nature portfolio

# Peer Review File

## Crinophagic granules in pancreatic β cells contribute to mouse autoimmune diabetes by diversifying pathogenic epitope repertoire

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**REVIEWER COMMENTS**

**Reviewer #1 (expert in immunopeptidome analysis in T1D):**

Wan and co-workers studied crinophagy, a traditionally overlooked process in endocrine cells for removal of excessive secretory granules, in generating islet beta cell specific antigens and mechanistically investigated their roles in T1D development in NOD mice. This work is conceptually novel, all experiments were well designed and executed, data interpretation is well reasoned, and the large body of comprehensive and complementary evidence (pharmacology, immunopeptidomics, antigen presentation assay, single cell RNAseq, antigen presentation specific T cell assay etc) support their conclusion. This paper is well written, data very well presented. It will be a highly impactful paper in islet beta cell crinophagy, beta cell autoimmunity and the pathogenic mechanism of T1D. It is recommended for publication with minor changes as suggested below.
Comments:
1. It is known that female NOD mice have higher incidence rate of spontaneous T1D than male. Is there a specific reason to use either male or female mice in certain experiments? A following question, did the authors identify sex-related differences in this work?
2. The number of cells used in mass spectrometry-based peptidomics analysis was not mentioned. Please add in Fig 2C.
3. Since as shown in Supp Fig 3a, both phosphorylation and deamidation were identified in the insulin C peptide. When assessing the immunogenicity of phosphorylated peptide, it is suggested to include the version with both phosphorylation and deamidation as controls, instead of only using native C-peptide as control.
4. A very large number of mice (155 WT NOD, 71 NOD.Rag1) were used for identifying the MHC-II peptides in thymus. Typically tens of millions of cells are used in this type of work. Can the authors estimate the number of thymic cells used in this experiment?
5. Conformational insulin. What does it refer to? Considering many forms of insulin may exist, a footnote is needed to describe the actual molecular identity of conformational insulin.
6. Experimental section, Mass Spectrometry. To my knowledge, there is no UltiMate 1000 HPLC system, did the authors mean UltiMate 3000?

**Reviewer #2 (expert in thymic selection, T1D):**

The authors have used an extensive array of techniques to address the role of crinophagic granules in T1D pathology. The studies are focused on the NOD mouse strain. It is important any study that uses this strain the diabetes incidence of the colony and the insulitis score over time is provided. If male and female mice are used interchangeable in different figures, a rationale should be provided as to why, and the T1D incidence and insulitis score for the sexes provided. This information brings clarity to the reader about the correlation in data gained with T1D pathology in the NOD strain.

The authors may wish to consider the following suggestions:

Figure 1. This reviewer does not have expertise in EM, so comments are restricted to the

non-EM data. The authors utilized male NOD mice to assess immunogenicity of crinosomes versus DCGs (Fig. 1d) in presence or absence of chloroquine, but used female NOD mice for the diabetes development study (Fig.1e). Male and female NOD mice are quite distinct in T1D pathology, and for robustness, the authors should consider including female NOD mice as a comparison in Fig 1d. It is also important to be transparent regarding the insulitis score for the male and female NOD mice in the colony. Fig1e is unfortunately difficult to interpret; in the methodology it states that treatment started at 21 days (3 weeks) but Fig1e states treatment started at 28 days (4 weeks). There is some difference between 3-4 weeks of age in female NOD mice, a point when beta cell defects have occurred and autoreactive immune cells are being primed in the PLN- a process that chloroquine may impede, irrespective of its role in impeding crinophagic immunogenicity. In addition, 30 mice were used for the chloroquine treatment group but only 17 for the PBS control. It is important to have as close as possible equal numbers of test and control animals in these types of studies to avoid bias in incidence readout. It would appear that 65% of chloroquine-treated mice developed T1D compared to 85% of PBS controls. It is more correct to say that the treatement slowed T1D development and slightly decreased T1D incidence, but it was not to the extent that the authors state. Please think about rewording the conclusion for this figure.

Fig. 2a: Here male NOD mice are used, it would have been more beneficial to include female NOD mice too as a comparison. Unfortunately, the control of the B:9-23 peptide pulse does not prove that chloroquine does not impede normal APC activity of the macrophages, only crinophagy. A control culture of islet cells (macrophages) pulsed with B:9-23 in the presence or absence of chloroquine is needed to strengthen this conclusion.

Figure 2b: can the authors please provide information whether female or male NOD mice are used in the study, and the age of the mice.

Fig. 3a. The FACS data for mTEC isolation (Suppl Fig.4) shows CD45lo cells included in the sort. Some immune cells express EPCAM, so caution must be used when isolating mTECs solely on CD45/EPCAM (Ly51-) profiles. Inclusion of UEA-1 is an additional marker that is more specific for mTECs would benefit the experiment. Can the authors clarify whether male or female NOD mice are used, and the age of the animals.

Fig3b. This reviewer is struggling to understand how the SD for E21 NOD cultures is so low. By the reviewers calculations, the outlier brings the SD closer to 1350 (upper range). This would decrease significance. To avoid confusion, please provide the mean +/- SD values for all the samples. You may wish to provide this information in the Figure legend for all figures of similar style.

Fig 3e. The thymus of NOD mice changes quite significantly as T1D progresses. In particular a significant increase in thymic B cells, and a change in insulin responses. Whilst the data presented in this figure is interesting, it represents a snap shot of a thymus. Therefore, the authors may wish to repeat the experiment as a time-course over the course of the T1D process to confirm G20 is never presented by thymic APC.

Fig. 3i: can the authors please explain why islet infiltrating B cells were excluded from the assay considering these cells are the most prominent APC in the islets of female mice at this age. Considering Fig 3 is assessing variable insulin peptide presentation between thymus and islets, it would have benefited the paper to do a side by side comparison of the thymus and islets from the same mice to keep consistent the sex, age and disease course of each mouse used.

Figure 5 & Suppl Fig.6. The authors are complimented on the range of tetramers made. However, Suppl Fig. 6d is not convincing in terms of thymic peptide epitope binding tetramers. Could the authors please provide data of control tetramers with scrambled epitopes to enable clarity of what is true binding to the CD4 T cell, and what is unfortunately noise.

Fig. 6, could the authors please provide a rationale for the 2-cycle expansion. Please do

remember that the readership of nature Communications is diverse.

The authors have written a good discussion, particularly the transparency in discussing the caveats of their hypothesis. There is a lot of interesting data regarding beta cell functionality in human T1D, (https://npod.org) and it would be interesting for the authors to include human islets in their studies.

**Reviewer #3 (expert in immunological tolerance and antigen presentation in T1D):**

This research article follows up on a previous study published by the authors in 2020 that characterized the MHC-II peptidome on pancreatic islets of NOD mice. A few years before, Dr. Unanue's lab described the existence of beta cell crinosomes as a source of antigenic insulin peptides. In this paper, the authors convincingly show that the content of these crinosomes is indeed driving pathogenic T cell responses and differs from peptides presented in the thymus. There is a clear distinction between T cells specific to thymically generated peptides and crinosome-derived peptides. These data are significant because they finally demonstrate something that has long been suspected, namely that pathogenic T cells recognize epitopes that are distinct from those displayed during thymic development. As a result, this pool of T cells has not been subjected to thymic deletion or regulatory T cell selection.
This is a well-written article, easy to follow with most experimental approaches summarized in a simple drawing next to the data. There are 4 key notable results:
1- Demonstration that chloroquine significantly reduces the number of crinosomes and their peptide content (including neoepitopes), resulting in reduced stimulation of T cells reactive to crinosome-specific insulin peptides, and ultimately, reduced NOD disease incidence.
2- Demonstration that thymic APCs present antigen to some insulin-reactive T cells, but not to those that are reactive to crinosome peptides.
3- Characterized the MHC-II immunopeptidome of thymic APCs in NOD mice, a truly heroic undertaking given the limited material.
4- Generated sets of MHC tetramers for both thymic epitopes and crinosome epitopes and demonstrated a greater abundance of T cells reactive to crinosome epitopes, with higher Teff/Treg ratio, while the fewer thymic epitope-specific T cells had a lower Teff/Treg ratio.

I do not find major issues with the paper; it is very comprehensive, meaningful controls are used throughout (e.g., B16A mice, HEL-reactive T cells, etc), and multiple T cell clones and tetramers are also used for robustness. As acknowledged in the discussion, the effect of chloroquine treatment on disease incidence may result from multiple effects beside reduced crinophagy, but the antigen presentation assays in Fig.2 are convincing. Based on the authors' claims, one would expect chloroquine not to affect presentation by mTECs and other thymic APCs (Fig.3). Given the different antigen processing mechanisms of mTECs and thymic DCs, this could be interesting to look at that, or at least discuss.

There is a major question that is not addressed or discussed, which is whether crinophagy itself is abnormal in NOD mice. One expects that crinophagy is a physiological phenomenon, but it may be exacerbated in NOD mice, which may contribute to the predominant autoimmune diabetes pathology in these mice (as they are also prone to other autoimmune manifestations). Using the crinosome fraction from the islets of diabetes-resistant control strains in the system described in Fig.1d would help shed light on this. Since C3.g7 cells are used in this assay, the MHC haplotype of the control strains would not matter, only the crinosome content.

Minor points:
-Fig.1b shows the quantification of crinosomes based on multivesicular bodies. Is it not possible to quantify the relative abundance of insulin granules, crinosomes and lysosomes using the immunogold technique?
-The results from Fig.3c are somewhat unexpected and suggest that mTECs can acquire exogenous insulin, process it and present it to T cells just as efficiently as using pulsed B:9-23 peptide within the timeframe of an overnight culture.
-Fig.S4d: as a word of caution, the B220+ CD11c+ gate contains pDCs, but may also contain CD11c+ B cells, which have been previously described.
-The authors are commended for the technical prowess of identifying beta cell antigen epitopes presented in the NOD thymus. Although few peptides (1-2) were identified for each beta cell antigen, these data were successfully leveraged to produce additional MHC tetramers for thymic epitopes. Do the authors think that these epitopes all result from thymic expression or may also be brought in by migrating APCs? For example, there is no convincing evidence that ZnT8 is expressed in the thymus of mice and humans, and evidence suggesting that it is in fact not expressed in the human thymus (Mallone & Kyewski).
-scRNAseq data in Fig.5c shows expression of early activation transcripts in all subsets but naïve T cells. Is it possible that some of these signals are the direct result of TCR stimulation by MHC tetramers during enrichment/sorting rather than reflecting a preexisting signature? Unlike the naïve T cells, antigen-experienced T cells may be less dependent on costimulation and respond to TCR stimulation alone.
-T cells that are thymic epitope-specific seem to be perfectly capable of suppressing each other (as evidenced by blocking experiments in Fig.6) but incapable of suppressing crinosome epitope-specific T cells. Is it possible that these two sets of epitopes are presented by different APCs in the periphery in vivo?
-On page 22, authors should replace “PTAs encoded by Aire, Fezf2 and Chd4” (incorrect) by PTAs controlled (or regulated) by Aire, Fezf2 and Chd4.

**Reviewer #4 (expert in pancreatic β cell biology and crinophagy):**

In this manuscript, Hu et al aim to evaluate the role of beta-cell-derived crinophagic vesicles in type 1 diabetes pathogenesis through a combination of in vivo, ex vivo, and in vitro studies. While these studies have potential functional significance and may provide conceptually important information for the field, several components of the data seem to be over-interpreted or perhaps even misinterpreted and some of the critical relevant literature is not discussed or is not correctly referenced. This is particularly troublesome given the bold claims of the manuscript that are not fully supported by the data as presented. Specific details are listed below:

1. There is a disconnect between the goal of studying the beta cell crinosome specifically (which arises from a direct fusion of the lysosome with secretory vesicles, likely without involvement of the autophagosome) and the use of chloroquine, which the authors state “…has been previously shown to inhibit the formation of autophagolysosomes by impairing the fusion of autophagosomes to lysosomes without affecting the acidification of these organelles.” It is therefore unclear why chloroquine would be used in these experiments unless the authors think that it is perhaps also affecting the lysosome directly in the islets. If this is the case, then the lysosome function must be evaluated more directly (e.g., is

lysosome acidity, localization, or function affected, etc.?). This is particularly relevant given the links between cathepsin function and hybrid insulin peptide formation demonstrated in the literature (PMID 36041196, which does not appear to be cited by the authors).
2. Previous literature suggested that systemic administration of chloroquine may prevent STZ-induced diabetes in a rat model through effects on immune cells. However, a recent clinical trial in autoantibody positive patients failed to observe any benefits of hydroxychloroquine treatment with respect to delaying or preventing diabetes development. This is of course in contrast to the hypothesis and implications that are put forth by the authors in the current study. They do discuss this work, but do so at a somewhat superficial level and misstate the dosing used in the study, indicating that a single dose was used, which is not true. They also implicate that chloroquine would directly impact crinophagy without providing data that it does so (see 1 above). In addition, they fail to discuss critical relevant literature showing that lysosomes appear to be dysfunctional in beta cells of human autoantibody positive organ donors and that islet crinophagy is impaired in the context of human type 1 diabetes (PMID 33515072). This is especially important given that the authors suggest that inhibiting beta cell crinophagy is a route to diabetes delay or prevention.
3. The altered islet crinosome peptide repertoire in animals treated with chloroquine is potentially interesting. However, there is no validation shown that the vesicles being evaluated are indeed fusions of lysosomes with insulin secretory vesicles. Is Lamp1 present in the mass spec dataset? In addition, the comparison of data in Fig. 2E should include an explanation of why what appears to be undetectable data (zero on the y axis) is included in the statistical analysis.
4. With respect to the in vivo chloroquine treatments, there seem to be a number of different dosing schemes used, and the duration of treatment differs between the animals used for monitoring mechanistic changes and those used for monitoring diabetes development. Considering that the authors suggest the human trials may have failed because of insufficient dosing, it would be beneficial to present data using a more standardized dosing regimen. Further, given that systemic chloroquine administration can elicit changes in islet autophagy within mere hours of dosing, it would be beneficial to understand whether chronic dosing is unique in the effects on the crinosome repertoire in comparison to effects that might be realized in more acute settings.
5. The authors show that presentation of insulin B chain peptides by circulating leukocytes is altered in chloroquine treated mice is altered. Does this also occur in a non-diabetes prone mouse model such as the NOD.B16A mouse or the NOR mouse?
6. In the experiments to evaluate in vivo presentation by islet macrophages, can the authors rule out the potential that accidental lysis of islet cells might be contributing to the identification of the G20 epitope?

Minor:
1. It is unclear how crinosomes are specifically defined and quantified from the electron microscopy data in Figure 1C. These details should be included.
2. References 34 and 35 are duplicates from the same paper.

## Point-by-Point Responses to the Reviewers

We sincerely thank all the reviewers for your thorough and insightful reviews of our manuscript. We have carefully considered all the suggestions, which led us to perform extensive experiments to address the raised concerns. Below we provide our detailed, point-by-point responses to each comment. The reviewers' comments are in blue font, and our responses are in black. In the responses to each concern, we present a figure labeled as **Figure R1, R2…etc**., to illustrate newly generated data. In the revised manuscript, changes corresponding to these points have been highlighted in yellow. All references used in this response are cited at the end of this document.

## Reviewer #1 (expert in immunopeptidome analysis in T1D):

Wan and co-workers studied crinophagy, a traditionally overlooked process in endocrine cells for removal of excessive secretory granules, in generating islet β cell specific antigens and mechanistically investigated their roles in T1D development in NOD mice. This work is conceptually novel, all experiments were well designed and executed, data interpretation is well reasoned, and the large body of comprehensive and complementary evidence (pharmacology, immunopeptidomics, antigen presentation assay, single cell RNAseq, antigen presentation specific T cell assay etc) support their conclusion. This paper is well written, data very well presented. It will be a highly impactful paper in islet β cell crinophagy, β cell autoimmunity and the pathogenic mechanism of T1D. It is recommended for publication with minor changes as suggested below.

We deeply appreciate the reviewer's encouraging feedback and constructive suggestions to improve our study. We conducted additional experiments during this revision to address several important questions raised by the reviewer.

Comments:

1. It is known that female NOD mice have higher incidence rate of spontaneous T1D than male. Is there a specific reason to use either male or female mice in certain experiments? A following question, did the authors identify sex-related differences in this work?

Crinophagy has been viewed as a physiological process whereby endocrine cells catabolize excessive secretory granules. Our previous studies observed crinosome formation across both diabetes-resistant B6 and B6g7 (B6 mice expressing I-Ag7 instead of I-Ab) mice, as well as in NOD mice. We detected the production of free insulin B:9-23 peptide in both NOD.Rag1-/- and B6g7.Rag1-/- mice[1]. These results support the notion that crinophagy occurs in β cells irrespective of islet autoimmunity. In these early experiments, we did not observe notable sex-related differences and obtained comparable results between male and female B6 and B6g7 mice. In the current study, we wanted to find an approach to inhibit crinophagy from the initial stage of diabetes development, considering peptides made in crinosomes can be available from the very beginning of the disease. We thought that young NOD male mice would be relevant to assess the effects of chloroquine in this physiological process, while minimizing the effects of autoimmune responses potentially confounding the results.

We fully agree with the reviewer that it is essential to substantiate our data using female NOD mice. We have repeated all the functional experiments on crinosome formation using young female (4-week-old) NOD mice. The results are consistent with those obtained from male NOD mice shown in the original manuscript.

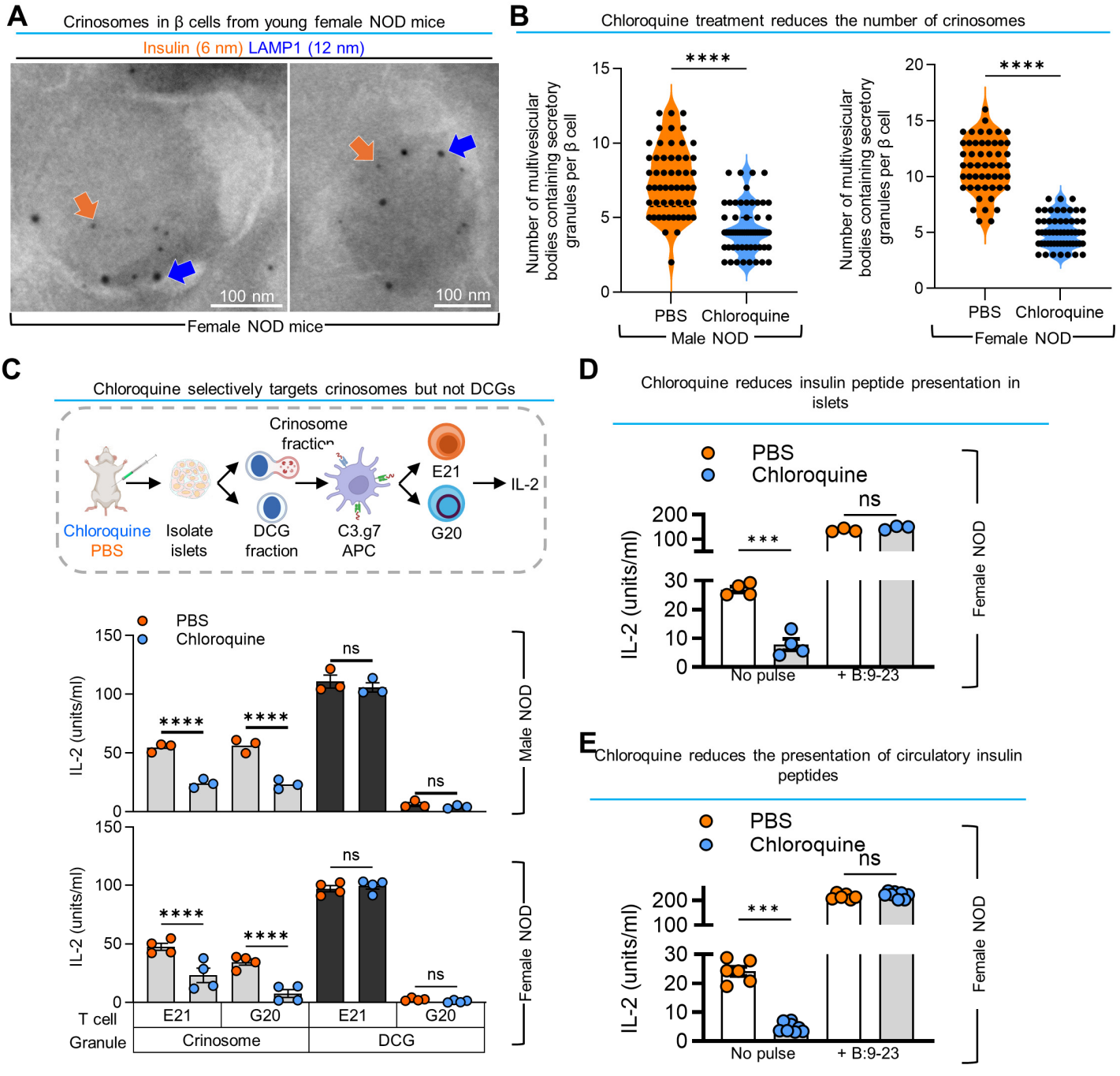
First, using immunogold EM, we identified crinosome-like granules containing both insulin and LAMP1 in β cells from young female NOD mice (**Figure R1A**).

Second, we applied the same short-term chloroquine treatment to female NOD mice and observed a reduction in crinosomes (multivesicular bodies containing secretory granules), as examined by regular electron microscopy (**Figure R1B**).

Third, using the granule-based antigen presentation assay, we confirmed that the chloroquine treatment decreased the production of insulin peptides in the crinosome fraction isolated from female NOD mice, without affecting the regular dense core granules (**Figure R1C**).

Fourth, female NOD mice treated with chloroquine also exhibited a similar reduction in the presentation of the G20 epitope in pancreatic islets (**Figure R1D**) and blood leukocytes (**Figure R1E**) in vivo.

**Figure R1. Consistent effects of chloroquine treatment between young male and female NOD mice.**



**A.** Immunogold Electron microscopy depicting two representative granules containing both insulin (6 nm) and LAMP1 (12 nm) from 4-week-old female NOD mice.

**B.** The violin plots (lower) summarize the numbers of the crinophagic granules per β cell in 6-week-old male or 4-week-old female NOD mice. Each symbol represents individual β cells. ****P < 0.0001; Mann-Whitney test.

**C.** Schematic (upper) depicting an antigen presentation assay designed for testing the presentation of the insulin epitope G20 or E21 contained in crinosome or DCG subcellular fractions isolated from islets of NOD mice given PBS or chloroquine. The bar graphs (lower) summarizes results (mean ± s.e.m) from three independent experiments in 6-week-old male or 4-week-old female NOD mice. Each symbol represents one biological replicate including 2-5 mice. ns, not significant; ****P < 0.0001; Mann-Whitney test.

**D.** Presentation of G20 by dispersed islet cells (islet resident macrophages) from 4-week-old female NOD mice given PBS or chloroquine without antigen pulse or during pulse with the B:9-23 peptide (10 μM). Data (mean ± s.e.m) summarize results from two independent experiments; each symbol represents one biological replicate including 2-5 mice. ns, not significant; ***P < 0.001; Mann-Whitney test.

**E.** Spontaneous presentation of G20 by blood leukocytes isolated from 4-week-old female NOD mice given PBS or chloroquine without antigen pulse or during pulse with the B:9-23 peptide (10 μM). Data (mean ± s.e.m) summarize results from two independent experiments; each symbol represents results from an individual mouse. ns, not significant; ***P < 0.001; Mann-Whitney test.

In summary, these new experiments indicate that crinosome formation is similar between young male and female NOD mice, which can be inhibited by treatment with chloroquine. Consistent with our previous studies, we did not observe notable sex-related differences in these experiments. We have included these results in the revised Figure 1c, 1d, 1e and Supplementary Figure 2a, 2b.

2. The number of cells used in mass spectrometry-based peptidomics analysis was not mentioned. Please add in Fig 2C.

In this analysis, each condition (PBS or chloroquine) included 1.2 x $10^6$ live β cells from ~1,500 islets purified from 10 mice. We have added this information to the corresponding figure legends.

3. Since as shown in Supp Fig 3a, both phosphorylation and deamidation were identified in the insulin C peptide. When assessing the immunogenicity of phosphorylated peptide, it is suggested to include the version with both phosphorylation and deamidation as controls, instead of only using native C-peptide as control.

In the crinosome peptidome, we identified a plethora of C-peptide sequences harboring phosphorylation and/or deamidation. Most of these harbored either phosphorylation or deamidation, with very few containing both changes. For the purpose of brevity, we presented the spectrum of a peptide having both phosphorylation and deamidation in Supplementary Fig. 3a, as an example. We recognize that our original manuscript did not clearly convey that most peptides contained either phosphorylation or deamidation, not both. We have revised the text to clarify this point.

In line with studies in human C-peptide, we previously found that the full-length native proinsulin-1 C-peptide conferred immunogenicity in NOD mice[2]. Moreover, the deamidation-induced Q-to-E conversion in the native C-peptide sufficiently enhanced its binding affinity to I-Ag7, leading to elevated T cell autoreactivity[2]. In this study, we sought to determine whether the phosphorylation change (phosphorylated serine) would similarly alter the immunogenicity of the native C-peptide. The initial results showed that T cell responses to the phosphorylated C-peptide were comparable to those to the native C-peptide, indicating a minimal effect of phosphorylation on boosting autoreactivity.

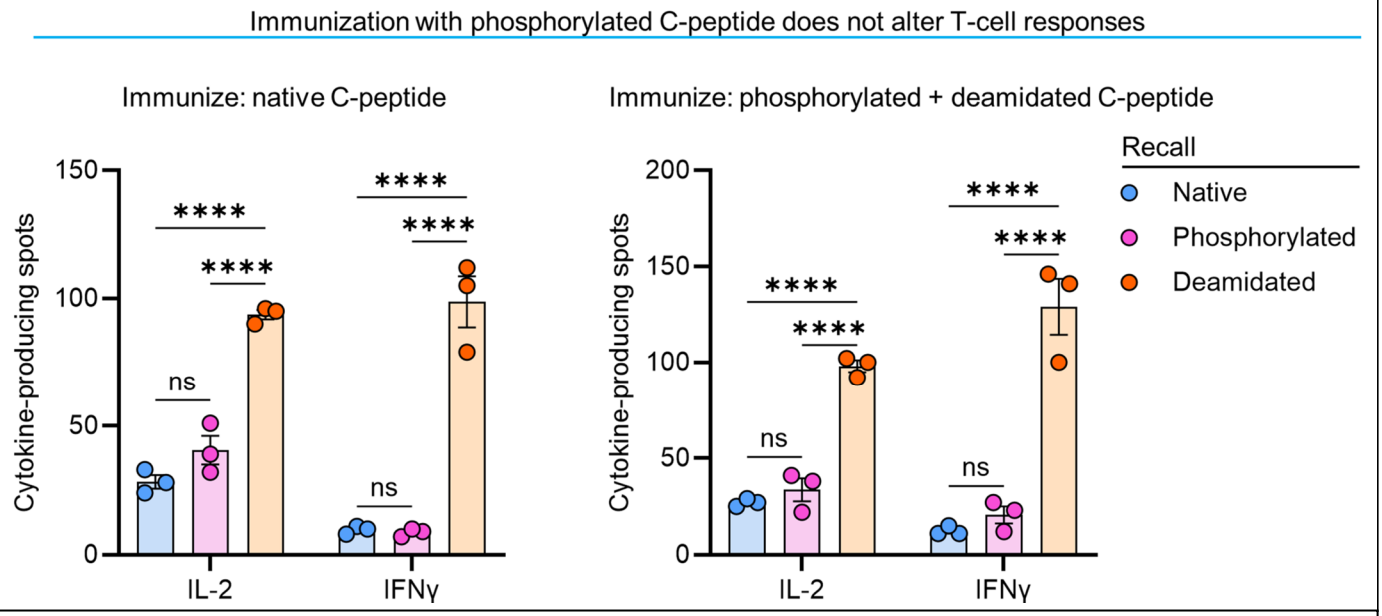
We agree with the reviewer that this conclusion needs to be substantiated by a side-by-side comparison among the deamidated, phosphorylated, and native C-peptide. To this end, we first expanded our original experiment where we immunized NOD mice with the native C-peptide

(EVEDPQVEQLELGGSPGDLQTLAEVARQ). In addition to recalling with the native and phosphorylated C-peptide (EVEDPQVEQLELGGsPGDLQTLAEVARQ), we also included a representative deamidated C-peptide (GDLQTLAEVARE). We detected robust responses to the deamidated C-peptide, while the reactivities to both the native and phosphorylated peptides were comparable and significantly lower (**Figure R2, left**).

In parallel, we performed new experiments in which NOD mice were primed by immunization with a mixture of the phosphorylated and deamidated C-peptides, followed by recalling with the native, phosphorylated, and deamidated peptides individually. In this context, the deamidated peptide again triggered strong T cell responses, in contrast to significantly weaker responses from either the native or phosphorylated peptide (**Figure R2, right**).

In summary, unlike deamidation, the identified phosphorylation in C-peptide did not significantly alter T cell reactivity. We have included these results as the new Supplementary Fig 3b.

**Figure R2. T cell responses to native, phosphorylated, and deamidated C-peptides.**




ELISPOT assay assessing T cell responses during recall with the full-length native insulin-1 C-peptide (EVEDPQVEQLELGGSPGDLQTLAEVARQ), the phosphorylated C-peptide (EVEDPQVEQLELGGsPGDLQTLAEVARQ), or the deamidated C-peptide (GDLQTLAEVARE), or the modified version with a phosphorylated serine, in mice immunized with either the native C-peptide (left) or a mixture of the phosphorylated and deamidated C-peptides (right). Data (mean ± s.e.m) summarize results from individual mice (each symbol). ns, not significant; Two-way ANOVA analysis with Tukey's multiple comparisons test.

4. A very large number of mice (155 WT NOD, 71 NOD.Rag1) were used for identifying the MHC-II peptides in thymus. Typically tens of millions of cells are used in this type of work. Can the authors estimate the number of thymic cells used in this experiment?

We apologize for not including the cell numbers in the original manuscript. For the 155 WT NOD experiment, we partially removed the thymocytes by gentle agitation and obtained about 1 x $10^9$ cells per mouse. The total cell count from all 155 mice was approximately 150 x $10^9$ cells. For the experiments using 71 unmanipulated NOD.Rag1-/- mice or 71 NOD mice with further thymocyte removal, we obtained about 3-4 x $10^7$ cells per

mouse. Thus, each experiment used about 3 x $10^9$ cells in total. We have included this information in the Methods.

5. Conformational insulin. What does it refer to? Considering many forms of insulin may exist, a footnote is needed to describe the actual molecular identity of conformational insulin.

We initially used the term 'conformational insulin' to refer to intact insulin that retains its native three-dimensional structure, in contrast to denatured or degraded insulin peptides. We acknowledge that the term 'conformational insulin' was not precise enough. We have revised the text to use 'intact insulin' consistently, which more accurately describes the molecular identity of the insulin being discussed.

6. Experimental section, Mass Spectrometry. To my knowledge, there is no UltiMate 1000 HPLC system, did the authors mean UltiMate 3000?

Thanks for pointing out this error. Yes, we intended to mean “UtiMate 3000”. This has been corrected.

## Reviewer #2 (expert in thymic selection, T1D):

The authors have used an extensive array of techniques to address the role of crinophagic granules in T1D pathology. The studies are focused on the NOD mouse strain. It is important any study that uses this strain the diabetes incidence of the colony and the insulitis score over time is provided. If male and female mice are used interchangeable in different figures, a rationale should be provided as to why, and the T1D incidence and insulitis score for the sexes provided. This information brings clarity to the reader about the correlation in data gained with T1D pathology in the NOD strain.

The authors may wish to consider the following suggestions:

1. Figure 1. This reviewer does not have expertise in EM, so comments are restricted to the non-EM data. The authors utilized male NOD mice to assess immunogenicity of crinosomes versus DCGs (Fig. 1d) in presence or absence of chloroquine, but used female NOD mice for the diabetes development study (Fig.1e). Male and female NOD mice are quite distinct in T1D pathology, and for robustness, the authors should consider including female NOD mice as a comparison in Fig 1d. It is also important to be transparent regarding the insulitis score for the male and female NOD mice in the colony. Fig1e is unfortunately difficult to interpret; in the methodology it states that treatment started at 21 days (3 weeks) but Fig1e states treatment started at 28 days (4 weeks). There is some difference between 3-4 weeks of age in female NOD mice, a point when β cell defects have occurred and autoreactive immune cells are being primed in the PLN- a process that chloroquine may impede, irrespective of its role in impeding crinophagic immunogenicity. In addition, 30 mice were used for the chloroquine treatment group but only 17 for the PBS control. It is important to have as close as possible equal numbers of test and control animals in these types of studies to avoid bias in incidence readout. It would appear that 65% of chloroquine-treated mice developed T1D compared to 85% of PBS controls. It is more correct to say that the treatement slowed T1D development and slightly decreased T1D incidence, but it was not to the extent that the authors state. Please think about rewording the conclusion for this figure.

We agree with the reviewer that it is essential to evaluate the effects of chloroquine in crinosome formation in female NOD mice. We have repeated all the functional experiments on crinosomes using young (4-week-old) female NOD mice. These new experiments are detailed in our response to a similar concern raised by Reviewer 1. Please see our response to comment #1 of Reviewer 1 (**Figure R1**).

The new data obtained from young female NOD mice are consistent with those from male mice shown in the original manuscript. We have included these results as the new Figure 1c, 1d, 1e and Supplementary Figure 2a, 2b in the revised manuscript. Below we briefly summarize these findings:

- By immunogold EM, we found crinosome-like granules in β cells from female NOD mice, which contained both insulin and LAMP1 (**Figure R1A**).
- By regular EM, we observed a similar of reduction in crinosomes in female NOD mice given the chloroquine treatment (**Figure R1B**).
- By granule-based antigen presentation assay, we found that the chloroquine treatment specifically diminished insulin peptide production in crinosome fractions purified from female NOD mice, while having minimal influences in DCGs (**Figure R1C**).
- By in vivo presentation, treatment with chloroquine resulted in a reduction in the presentation of the G20 epitope in either pancreatic islets (**Figure R1D**) or blood leukocytes (**Figure R1E**).

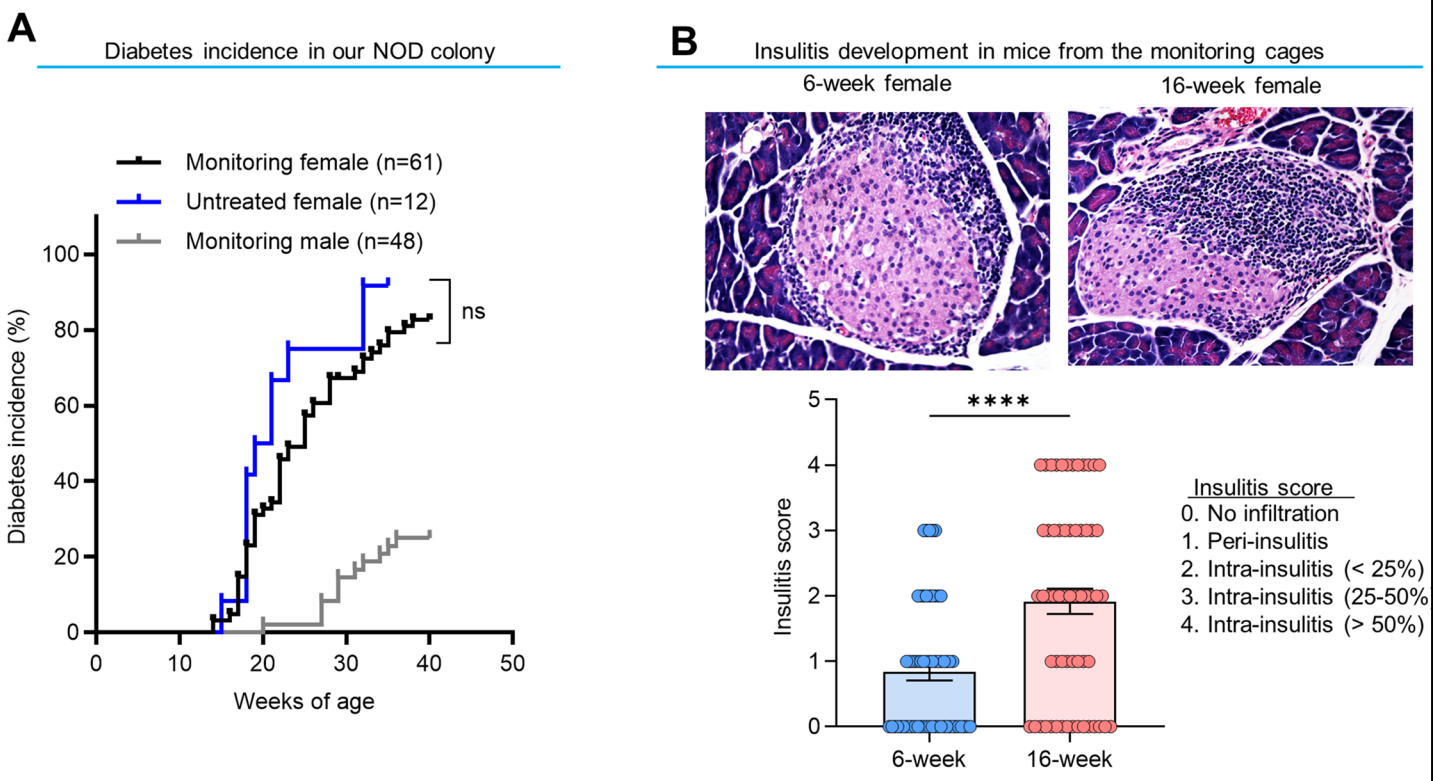
We also agree that diabetes incidence in NOD mice is variable between colonies. We have been paying extra caution to this issue. We routinely refresh our NOD breeders with JAX mice, to ensure minimal genetic drifting in our colony. We also set up diabetes monitoring cages on a rolling basis, beside any other experimental mice. Here we show the cumulative diabetes incidence of female NOD mice in our monitoring cages over the past three years (61 mice), when most of the experiments in this paper were done (**Figure R3A**). We also routinely perform H&E staining in pancreatic tissues from mice in our monitoring cages. As shown in **Figure R3B,** there is progressive insulitis development between 6- and 16-week-old female prediabetic NOD mice. Please also note the incidence of male NOD mice (n=48) in our monitoring cages. Thus, our NOD colony does not show genetic drift impacting on diabetes development. Notably, the incidence in the female NOD mice in the monitoring cages largely overlaps with that of the 12 untreated mice which were monitored along with those given PBS or chloroquine (**Figure R3A**). Thus, diabetes incidence in our colony has been consistent, which justify changes observed in the chloroquine experiment. We have included these results as the new Supplementary Figure 1f and 1g in the revised paper.

The treatment with chloroquine was started in 3-week-old (21 days) female NOD mice. We apologize for mistakenly stating the wrong age in the result text. The description in the Methods is correct. We agree that 3 weeks of age is a critical priming time point in the pLN, as demonstrated by a previous fundamental study[3]. In our earlier studies, we found that crinosome-derived insulin peptides (G20 epitope) are released from islets to circulation, whereby they can enter peripheral lymph nodes for recognition by T cells[1,4]. In this study, we found that mice given chloroquine had reduced circulatory insulin peptides that were presented by blood leukocytes, suggesting impaired peripheral priming. This could be explained by results showing that chloroquine directly diminished insulin peptide production in crinosomes. However, as stated in the discussion, a notable caveat in this study is potential off-target effects by chloroquine. We could not exclude the possibility that chloroquine may directly affect peripheral priming. We have acknowledged the reviewer's perspective in the revised discussion.

Regarding the diabetes monitoring experiments, beside the PBS control (n = 17), we also included 12 untreated mice as control. Diabetes onset and incidence in the untreated mice were comparable the PBS group. We consider this a meaningful result because it minimizes our concern whether continuous intraperitoneal injections may artificially alter diabetes incidence. Compared to either the PBS or untreated control, diabetes incidence in the chloroquine treatment group is statistically different. Also, the Log-rank (Mantel-Cox) test used to calculate statistics significance is known for its power to handle different numbers of animals in different groups, and the P-value reflects both onset time and incidence. Therefore, we believe that our approach is valid.

We stated in our original manuscript that diabetes incidence was reduced by about 50% in the chloroquine treatment group. We meant to express that the incidence of the chloroquine group was approximately half of the PBS or untreated group in most time points during the monitoring period. We recognize that this statement could be confusing if readers interpret it as of the incidence at the terminal time point and have revised this sentence as "*while all the PBS-treated mice eventually became diabetic, about 33% of mice in the chloroquine group remained diabetes free by 35-weeks of age*". In the revised discussion, we also acknowledged that the protection is partial and discussed this phenotype in the context of crinophagy in advanced diabetes stages as well as in humans.

**Figure R3. Diabetes incidence and insulitis development in our colony.**




**A.** Diabetes incidence in female NOD mice in separate monitoring cages versus untreated control mice that were monitored alongside mice given PBS or chloroquine. Diabetes incidence of male NOD mice in the monitoring cages is also shown. ns, not significant; Log-rank (Mantel-Cox) test.

**B.** Insulitis development between 6-week and 16-week non-diabetic female NOD mice in the monitoring cages. Shown are representative H&E images and quantification of insulitis scores of 60 islets from five mice per age. ****P < 0.0001; Mann-Whitney test.

2. Fig. 2a: Here male NOD mice are used, it would have been more beneficial to include female NOD mice too as a comparison. Unfortunately, the control of the B:9-23 peptide pulse does not prove that chloroquine does not impede normal APC activity of the macrophages, only crinophagy. A control culture of islet cells (macrophages) pulsed with B:9-23 in the presence or absence of chloroquine is needed to strengthen this conclusion.

The experiment has been reproduced in 4-week-old female NOD mice. Considering that islet macrophages may be exposed to chloroquine in vivo, we intended to use the B:9-23 peptide control to measure whether the chloroquine treatment may alter their ability to present exogenous free insulin peptide. The effect of in

vitro chloroquine treatment in the presentation of insulin peptides vs protein has been extensively tested in our previous studies in different APCs. In one study, we examined presentation of B:9-23 versus intact insulin in CD11c+ splenic DCs that were exposed to chloroquine in vitro. The data indicate that chloroquine only

[Redacted]

**A.** Control experiments using DCs incubated with insulin or peptide in the presence of chloroquine.
**B.** Antigen presentation assay showing responses of an Ins1C-specific CD4+ T cell hybridoma to C3g7 APCs treated with or without chloroquine and pulsed with the full-length 29-residue Ins1C:33-61.
**C.** Antigen presentation assay showing responses of IIT-3 and 9B9 T cells to ConA-activated peritoneal macrophages pulsed with indicated concentrations of intact insulin. Before antigen pulse, the macrophages were treated with PBS or chloroquine for 2 hours followed by washing.
**D.** Procedures are as described in C. ConA-activated peritoneal macrophages were pulsed with free B:9-23 peptide instead of intact insulin.

blocks processing and presentation of intact insulin to the E21-specific IIT-3 T cell, without any influence free B:9-23 (measured by the G20-specific 8F10 T cell) (**Figure R4A**). In a more recent study, we found that chloroquine has no influence in the presentation of the 29mer full-length C-peptide, indicating that even a long C-peptide can bypass internal processing (**Figure R4B**). This experiment used the C3g7 APC, a standard B-cell lymphoma APC line expressing I-Ag7.

It is technically challenging to perform in vitro chloroquine treatment and associated antigen presentation assay in islet cells (macrophages). We typically use 5 x $10^4$ islet cells per well, which contains about 500 islet macrophages. We found that during chloroquine treatment (our standard protocol is to treat APCs for 2 hours followed by washing), most islet macrophages will become adherent to culture wells. This issue, along with subsequent washing steps, results in a loss of most islet macrophages. To further address the reviewer's concern, we used ConA activated peritoneal macrophages, which mimic the activated features of the islet macrophages. In vitro chloroquine only inhibited presentation of E21 (by IIT-3) by peritoneal macrophages pulsed with intact insulin (**Figure R4C**) but did not affect the presentation of free B:9-23 peptide to both IIT-3 and 9B9 T cells (**Figure R4D**). These results are highly consistent with those observed in our previous studies.

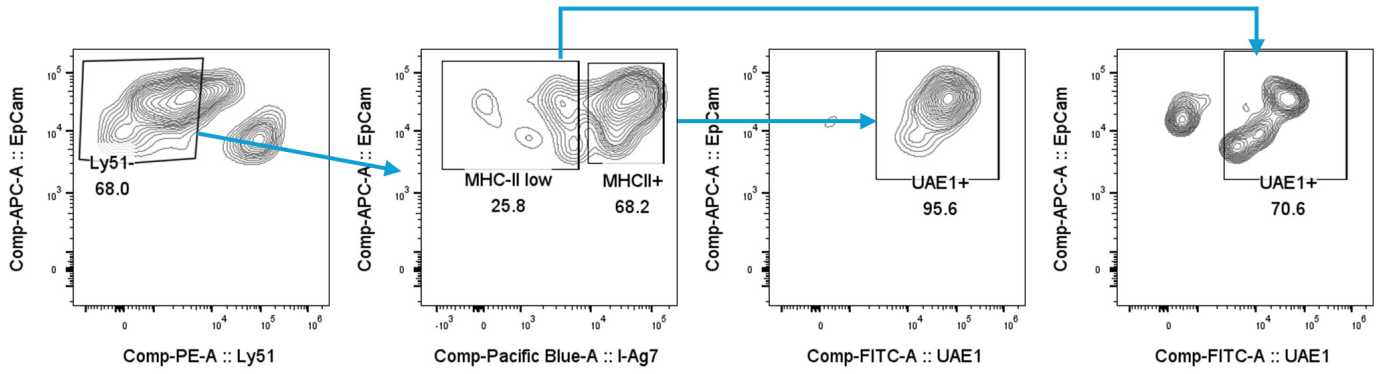
3. Figure 2b: can the authors please provide information whether female or male NOD mice are used in the study, and the age of the mice.

   The original experiments used 6-week-old male NOD mice and the results are reproduced in 4-week-old female NOD mice, as shown in **Figure R1E**.

4. Fig. 3a. The FACS data for mTEC isolation (Suppl Fig.4) shows CD45lo cells included in the sort. Some immune cells express EPCAM, so caution must be used when isolating mTECs solely on CD45/EPCAM (Ly51-) profiles. Inclusion of UEA-1 is an additional marker that is more specific for mTECs would benefit the experiment. Can the authors clarify whether male or female NOD mice are used, and the age of the animals.

   We appreciate this concern. Here we demonstrate a control experiment which was performed to decide the sorting strategy of I-Ag7+ mTECs. In this experiment, we used UEA-1 to verify the identity of mTECs. As shown in **Figure R5**, a vast majority of MHC-II+ mTECs (95.6%) also expressed UEA-1. We indeed observed less UEA-1+ cells in the MHC-II-negative or low population. Based on these results, we reasoned that including MHC-II (I-Ag7) would largely ensure the specificity of mTECs, which also aligns with the goal of the experiments to measure antigen presentation. We have noted this in the corresponding Figure legend. For FACS-sorting, due to limited space for markers, we could not include UEA-1. For FACS-sorting, we faced

**Figure R5. Further validation of mTEC sorting.**



constraints in the number of markers that could be included. Due to limited space for markers, we could not include UEA-1 in our primary experiments.

All the thymic antigen presentation shown in Figure 3 involved 3-6-week-old female and male NOD mice, when thymic selection is active. Since a big number of mice are used in these experiments (detailed more in the response to comment #7), we included both female and male mice between 3 and 6 weeks of age.

5. Fig3b. This reviewer is struggling to understand how the SD for E21 NOD cultures is so low. By the reviewers calculations, the outlier brings the SD closer to 1350 (upper range). This would decrease significance. To avoid confusion, please provide the mean +/- SD values for all the samples. You may wish to provide this information in the Figure legend for all figures of similar style.

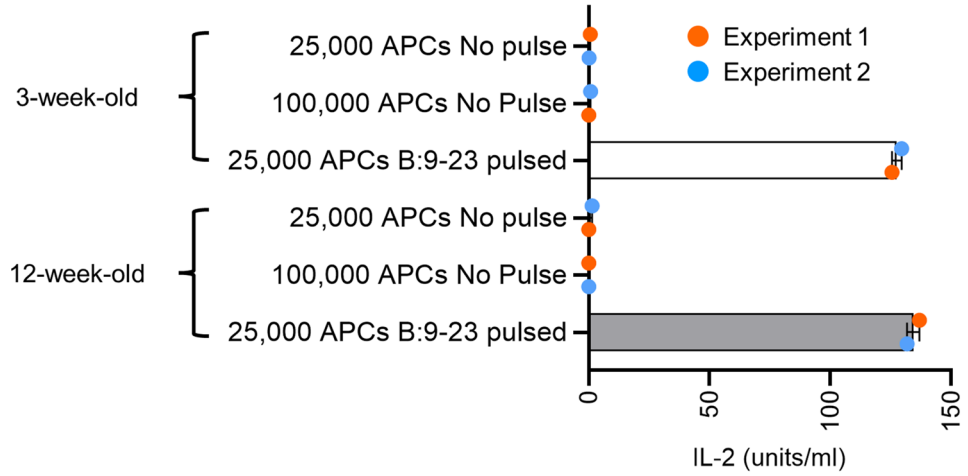
   As stated in the Figure legends, the error bars represent SEM rather than SD. Since the experiments were repeated for multiple times, SEM would appear lower than SD (should SD was used). In our view, it is expected to observe fairly big levels of variation in the spontaneous presentation of mTECs, due to the following reasons. First, we aimed to measure spontaneous presentation by mTECs without any additional antigen pulse, therefore the observed the responses solely depend on the amount of the epitope that was being presented by mTECs naturally at the time when the experiment was performed. Second, in these experiments, we sought to detect the presentation of a single insulin epitope, which is expected to be at a low level by mTECs. Third, the statistical significance was based on at least four independent experiments, which justifies the use of SEM. Lastly, in addition to internal controls, we included mTECs from B16A mice as a critical biological control. The fact that E21 presentation was below the detection limit in B16A mice further validates the results showing E21 presentation in mTECs from WT NOD mice. Previous studies have suggested that only 1-3% mTECs may express insulin at a given time[5]. Given these findings, we feel that the range of the data points in Figure 3b reflect the variable nature of spontaneous presentation of self-insulin epitopes by mTECs. The raw data points (E21 presentation by NOD mTECs) are as follows: 881, 1681, 768, 695, 406. SEM: 213.6. We have also submitted Source data files including raw numbers of all the data.

6. Fig 3e. The thymus of NOD mice changes quite significantly as T1D progresses. In particular a significant increase in thymic B cells, and a change in insulin responses. Whilst the data presented in this figure is interesting, it represents a snap shot of a thymus. Therefore, the authors may wish to repeat the experiment as a time-course over the course of the T1D process to confirm G20 is never presented by thymic APC.We fully agree that a time course analysis would offer valuable insights. However, executing such an experiment is challenging due to the labor-intensive nature of FACS-sorting sufficient individual thymic APC subsets to test the spontaneous presentation of single insulin epitopes (as detailed in the response to comment #7 below). Including multiple time points while sorting various APC subsets in the same experiment is particularly difficult. To address this concern, we conducted new experiments where we FACS-sorted bulk CD45+MHC-II+ thymic APCs from either 3- or 12-week-old female NOD mice. We found that an exogenous pulse with B:9-23 induced strong presentation of G20, as expected (**Figure R6**). However, spontaneous G20 presentation by either 25,000 or 100,000 APCs was not detected in young and old NOD mice (**Figure R6**). These results were consistently observed across two independent experiments.

   While these results suggest that thymic presentation of G20 is minimal even at an advanced disease stage, we respectfully request to include these findings in a future study. As discussed in the original manuscript, it is challenging to conclude that a given epitope is completely absent in the thymus due to sensitivity limitations in thymic antigen presentation and immunopeptidome analysis. We believe that such data needs to be supported by T cell biology experiments in parallel, as shown in the current study. We have an ongoing project in which we are following phenotypic changes in all epitope-specific CD4 T cells (both thymic and islet sets)

over the disease course. We feel that the results shown in Figure R7 would be more suitable to include in this ongoing study, where a comprehensive time-course analysis can be more thoroughly explored.

**Figure R6. Thymic APC presentation in 3- and 12-week-old female NOD mice**




CD45+MHC-II+ APCs were FACS-sorted from thymic of 3- or 12-week-old female NOD mice. Indicated numbers of the APCs were used to culture with the 9B9 T cell hybridoma with or without pulse with the B:9-23 peptide.

7. Fig. 3i: can the authors please explain why islet infiltrating B cells were excluded from the assay considering these cells are the most prominent APC in the islets of female mice at this age. Considering Fig 3 is assessing variable insulin peptide presentation between thymus and islets, it would have benefited the paper to do a side by side comparison of the thymus and islets from the same mice to keep consistent the sex, age and disease course of each mouse used.

We appreciate the reviewer's insight regarding islet-infiltrating B cells in Fig. 3i. We and other investigators have examined insulin MHC-II presentation by B cells, especially in the context of insulin-specific B cells[6–12]. In an earlier study, we showed that insulin-specific B cells from germinal centers of various lymph nodes present both E21 and G20, leading to production of class-switched insulin autoantibodies[6]. Similarly, circulating B cells were able to capture insulin peptides released from islets and present both E21 and G20[4], an aspect further examined in the current study in the context of chloroquine treatment. Based on these results, we expected that intra-islet B cells would spontaneously present both E21 and G20. Considering that presentation by other islet APCs, like macrophages, DC1, and DC2, remain less clear, we prioritized these APCs in this study. Examining MHC-II antigen presentation in T1D by professional and non-professional APCs has been a long-lasting interest in the lab.

In the experiment depicted in Fig. 3i, the FACS sorter in our facility could allow for sorting four populations. In addition to intra-islet macrophages, DC1, and DC2, we opted to examine MHC-II+ intra-islet endothelial cells (CD45-CD31+). In our other ongoing experiments, intra-islet endothelial cells (CD45-CD31+) do not express MHC-II+ at steady state but start to upregulate MHC-II and costimulatory molecules during islet autoimmunity. This unexpected finding led us to explore the role of endothelial cell in presenting T1D-related antigens by MHC-II. Therefore, we respectfully ask that we report these results in a different manuscript.

We also agree that the best approach would be to isolate thymic and islet APCs from the same mice and perform a side-by-side comparison. Please note that most of the experiments shown in Figure 3 were aimed at detecting spontaneous presentation rather than conventional exogenous antigen pulse. This endeavor requires a substantial number of APCs involving many mice per experiment. For example, detecting spontaneous presentation of a single insulin epitope by mTECs required including 150,000 FACS-sorted I-Ag7+ mTECs per well, involving about five mice. This estimation was based on the fact that only 1-3% of

mTECs may express insulin at any given time. Each experiment shown in Figure 3b simultaneously tested mTECs from two strains (NOD and B16A) for two insulin epitopes (G20 and E21), which required at least 20 mice. Similarly, isolating pancreatic islets required injecting collagenase via the common bile duct, a microsurgery-like procedure using a dissection microscope, after which, individual islets needed to be handpicked under a regular microscope, dispersed, and used for FACS-sorting. It is also worth noting that MHC-II+ APCs only constitute about 5-10% of the total islet cells[13], with even smaller numbers for specific macrophages, DC1s, and DC2s. Therefore, because isolation of either thymic or islet APCs is highly labor intensive, with each procedure taking about 8-10 hours to finish, the current scale of our laboratory is unable to do both at the same time.

8. Figure 5 & Suppl Fig.6. The authors are complimented on the range of tetramers made. However, Suppl Fig. 6d is not convincing in terms of thymic peptide epitope binding tetramers. Could the authors please provide data of control tetramers with scrambled epitopes to enable clarity of what is true binding to the CD4 T cell, and what is unfortunately noise.

We appreciate the reviewer's feedback and recognition of our work on tetramers. The data in the original Supplementary Fig. 6d show each tetramer-binding T cell population in the thymus, whereas their counterpart populations in the secondary lymphoid organs (SLOs) are depicted in Fig. 5a. We agree that the populations in the thymus did not appear as prominent as those in the periphery. This could be due to the global thymic T cell repertoire being more diverse than the peripheral repertoire, leading to a lower frequency of tetramer-binding T cells in the thymus. This phenomenon is particularly evident for tetramer-binding T cells recognizing the four thymic epitopes, which may undergo a certain level of clonal deletion.

I-Ag7 is a notoriously difficult MHC-II molecule to produce tetramers, due to its "promiscuous" binding property. Most of the tetramer work in this study used I-Ag7 based monomers incorporating the G20 or E21 insulin epitope. These reagents were produced in house (in the Teyton laboratory) and have been carefully validated in previous publications[14,15]. In our experience, the most reliable validation approach is a functional assay: testing whether plate-bound I-Ag7:peptide monomers could activate their respective epitope-specific CD4 T cell hybridomas. This approach was used in the original study[14] (Teyton group; **Figure R7A**) and in the present study (original Supplementary Fig. 6c; **Figure R7B**). Results from both laboratories show that the two insulin epitope monomers specifically activate their respective epitopes without cross-reactivity, despite that the two epitopes only differ by a single residue shift. We also demonstrate that none of the I-Ag7:insulin epitope molecules were able to stimulate an HEL-specific hybridoma specific for the HEL11-27 peptide (a defined I-Ag7 binder).

To our knowledge, using scrambled epitopes may cause two issues. First, a "randomized" scrambled epitope may not have MHC-II binding affinity to stabilize the MHC-II molecules, leading to failure of tetramer production. Second, a given scrambled peptide with binding capacity may be recognized as a foreign peptide. Thus, its corresponding tetramer may still detect a population of CD4 T cells and cannot be used for control purposes. For control purpose, the Teyton lab produced the I-Ag7:HEL11-27 tetramer. In a recent publication[16], the I-Ag7:HEL11-27 tetramer stained less cells than the I-Ag7:G20 tetramer (**Figure R7C**), providing validation with a T1D-irrelevant epitope. In our hands, we had conducted control experiments in which the two I-Ag7:G20/E21 tetramers were used to stain peripheral cells from NOD.H4 mice, which express I-Ak instead of I-Ag7. There were no detectable T cells in the NOD.H4 mice (**Figure R7D**), confirming that the tetramer reagent is largely I-Ag7 specific.

[Redacted]

**A.** Register-specific activation of type A and B hybridoma T cells using recombinant I-Ag7/peptide–coated wells. This result is representative of a minimum of five similar experiments. Each point is a biological triplicate (SDs are represented for each triplicate). cpm, counts per minute.
**B.** Responses of the E21- and G20-specific CD4+ T cell hybridomas to plate-coated E21:I-Ag7 and G20-20:I-Ag7 monomers. An HEL-specific T cell hybridoma was included as a negative control.
**C.** Typical example of a I-Ag7Ins12-20 tetramer staining on peripheral blood with or without magnetic enrichment. The HEL11-27 peptide was used as a control.
**D.** An example of minimal staining with the I-Ag7:G20 and I-Ag7:E21 tetramers in NOD.H4 mice.

9. Fig. 6, could the authors please provide a rationale for the 2-cycle expansion. Please do remember that the readership of nature Communications is diverse.

Thank you for this suggestion. We have revised the text to make the rationale and methodology clearer. Specifically, each stimulation cycle is 7 days and we used two cycles (total 14 days). We have also added a citation of a previous study where we used the two-cycle stimulation to detect epitope-specific T cell responses without immunization.

10. The authors have written a good discussion, particularly the transparency in discussing the caveats of their hypothesis. There is a lot of interesting data regarding β cell functionality in human T1D, (https://npod.org) and it would be interesting for the authors to include human islets in their studies.

We appreciate the reviewer's perspective on human β cells and agree that this would be a necessary direction, should crinophagy research be continued. Currently, since little is known about how crinophagy functions in β cells, few studies have touched upon this area in human β cell biology. In one of our earlier studies, we found that the crinosome subcellular fractions from non-diabetic human islets (from Prodo Laboratory) also contained several shorter insulin peptides, like the mouse islets (Extended Figure 4 in ref[1]). It is therefore possible that similar and common observations could be obtained in human islets. Others have shown that already diabetic patients have reduced crinophagy than healthy controls[17], but little is known about crinophagy in steady state and early diabetes stage in humans. We hope that the current study will be a starting point leading us to move forward to human crinophagy research, especially its implications in T1D patients. We are preparing a proposal to nPOD, based on data presented in this manuscript, hoping to acquire T1D islet tissues for initial experiments.

## Reviewer #3 (expert in immunological tolerance and antigen presentation in T1D):

This research article follows up on a previous study published by the authors in 2020 that characterized the MHC-II peptidome on pancreatic islets of NOD mice. A few years before, Dr. Unanue's lab described the existence of β cell crinosomes as a source of antigenic insulin peptides. In this paper, the authors convincingly show that the content of these crinosomes is indeed driving pathogenic T cell responses and differs from peptides presented in the thymus. There is a clear distinction between T cells specific to thymically generated peptides and crinosome-derived peptides. These data are significant because they finally demonstrate something that has long been suspected, namely that pathogenic T cells recognize epitopes that are distinct from those displayed during thymic development. As a result, this pool of T cells has not been subjected to thymic deletion or regulatory T cell selection.

This is a well-written article, easy to follow with most experimental approaches summarized in a simple drawing next to the data. There are 4 key notable results:

1- Demonstration that chloroquine significantly reduces the number of crinosomes and their peptide content (including neoepitopes), resulting in reduced stimulation of T cells reactive to crinosome-specific insulin peptides, and ultimately, reduced NOD disease incidence.

2- Demonstration that thymic APCs present antigen to some insulin-reactive T cells, but not to those that are reactive to crinosome peptides.

3- Characterized the MHC-II immunopeptidome of thymic APCs in NOD mice, a truly heroic undertaking given the limited material.

4- Generated sets of MHC tetramers for both thymic epitopes and crinosome epitopes and demonstrated a greater abundance of T cells reactive to crinosome epitopes, with higher Teff/Treg ratio, while the fewer thymic epitope-specific T cells had a lower Teff/Treg ratio.

We deeply appreciate reviewer's positive, supportive, and encouraging comments. These remarks have not only provided insightful perspectives on the findings presented in this manuscript but also allowed us to expand our discussion in meaningful ways. Many points raised by the reviewer align with directions we are actively pursuing in the lab. We have also performed additional experiments to further elaborate on the issue of crinophagy being a physiological process.

1. I do not find major issues with the paper; it is very comprehensive, meaningful controls are used throughout (e.g., B16A mice, HEL-reactive T cells, etc), and multiple T cell clones and tetramers are also used for robustness. As acknowledged in the discussion, the effect of chloroquine treatment on disease incidence may result from multiple effects beside reduced crinophagy, but the antigen presentation assays in Fig.2 are convincing. Based on the authors' claims, one would expect chloroquine not to affect presentation by mTECs and other thymic APCs (Fig.3). Given the different antigen processing mechanisms of mTECs and thymic DCs, this could be interesting to look at that, or at least discuss.

   We fully agree with the reviewer that assessing the effects of chloroquine on thymic APCs is important. In our earlier studies[2,18], we found that chloroquine had no effect on the presentation of free insulin peptides, including B:9-23 (giving rise to G20; please see published data in **Figure R4A** and new experiments in **Figure R4C**) and the 29-mer full-length insulin-1 C-peptide (giving rise to CP1; **Figure R4B**). In the current study, we did not find detectable presentation of both G20 and CP1 by mTECs and other thymic APCs. These findings align with the reviewer's assessment that in vivo chloroquine treatment may not affect the presentation of these crinosome-derived epitopes in the thymus.

   Additionally, we found that mTECs specifically present E21, an epitope generated via internal processing of intact insulin. We previously observed that in vitro chloroquine treatment could block the presentation of E21 in APCs (splenic DCs and ConA-activated peritoneal macrophages) during pulse with exogenous insulin (**Figure R4A** and **Figure R4C**)[18]. This suggests that in vivo chloroquine treatment used in this study may diminish E21 presentation by thymic APCs. Although this may potentially affect the differentiation of E21-specific Tregs, the overall outcome of chloroquine treatment was to suppress diabetes development. These results suggest that the influence of chloroquine in islet presentation outweighs its potential effect in thymic presentation. We have acknowledged the reviewer's perspective in the revised discussion.

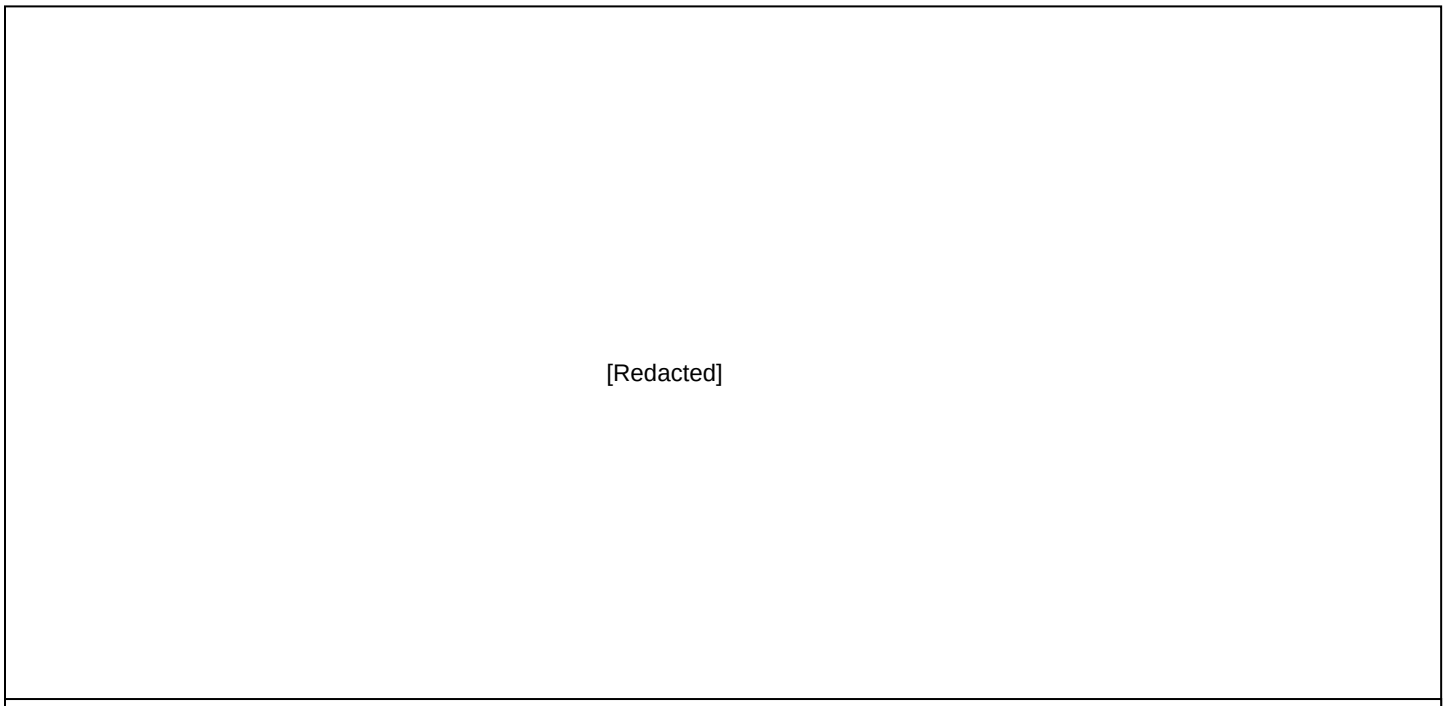
2. There is a major question that is not addressed or discussed, which is whether crinophagy itself is abnormal in NOD mice. One expects that crinophagy is a physiological phenomenon, but it may be exacerbated in NOD mice, which may contribute to the predominant autoimmune diabetes pathology in these mice (as they are also prone to other autoimmune manifestations). Using the crinosome fraction from the islets of diabetes-resistant control strains in the system described in Fig.1d would help shed light on this. Since C3.g7 cells are used in this assay, the MHC haplotype of the control strains would not matter, only the crinosome content.

   The question how autoimmune responses may further influence crinophagy, as pointed out by the reviewer, is considerably important and is being actively pursued in the laboratory. A recent development in this regard is our collaboration with Dr. Katie Haskins laboratory, in which we used a specific monoclonal antibody recognizing the 6.9HIP to map this peptide in β-cell granules[19]. We found that the presence of 6.9HIP was much more pronounced in β-cell granules, including crinosomes, from older NOD mice. Supporting this, using granule fractions from β cells after ER stress, we observed a much higher level of presentation of 6.9HIP. This study supports the notion that crinophagy could be exacerbated by autoimmune signals, such as ER stress, leading to increased production of pathogenic epitopes. We have added discussion of these points in the revised manuscript.

   We also appreciate the suggestion of comparing crinosome presentation between NOD and diabetes resistant strains. So far, our data suggest that crinosomes from young NOD mice are similar to diabetes-resistant strains, such as regular B6 and B6.g7 mice. In our initial studies[1], we isolated the crinosome fractions from NOD (3-week female), B6.g7, and B6 mice, and analyzed the granule contents by mass spectrometry (**Figure R8A**). In all three strains, the crinosome fraction contained diverse short peptides from insulin B-chain (**Figure R8A**), suggesting that peptide degradation is a common process between diabetes-prone and -resistant strains and in the context of NOD diabetes, potentially immunogenic peptides could be available from the very beginning. We therefore started the chloroquine treatment in 21-day-old female NOD mice, hoping to target

crinophagy at the initial disease stage. We have an ongoing project involving a time-course experiment to examine peptide production in crinosomes at different disease stages.

To further test crinophagy in non-diabetic conditions, we performed new experiments by treating the Min6 β cell line (derived from B6 mice) with chloroquine in vitro (**Figure R8B**). Chloroquine similarly diminished insulin peptide production in crinosome fractions from Min6 cells (**Figure R8B**). The effects of chloroquine, again, were not seen in the DCG fraction (**Figure R8B**). Considering that these results are very similar with data presented in the manuscript using in vivo chloroquine injections in young male or female NOD mice, we did not include them in the revised manuscript.




**A.** Peptide coverage of insulin B chain by sequences identified in 25k (red) and 5k (blue) β-cell granules using nLC–MS/MS analysis. Each line represents the alignment of individual peptides with insulin-2 B:1–30. Data are from four independent analyses using islets from 8–10 mice per strain.

**B.** Presentation of the insulin epitope G20 or E21 contained in crinosome or DCG subcellular fractions isolated from Min6 cells that were treated with chloroquine or PBS in vitro followed by extensive wash. Each point represents an independent experiment.

Minor points:

3. Fig.1b shows the quantification of crinosomes based on multivesicular bodies. Is it not possible to quantify the relative abundance of insulin granules, crinosomes and lysosomes using the immunogold technique?

We agree that immunogold EM would be better for quantifying different vesicles in β cells granules, as it uses more specific markers. However, immunogold EM is known to have some limitations in sensitivity, which can result in some granules not being labeled effectively. This is due to the complex and time-consuming nature of the immunolabeling process, which requires careful optimization of fixation, antibody concentrations, and enhancement steps to ensure accurate labeling. Because of these potential limitations and the concern for missing some granules, we used regular EM for quantification, which offers a more straightforward approach despite the trade-off in specificity.

4. The results from Fig.3c are somewhat unexpected and suggest that mTECs can acquire exogenous insulin, process it and present it to T cells just as efficiently as using pulsed B:9-23 peptide within the timeframe of an overnight culture.

The results showing that mTECs can efficiently process exogenous insulin were somewhat unexpected to us as well. One potential explanation for these results is that we purified mature MHC-II+ mTECs for these experiments. Mature mTECs may possess an active antigen processing and presentation machinery, which could facilitate the efficient processing of exogenous insulin. This also aligns with the demonstrated role of mature mTECs in presenting self-antigens during central tolerance. Additionally, we previously found that in vivo processing of insulin molecules by APCs largely depends on insulin receptor mediated uptake[1]. It is possible that mTECs efficiently take up insulin due to their high expression of insulin receptors.

5. Fig.S4d: as a word of caution, the B220+ CD11c+ gate contains pDCs, but may also contain CD11c+ B cells, which have been previously described.

   Thanks for pointing out this oversight. We have clarified this in the revised text.

6. The authors are commended for the technical prowess of identifying β cell antigen epitopes presented in the NOD thymus. Although few peptides (1-2) were identified for each β cell antigen, these data were successfully leveraged to produce additional MHC tetramers for thymic epitopes. Do the authors think that these epitopes all result from thymic expression or may also be brought in by migrating APCs? For example, there is no convincing evidence that ZnT8 is expressed in the thymus of mice and humans, and evidence suggesting that it is in fact not expressed in the human thymus (Mallone & Kyewski).

   We fully agree and believe that antigen transport from periphery to the thymus plays an important role in expanding the thymic epitope repertoire for central tolerance. It is entirely possible that some of the thymic epitopes identified in the current study were in fact brought to the thymus by migratory APCs. For example, we found that DC2 in the thymus naturally presented the insulin E21 epitope. Although this may be due to communication processes between DC2 and mTECs, we also suspect that it is a peripheral phenomenon. This corresponds to studies showing that multiple mechanisms, such as thymic entry of blood-borne small proteins[20], acquisition of circulating proteins by thymic transendothelial DCs[21], and antigen transport by migratory APCs[22,23], contribute to diversifying the thymic epitope repertoire. With respect to ZnT8, we completely agree that this may well be a thymic-homing peripheral antigen. In addition to the evidence pointed by the reviewer, the identical ZnT8 epitope was found in our previous analysis of both spleen and pancreatic lymph node MHC-II peptidomes[2]. Migration of peripheral APCs to the thymus may allow the presentation of the ZnT8 epitope in the thymus. We have expanded our discussion to include these points.

7. scRNAseq data in Fig.5c shows expression of early activation transcripts in all subsets but naïve T cells. Is it possible that some of these signals are the direct result of TCR stimulation by MHC tetramers during enrichment/sorting rather than reflecting a preexisting signature? Unlike the naïve T cells, antigen-experienced T cells may be less dependent on costimulation and respond to TCR stimulation alone.

   We are also intrigued by this “early activation” signature. As pointed out by the reviewer, we cannot completely exclude the possibility that tetramer engagement may contribute to this signature. When examining these early activation genes, we noticed that many were also upregulated in the G20-specific 8F10 TCR transgenic CD4 T cells during antigen encounter in the distal inguinal lymph nodes (comparing 8F10 T cells sourced from WT NOD vs B16A mice), as indicated in our previous report[1]. By two-photon imaging, we found that 8F10 T cells were constantly encountering antigens in various peripheral lymph nodes, as indicated by a reduction in motility[1].

   At the transcriptional level, these 8F10 T cells exhibited an “early activation” phenotype while lacking a complete activation profile. We interpreted this as “intermediate peripheral priming,” based on studies showing that T cells can integrate successive TCR signals during repeated antigen encounters[24,25]. Observing a similar signature in the tetramer-sorted T cells in the current study led us to wonder whether it reflects the previously

observed signature in 8F10 T cells. This was confirmed by Gene Set Enrichment Analysis (GSEA), which showed significant enrichment, as shown in Figure 5e.

Since the experiments using 8F10 T cells did not involve tetramer staining, it is possible that the “early activation” signature represents an intermediate differentiation stage of self-reactive CD4 T cells undergoing antigen encounters in the periphery. This suggests that the signature observed might be a reflection of ongoing antigen encounters rather than an artifact of the sorting process.

8. T cells that are thymic epitope-specific seem to be perfectly capable of suppressing each other (as evidenced by blocking experiments in Fig.6) but incapable of suppressing crinosome epitope-specific T cells. Is it possible that these two sets of epitopes are presented by different APCs in the periphery in vivo?

This is a very interesting question that we are actively investigating. We have been considering whether crinosomes could be handled in a cell-associated manner, which might involve a more specialized presentation mode compared to soluble antigens. Our current focus is on DC1, given their demonstrated ability to present cell-associated antigens. Crinosomes may preserve a portion of the membrane from insulin dense core granules, which express various molecules. These molecules could potentially facilitate ligand-receptor interactions, leading to uptake by DC1 compared to other APCs such as DC2, macrophages, and B cells.

We are testing this hypothesis to determine if different APCs exhibit varying levels of antigen presentation due to the nature and source of the antigenic materials. If crinosomes indeed interact preferentially with certain APCs, this could explain the observed differences in suppression capabilities of thymic epitope-specific T cells versus crinosome epitope-specific T cells. We appreciate the reviewer's insightful question and are conducting further experiments to explore this possibility.

9. On page 22, authors should replace “PTAs encoded by Aire, Fezf2 and Chd4” (incorrect) by PTAs controlled (or regulated) by Aire, Fezf2 and Chd4.

Thanks for pointing out this error. We have corrected this in the revised manuscript.

## Reviewer #4 (expert in pancreatic β cell biology and crinophagy):

In this manuscript, Hu et al aim to evaluate the role of β-cell-derived crinophagic vesicles in type 1 diabetes pathogenesis through a combination of in vivo, ex vivo, and in vitro studies. While these studies have potential functional significance and may provide conceptually important information for the field, several components of the data seem to be over-interpreted or perhaps even misinterpreted and some of the critical relevant literature is not discussed or is not correctly referenced. This is particularly troublesome given the bold claims of the manuscript that are not fully supported by the data as presented. Specific details are listed below:

We sincerely thank the reviewer for pointing out weaknesses in our original manuscript and for providing suggestions to improve the paper. As we understand, most of the reviewer’s concerns stem from perspectives on how autophagy, crinophagy, and lysosomes function in β cells. In our humble opinion, this is a largely understudied area with limited existing literature indicating potential molecular mechanisms underlying these complex biological processes. Our laboratory does not have adequate expertise in examining crinophagy or lysosome biology as physiological processes but is more oriented to identifying peptide antigens produced in crinophagic granules, which would enable further analysis of autoimmune T cells in the context of T1D.

Our primary focus in this paper is the role of crinosomes in producing T1D-relevant epitopes and whether this process contributes to T cell activation bypassing thymic selection. Therefore, most of our experiments on crinosomes used immunological readouts, such as the granule-based antigen presentation assay, which fall within our expertise. Also, we aimed to target crinophagy at the very initial stage of diabetes rather than the

hyperglycemia or diabetic stage when crinophagy may have undergone significant changes. Within these contexts, the effects of in vivo chloroquine treatment in inhibiting peptide production were consistent across different scenarios. That being said, we are eager to learn from β cell biologists and endocrinologists about advances in crinophagy biology and are much encouraged by recent studies revealing specific molecules involved in delivering DCGs to lysosomes, as mentioned in the Discussion of the paper. We believe that this research will guide us on examining the implications of crinophagy in autoimmune diabetes.

Below we present new validation experiments showing that our isolation of the crinosome subcellular fraction from β cells has minimal mixing with lysosomes. Additionally, we explain that the misinterpretation of the dosing in the hydroxychloroquine study was due to a technical error, as a portion of the originally submitted manuscript (including Discussion and References) used an older and incomplete version of our writing. For this reason, PMID 33515072, which we cited multiple times, was not listed in the submitted manuscript. This also corresponds to the reference duplication error noted by the reviewer. One reference that we intended to cite was PMID 33515072. We apologize for having caused this confusion.

1. There is a disconnect between the goal of studying the β cell crinosome specifically (which arises from a direct fusion of the lysosome with secretory vesicles, likely without involvement of the autophagosome) and the use of chloroquine, which the authors state "…has been previously shown to inhibit the formation of autophagolysosomes by impairing the fusion of autophagosomes to lysosomes without affecting the acidification of these organelles." It is therefore unclear why chloroquine would be used in these experiments unless the authors think that it is perhaps also affecting the lysosome directly in the islets. If this is the case, then the lysosome function must be evaluated more directly (e.g., is lysosome acidity, localization, or function affected, etc.?). This is particularly relevant given the links between cathepsin function and hybrid insulin peptide formation demonstrated in the literature (PMID 36041196, which does not appear to be cited by the authors).

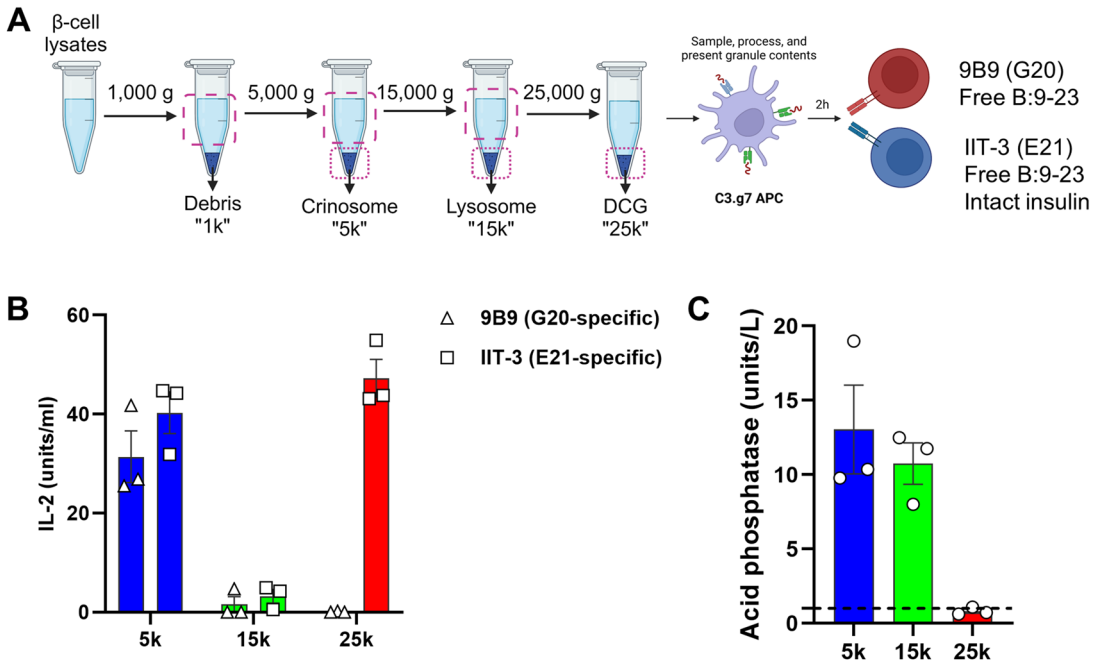
Based on our limited knowledge of β cell biology, autophagy, and crinophagy, both macroautophagy and crinophagy appear to involve a "fusion" step with lysosomes. Although molecular mechanisms controlling this fusion step still remains elusive, we thought that there might be some similarities between macroautophagy and crinophagy. If chloroquine can successfully inhibit the fusion of autophagosomes with lysosomes, it might also inhibit the fusion between DCGs and lysosomes, thereby diminishing crinosome generation. We completely agree with the reviewer that this was a very preliminary, simplistic, and perhaps naïve hypothesis. However, our functional assays demonstrated that chloroquine indeed inhibited the formation of crinosomes, and importantly, its impact on DCGs was minimal. Despite the limitations of chloroquine, as acknowledged in our discussion, this approach has allowed us to uncouple crinosomes from DCGs in the context of epitope generation, which is our primary goal.

The effect of chloroquine in lysosomes is an important question to consider. In previous studies, we found that crinosomes possess lysosomal activities, which degrade β cell proteins, resulting in generation of immunogenic peptide fragments[1,2]. Our immunogold EM experiments also suggest that crinosomes can be independently identified along with regular lysosomes. containing both registers (*Supplementary Fig. 1b*), demonstrating their comparable sensitivities. We previously found that the subcellular fraction obtained via 5,000 g was the only fraction capable of presenting the G20 epitope, suggesting that this "5k" fraction is particularly enriched for insulin peptide fragments and represents crinosomes. In contrast, the fraction obtained via 25,000 g (25k), only presented the E21 epitope, indicating that it mainly represented DCGs containing insulin molecules[18]. These results were later verified by examining granule contents through ELISA and mass spectrometry analysis[1]. For further validation, we performed new experiments and analyzed 5k, 15k (obtained via 15,000 g), and 25k subcellular fractions (from islets of 4-week-old female NOD mice) for both antigen presentation and acid phosphatase levels (to measure lysosome activity) (**Figure R9A**).

Presentation of G20 was again seen only in the 5k but not the 25k fraction, whereas the 25k fraction only presented E21 (**Figure R9B**). Also, the 5k but not the 25k fraction exhibited acid phosphatase activity (**Figure R9C**). Although presentation of either G20 or E21 was minimal in the 15k fraction (**Figure R9B**), we detected acid phosphatase in the 15k fraction (**Figure R9C**), suggesting the presence of lysosomes in this fraction. Overall, these results confirm that the 5k fraction, as the only fraction having both for insulin peptide production and lysosome activity, is compatible with features of crinosomes. Furthermore, the minimal antigen presentation in the 15k fraction suggested that the isolation of the crinosome fraction had minimal mixing with lysosomes. We have included these results as the new Supplementary Figures 1c-e in the revised manuscript.

We initially suggested crinosome as a site for the formation of HIPs[1,2]. Two subsequent studies from Dr. Kappler and Delong's groups revealed a mechanistic role of cathepsins in this process. HIP formation appears to require an acidic vesicular environment that concurrently provides source β cell peptides. This makes crinosomes, and to some extent, DCGs, suitable for making HIPs, whereas the contribution of lysosomes has yet to be determined. Our recent collaboration with Dr. Haskins's group further confirmed this notion[19]. All these papers, including PMID 36041196, were cited in the Introduction section of the original manuscript.

**Figure R9. Validation of the isolation of the crinosome fraction.**




A. Schematics of isolating subcellular fractions. After isolation, the fractions were offered to the C3.g7 APC for testing presentation to the 9B9 and IIT-3 T cells.

B. Responses of 9B9 or IIT-3 T cells to indicated granule fractions.

C. Levels of acid phosphatase in the granule fractions, as tested by ELISA.

2. Previous literature suggested that systemic administration of chloroquine may prevent STZ-induced diabetes in a rat model through effects on immune cells. However, a recent clinical trial in autoantibody positive patients failed to observe any benefits of hydroxychloroquine treatment with respect to delaying or preventing diabetes development. This is of course in contrast to the hypothesis and implications that are put forth by the authors in the current study. They do discuss this work, but do so at a somewhat superficial level and misstate the dosing used in the study, indicating that a single dose was used, which is not true. They also implicate that chloroquine would directly impact crinophagy without providing data that it does so (see 1 above). In addition, they fail to discuss critical relevant literature showing that lysosomes appear to be dysfunctional in β cells of human autoantibody positive organ donors and that islet crinophagy is impaired in the context of human type 1 diabetes (PMID 33515072). This is especially important given that the authors suggest that inhibiting β cell crinophagy is a route to diabetes delay or prevention.

In the clinical study of hydroxychloroquine trial, the authors stated that "*There are limitations to this clinical study. We tested a single dose of hydroxychloroquine that is on the low end of dosing for other diseases but was selected to minimize the risk of retinal toxicity*". Initially, we interpreted this as a single dose and discussed this point accordingly, and later realized that this was not the case. However, the Discussion section in the original submission was from an older version based on the misinterpretation. This has been corrected in the revised manuscript.

Although this clinical study did not observe delayed progression to stage 2 T1D from stage 1, hydroxychloroquine indeed reduced "*the acquisition of additional autoantibodies and the titers of autoantibodies to GAD and insulin*". Therefore, certain therapeutic benefits have been achieved. Given the complex nature of human T1D and the difficulty to organize a clinical trial (the authors noted that this study was disrupted by COVID 19), we consider this a positive result worth further investigation.

As mentioned above, we do not feel that the outcome of this clinical study contradicts our hypothesis. In our experience, it has always been difficult to relate a clinical trial in T1D to mouse studies that are performed under different conditions. The hydroxychloroquine trial started with stage 1 patients with multiple islet autoantibodies, also recognized as "*a point of no return*". This is considerably different from our mouse study that starts the chloroquine treatment in 3-week-old (21 days) female NOD mice (for monitoring diabetes incidence). Stage 1 patients already manifest epitope spreading and have a more diverse epitope repertoire which is difficult to be handled by a single therapeutic agent. In our study, we aimed to target crinophagy at the initial phase, a time point when most NOD mice do not show detectable autoantibody titers. We think that autoreactivity to diabetogenic epitopes at this stage would be more manageable and inhibiting epitope production in crinosomes may offer therapeutic benefits. We have discussed these points in the revised manuscript.

With respect to "They also implicate that chloroquine would directly impact crinophagy without providing data that it does so (see 1 above).", almost all the results in Figures 1 and 2 show that in vivo chloroquine treatment was effective in reducing crinosome numbers, which corresponds to the reduction in peptide production. This effect was not observed in DCGs. Please also note that we have repeated most of the functional experiments using young female NOD mice during this revision and the results are also consistent with those observed in male mice in the original manuscript (**Figure R1**).

We are aware of PMID 33515072 and have read it with care. We agree with the reviewer that this is critical paper. The authors used elegant imaging techniques to demonstrate a significant reduction in LC3-LAMP1 co-localization in islets of diabetic NOD mice compared to non-diabetic or NOR mice, indicating impaired autophagy. More related to crinophagy, proinsulin-LAMP1 complex also reduced in diabetic human subjects. We have indeed discussed these findings in the context of how crinophagy may be influenced in the terminal diabetic stage and in humans. However, when submitting the paper, we mistakenly did not use this more updated version of Discussion along with the correctly ordered references. We have corrected this error in the revised manuscript.

3. The altered islet crinosome peptide repertoire in animals treated with chloroquine is potentially interesting. However, there is no validation shown that the vesicles being evaluated are indeed fusions of lysosomes with insulin secretory vesicles. Is Lamp1 present in the mass spec dataset? In addition, the comparison of data in Fig. 2E should include an explanation of why what appears to be undetectable data (zero on the y axis) is included in the statistical analysis.

Please see the new experiments showing that the crinosome fraction (5k) was the only fraction that has both insulin peptide production and lysosomal activities (**Figure R9B-C**). This is in stark contrast with the DCG fraction (25k), which mostly has insulin molecules but not free peptide or lysosomal activity, and the lysosome

fraction (15k), which only has lysosomal activity but not insulin products (**Figure R9B-C**). These data validate that the 5k subcellular fraction indeed represents features of crinosomes.

The mass spec experiments were designed to examine granule contents. We needed to freeze and thaw the crinosome fraction for five times to burst open the granules, and the contents were desalted for mass spec analysis. In this experiment, we did not solubilize membrane proteins and will not expect to see LAMP1.

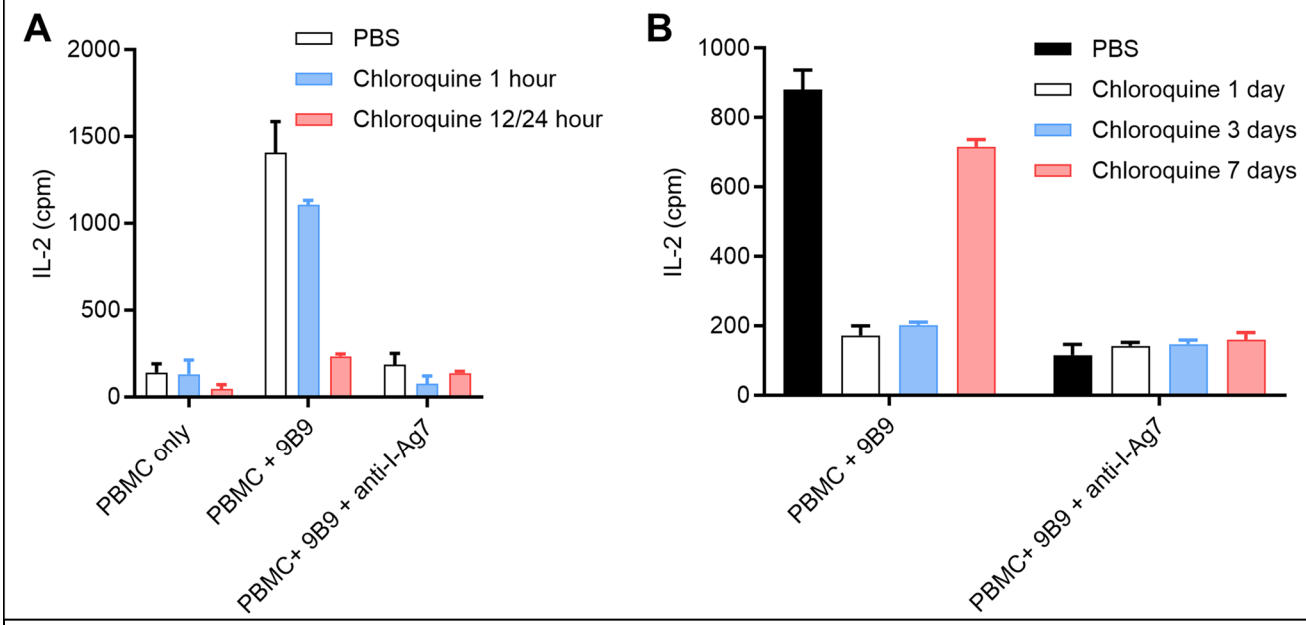
We appreciate the reviewer's concern for our inclusion of zero values and apologize that the data was not clearer. Each dot within the samples paired by condition (PBS control, chloroquine treatment) represents an individual peptide sequence, so there is an equal number of dots between the two samples, even if a specific sequence was not detected in one sample. For instance, in the panel for InsB, each dot represents a fragment of InsB. In those instances where a peptide was detected in one sample but not the other, a value of "0" was used in the sample where the peptide was not below the detection limit of our mass spec search. We had much serious debate and discussion surrounding the handling of this issue. If we generate an artificial number for those instances where a peptide was not detected, it improves the statistics. However, to us, this practice is deceptive. Thus, we decided that a value of "0" was the most honest representation of our data.

4. With respect to the in vivo chloroquine treatments, there seem to be a number of different dosing schemes used, and the duration of treatment differs between the animals used for monitoring mechanistic changes and those used for monitoring diabetes development. Considering that the authors suggest the human trials may have failed because of insufficient dosing, it would be beneficial to present data using a more standardized dosing regimen. Further, given that systemic chloroquine administration can elicit changes in islet autophagy within mere hours of dosing, it would be beneficial to understand whether chronic dosing is unique in the effects on the crinosome repertoire in comparison to effects that might be realized in more acute settings.

We primarily used two treatment regimens in this study. For most functional assays (peptidome, granule presentation, islet presentation), we used a "short-term" protocol, in which four doses of chloroquine were administered by intraperitoneal injections over a three-day period (two doses on the first day followed by one dose each on the next two days). The only variant of this short-term regimen was the testing for blood leukocyte presentation, wherein mice received two chloroquine injections at 12 and 24 hours before an in vivo glucose challenge. This adjustment was made in order to detect insulin peptides presentation by blood leukocytes; they need to be released into the circulation by glucose challenge. We previously found that such presentation could only be detected within a two-hour time window post-challenge. We therefore reduced the chloroquine injection frequency to align with this feature. For treating diabetes, we used an "intermittent" protocol, in which chloroquine was administered twice a week for three weeks, followed by a one-week break.

These protocols were designed according to several preliminary experiments. Initially, we found that there was no change in the presentation of the G20 epitope by blood leukocytes one hour after chloroquine treatment, whereas notable inhibition was observed in mice given two injections of chloroquine at 12 and 24 hours before the in vivo glucose challenge (**Figure R10A**). We then tested the duration of the effects in mice given two injections (at 12 and 24 hours). The inhibition was observed one and two days after the treatment but completely reversed on day seven (**Figure R10B**), indicating that the effects of chloroquine may start to decline after three days. Based on these results, we reasoned that a three-day time window would be appropriate for functional experiments. The intermittent protocol was also based on these results. We opted to inject twice a week to maintain the effect throughout the treatment period. For safety reasons, we chose to decrease the injection frequency and included a one-week break after a three-week treatment period. During this break, the effects of chloroquine should largely diminish, resetting the treatment. We have revised the Methods section to clarify these rationales.

**Figure R10. To determine the timing of the chloroquine treatment.**




**A.** Blood leukocyte presentation of G20 in NOD mice given PBS, chloroquine 1 hour before glucose challenge, or chloroquine 12 and 24 hours before glucose challenge.

**B.** NOD mice were given two injection of PBS or chloroquine within 24 hours and were tested for blood leukocyte presentation of G20 after 1 day, 3 days, and 7 days.

We completely agree with the reviewer that it would be ideal to use a standardized protocol for all the experiments. Considering the chronic nature of T1D and the relatively short effects of chloroquine, we thought that a prolonged regimen would be necessary for the purpose of treating the disease. On the other hand, since both the short-term and intermittent protocols are based on the observed effects for about three days, the results from the functional assays on changes in the crinosome peptidome and epitope presentation should be relevant in explaining the observed delay in diabetes development. This is further supported by subsequent evidence showing that the group of CD4 T cells recognizing epitopes derived from crinosomes manifested an effector phenotype and were readily responsive upon antigen stimulation, unlike those tolerant T cells recognizing thymic epitopes.

5. The authors show that presentation of insulin B chain peptides by circulating leukocytes is altered in chloroquine treated mice is altered. Does this also occur in a non-diabetes prone mouse model such as the NOD.B16A mouse or the NOR mouse?

Examining peptide production in crinosomes in diabetes-resistant conditions is an important question that we are actively pursuing. Reviewer 3 raised a similar question, please see our detailed response to comment #2 of reviewer 3, with data presented in **Figure R8**. In line with the concept that crinophagy is a physiological process, we previously found that production of shorter insulin peptide segments is a shared phenomenon across NOD and diabetes-resistant strains including regular B6 and B6.g7 (B6 background expressing I-Ag7) mice[1]. For example, using mass spec analysis, we found diverse shorter insulin B-chain peptides in the crinosome fraction (5k), whereas such material was much less in the DCG fraction (25k). This observation was consistent across NOD (3-week female), B6, and B6.g7 mice[1]. During this revision, we also performed new experiments showing that in vitro treatment of the Min6 β cell line (B6 derived) with chloroquine similarly diminished insulin peptide production in the crinosome fraction, without affecting DCGs (**Figure R8**).

These data in general suggest that peptide production is a common phenomenon between diabetes-resistant and -prone strains. Although these peptides can be available in both diabetes-resistant and -prone mice, there may be profound differences in their presentation and recognition by corresponding T cells. In the context of blood leukocyte presentation, we previously found that presentation of insulin peptides was about 30-50% less in B6.g7 mice as compared to young NOD mice (female and male show similar results)[4]. This suggested that APCs in B6.g7 mice may be intrinsically less potent in presenting potentially diabetogenic peptides, possibly due to expression of certain diabetes-resistant genes on the B6 background. We are currently investigating this process. Due to this complex issue, we have not examined the influence of chloroquine in diabetes-resistant strains like B6.g7 or NOR mice. B16A mice express a mutant insulin that would not be recognized by our T cell hybridomas that only recognize the native G20 or E21 epitope, so our system will not be able to detect presentation by blood leukocytes in B16A mice.

6. In the experiments to evaluate in vivo presentation by islet macrophages, can the authors rule out the potential that accidental lysis of islet cells might be contributing to the identification of the G20 epitope?

We have been paying attention to potential influence of β cell death in islet macrophage presentation. In our early studies, we found that islet macrophages actively sample and acquire antigenic materials from β cells[18]. In terms of MHC-II presentation, this process depends on close contact between macrophages and live β cells. We purposely killed all the isolated β cells by streptozotocin, and the presentation of G20 was negative[18]. These results suggest that G20 presented by islet macrophages results from a communication process between macrophages and β cells rather than an artifactual phenomenon.

Minor:

1. It is unclear how crinosomes are specifically defined and quantified from the electron microscopy data in Figure 1C. These details should be included.

We counted multi-vesicular bodies containing secretory granules (dense cores). The figures also denoted these vesicles by purple arrows.

2. References 34 and 35 are duplicates from the same paper.

This was also the mistake that was made during submitting the paper. The discussion and reference sections were from an older version. In this place, we intended to both Mauthe et al., Autophagy 2018 and PMID 33515072 noted by the reviewer, since both papers suggest that chloroquine can inhibit autophagosome/lysosome fusion. PMID 33515072 would be more relevant since it tested NOD mice. However, we mistakenly duplicated the citation of the Autophagy paper.

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**REVIEWER COMMENTS**

**Reviewer #1 (Remarks to the Author):**

All comments have been diligently addressed by the authors. It is recommended for publication.

**Reviewer #2 (Remarks to the Author):**

The authors have acknowledged and addressed this reviewer's concerns. This has resulted in new experiments added, or clarifications of existing data. The reviewer is sympathetic to the challenges of complex islet and thymic APC work, and understands how limits in laboratory equipment or time-scales for performing experiments can make some reviewer suggestions difficult to address. It is this reviewer's belief that publication of research data should not be impeded in such circumstances should the majority of the data presented be robust and caveats of data that could need some more substantiation is tackled in the discussion. The authors have satisfied this issue and the reviewer thanks them for their measured and professional responses to the concerns raised.

**Reviewer #3 (Remarks to the Author):**

The authors have done an excellent job with revising and improving their manuscript and addressing the reviewers' critiques.

I still have a few minor points to make:

Although Fig.R4C.D is not included in the paper, I am perplexed as to what the B:9-23 concentrations in panel C mean (as opposed to panel D) if only intact insulin is used.

I did not see where the possible effect of chloroquine on thymic APCs and the idea that the influence of chloroquine in islet presentation outweighs its potential effect in thymic presentation are discussed/mentioned in the discussion.

Data in Fig.R8A are not quantitative. Any knowledge or speculation on whether B6 or B6g7 beta cells produce the same number of crinosomes or the same amount of peptides per crinosome as NOD beta cells? MS only gives a sense of the peptidome's diversity, which appears to be the same.

In their rebuttal, the authors agree that the possibility that tetramer engagement may contribute to the early activation signature, but do not acknowledge it in the manuscript. On the other hand, I recognize that HEL-reactive enriched T cells have a naïve phenotype contrasting drastically with the signature of T cells enriched with the T1D epitope tetramers, and arguing against tetramer binding as a cause for this early activation signature. Having said that, I have a hard time reconciling the statement that "I-Ag7:HEL11-27 tetramer stained less cells than the I-Ag7:G20 tetramer" (Fig.R7C) in response to Reviewer #2 and the data presented in Fig.5b indicating that more HEL-reactive T cells were isolated than the combination of G20-, 6.9HIP-, 2.5HIP- and CP1d-reactive T cells (islet epitope set). These

data appear contradictory.

**Reviewer #4 (Remarks to the Author):**

My concerns have been addressed in this revised manuscript and accompanying response to reviewers.

Reviewer #3 (Remarks to the Author):

The authors have done an excellent job with revising and improving their manuscript and addressing the reviewers' critiques.

We thank the reviewer for the suggestions. Here, we provide additional data and further clarification to address these points.

Although Fig.R4C.D is not included in the paper, I am perplexed as to what the B:9-23 concentrations in panel C mean (as opposed to panel D) if only intact insulin is used.

We apologize for having mislabeled the figure panel. In panel C, we only used intact insulin (10 μm or 1 μM), while in panel D, we used B:9-23 free peptide (10 μm or 1 μM). Please see the corrected Figure R4 CD below. Consistent with our previous results, in vitro chloroquine only inhibited presentation of E21 (by IIT-3) by peritoneal macrophages pulsed with intact insulin (Figure R4C) but did not affect the presentation of free B:9-23 peptide to both IIT-3 and 9B9 T cells (Figure R4D).

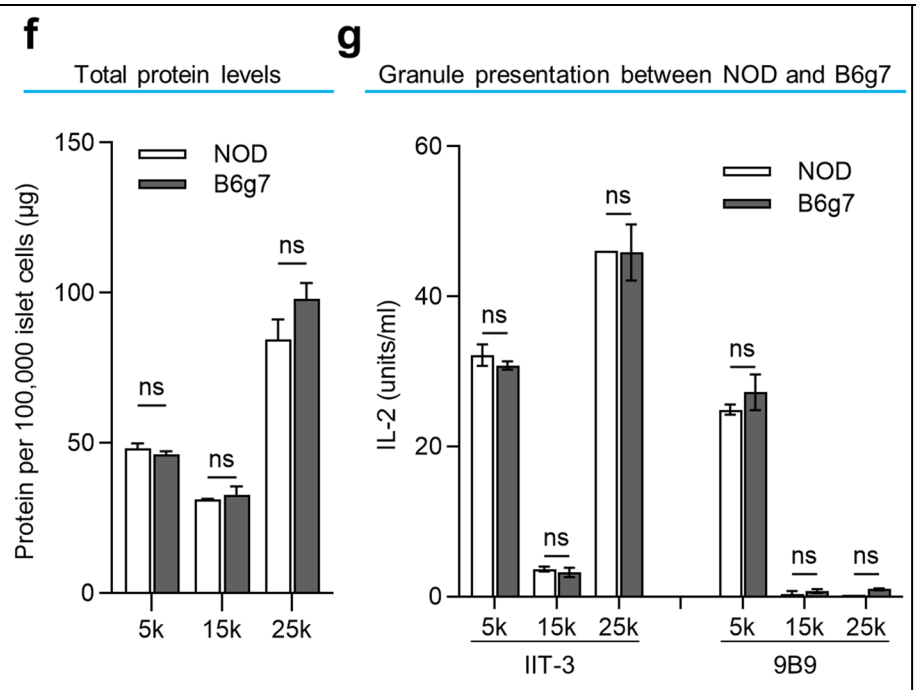
| | |
|---|---|
| [Redacted] | **A.** Control experiments using DCs incubated with insulin or peptide in the presence of chloroquine.<br>**B.** Antigen presentation assay showing responses of an Ins1C-specific CD4+ T cell hybridoma to C3g7 APCs treated with or without chloroquine and pulsed with the full-length 29-residue Ins1C:33-61.<br>**C.** Antigen presentation assay showing responses of IIT-3 and 9B9 T cells to ConA-activated peritoneal macrophages pulsed with indicated concentrations of intact insulin. Before antigen pulse, the macrophages were treated with PBS or chloroquine for 2 hours followed by washing.<br>**D.** Procedures are as described in C. ConA-activated peritoneal macrophages were pulsed with free B:9-23 peptide instead of intact insulin. |

I did not see where the possible effect of chloroquine on thymic APCs and the idea that the influence of chloroquine in islet presentation outweighs its potential effect in thymic presentation are discussed/mentioned in the discussion.

Thank you for this suggestion. We have revised the discussion to reflect this point in the context of the limitation in this study. Please see the revised discussion: "Nevertheless, a caveat in our experiments lies in potential off-target effects by chloroquine. Although our data suggest that the influence of chloroquine in islet presentation may outweigh its potential effect in thymic selection, the observed delay in diabetes development may result from chloroquine's effects in other tissue sites."

Data in Fig.R8A are not quantitative. Any knowledge or speculation on whether B6 or B6g7 beta cells produce the same number of crinosomes or the same amount of peptides per crinosome as NOD beta cells? MS only gives a sense of the peptidome's diversity, which appears to be the same.

To address this question, we performed two side-by-side comparison experiments between age/sex-matched NOD and B6g7 mice (4-week-old female). First, we measured the total protein amounts by BCA assay in the contents of different granule subcellular fractions (granule contents are released by repeated freezing and thawing). The results show that the crinosome (5k) and DCG (25k) between NOD and B6g7 mice have similar total protein levels. As expected, the DCG fraction had higher protein levels than the lysosome (15k) and crinosome (5k) fractions, further validating our granule isolation. Second, we did the granule antigen-presentation experiments simultaneously in NOD and B6g7 mice. In both crinosome and DCGs, the responses of IIT-3 and 9B9 T cells were similar. We have included these results as the new Supplementary Figure 1f and 1g, which are also demonstrated below. In the discussion, we also noted that "Young NOD mice showed similar insulin peptide levels in the crinosome fraction with B6g7 mice, suggesting that crinophagy function normally at the onset of autoimmunity."




**f.** Bicinchoninic acid (BCA) protein assay of the contents from indicated subcellular fractions isolated from 4-week-old female NOD and B6g7 mice. Data (mean ± s.e.m) summarize results from two independent experiments. ns, not significant; Two-way ANOVA test.

**g.** Response of IIT-3 and 9B9 T cells to indicated subcellular fractions isolated from 4-week-old female NOD and B6g7 mice. Data (mean ± s.e.m) summarize results from two independent experiments. ns, not significant; Two-way ANOVA test.

In their rebuttal, the authors agree that the possibility that tetramer engagement may contribute to the early activation signature, but do not acknowledge it in the manuscript. On the other hand, I recognize that HEL-reactive enriched T cells have a naïve phenotype contrasting drastically with the signature of T cells enriched with the T1D epitope tetramers, and arguing against tetramer binding as a cause for this early activation signature. Having said that, I have a hard time reconciling the statement that "I-Ag7:HEL11-27 tetramer stained less cells than the I-Ag7:G20 tetramer" (Fig.R7C) in response to Reviewer #2 and the data presented in Fig.5b indicating that more HEL-reactive T cells were isolated than the combination of G20-, 6.9HIP-, 2.5HIP- and CP1d-reactive T cells (islet epitope set). These data appear contradictory.

As we demonstrated in the manuscript (Figure.5e), the "early activation" signature in tetramer-binding T cells is very similar to the previously observed signature in the G20-specific 8F10 TCR transgenic T cells, which underwent weak antigen encounter while acquiring a similar "early activation" gene profile. Given that these findings were made in non-tetramer settings, we considered that the early activation signature in the tetramer-binding CD4 T cells occurred in vivo. However, we do agree with the reviewer that it would be better to state this point clearly. We have added the following sentence in the results part: "Thus, although the early activation signature may result from artificial tetramer engagement, it also mirrored a previously observed in vivo signature."

The HEL-reactive T cells included in scRNAseq came from a TCR transgenic mouse generated by us. In our rebuttal, we used data of I-Ag7:HEL11-27 tetramer to address the specificity issue of the tetramers. It is impossible to obtain enough HEL tetramer-binding T cells for scRNAseq due to their extremely low frequency. In our manuscript, the source of the HEL TCR transgenic cells has been clearly stated.

**REVIEWERS' COMMENTS**

**Reviewer #3 (Remarks to the Author):**

All of the questions have been addressed to the satisfaction of the reviewer.