TLR2 and Dectin 1-associated innate immune response modulates T cell response to pancreatic beta-cell-antigen and prevents Type 1 diabetes

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Abstract

The progression of autoimmune diseases is dictated by deviations in the fine balance between pro-inflammatory versus regulatory responses and pathogen recognition receptors (PRRs) play a key role in maintaining the balance. Previously, we have reported that ligation of TLR2 and Dectin 1 on APCs by zymosan results in a regulatory immune response that prevents type 1 diabetes (T1D). Here, we show that TLR2 and Dectin 1 engagement by zymosan promotes regulatory T cell (Treg) responses against the pancreatic β-cell-specific Ag. Unlike the TLR4 ligand, bacterial lipopolysaccharide, which induced pro-inflammatory cytokines and pathogenic T cells, zymosan induced a mixture of pro- and anti-inflammatory factors and regulatory T cells, both in vitro and in vivo. Ag-specific T cells that are activated using zymosan-exposed dendritic cells (DCs) expressed Foxp3 and produced large amounts of IL-10, TGF-β1 and IL-17. NOD mice that received β-cell-Ag loaded, zymosan-exposed DCs showed delayed hyperglycemia. Injection of NOD mice at pre-diabetic age and early-hyperglycemic stage with β-cell-Ag, along with zymosan, results in a superior protection of the NOD mice from diabetes as compared to mice that received zymosan alone. This therapeutic effect was associated with increased frequencies of IL-10-, IL-17-, IL-4-, and Foxp3-positive T cells, especially in the pancreatic lymph nodes. These results show that zymosan can be used as an immune regulatory adjuvant for modulating the T cell response to pancreatic β-cell-antigen and reversing early stage hyperglycemia in T1D.

Key words: Toll-like receptor 2; Dectin 1; autoimmunity; adaptive immunity; innate immunity; Type 1 diabetes; pancreatic β-cell-antigen, immune modulation/tolerance/suppression.
**Introduction**

Innate immunity, initiated primarily by environmental factors such as microbes, plays a key role in initiating or preventing the T cell response to pancreatic β-cell-Ag in type 1 diabetes (T1D). While it has been suggested that the pro-inflammatory response mediated by pathogen recognition receptors (PRRs) facilitates β-cell-Ag presentation by activated APCs (1), environmental factors such as bacterial and viral infections are known to have a protective effect in T1D (2-5). Innate immune response is mediated by an array of PRRs such as Toll-like receptors (TLRs) and c-type lectin receptors (CLRs) that primarily recognize microbial products. In recent years, studies including ours have shown that innate immune responses induced through TLR2 and Dectin 1 using zymosan, a fungal cell wall component, are regulatory in nature and involves, in addition to pro-inflammatory factors, the expression of IL-2, IL-10, TGF-β1 and retinaldehyde dehydrogenase 1A2 (Raldh1A2) by one or other type of APCs (6-13). Importantly, the innate immune response induced by zymosan has the ability to prevent/delay disease in T1D and EAE models, even upon disease onset (6-11).

In this report, we show that zymosan-induced innate immune response facilitates Treg induction and/or expansion and Th1 to Th17 skewing of the T cell response to pancreatic β-cell-Ag. Importantly, treatment with zymosan along with β-cell-Ag resulted in a significant delay in hyperglycemia in NOD mice even when the treatment was initiated at an early-hyperglycemic stage as compared to treatment with zymosan alone. These observations show that zymosan has therapeutic values as a tolerogenic adjuvant and it can be used for promoting β-cell antigen specific tolerance and to reverse early stage hyperglycemia in T1D.
Materials and Methods

Mice

Wild-type NOD/LtJ, NOD-BDC2.5-TCR-transgenic (TCR-Tg), NOD-Scid, C57BL/6 and OT-II-TCR-transgenic (OT-II-TCR-Tg) mice were purchased from the Jackson laboratory (Maine, USA). OT-II-TCR-Tg-Foxp3-GFP-knockin (ki), NOD-Foxp3-GFP and NOD-BDC2.5-Foxp3-GFP mice were generated at our animal facilities. To detect hyperglycemia in NOD mice, glucose levels in blood collected from the tail vein of wild-type NOD-Ltj or NOD-Scid mice were monitored using the Ascensia Micro-fill blood glucose test strips (Bayer, USA). All animal studies were approved by the animal care and use committee of UIC and MUSC.

Peptide antigens, cell lines, and antibodies

Immunodominant β-cell-antigen peptides [viz., 1. Insulin B (9-23), 2. GAD65 (206-220), 3. GAD65 (524-543), 4. IA-2beta (755-777), 5. IGRP (123-145), 6. BDC2.5 TCR reactive peptide (YVRPLWVRME; referred to as BDC peptide), and 7. OVA (323-339) peptides] were described in our earlier studies (14-16). Peptides 1-5 were pooled at an equal molar ratio and used as β-cell-Ag for in vitro and in vivo experiments.

Purified zymosan A of Saccharomyces cerevisiae was prepared as described earlier (6; 7). Bacterial lipopolysaccharide (LPS; Escherichia coli origin; ion-exchange purified), Curdlan, PMA, ionomycin, Brefeldin A, and monensin were purchased from Sigma-Aldrich, BD Biosciences, eBioscience, Invivogen, and Invitrogen. Normal rat serum, various fluorochrome-conjugated reagents and antibodies (Abs), and isotype control Abs (Invitrogen, BD Biosciences, eBioscience, R&D Systems, and Biolegend Laboratories) were used for FACS. Magnetic bead-based total and CD4+ T cell and CD11c+ dendritic cell (DC) isolation kits (Miltenyi Biotec and
Invitrogen) were used for enriching or depleting T cells and DCs. Paired Abs and standards for ELISA were purchased from R&D Systems, BD Biosciences, Invitrogen, and eBioscience.

**Treating NOD mice with zymosan and β-cell-Ag**

Twelve-week-old euglycemic (glucose levels <110 mg/dl; pre-diabetic age) and 10-20 week-old early- hyperglycemic (glucose levels between 140-250 mg/dl; early-hyperglycemic stage) wild-type female NOD/Ltj mice were treated with zymosan and/or β-cell-Ag. Although insulitis in NOD mice is very heterogeneous at any given age, 12-week-old euglycemic mice represent the pre-diabetic stage because the hyperglycemia begins to appear at this stage. Mice were injected with zymosan (i.v.; 25 μg/mouse/day on days 1, 3, 5, 16, 18 and 20) in PBS. Some groups of mice were injected i.v. with β-cell-Ag (0.5 μg/mouse/day on days 5 and 20) in PBS. Mice with glucose levels >250 mg/dl for two consecutive weeks were considered diabetic.

**Experiments using NOD-BDC2.5 TCR-Tg mice and T cells**

Four-week-old NOD-BDC2.5-Foxp3-GFP mice were injected i.v. with bacterial LPS (5 μg/mouse/day) or zymosan (25 μg/mouse/day) for 3 consecutive days, and BDC2.5 peptide (5 μg/mouse) on day 3. Spleen cells from these mice (2x10^6/mouse) were injected into 4-week old WT NOD mice and examined for blood glucose levels every other day. In a separate experiment, 4-week-old WT NOD mice were treated with zymosan and/or β-cell-Ag as described above, and injected with 2x10^6 ex vivo activated BDC2.5 TCR-Tg T cells. The recipients were monitored for hyperglycemia every other day. In one set of experiments, 12-week-old euglycemic WT NOD mice were treated with zymosan and/or β-cell-Ag as described above, and CFSE labeled BDC2.5
TCR-Tg T cells were injected i.v. (2x10^6/mouse). After 96 h, spleen and PnLN cells from these mice were stained for Vβ4 and CD4 and examined for proliferating T cells by FACS.

*Dendritic cells, T cells and in vitro assays*

Splenic CD11c+ cells and total T cells were enriched using magnetic separation reagents. Bone marrow (BM)-derived DCs (BM DCs) were generated as described before (6). Splenic CD11c+ DCs and BM DCs were incubated with zymosan (25 µg/ml) or LPS (2 µg/ml) for different time points. Pilot experiments using varying amounts of these agents were performed to determine the optimum culture conditions based on TNF-α production. cDNA prepared from DCs was subjected to qualitative and real-time quantitative PCR using CYBR-green PCR mastermix (ABI prism). DCs from 36 h cultures were examined for the levels of surface activation markers after staining with fluorochrome-labeled specific Abs. Spent media were tested for cytokine levels by ELISA.

Purified total T cells (1x10^5 cells/well) were incubated with zymosan-exposed or unexposed DCs (2x10^4 DCs/well) in the presence of anti-CD3 Ab (2 µg/ml). In some assays, DCs were incubated with β-cell-Ag (5 µg/ml) and zymosan (25 µg/ml) or LPS (2 µg/ml) agents overnight, washed and cultured with CD4+ T cells. In some assays, spleen and PnLN cells (2 x 10^5 cells/well) from treated and control mice were stimulated with anti-CD3 Ab (2 µg/ml) or β-cell-Ag (5 µg/ml) for 48h. Spent media from these cultures were tested for cytokines.

*FACS analysis*

Freshly isolated and *ex vivo* cultured cells were washed using PBS supplemented with 2% FBS and 10 mM EDTA (pH 7.4) and blocked with anti-CD16/CD32 Fc block Ab or 5% rat serum on
ice for 15 min. For surface staining, cells were incubated with FITC-, PE-, and PECy5 or PE-TR-labeled appropriate Abs, in different combinations, on ice for 45 min and washed three times before analysis. Cells were also stained using isotype-matched control Abs for determining the background.

**DC and T cell transfer experiment**

BMDCs were cultured in the presence of zymosan (25 µg/ml) or LPS (2 µg/ml) with β-cell-Ag (5 µg/ml) or Ag alone for 24h, washed, and injected into 8-week-old pre-diabetic female NOD mice (i.v. 5x10⁶ cells/mouse). In some experiments, total splenocytes (5x10⁶ cells/mouse) or purified T cells (2x10⁶ cells/mouse) from the spleens of control and treated mice (15 days post-treatment) were transferred into euglycemic female NOD mice and monitored as described above. In some experiments, freshly isolated T cells from BDC2.5-TCR-Tg mice were labeled with CFSE and injected into zymosan and/or β-cell Ag treated WT NOD mice. The recipients were euthanized 4 days post- T cell transfer to determine the proliferation of donor T cells. In addition, BDC2.5-TCR-Tg T cells were activated using control and zymosan exposed and BDC2.5 peptide pulsed DCs, and T cells purified from these cultures were injected into 6-week-old WT mice (i.v).

**Histochemical analysis and examination of inflammatory response**

Pancreata were fixed in 10% formaldehyde, 5-µm paraffin sections were made, and stained with hematoxylin and eosin (H&E). Stained sections were analyzed using a grading system in which 0 = no evidence of infiltration, 1 = peri-islet infiltration (<5%), 2 = 5-25% islet infiltration, 3 = 25–50% islet infiltration, and 4 = >50% islet infiltration as described in our earlier studies (6; 14-17). About 150 islets were examined for every group. In some experiments, pancreatic sections
were stained using anti-insulin and anti-glucagon antibodies followed by Alexa fluor 488- and 568- linked secondary antibodies and DAPI, and scored for insulitis based on DAPI-positive cells in islet areas and insulin expression. Insulitis was scored as described for H&E stained sections and insulin positive and negative islets were counted. Areas that appeared to have completely lost islets were considered as grade 5 and included in this grading approach. In some experiments, H&E stained kidney and liver sections were examined for inflammation and tissue damage. Further, serum samples collected from zymosan treated and control mice were tested for c-reactive protein levels by ELISA (R&D Systems).

Statistical analysis

Mean, SD, and statistical significance (p-value) were calculated using Microsoft Excel, GraphPad online, and/or other online statistical applications. Two-tailed t test was employed unless specified for values from in vitro and ex vivo assays. Log-rank analysis was performed to compare T1D incidence (hyperglycemia) of the test group with that of the control group. Fisher’s exact test was used for comparing the total number of infiltrated islets in test vs. control groups. A p value of \( \leq 0.05 \) was considered significant. In most experiments, unless specified, individual treated groups were compared to non-treated group for calculating p-values.

Results

**TLR2 and Dectin 1 engagement by zymosan modulates the pro-inflammatory response of DCs**

In this study, we compared the innate immune response induced by zymosan through TLR2 and Dectin 1 with that induced through TLR4 by bacterial LPS. BM derived (generated using GM-CSF and IL-4; represents primarily myeloid DCs) and peripheral DCs were activated using LPS or zymosan and examined for the amount of cytokines secreted. As observed in Fig. 1A, while
zymosan-exposed DCs produced large amounts of the immune regulatory cytokines, IL-2, IL-10 and TGF-β1 along with pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β, IL-12), LPS induced only pro-inflammatory cytokines in these DCs. We have also examined the ability of zymosan-exposed or unexposed DCs from NOD mice to express Raldh1A2 in comparison with DCs that are treated with LPS. As observed in Fig. 1B, exposure to zymosan, but not LPS, upregulated Raldh1A2 expression in both splenic and BM DCs, albeit at much lower level (~10-fold induction from basal level) than previously reported (~200-fold induction from basal level) (10). However, expression of this enzyme, in combination with the observed immune regulatory cytokines, by DCs suggests that zymosan-exposed APCs may promote a regulatory T cell response upon antigen presentation.

Ag presentation by zymosan-exposed DCs induces Foxp3+ and IL-10-secreting T cells

Next, zymosan DCs were examined for their ability to modulate T cell response using purified T cells from OT-II TCR-Tg mice. As observed in Fig. 1C, DCs that were exposed to LPS and zymosan induced similar levels of proliferation in Ag specific T cells. However, the zymosan-exposed DCs induced significantly higher frequencies of Foxp3+ cells in culture as compared to unexposed- or LPS-exposed DCs. Examination of the cytokine profiles revealed that T cells activated using DCs that were exposed to zymosan produced significantly higher amounts of IFN-γ, IL-17, and IL-10 and reduced amounts of IL-4 as compared to untreated control DC activated T cells (Fig. 1D). However, T cells that were activated using the TLR4 ligand produced high amounts of IFN-γ and IL-17 and lower amounts of TGF-β1, compared to control DC activated T cells. These observations show that TLR2 and Dectin-1 engaged DCs and TLR4 engaged DCs modulate T cell function differently.
NOD mouse T cells that were activated in the presence of zymosan-exposed APCs show regulatory phenotype

Both BDC2.5 TCR-Tg cells and T cells from WT NOD mice demonstrate diabetogenic properties upon activation (14; 17). Therefore, to further understand the effect of TLR2 and Dectin-1 mediated innate immune response on T cells in a type 1 diabetes model, BDC2.5 TCR-Tg and WT NOD-mouse T cells were activated in the presence of zymosan-exposed splenic DCs. The zymosan-exposed DCs induced significantly higher frequencies of Foxp3+ cells in the cultures of WT NOD mouse T cells as compared to unexposed or LPS-exposed DCs (Fig. 2A). On the other hand, when LPS-exposed DCs were used, considerably low frequencies of T cells in the cultures expressed Foxp3 as compared to controls (Fig. 2A). T cells from WT NOD mice produced higher IL-10 and TGF-β1 when activated in the presence of zymosan-exposed, but not LPS-exposed, DCs. (Fig. 2B). Similar to WT T cells, zymosan-, but not LPS-, exposed DCs (both splenic and BM DCs), induced an increase in the frequency of Foxp3+ cells in BDC2.5 TCR-Tg T cell cultures (Fig. 2C). BDC2.5 TCR-Tg T cell cultures also showed significantly higher amounts of IL-10, TGF-β1 and IL-17 along with IFN-γ when zymosan-exposed DCs were used for antigen presentation (Fig. 2D). These observations show that innate immune response induced by zymosan, but not LPS, has the ability to modulate the properties of diabetogenic T cells and induce T cells with a regulatory phenotype.

Zymosan-exposed β-cell-Ag-pulsed DC treatment results in suppressed insulitis and delayed hyperglycemia in NOD mice

To assess the immune regulatory nature of Ag presentation by zymosan exposed DCs in vivo as compared to TLR4-engaged DCs, 8-week-old NOD mice were injected with zymosan or LPS-
exposed β-cell-Ag-pulsed BMDCs and monitored for hyperglycemia. NOD mice that received zymosan-exposed, but not LPS-exposed, β-cell-Ag-pulsed DCs showed a significant delay in hyperglycemia as compared to β-cell-Ag loaded control DC recipient mice (Fig. 3A). One set of euglycemic mice from a similar experiment was euthanized 30-days post-treatment and pancreatic tissues were examined for insulitis. As observed in Fig. 3B, mice that received zymosan-exposed and β-cell-Ag-pulsed DCs had significantly higher numbers of islets with less severe insulitis or no immune cell infiltration compared to untreated or control DC recipient mice. Spleen cells from one set of mice that were euthanized 15-days post-treatment were examined for the frequency of Foxp3+ T cells. As shown in Fig. 3C, the number of Foxp3-expressing CD4+ T regulatory cells (Tregs) is significantly higher in mice that were injected with β-cell-Ag-pulsed zymosan-exposed-DCs as compared to β-cell-Ag loaded (none) DC recipients or mice that received DCs that were not pulsed with β-cell-Ag (control). Although LPS-exposed β-cell-Ag-loaded DC recipients showed a modest increase in Foxp3+ T cells compared to controls, they developed more or less similar level of insulitis as the control DC recipients. These results suggest that β-cell-Ag presentation by the zymosan-exposed DCs has the ability to promote induction/expansion of Tregs in vivo.

_T cells from LPS, but not zymosan, treated BDC2.5 TCR-Tg mice induce hyperglycemia in young NOD mice_

To assess whether zymosan and LPS induce pro-inflammatory adjuvant effects in vivo and promote the generation of diabetogenic T cells, young BDC2.5-Foxp3-GFP mice were injected with bacterial LPS or zymosan along with the BDC2.5 peptide. Zymosan injected NOD-BDC2.5-Foxp3-GFP mice showed a profound increase in the CD4+GFP+T cell numbers,
compared to control group of mice (Fig. 3D). On the other hand, LPS recipients showed lower CD4+GFP+ T cell frequency compared to control mice. Importantly, T cells from only LPS, but not zymosan, recipient mice could induce hyperglycemia in young NOD mice (Fig. 3E). T cells from mice that received BDC2.5 peptide alone did not induce hyperglycemia, perhaps due to tolerogenic antigen presentation by steady state APCs in the absence of adjuvant treatment in these peptide-recipient mice. Whether i.v. injection with zymosan can lead to a strong inflammatory response was also assessed by determining the serum levels of CRP in zymosan treated mice. As observed in Supplemental Fig. 1, a marginal increase in the serum CRP levels was detected 48h after zymosan treatment. On the other hand, i.v. injection with LPS induced a 5 fold increase in the serum CRP levels as compared to control. These results indicate that zymosan does not have a strong pro-inflammatory adjuvant property, as opposed to that of LPS.

*Zymosan induced innate immune response does not suppress β-cell-Ag specific T cell proliferation, but modulates their diabetogenic function.*

Next, we examined whether zymosan treatment induced modulation of immune response to β-cell-Ag affects T cell proliferation in vivo. Twelve-week old (pre-diabetic age) NOD mice were treated with zymosan and/or β-cell-Ag, injected with CFSE labeled BDC2.5 TCR-Tg T cells alone or along with BDC2.5 peptide, and examined for CFSE dilution in donor cells by FACS. As anticipated, PnLN, but not the spleen, of control mice showed profound proliferation of BDC2.5 T cells in the absence of BDC2.5 peptide injection (Fig. 4A). Importantly, PnLN of all groups of mice that were treated with zymosan and/or β-cell-Ag also showed significant number of proliferating BDC2.5 T cells. PnLN from all BDC2.5 peptide injected mice showed more or less similar extent of BDC2.5 T cell proliferation (Fig. 4A). In addition, upon peptide injection,
spleen cells from zymosan treated and untreated mice showed similar extent of BDC2.5 T cell proliferation. These results, in association with the observations of Figs. 2 and 3, suggest that while the zymosan induced innate immune response may not have a significant suppressive effect on T cell proliferation, it may influence the functionality of these T cells.

Our previous report (14) has shown that young WT NOD mice injected with \textit{in vitro} activated TCR-Tg T cells can serve as an effective adoptive transfer model for T1D to assess if the Ag-specific T cells are pathogenic. In this model, if the TCR-Tg T cells are pathogenic, then 100% of recipient mice develop overt hyperglycemia within 6-8 days. If the T cells are not pathogenic in nature, then the recipients fail to develop T1D for months. Therefore, to further assess the regulatory nature of TLR2 and Dectin 1 dependent innate immune response, young WT NOD mice were treated with zymosan and/or \(\beta\)-cell-Ag, followed by injection of \textit{in vitro} activated diabetogenic BDC2.5 TCR-Tg T cells. As observed in Fig. 4B, diabetogenic BDC2.5 TCR-Tg induced overt-hyperglycemia in control and \(\beta\)-cell-Ag treated mice within 8 days. However, these T cells failed to induce hyperglycemia in zymosan and zymosan plus \(\beta\)-cell-Ag treated groups of mice. These results suggest that zymosan induced innate immune response alters the function of diabetogenic T cells \textit{in vivo}.

\textit{Zymosan treatment does not produce side effects such as global immune suppression, but causes increased cell death in PnLN}

Various effects of TLR2 and Dectin 1 dependent innate immune response on secondary lymphoid organs as well as non-lymphoid organs were examined to further understand the potential side effects of zymosan treatment. Twelve-week-old (pre-diabetic age) mice were treated with zymosan and various tissues were examined for cell death. Cells from the pancreas,
PnLN and spleen were examined for dead/dying (both apoptotic and necrotic) cells by Annexin V and 7-AAD staining. While spleen cells from zymosan and zymosan plus β-cell-Ag recipient mice did not show a difference in cell death, PnLN of these treated mice had higher number of Annexin V and/or 7-AAD positive cells compared to control mice (Supplemental Fig. 2A). Importantly, CD4+ T cells from pancreas and PnLN of only zymosan plus β-cell-Ag recipient mice, but not zymosan alone or control group of mice, showed considerable cell death (Supplemental Fig. 2B). These results suggest that zymosan treatment causes immune cell death at sites of high inflammation, and in addition to the regulatory cytokine mediated effect, apoptotic elimination of activated immune cells including T cells could be a feature of zymosan-induced immune modulation.

To assess whether the zymosan treatment affects DC numbers and their functionality, leading to T cell death, pancreatic, PnLN and spleen cells were examined for the frequencies of DCs. As observed in Supplemental Fig. 3A, treatment with zymosan at the dose selected for this study did not induce a considerable increase in the number of CD11c+ DCs in pancreas, PnLN or spleen. Further, the expression levels of apoptotic ligands FAS-L and TRAIL were comparable in pancreas, PnLN or spleen DCs of control and zymosan treated groups of mice. Further analysis revealed that myeloid (CD8α-), lymphoid (CD8α-) and plasmacytoid (PDCA1) DC frequencies were not noticeably different in the spleen and PnLN of control and zymosan treated mice (Supplemental Fig. 4). Although the antigen presenting function of DCs from zymosan treated and control mice may be different in vivo, these observations indicate that the frequency of DCs is not affected by zymosan treatment and they may not have a direct role in causing T cell death in the pancreatic microenvironment of zymosan treated mice.
We have also examined whether zymosan treatment induces side effects such as inflammation in non-lymphoid organs like kidney and liver. As observed in Supplemental Fig. 5, i.v. injection with low dose zymosan does not cause noticeable inflammation in the non-lymphoid organs such as kidney and liver. To assess whether zymosan and β-cell-Ag treatment induces general immune suppression, the ability of T cells from treated NOD mice to proliferate against alloantigen in an MLR assay was tested. As observed in Supplemental Fig. 6, T cells from zymosan and zymosan plus β-cell-Ag treated mice showed a more pronounced proliferative response than T cells from untreated or β-cell-Ag treated NOD mice. While this enhanced proliferative ability of T cells from zymosan treated mice, compared to control T cells, could be an effect of the cytokines IL-2 and TNF-α produced by zymosan exposed APCs, this result suggests that zymosan treatment does not produce general immune suppression.

**β-cell-Ag delivery along with zymosan results in the prevention of T1D**

As shown in our previous report (6), the innate immune response induced in NOD mice by injecting higher dose of zymosan (100 µg/injection; i.p.) could achieve long-term protection from T1D. Results from experiments using zymosan-exposed DCs presented in Fig. 3 suggest that a relatively short-term treatment, with smaller doses of zymosan, may be sufficient to achieve a long-lasting therapeutic effect in conjunction with the β-cell-Ag. Twelve-week-old pre-diabetic age NOD mice were co-administrated with zymosan and β-cell-Ag and monitored for hyperglycemia. As observed in Fig. 5A, mice that were treated with zymosan and β-cell-Ag remained diabetes-free for a significantly longer duration than the mice that received zymosan alone. In addition, as observed in our previous study (13), mice that received zymosan also showed significant protection from T1D as compared to untreated mice.
The pancreatic islets of pre-diabetic mice that received zymosan and β-cell-Ag showed significantly less severe immune cell infiltration and insulitis compared to peptide treated and untreated control mice within 30 days post-treatment (Fig. 5B). While the mice that received low dose zymosan alone also showed significantly less severe insulitis compared to untreated mice, the difference in insulitis observed between pancreatic islets of mice that received β-cell-Ag alone and untreated controls was not statistically significant, at 30 days post-treatment. These results suggest that exposure of APCs to zymosan in vivo leads to the skewing of T cell response from pathogenic to protective type.

*Early hyperglycemic mice show prolonged protection from diabetes when β-cell-Ag is delivered along with zymosan*

Encouraged by the results of Fig. 5, we examined whether modulating the immune response against β-cell-Ag, with the help of zymosan-induced innate immune response can control autoimmunity in early hyperglycemic mice and restore euglycemia. Early hyperglycemic (glucose: 140-250 mg/dl) mice were treated with zymosan alone or along with β-cell-Ag and examined for blood glucose levels every 3rd day. One hundred percent of non-injected control mice were diabetic within 6 weeks post-treatment. However, majority of the mice that received zymosan along with β-cell-Ag showed normal glucose levels and remained diabetes-free for more than 20 weeks post-treatment (Fig. 6A and Supplemental Fig. 7). Although less profound, mice that received zymosan alone also showed significant protection from diabetes compared to control mice.

Examination of the pancreatic tissue from one set of these mice, 30 days post-treatment, revealed that zymosan and β-cell-Ag-treated mice have significantly higher number of infiltration-free
islets or islets with less severe insulitis compared to other groups after 30 days post-treatment (Supplemental Fig. 8A). Another set of mice from a similar experiment were euthanized on day 30, and the pancreatic tissue sections were stained for insulin and glucagon to assess islet function. While differences in the overall insulitis severities between zymosan and zymosan plus β-cell-Ag groups were not statistically significant in Supplemental Fig. 6, the latter group showed a profound increase in the number of insulin positive islets compared to zymosan recipients (Fig. 6C). Overall, the percentage of insulin positive islets were significantly low in control and β-cell-Ag treated mice compared to zymosan and zymosan plus β-cell-Ag recipients. These results suggest that the zymosan-induced innate immune response can significantly affect the infiltration of lymphocytes into the pancreatic islets, resulting in protection of insulin-producing islets from autoimmune destruction.

*T cells from zymosan and β-cell-Ag treated mice proliferate against β-cell-Ag and produce IL-17 and IL-10*

Spleen and PnLN cells obtained from mice that were euthanized 30 days post-treatment were examined for their ability to respond to an *ex vivo* challenge with β-cell-Ag. Both CD4+ and CD8+ T cells from mice that received β-cell-Ag or zymosan plus β-cell-Ag showed comparable extent of CFSE dilution upon *ex vivo* challenge, which was significantly higher than that of their counterparts from untreated control mice (Supplemental Fig. 8B). However, the cytokine profiles of spleen and PnLN cells from zymosan and β-cell-Ag recipient groups were significantly different when challenged with β-cell-Ag *ex vivo* (Fig. 7A). While all β-cell-Ag recipient groups produced comparable levels of IFN-γ, which is significantly higher than mice that have not received β-cell-Ag, zymosan plus β-cell-Ag recipients produced significantly higher amounts of
IL-10 and IL-17 as compared with mice that received β-cell-Ag alone. Further, as observed in Fig. 7B, while the CD4+IFN-γ+ T cell frequencies were comparable in all groups upon non-specific activation using PMA and ionomycin, similar to the observations of Fig. 7A, all zymosan-recipient groups had significantly higher frequencies of IL-10- and IL-4-expressing cells. Importantly, CD8+ T cells not only showed a similar trend, but the difference in frequencies of IL-10 and IL-4 producing cells was more pronounced among these T cells in zymosan plus β-cell-Ag treated mice, compared to untreated controls. Moreover, zymosan β-cell-Ag recipient mice showed higher number of IL-17-producing CD4+ and CD8+ T cells as compared to control groups. On the other hand, higher numbers of CD8+IFN-γ+ T cells were detected in β-cell-Ag, but not zymosan plus β-cell-Ag, treated mice. These observations suggest that zymosan-exposed APCs skew the T cell response against β-cell-Ag from IFN-γ production to IL-17, IL-10 and/or IL4 production (from pathogenic Th1 and TC1 to less pathogenic or T1D protective Th17 & TC17 and Th2 & TC2) in vivo.

_Treatment with zymosan and β-cell-Ag leads to increase in T cells with regulatory phenotype_

To realize whether zymosan and/or β-cell-Ag treatment can induce and/or expand T cells with regulatory properties, spleen and PnLN cells from zymosan and/or β-cell-Ag treated mice were examined for Foxp3+ and LAP+ T cells by FACS. As observed in Fig. 8A, significantly higher numbers of Foxp3+CD4+ T cells were found in the PnLN of mice treated with zymosan and β-cell-Ag as compared to control mice. However, the splenic Foxp3+CD4+ T cell frequencies in zymosan and β-cell-Ag treated mice were not different. Significantly higher numbers of splenic CD4+ T cells from mice that were treated with zymosan alone or along with β-cell-Ag were positive for surface LAP and most of these LAP-positive cells were found to be CD25- (not
shown). Splenic regulatory T cells from the mice that were euthanized 15-days post-treatment were adoptively transferred into 8- and 10-week-old female NOD mice to determine their potential to prevent hyperglycemia. CD4+CD25+ and LAP+ T cells isolated from the spleen of treated and untreated mice did not show significant differences in their ability to suppress autoimmunity, when transferred into young WT female mice (not shown), suggesting that the per cell suppressive abilities of these purified populations from treated mice are not superior to their control counterparts. On the other hand, as observed in Fig. 8B, pre-diabetic (8-week-old mice) that received total T cells from the spleen of zymosan plus β-cell-Ag treated mice showed higher protection from T1D compared to mice that received T cells from control groups of mice. Ten-week-old mice that received T cells from zymosan alone or along with β-cell-Ag also developed hyperglycemia relatively slowly compared to mice that received T cells from control mice. Moreover, WT NOD mice that received total spleen cells from treated and untreated mice (Supplemental Fig. 9) and NOD-Scid mice that received purified T cells also showed similar disease incidence trend as that of WT NOD mice (not shown). These observations, in association with Fig. 7, not only suggest that T cells play a key role in zymosan plus β-cell-Ag treatment-induced protection from T1D, but also indicate that the overall increase in multiple populations of T cells with regulatory phenotype, and not the superior ability of specific subsets, contributes to this disease protection.
Discussion

Previously, we and others have reported that inducing innate immune response through PRRs like TLR2 and Dectin 1 using the fungal cell wall agent, zymosan could suppress autoimmunity and prevent T1D in NOD mice (6; 8). Here, we show that co-administration of β-cell-Ag and zymosan leads to prolonged protection of NOD mice from diabetes and this effect appears to be dependent on a combination of zymosan-induced regulatory innate immune response involving IL-10, TGF-β1, IL-2 and Raldh1A2 and modulation of T cell response by zymosan-exposed APCs.

It has been shown that engagement of TLR2 by its ligands induces both pro- and anti-inflammatory cytokine responses by APCs and IL-10 in T cells (18-22). However, our observations show that the TLR2 ligands, Pam3Cys and Pam2Cys, do not induce IL-2 and TGF-β1 in APCs independently of Dectin 1 engagement (Supplemental Fig. 10). The synergistic signaling through TLR2 and Dectin 1 (23-26), although does not induce significant increase in the expression of co-inhibitory ligands such as PD-L1 and PD-L2, triggers the production of high levels of immune regulatory factors IL-10, TGF-β1, IL-2 and Raldh1A2 (6; 7; 10). Considering the involvement of these factors in immune regulation (10; 27-30), we hypothesized that Ag presentation by zymosan-exposed APCs that produce these factors can skew the T cell response from pro-inflammatory to anti-inflammatory/regulatory type. Our observations that Ag presentation by zymosan-exposed DCs can promote IL-10 and Foxp3 expression in T cells support this notion.

Since T1D is the result of gradual expansion of autoreactive T cells and increase in inflammation, exposure of APCs to the immunodominant self β-cell-Ag specific peptides in
early hyperglycemic NOD mice can result in the induction and/or expansion of pathogenic T cells, leading to early hyperglycemia (14; 15). Our observation that treatment with β-cell-Ag and zymosan induces protection from T1D in pre-diabetic NOD mice, more significantly, as compared to treatment with zymosan or β-cell-Ag alone suggests that the zymosan-exposed APCs modulate T cell response against β-cell-Ag in vivo, and this modulated response is protective in nature. This notion is substantiated by the observation that zymosan-exposed, β-cell-Ag loaded DCs could induce better Treg response and prevent T1D compared to β-cell-Ag loaded non-treated DCs in NOD mice. Importantly, although the treatment of NOD mice with low dose zymosan does not cause an increase in the frequencies of DCs or their subsets in spleen and pancreatic microenvironment, antigen presenting ability of these cells may profoundly be altered by exposure to zymosan in vivo.

The protective nature of T cells from zymosan and β-cell-Ag treated mice was evident from higher frequencies of T cells with regulatory phenotype, in pancreatic LNs in particular, and their ability to produce IL-10 and IL-4 upon challenge/exposure to β-cell-Ag. Interestingly, T cells from mice that were treated with zymosan and β-cell-Ag also produce relatively higher amounts of IL-17. These observations indicate that zymosan induced innate immune response contributes to the skewing of T cell response against β-cell-Ag from IFN-γ production (pathogenic Th1 and TC1) to IL-17, IL-10, and/or IL4 production (less pathogenic or T1D protective Th17 & TC17 and Th2 & TC2) in vivo. While T1D is generally considered to be a Th1 mediated autoimmune disease, skewing immune response to Th17 and Th2 type results in protection of NOD mice from the disease (31; 32). Recent studies, including ours, have shown that a shift in the immune response in NOD mice from Th1 to Th17 under various conditions can lead to prevention of the disease or delayed hyperglycemia (31; 32). On the other hand, the potential contribution of shift
in the T cell response towards IL-4 production when peptide vaccine, oral tolerance and adjuvant therapy approaches are employed has been well recognized for many years (33-37). Interestingly, we observed that the percentages of IL-10- and IL-4-positive T cells were higher in the spleen of both zymosan alone and zymosan plus β-cell-Ag groups indicating that these cells may be the result of zymosan-induced innate immune response. On the other hand, increase in the percentage of IL-17-producing cells was observed primarily in zymosan plus β-cell-Ag treated mice indicating a T cell response specific to this antigen under conditions of zymosan-induced innate immune response. In addition to the difference in cytokine profiles of T cells from zymosan and β-cell-Ag treated and control mice, these treated mice showed higher Foxp3+ T cell frequencies in the PnLN, but not spleen compared to zymosan treated mice. This suggests selective trafficking of Foxp3+ cells to the pancreatic microenvironment.

Treatment with pro-inflammatory agents such as bacterial LPS (TLR4 ligand) and poly I:C (TLR3 ligand) can delay hyperglycemia in NOD mice when the treatment is initiated at early stages (38-44). Although the potential mechanism is not known, a previous study has shown that the prolonged treatment of NOD mice at different stages of disease progression with LPS from Salmonella enterica results in an increase in the regulatory T cell numbers and protection from the T1D (39). Our observations show that treatment with E. coli LPS-exposed DCs can also result in a modest increase in Foxp3+ cells in vivo, but they fail to produce protection of the NOD mice from T1D. A role for TNF-α produced by APCs upon exposure to these TLR ligands and other microbial agents and a better immune regulation achieved through immune potentiation and activation of Tregs, especially at early stages of insulitis, have been suggested (45). In fact previous reports have shown that TNF-α has contradictory effect at different stages of disease development in NOD mice (46; 47). On the other hand, we and others have reported
the unique ability of zymosan to induce the expression of IL-10, TGF-β1, IL-2, PD-L1, and/or Raldh1A2 in APCs (6-10; 48) and promote protection from autoimmune diseases, even at prediabetic age and early hyperglycemic stage. Increased cell death in the PnLN, but not spleen, of zymosan treated mice suggests an additional mechanism to eliminate inflammatory immune cells and suppress autoimmunity. Lack of significant difference in the expression levels of FAS-L and TRAIL on DCs from zymosan treated mice suggests that cell death in the pancreatic microenvironment could be DC independent. On the other hand, our current observations that only zymosan, but not LPS, could induce immune regulatory factors such as IL-2, IL-10 and TGF-β1 in DCs, suppress the diabetogenic T cell function, and promote protection from T1D further demonstrate the regulatory nature of zymosan-associated innate immune response.

In spite of the disease promoting effect of zymosan in genetically susceptible mouse model of arthritis (49), the ability of zymosan to ameliorate and/or prevent disease progression has been demonstrated in T1D and EAE models (6-11). While low dose zymosan protected mice from EAE, treatment with high dose caused initiation of EAE through Th17 cells (11; 50). Th17 cells, unlike in T1D model where they are protective/less pathogenic (31; 32), play a pathogenic role in EAE, arthritis and many other autoimmune conditions (51-53). However, in association with previous reports including ours (31; 32), we demonstrate that skewing the T cell response to IL-17 production instead of IFN-γ, in combination with other factors such as Tregs, IL-10 and IL-4, results in suppressed autoimmunity and delayed hyperglycemia in NOD mice.

In conclusion, our observations show that TLR2 and Dectin 1 dependent innate immune response induced by zymosan is significantly different from that induced by a TLR4 ligand E. coli LPS. While co-operative signaling though TLR2 and Dectin 1 induced by zymosan (23-26) involves both pro- and anti-inflammatory responses, signaling through TLR4 induces primarily
pro-inflammatory responses in DCs. While the role of zymosan induced cell death at the site of inflammation in reduced insulitis and protection of NOD mice from T1D requires further investigation, our current study shows that the synergistic innate immune response mediated by TLR2 and Dectin 1 not only has the unique ability to produce regulatory innate immunity but also modulate T cell response to β-cell-Ag and protect NOD mice from T1D. Importantly, our observations show that an overall increase in T cells with regulatory properties and a skewing in the immune response (cells that express Foxp3, LAP, IL-10, IL-17 and IL-4), rather than the superior suppressive ability of an individual population, is responsible for the protection of NOD mice from T1D. We conclude that zymosan has the potential to be used as a tolerogenic / immune regulatory adjuvant for promoting β-cell-Ag specific immune modulation and reverse hyperglycemia, at least at early stages.
Conflict of Interest statement: Authors do not have any conflict(s) of interest to disclose.

Author contribution:  S.K.M. designed experiments, researched and analyzed data, M.H.S. researched data, R.G. researched and analyzed data and reviewed/edited the manuscript, B.M.J researched data and reviewed/edited the manuscript, N.P. researched data, and C.V. designed experiments, analyzed data, and wrote/reviewed/edited manuscript.

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Figure legends

FIGURE 1: Zymosan exposed DCs produce regulatory innate immune response and modulate T cell response. Immature DCs, generated in vitro from BM cells (BMDCs) using GM-CSF and IL-4 and freshly isolated splenic CD11c+ DCs were left untreated or exposed to zymosan (25 µg/ml) or LPS (1µg/ml) for different durations. A) Cytokine levels (as indicated) were measured by ELISA in supernatants obtained from the above cultures at 48h time point. Mean±SD of values from three to four individual experiments carried out in duplicate or triplicate are shown. B) Cells harvested after 12h were used in real-time quantitative PCR assays. Expression levels were calculated relative to house-keeping gene (actin) expression and the values of zymosan- and LPS-treated samples were compared against the value of untreated (none) sample which was considered as 1. This assay was repeated at least 3 times in duplicate and the mean values are shown. C) splenic DCs were incubated with zymosan or LPS and OVA (323-339) peptide for 24h, washed and incubated with purified T cells (CFSE-labeled or unlabeled) from OT-II TCR-Tg mice for 96 h. CFSE labeled cells were stained using fluorochrome-labeled anti-mouse CD4 Ab and examined for CFSE dilution by FACS. Cells, from wells where unlabeled T cells were used, were also stained for surface CD4 and intracellular Foxp3 and examined by FACS. Representative FACS graphs (upper panels) and mean ± SD of % values (lower panels) of CD4+ T cells with CFSE dilution and Foxp3 expression are shown. This experiment was repeated at least 4 times with similar results. D) Cell-free supernatants from these T cell cultures were tested for cytokine levels by ELISA. Mean±SD of values from three individual experiments carried out in duplicate or triplicate are shown. Statistical significance was assessed by comparing the values of zymosan and LPS groups separately with that of control group by t-test. *, p <0.05; **, p <0.01; ***, p <0.001.
FIGURE 2: **Zymosan, but not LPS, exposed NOD mouse DCs induce Foxp3+ and IL-10+ T cells.**

A) Purified T cells from NOD-Foxp3-GFP mice were activated using anti-CD3 Ab in the presence of zymosan- or LPS-exposed splenic DCs for 96h. Cells from these cultures were stained for surface CD4 and examined for GFP+ T cells. B) Supernatants from these NOD-Foxp3-GFP T cell cultures were examined for the amounts of secreted cytokines by ELISA. C) Splenic and BMDCs were prepared, cultured without or with zymosan and LPS for 24h and BDC2.5 peptide, washed and incubated with purified T cells from NOD-BDC2.5 TCR-Tg or NOD-BDC2.5-Foxp3-GFP mice for 96 h. NOD-BDC2.5-Foxp3-GFP T cells were stained for CD4 and examined for CD4+GFP+ T cells. D) Supernatants from primary cultures of NOD-BDC2.5 TCR-Tg cells were examined for the amounts of secreted cytokines by ELISA. These experiments were repeated at least 3 times with similar results. Representative FACS graphs (left panels) and mean ± SD of % values (right panel) of CD4+ T cells with GFP (Foxp3) are shown for panels A and C. Mean± SD values are shown for panels B and D. Statistical significance was assessed by comparing the values of zymosan and LPS groups separately with that of control group by t-test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

FIGURE 3: **Zymosan does not have pro-inflammatory adjuvant activity like bacterial LPS.**

Eight-week old euglycemic female NOD mice were injected (i.v.) with untreated (control group), β-cell-Ag pulsed (none group), β-cell-Ag pulsed zymosan-exposed (zymosan group), or β-cell-Ag pulsed LPS-exposed (LPS group) BM DCs (2x10^6 cells/mouse). A) Mice were bled every week post-injection to examine blood glucose levels. The group that received zymosan and/or LPS exposed DCs was compared in a log-rank test to control group for calculating $p$-value. B) One set of treated and control mice from parallel experiments were euthanized 30 days after the last injection. Pancreatic tissues were processed for H&E staining to evaluate insulitis as...
described in materials and methods. Islets with representative insulitis grade (upper panel) and the percentages of islets with different grades of lymphocyte infiltration plotted as bar diagram (lower panel) are shown. Sections of pancreatic tissues from 3 mice/group were examined for insulitis and the insulitis score of at least 150 islets/group was plotted as bar diagram. C) One set of mice were euthanized 15 days post-treatment and examined for CD4+Foxp3+T cell frequency in the spleen by FACS. Representative FACS graphs (left panel) and mean ± SD of values from 3 mice per group tested independently (right panel) are shown. Statistical significance was assessed by t-test. D) Four week old NOD-BDC2.5-Foxp3-GFP mice were injected i.v. with zymosan (25 µg/mouse/day) and bacterial LPS (5 µg/mouse/day) for 3 consecutive days or left untreated. All 3 groups of mice received BDC2.5 peptide (5 µg/mouse) on day 3. Spleen cells from these mice were tested for GFP expression in CD4+ T cells by FACS. Representative FACS graphs (left panel) and mean ± SD of % values (right panel) of CD4+ T cells with GFP (Foxp3) expression are shown. *, p <0.05; ***, p <001. E) T cells from the groups of mice described for panel C were injected into 4-week old WT NOD mice (2x10^6 cells/mouse) and tested for blood glucose levels every other day and mice with blood glucose level of 250 mg/dl for two consecutive tests were considered diabetic. Log-rank test was employed to calculate p-values.

FIGURE 4: Zymosan induced innate immune response does not suppress β-cell-Ag specific T cell proliferation, but modulate their diabetogenic function in vivo. Twelve-week-old pre-diabetic age WT NOD mice were injected i.v. with zymosan (25 µg/mouse/day) for 3 consecutive days or left untreated. One set of treated and untreated mice received β-cell-Ag (0.5 µg/mouse) on day 3. A) On day 5, these mice were injected i.v. with CFSE labeled T cells from NOD-BDC2.5 TCR-Tg mice (2x10^6 cells/mouse) and euthanized on day 9, CFSE dilution in
CD4+Vβ4+ T cells was examined by FACS. Vβ4+ cells were gated for the graphs shown. Please note that 100% CD4+ T cells from donor BDC2.5 mice are Vβ4+, approximately 7.0% WT NOD (recipient) mice T cells are also Vβ4+. Therefore, the indicated approach for analysis has been employed to calculate % CFSE low cells and mean fluorescence intensity (MFI) values for each graph, as shown in the extreme left panel. Representative graphs from two independent experiments are shown. B) Zymosan and β-cell-Ag treated and control mice were injected with ex vivo BDC2.5-peptide activated T cells from NOD-BDC2.5 TCR-Tg mice (2x10^6 cells/mouse) on day 5 and the recipient mice were monitored for hyperglycemia as described for Fig. 4. p-values were calculated by log-rank test.

FIGURE 5: Treatment using zymosan and β-cell-Ag resulted in better protection of NOD mice from hyperglycemia as compared to treatment with zymosan alone. Twelve-week old euglycemic female NOD mice were pooled, randomly picked, and left untreated (control) or treated with zymosan (on days 1, 3, 5, 13, 15, 17, 25, 27 and 29 with 25 µg/mouse/day). Some group of animals received β-cell-Ag on days 5, 17 and 29. A) Mice were checked every week for hyperglycemia and blood glucose level of 250 mg/dl for two consecutive weeks was considered diabetic. Log-rank test was performed to compare different groups of mice and the p-value is shown on each graph. The group that received β-cell-Ag was also compared to control mice. B) One set of treated and control mice from parallel experiments were euthanized 4 weeks after the last injection, pancreatic tissues were processed for H&E staining to evaluate insulitis as described in materials and methods. The percentages of islets with different grades of insulitis plotted as bar diagram are shown. Sections of pancreatic tissues from at least 4 mice/group were examined for insulitis and the insulitis score of at least 150 islets/group were plotted as bar diagram.
FIGURE 6: Treatment using zymosan and β-cell-Ag resulted in better protection of early hyperglycemic mice from diabetes as compared to mice that were treated with zymosan. A) Early hyperglycemic mice (glucose levels between 140 and 250 mg/dl; screened from 12-20-week-old mice, pooled, and randomly picked) were left untreated or treated (i.v.) with zymosan (on days 1, 3, 5, 13, 15, 17, 25, 27 and 29 with 25 µg/mouse/day). Some animals received β-cell-Ag on days 5, 17 and 29. Mice were bled every 3 days for glucose levels. Mice with glucose levels 250 mg/dl for two consecutive weeks were considered diabetic. Log-rank test was performed to compare the hyperglycemia incidence in treated and control groups of mice and the significant p-values are shown. Glucose levels of each group are shown in Supplemental Fig. 5. B) Pancreatic tissue obtained from a parallel set of euglycemic treated and control mice*, 4-weeks after the final injection, were sectioned and stained using anti-insulin antibody followed by anti-rat-IgG-Alexa fluor 488 reagent and anti-glucagon antibody followed by anti-rabbit-IgG Alexa fluor 568 reagent and mounted using antifade containing DAPI. Insulitis was scored based on DAPI staining as described in Materials and Methods. Representative islet areas (left panel) and the percentages of insulin positive and negative islets with different grades of insulitis plotted as bar diagram (right panel) are shown. At least 60 islets from a total of 3-4 mice were examined for each group. Statistical significance was calculated by comparing insulin positive islets with less than 50% infiltration; grade ≤3 insulitis. * Since 100% control mice developed overt-hyperglycemia within 5 weeks post-treatment initiation (no islet structures were left at this stage), fresh batch of mice with glucose levels between 140 and 250 mg/dl at the time of testing were included as surrogate control. Insulin and glucagon positive islets areas were not detected in pancreatic tissues from overt-hyperglycemic mice (not shown). Yellow fluorescence appears to be non-specific staining and/or auto-fluorescence.
FIGURE 7: Treatment using zymosan and β-cell-Ag modulates the T cell response in NOD mice. Twelve-week-old NOD mice were treated with zymosan and/or β-cell-Ag as described for Fig. 5, euthanized 30 days post-treatment, and spleen and PnLN cells were examined for proliferative and cytokine responses. A) Spleen and PnLN cells were cultured with β-cell-Ag for 72h and cell-free supernatants were tested for cytokine levels by ELISA. Mean±SD of values from at least three mice/group tested in triplicate are shown. B) Freshly isolated cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 4h and stained for CD4+ (upper panels) CD8+ (lower panels) cells and intracellular cytokines for FACS analysis. Representative FACS graphs of splenic CD4+ and CD8+ cells with specific cytokine expression (left panels) and mean±SD values of cells from 4-5 mice tested independently (right panels) are shown. Statistical significance was assessed by comparing the values of treated groups with that of untreated control group by t-test. *, p <0.05; **, p <0.01; ***p <001.

FIGURE 8: Treatment using zymosan and β-cell-Ag results in the induction of Foxp3+ and LAP+ T cells. Twelve-week-old NOD mice were treated with zymosan and/or β-cell-Ag as described for Fig. 5. A) One set of mice were euthanized 30 days post-treatment, and spleen and PnLN cells were examined for surface CD4 and intracellular Foxp3 and analyzed by FACS (upper panels). Cells were also stained surface CD4 and LAP for FACS analysis (lower panels). CD4+ population was gated for all panels shown. Representative FACS graphs (left panels) and mean±SD values of cells from at least 5 mice/group tested independently (right panels) are shown. Statistical significance was assessed by comparing the values of treated groups with that of untreated control group by t-test. *, p <0.05; **, p <0.01; ***p <001. B) Splenic T cells were purified from treated and untreated groups of mice and injected (i.v.) into 8-week-old (left panel) and 10 week-old (right panel) female NOD mice (2x10⁶ cell/mouse; 5 mice/group). These T cell
recipients were tested for blood glucose levels every week and the mice that showed glucose levels $>250$ mg/dl for two consecutive weeks were considered diabetic. Log-rank test was employed to assess statistical significance.
Diabetes

205x253mm (300 x 300 DPI)
A

Spleen

None  
BDC2.5peptide

19.8
218
69.2
161

15.5
191
61.2
151

17.9
206
63.8
200

20.5
189
56.0
150

PnLN

None  
BDC2.5peptide

67.7
173
90.2
95

63.0
183
84.9
85

55.0
224
87.3
129

72.6
134
88.1
109

CFSE

CD4

% CFSE low
MFI

100%

B

% Diabetic mice

0 2 4 6 8 10 12 14 16
Days (post-transfer)

None (n=5)  
β-cell Ag (n=5)  
Zymosan (n=5)  
Zymosan+  
β-cell-Ag (n=5)

p<0.0016  
p<0.0016  
p<0.0016
Diabetes

A

| Control | β-cell Ag | Zymosan | Zymosan + β-cell Ag |
|---------|-----------|---------|---------------------|
| Exp.2   | 11.2%     | 10.9%   | 12.3%               |
| CD25    | 4.01%     | 6.57%   | 8.13%               |

% Polk Cells

B

% Diabetes free mice

Week (post-transfer)

B

% Diabetes free mice

Week (post-transfer)
Supplemental Fig. 1: Treatment using bacterial LPS, but not zymosan, induces CRP. Pre-diabetic age NOD mice were treated with zymosan and LPS as described for Fig. 3. Serum samples collected 48 h after treatment were tested for CRP levels by ELISA. Shown are the mean ±SD values of three mice/group tested in triplicate. ***p <0.001
Supplemental Fig. 2: Effect of treatment using zymosan in NOD mice. Pre-diabetic age NOD mice were treated with zymosan and/or β-cell-Ag as described for Fig. 5, and euthanized 24 h post-treatment for obtaining the spleen, PnLN and pancreatic cells. A) Single cell suspensions were stained using Annexin V-PE, 7-AAD and CD4 examined for dead/dying cells (apoptotic and necrotic cells) by FACS. Total events (A) and events that are gated for CD4+ population (B) are shown. Representative FACS graphs of 2 mice/group tested in triplicate are shown. These experiments were done twice.
Supplemental Fig. 3: Effect of treatment using zymosan in NOD mice. Pre-diabetic age NOD mice were treated with zymosan and/or β-cell-Ag as described for Fig. 5, and euthanized 24 h post-treatment for obtaining spleen, PnLN and pancreas. A) Single cell suspensions of spleen and PnLN were stained using CD11c and CD45 specific antibodies and examined for DC frequencies by FACS. Pancreatic tissues were digested using collagenase followed by trypsin, and stained using CD11c specific antibody. B) Cell suspensions were also stained for CD11c, FAS-ligand and TRAIL expression and examined by FACS. CD11c+ events were gated for examining FAS and TRAIL expression on DCs.
Supplemental Fig. 4: Effect of treatment using zymosan in NOD mice. Pre-diabetic age NOD mice were treated with zymosan and/or β-cell-Ag as described for Fig. 5, and euthanized 24 h post-treatment for obtaining spleen and PnLN. Single cell suspensions were stained using CD11c, CD8a and PDCA1 specific antibodies and examined for CD8a+ (lymphoid), CD8a- (myeloid) and PDCA1+ (plasmacytoid) DC frequencies by FACS.
Supplemental Fig. 5: Effect of treatment using zymosan in NOD mice. Pre-diabetic age NOD mice were treated with zymosan and or β-cell-Ag as described for Fig. 5, and euthanized 24 h post-treatment. Liver and kidney tissue sections were stained using H&E. Images were acquired using a light microscope under a 20x objective. Images of representative areas of each tissue are shown. Two mice were tested/group.
Supplemental Fig. 6: Effect of zymosan treatment on the ability of NOD mouse T cells to respond to third party antigen. Pre-diabetic age NOD mice were treated with zymosan and/or β-cell-Ag as described for Fig. 5 and euthanized 24 h post-treatment for obtaining spleen cells. CFSE labeled spleen cells were cultured in the presence of spleen cells from C57BL6 mice in an MLR assay at different effector:target ratios. After 96 h, CFSE dilution in effector T cells was examined by FACS. Cells from two mice were tested in triplicate for each group and representative FACS graphs are shown.
Supplemental Fig. 7: Blood glucose levels of mice used in Fig. 6 during treatment and monitoring period. Mice with overt-hyperglycemic mice were euthanized within two weeks. Mice with blood glucose levels appeared as HI in the glucose meter are considered having 600 mg/dl for generating these graphs.
Supplemental Fig. 8A: **Insulitis in early-hyperglycemic mice that were treated using zymosan and β-cell Ag.** Mice were treated as described in Fig. 6. Pancreatic tissue obtained from a parallel set of euglycemic treated and control mice, *4 weeks after the final injection*, were processed for H&E staining to evaluate insulitis as described for Fig. 4. The percentage of islets with different grades of insulitis plotted as bar diagram are shown. At least 150 islets from a total of 4-5 mice were examined for each group. * Since 100% control mice developed overt hyperglycemia within 5-6 weeks post-treatment initiation (no islets left at this stage), fresh batch of mice with glucose levels between 140 and 200 mg/dl at the time of testing were included as surrogate control.

Supplemental Fig. 8B: Proliferative ability of T cells from zymosan and β-cell-Ag treated mice. Twelve-week-old NOD mice were treated with zymosan and/or β-cell-Ag as described for Fig. 5, euthanized 30 days post-treatment, and spleen and PnLN cells were examined for proliferative responses. CFSE-labeled spleen and PnLN cells were cultured with β-cell-Ag for 96h and examined for CD4 and CD8 cells having CFSE dilution by FACS. Representative FACS graphs (left panels) and mean±SD values of cells from 4-5 mice tested independently (right panels) are shown.
Supplemental Fig. 9: Adoptive transfer of spleen cells from zymosan and β-cell-Ag treated mice results in a significant delay of hyperglycemia in NOD mice. Total spleen cells were from treated and untreated groups of mice were i.v. injected into 8-week-old female NOD mice (5x10^6 cell/mouse; 5 mice/group). The recipients were tested for blood glucose levels every week and the mice that showed glucose levels >250 mg/dl for two consecutive weeks were considered diabetic. Log-rank test was employed to assess statistical significance.
Supplemental Fig. 10

Supplemental Fig. 10B: TLR2 and Dectin-1 co-engagement, but not TLR2 engagement alone, induces IL-2 and TGF-β1 in DCs. Immature DCs, generated in vitro from BM cells (BMDCs) using GM-CSF and IL-4, were cultured with zymosan (25 µg/ml) or LPS (2 µg/ml) for 48 h and tested for PD-L1 and PD-L2 expression by FACS. Mean fluorescence intensity (MFI) values are shown for each histogram.

Supplemental Fig. 10A: PD-1 ligand expression on zymosan exposed DCs. BMDCs, generated in vitro from BM cells (BMDCs) using GM-CSF and IL-4, were cultured with zymosan (25 µg/ml) or LPS (2 µg/ml) for 36 h and tested for PD-L1 and PD-L2 expression by FACS. Mean fluorescence intensity (MFI) values are shown for each histogram.

Supplemental Fig. 10: TLR2 and Dectin-1 co-engagement, but not TLR2 engagement alone, induces IL-2 and TGF-β1 in DCs. Immature DCs, generated in vitro from BM cells (BMDCs) using GM-CSF and IL-4, were cultured with zymosan (25 µg/ml), Pam3Cys (1.0 µg/ml), Pam2Cys (1.0 µg/ml), and Curdlan (5 µg/ml) for 48 h. Cytokine levels were measured in supernatants obtained from the above cultures by ELISA. Mean±SD of values from 2 individual experiments carried out in triplicate are shown. Zymosan treated groups were compared separately to that of untreated control (none) group by t-test. *, p <0.05; **, p <0.01; ***, p <0.001.