Reply to "Tolerogenic insulin peptide therapy precipitates type 1 diabetes"

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In this issue of JEM, Bergman et al. (https://doi.org/10.1084/jem.20160471) challenge the data published in our previous JEM paper on the preventive effect of tolerogenic vaccination with a strong agonist insulin mimetope in type 1 diabetes. Here, we provide a response to these data and suggest that appropriate subimmunogenic conditions are required to induce Foxp3$^+$ regulatory T cell conversion.

Foxp3$^+$ regulatory T (T reg) cells are important for the establishment of immunological tolerance. Several independent studies originally demonstrated that a strong-agonistic ligand provided under subimmunogenic conditions results in efficient conversion of naive CD4$^+$ T cells into Foxp3$^+$ T reg cells (Kretschmer et al., 2005; Daniel et al., 2010; Gottschalk et al., 2010; Bilate and Lafaille, 2012).

We have previously reported that a strong-agonistic variant of a weak-agonistic insulin epitope provided under appropriate subimmunogenic conditions conferred efficient insulin-specific T reg cell induction in nonobese diabetic (NOD) mice (Daniel et al., 2011). Despite this, and given our growing appreciation regarding the complexity and heterogeneity of immune activation processes in type 1 diabetes (T1D; von Herrath et al., 2016), it appears reasonable to argue that T reg cell induction per se cannot be equated to efficacy of T1D prevention in NOD mice.

In the study by Bergman et al., the application of the weak-agonistic insulin B:9-23 peptide (Stadinski et al., 2010) promotes significant disease worsening, suggesting that therapeutic application of such an approach during ongoing islet autoimmunity can be prone to undesired consequences.

Given a setting prone to autoimmune activation as in T1D, one important challenge for efficient T reg cell conversion is to maintain appropriate subimmunogenic conditions, which are critically influenced by antigen dose and activation status of antigen-presenting cells and T cells (Haxhinasto et al., 2008; Sauer et al., 2008) as well as by intrinsic T cell sensitivity to antigenic stimulation. Although we agree that the selection of a 5 µg/day insulin peptide dose was a reasonable starting point to attempt T1D prevention in NOD mice, analyses of T cell–related vaccination responses (biomarkers of vaccination efficacy [Rekers et al., 2015]) would have helped to understand whether in the present experimental setup by Bergman et al. (2017) the insulin therapy was indeed tolerizing. Accordingly, it has been proposed that efficient T reg cell induction requires individual optimizations of ligand dose in a given experimental environment (Kretschmer et al., 2005; Gottschalk et al., 2010) and is supported by the assessment of naive versus activated CD4$^+$ T cells before antigen application because activated CD4$^+$ T cells are resistant to Foxp3$^+$ T reg cell conversion. Therefore, the observation by Bergman et al. (2017) that already the weak-agonistic insulin B:9-23 peptide promotes a disease worsening might suggest that in these experimental conditions the overall setting might be predisposed to immune activation besides the autoimmune NOD phenotype per se.

Concerning antigen application by osmotic mini-pumps, in our previous study, control animals were not left unmanipulated, as erroneously assumed by Bergman et al. (2017), but were implanted with pumps filled with vehicle to match treatment conditions. For all groups, the pumps remained longer than 2 wk in the animals but were not left in place for the full length of the experiment (40 wk). Instead, for all groups, the pumps were explanted after a total time of 4 wk by aseptic surgery. The fact that in our previous study NOD mice implanted with vehicle filled pumps (controls) as well as animals treated with the weak-agonistic insulin peptide B9:23 were not protected from disease excludes a protective effect of "pump-induced inflammation."

We agree with Bergman et al. (2017) that in addition environmental factors might critically contribute to study outcomes and prevention efficacy. Bergman et al. (2017) used NOD mice originally obtained from The Jackson Laboratory. To study diabetes prevention after insulin-specific tolerogenic vaccination, we had previously used NOD mice obtained

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Abbreviations used: NOD, nonobese diabetic; SFB, segmented filamentous bacteria; T1D, type 1 diabetes.

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from Taconic and offspring of these Taconic-derived NOD animals bred at our local animal facility.

Currently, the exact contribution of the origin of NOD mice, whether from The Jackson Laboratory or Taconic, in affecting tolerance induction and efficacy of disease intervention remains unclear. Despite this, careful studies have recently compared gut microbiota between colonies of NOD mice derived from NOD/Jackson and NOD/Taconic. Microbiota composition revealed major differences on genus level and much more diverse microbiota in NOD/Taconic mice. More recent data support the notion that specific species within microbiota, e.g., *Akkermansia muciniphila*, *Allobaculum*, and *Mucispirillum*, carry diabetes-delaying and immune-modulating properties (Hansen et al., 2012). Bergman et al. (2017) describe the presence of segmented filamentous bacteria (SFB) in their study colony. Recent findings suggest that SFB boost and exacerbate autoimmune activation by promoting differentiation and migration of Peyer's patch T follicular helper cells, e.g., in autoimmune arthritis (Teng et al., 2016). Therefore, evidence that specific elements of the microbiota impinge on peripheral T reg cell induction (Lathrop et al., 2011) and on autoimmune activation is beginning to emerge (Mathis and Benoist, 2012; Pearson et al., 2016) and could also contribute to the observed different outcomes between the two studies discussed here.

Bergman et al. (2017) used the analysis of insulin autoantibodies to assess autoimmune activation in the respective NOD mouse colony. However, based on recent evidence, differences in proinflammatory polarization of innate immune cells and the local microenvironment between the NOD colonies of the two studies may in addition critically impinge on local T reg cell induction potential and thereby on outcomes of antigen-specific therapy and disease prevention efficacy (Kolb and von Herrath, 2017).

To broaden the window of subimmunogenic ligand application and to increase robustness at a given antigen dose, we also had used a combination of insulin peptide application together with the mTOR inhibitor everolimus (Daniel et al., 2011). This combination showed superior effects also on insulitis scores (Daniel et al., 2011). Approaches that can inhibit the signaling intermediates of T cell activation and/or strategies that can impact β cell/metabolic function combined with antigenic application therefore may function to strengthen the robustness of treatment outcomes (Kolb and von Herrath, 2017).

In summary, we previously concluded that (a) a strong agonistic insulin mimetope induces stronger T cell proliferation and (b) when provided under appropriate subimmunogenic conditions is superior in inducing Foxp3$^+$ T reg cells when compared with insulin B:9-23. We also found that (c) the combination of the insulin-mimetope at subimmunogenic doses with the mTOR inhibitor everolimus is superior in inducing Foxp3$^+$ T reg cells, that (d) at an appropriate subimmunogenic dose the insulin mimetope can prevent progression of diabetes development, and that (e) the combination of antigenic therapy together with mTOR inhibition strengthens the tolerizing potential of T reg cell induction in NOD mice and reveals superior effects also on insulitis scores (Daniel et al., 2011).

Recent experiments by Bergman et al. (2017) confirm the notion (d) that individual subimmunogenic ligand dose identification for T reg cell conversion supports efficient tolerance induction and thereby can impact disease outcomes. In addition, their findings confirm our point that biological and environmental factors can impinge on antigenic dosing requirements and disease prevention efficacy. The other discoveries and conclusions we reported were not addressed by these authors, but independently shown by several studies (Stadinski et al., 2010; Crawford et al., 2011) using different models and animal facilities.

More mechanistic studies are required to gain an improved understanding of how antigen application for the efficient and stable induction of T reg cells can be best achieved in the setting of T1D (Peakman, 2012). Combinatorial strategies inhibiting the signaling intermediates of T cell activation might help to increase the robustness of tolerance induction to reduce T1D incidence (von Herrath et al., 2016).

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