omnibus normality test and/or Shapiro-Wilk normality test in GraphPad Prism software to determine whether they are normally distributed. If one of the data sets was not normally distributed or the sample number n was too small to perform the normality tests, non-parametric tests were used to calculate p-values.

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Is there an estimate of variation within each group of data?	All experiments were reliably reproduced and results are represented as mean +/- SD as indicated in figure legends.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies used in the study were listed with clone number and manufacturer in the methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Species: mouse, strains: C57BL/6, Igalpha f/f mb1cre, gender: female animals were used, age: 8 - 30 weeks old mice were used.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were done in compliance with the German Animal Welfare Act after being reviewed and approved by the Animal Care and Use Committees of Ulm University and the German animal welfare office. (permission: 1484).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	All animal experiments were done in compliance with the German Animal Welfare Act after being reviewed and approved by the Animal Care and Use Committees of Ulm University and the German animal welfare office. (permission: 1484).

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
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15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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