Immudex MHC I & MHC II Monomers
Superior quality and broad selection of ready-to-use and peptide-receptive monomers RUO and GMP available

The Journal of Immunology

This information is current as of March 4, 2022.

Roles for TGF-β and Programmed Cell Death 1 Ligand 1 in Regulatory T Cell Expansion and Diabetes Suppression by Zymosan in Nonobese Diabetic Mice

Oliver T. Burton, Paola Zaccone, Jenny M. Phillips, Hugo De La Peña, Zoltán Fehérvári, Miyuki Azuma, Sarah Gibbs, Brigitta Stockinger and Anne Cooke

J Immunol 2010; 185:2754-2762; Prepublished online 30 July 2010;
doi: 10.4049/jimmunol.1001365
http://www.jimmunol.org/content/185/5/2754

References This article **cites 71 articles**, 44 of which you can access for free at:
http://www.jimmunol.org/content/185/5/2754.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2010 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on March 4, 2022

Roles for TGF-β and Programmed Cell Death 1 Ligand 1 in Regulatory T Cell Expansion and Diabetes Suppression by Zymosan in Nonobese Diabetic Mice

Oliver T. Burton,*,[1] Paola Zaccone,*,[1] Jenny M. Phillips,* Hugo De La Peña,*,† Zoltán Fehérvári,* Miyuki Azuma,‡ Sarah Gibbs,* Brigitta Stockinger,† and Anne Cooke*

Zymosan is a complex fungal component shown to be capable of both promoting and suppressing the development of autoimmune disorders in mice. In this study, we show that a single injection of zymosan just prior to diabetes onset can significantly delay the progression of disease in NOD mice. Zymosan treatment of NOD mice induced the production of biologically active TGF-β from cells infiltrating the pancreas and was associated with expansion of programmed cell death 1 ligand 1$^+$TGF-β$^+$ macrophages and Foxp3$^+$ regulatory T cells in vivo. Neutralization of either TGF-β or programmed cell death 1 ligand 1 abrogated the protective effects of zymosan. Zymosan acted through TLR2 as well as ERK and p38 MAPK to induce macrophage secretion of TGF-β and promotion of Foxp3$^+$ regulatory T cells in vitro and in vivo. ***The Journal of Immunology*****, 2010, 185: 2754–2762.**

The development of type 1 diabetes in the NOD mouse is a spontaneous autoimmune process influenced by genetic and environmental factors, with significant roles for CD4 and CD8 T cells (1–4), B cells (5, 6), and innate APCs (7–9). APCs have been suggested to be involved during both initiation and effector stages of disease. For instance, roles for both B cells (5, 6) and CD11c$^+$CD11b$^+$ dendritic cells (DCs) (8) have been reported for priming autoreactive T cells in diabetes. Consistent with this, surgical removal of the pancreatic draining lymph nodes effectively prevents the onset of diabetes when performed on young NOD mice, but not in older mice (>10 wk of age) that already show substantial cellular infiltrates into the islets of Langerhans cells (10, 11). T cells that infiltrate the pancreas recruit CD11b$^+$ macrophages (Mϕs) (12), which are required within the pancreatic lesion alongside T cells to effect the destruction of the insulin-producing β cells (7).

Altering the environment in which autoantigens are presented strongly influences the balance of T cell responses and the progression of autoimmunity in NOD mice. Th1 cells are widely thought to mediate type 1 diabetes in NOD mice, and, accordingly, increases in cytokines such as IL-10, IL-2, or IL-4 can favor other T cell populations, including Th2 or Foxp3$^+$ regulatory T cells (Tregs) and inhibit autoimmune diabetes (13–16). TGF-β potently reduces the maturation status of APCs and inhibits Th1 responses (17, 18) and, accordingly, suppresses the onset of diabetes when expressed as a transgene in the islets or in DCs (19–22). Foxp3$^+$ Tregs have been invoked to explain the inhibitory effects of TGF-β on diabetes progression (18, 22, 23), and it has been shown in multiple systems that effector cells need to be able to respond to Treg-produced TGF-β for the Tregs to control diabetes development (17, 24, 25). Perhaps uniquely among regulatory cytokines, TGF-β may even assist in establishing tolerance in overtly diabetic mice, as has been suggested in the context of islet transplantation (23) and anti-CD3 therapy (26, 27).

Specialized tolerogenic DCs that express low levels of costimulatory molecules, or negative costimulatory molecules such as programmed cell death 1 ligand 1 (PD-L1), may preferentially induce Foxp3$^+$ Tregs (28–32). In the face of potent inhibitory signals, T cells may even become unresponsive, as has been shown to occur when PD-L1 interacts with its receptor, programmed cell death 1 (PD-1), on activated T cells (33, 34). In NOD mice, PD-L1 provides a critical check on effector T cells in the pancreas of NOD mice, and its absence results in aggressive infiltration of the pancreas and rapid onset of diabetes (35–38).

Exposure of NOD mice to active infections or antigenic preparations of bacteria, viruses, or helminth parasites can alter both APC and T cell phenotypes, suppressing the progression of autoimmunity (39), but until recently the effects of fungal Ags on diabetes onset were unknown (40). Interactions between fungal Ags and the cells of the innate immune system are capable of strongly polarizing T cell responses, giving rise to either proinflammatory subsets such as Th1 and Th17 cells or suppressive populations such as Tregs or IL-10–producing T cells (40–44). At the crux of this dichotomy lies the ability of fungal Ags to trigger a complex mixture of cytokine secretion from APCs, including IL-1, IL-6, TNF, and members of the IL-12 family alongside the suppressive cytokines IL-10 and TGF-β (40, 42, 43). The

*Department of Pathology, University of Cambridge, Cambridge; †Department of Molecular Immunology, Medical Research Council National Institute for Medical Research, London, United Kingdom; and ‡Department of Molecular Immunology, Tokyo Medical and Dental University, Tokyo, Japan

[1]O.T.B. and P.Z. contributed equally to this work.

Received for publication April 26, 2010. Accepted for publication June 26, 2010.

This work was supported by the Wellcome Trust, the Biotechnology and Biological Sciences Research Council, and the Juvenile Diabetes Research Foundation. O.T.B. was funded by a Herchel Smith Fellowship.

Address correspondence and reprint requests to Dr. Oliver Burton, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, United Kingdom. E-mail address: olivertburton@gmail.com

The online version of this article contains supplemental material.

Abbreviations used in this paper: Aldh1a1, retinaldehyde dehydrogenase; C_T, threshold cycle; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; *Hprt1*, hypoxanthine phophoribosyl transferase 1; LAP, latency-associated peptide; Mϕ, macrophage; PD-1, programmed cell death 1; PdBu, phorbol 12,13-dibutyrate; PD-L1, programmed cell death 1 ligand 1; pMϕ, peritoneal macrophage; Treg, regulatory T cell.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1001365

Downloaded from http://www.jimmunol.org/ by guest on March 4, 2022

induction of TGF-β is particularly important for driving Th17 and Foxp3$^+$ Treg responses, which have been implicated in the eradication (45–47) and persistence (48, 49), respectively, of fungal pathogens. Fungal Ags can also act as adjuvants, and zymosan, a complex fungal cell wall component, drives Th17 responses that promote autoimmunity in mice (44, 50). Recently, however, zymosan has been shown to initiate events favoring the generation of Foxp3$^+$ Tregs and suppressing the development of experimental autoimmune encephalomyelitis (EAE) (41, 42), and it has also been shown to prevent diabetes onset in NOD mice (40). In this paper, we investigate the ability of zymosan to drive innate immune responses favorable to Foxp3$^+$ T cell development in NOD mice, and we assess the contributions of specific molecules in the regulation of diabetes onset by zymosan and Tregs.

Materials and Methods

Mice

Female NOD/Tac mice were housed and barrier bred under specific pathogen-free conditions in the Pathology Department, University of Cambridge animal facilities (Cambridge, U.K.). Mice were used between 10 and 12 wk of age. All animal work was conducted under United Kingdom Home Office project license regulations after approval by the Ethical Review Committee of the University of Cambridge. Mice were routinely monitored for glucosuria using Diastix (Bayer Diagnostics, Basingstoke, U.K.).

Cell culture and reagents

All cells were cultured in IMDM supplemented with 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS. Anti-CD3 clone 145.2C11 (2 μg/ml) was grown and affinity purified in house. Cells were stimulated in the presence of zymosan from *Saccharomyces cerevisiae* (50 μg/ml; Sigma-Aldrich, St. Louis, MO), LPS from *Samonella enteritidis* (1 μg/ml; Sigma), FSL-1 (1 μg/ml; InvivoGen, San Diego, CA), or Pam_3CysK_4 (1 μg/ml; InvivoGen). Fluorescently conjugated Alexa 488–zymosan A bioparticles were purchased from Invitrogen (Paisley, U.K.). The retinoic acid receptor antagonist LE540 (1 μM; Wako Pure Chemicals, Osaka, Japan) and the ERK inhibitor U0126 (10 μM, Invivogen) were dissolved in DMSO, whereas the p38 MAPK inhibitor SB202190.HCl (5 μM; Enzo Life Sciences, Farmingdale, NY) was prepared in aqueous solution.

TGF-β bioassay

The MLE/PAI-L cell line is derived from mink lung epithelial cells and contains firefly luciferase under PAI-1 promoter control (51). MLE cells were cultured with 50 μl tissue culture supernatants or a double-dilution standard curve derived from recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) for 18 h. Cells were then lysed and luciferase activity was measured using a luciferase assay kit (Biotium, Hayward, CA) and a BetaLux scintillation counter (Wallac, PerkinElmer, Wellesley, MA).

Abs for neutralization and receptor blockade

Anti–TGF-β (1D11.16), anti–IL-6 (6B4), anti–IL-6R (15A7), anti–IL-10R (1B1.3a), and anti–PD-L1 (MIH5) were grown in-house and were precipitated from hybridoma supernatants and dialyzed against PBS before use. For in vivo cytokine neutralization experiments, Abs were injected i.p. 2 h prior to zymosan injection, then again at 48 h and on day 5. Other Abs were purchased, including anti-TLR2 (T2.5; eBioscience, San Diego, CA), anti-SIGNR1 (ER-TR9; Hycult Biotechnology, Uden, The Netherlands), and anti–dectin-1 (2A11; AbD Serotec, Oxford, U.K.), and were used at 20 μg/ml in vitro.

Isolation of pancreatic-infiltrating mononuclear cells and Mϕs

Whole pancreas samples were harvested and processed from individual mice. Briefly, pancreases were torn into smaller pieces in cold PBS containing 5% FCS, 56 mM glucose, and Complete Mini protease inhibitors (Roche Diagnostics, Basel, Switzerland). The tissues were washed twice in cold PBS before incubation in 2 ml prewarmed PBS containing 15% FCS, 10 μg/ml DNase I (Sigma-Aldrich), and 330 μg/ml Liberase CI (Roche Diagnostics). After digestion, tissues were washed and cell suspensions were prepared by forcing through a 70-μm cell strainer. Cells were then centrifuged through a 33% Percoll (GE Healthcare, Chalfont St. Giles, U.K.) gradient to obtain leukocyte populations.

Peritoneal Mϕs were flushed from the peritoneal cavity by lavage with cold PBS and isolated to >99% CD11b$^+$ Mϕs by adherence to plastic plates over 4 h at 37°C in IMDM with 10% FCS. Pancreatic Mϕs from zymosan-treated mice were separated from total pancreatic mononuclear cells similarly, reaching >95% CD11b$^+$CD11c$^-$ cells.

Flow cytometry

Cells were washed and resuspended in staining buffer (PBS, 2.5% FCS, and 0.05% NaN_3). Nonspecific binding was blocked using 2.4G2 anti-FcγR supernatant (prepared in-house). Cells were stained with appropriate combinations of the following Abs: CD3ε (2C11), CD4 (RM4-5), CD11b (M1/70), CD11c (HL3), Gr-1 (RB6-8C5), Foxp3 (FJK-16s), PD-L1 (MIH5), PD-1 (J43), IL-4 (11B11), IL-17 (TC11-18H10), and IFN-γ (XMG1.2). Biotinylated polyclonal goat anti-human latency-associated peptide (LAP) and biotinylated normal goat serum (R&D Systems) were used in conjunction with streptavidin-PE (BD Biosciences, San Diego, CA). For live cell discrimination, 7-aminoactinomycin D (BD Biosciences) was used.

Intracellular Foxp3 staining was performed according to the manufacturer's instructions (Foxp3 anti-mouse/rat Foxp3 staining set; eBioscience). For intracellular cytokine staining, cells were stimulated for 4 h with 500 ng/ml phorbol 12,13-dibutyrate and ionomycin, and cytokine secretion was blocked using 1 μg/ml brefeldin A (all from Sigma-Aldrich). Cells were fixed promptly in 1% paraformaldehyde-PBS and permeabilized with 0.5% saponin (Sigma-Aldrich) in staining buffer.

For CFSE dye dilution experiments, splenocytes (2×10^7) were labeled in 1 ml PBS containing 5 μM CFSE (Invitrogen) by incubating for 15–20 min at 37°C. The reaction was stopped and excess CFSE removed by washing with 10% FCS IMDM.

For detection of phosphorylated epitopes, Mϕs were stimulated for 45 min with Ags and then immediately fixed with 4% paraformaldehyde-PBS for 30 min at room temperature. Cells were permeabilized with 70% ice-cold methanol for at least 30 min. Methanol was later removed by two wash steps, and cells were exposed to Abs in staining buffer for 30 min using anti–phospho-p38 MAPK Thr^{180}/Tyr^{182} (28B10)-Alexa 488 and anti–phospho-p42/p44 Erk1/2 Thr^{202}/Thr^{204} (E10)-Alexa 647 (Cell Signaling Technologies, Danvers, MA). General staining procedures were adapted from work by Krutzik et al. (52).

Cells were acquired using a BD FACSCalibur, a BD FACScan (BD Biosciences), and a CyAn ADP (Beckman Coulter, Fullerton, CA) and were analyzed using FlowJo (Tree Star, Ashland, OR) software.

RNA isolation and real-time RT-PCR

Total RNA was isolated using an RNeasy kit, converted to cDNA using a reverse transcription kit, and quantified in real-time using Fast SYBR Green PCR (all from Qiagen, Valencia, CA). RNA was normalized prior to conversion into cDNA, and equal amounts of cDNA were analyzed in real-time. cDNA was analyzed in duplicate reactions with amplification of target gene and housekeeping gene (hypoxanthine phophoribosyl transferase 1 [*Hprt1*]) transcripts performed on the same reaction plate on a 7500 Fast real-time PCR system (Applied Biosystems). Proprietary QuantiTect primer assays for all genes were purchased from Qiagen. Relative gene expression was normalized to *Hprt1* according to the change in threshold cycle (C_T) method, $C_T^{Gene1} - C_T^{Hprt1}$.

Statistics

Statistical analyses were routinely performed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA). Diabetes progression was analyzed by plotting Kaplan-Meier survival curves and performing log-rank tests between the cohorts. The Mann-Whitney *U* test was used to assess the significance of nonparametric data, while unpaired *t* tests were used for parametric data sets. For comparisons between multiple samples, one-way ANOVA (for independent data sets) or repeated measures ANOVA (for paired data) was used. Tests performed and calculated two-tailed *p* values are indicated in the individual figure legends.

Results

Female NOD mice in our colony develop an incidence of diabetes that exceeds 70% penetrance by 20 wk of age, while substantial pancreatic infiltration and insulitis are already detectable by 10 wk of age. When 10- to 11-wk-old female prediabetic NOD mice received a single i.p. injection of zymosan, diabetes onset was significantly delayed, whereas earlier administration had little effect on the kinetics of diabetes onset (Fig. 1 and data not shown). Previous studies indicated a need for multiple immunizations to

Downloaded from http://www.jimmunol.org/ by guest on March 4, 2022

achieve tolerance (40); however, we found that a single treatment was sufficient to considerably delay diabetes progression.

Delay of diabetes onset by zymosan depends on TGF-β and PD-L1

The anti-inflammatory cytokines IL-10 and TGF-β have been shown to be produced in response to zymosan by both APCs and T cells (40), and IL-10 was found to be involved in the suppression of EAE by zymosan (41). Overexpression of TGF-β can ameliorate or prevent diabetes in the NOD mouse (21), as can expansion or activation of Tregs in vivo (53), and therefore we examined the contributions of TGF-β and IL-10 to the inhibition of diabetes onset by zymosan. We found that Ab neutralization of TGF-β abrogated the ability of zymosan to delay diabetes (Fig. 2*A*). IL-10 has also been suggested to assist in Treg induction by zymosan (41, 42), but IL-10R blockade did not affect the onset of diabetes in zymosan-treated NOD mice (Fig. 2*B*). In addition to driving Tregs via TGF-β (41), zymosan has been shown to initiate autoimmune pathologies by inducing TGF-β– and IL-6–dependent Th17 cells (44, 50). IL-6 had no role in zymosan-mediated inhibition of diabetes onset (data not shown), which suggested a lack of involvement of a Th17 response.

PD-L1–PD-1 interactions limit the progression of diabetes in NOD mice (35), and therefore we examined whether PD-L1 was involved in the delay of diabetes in zymosan-treated mice. We observed that irrespective of previous zymosan treatment, neutralization of PD-L1 triggered rapid onset of diabetes in NOD mice (Fig. 2*C*).

As substantial islet infiltrates were already present in pancreas of zymosan-injected mice, its mode of action was unlikely to be prevention of infiltration, but rather it suggested alterations in immune regulation within the pancreas involving rapidly responding innate immune cells. We therefore examined the composition and phenotypes of the cells recovered from the pancreas of zymosan-treated mice. The most remarkable and consistent changes we observed were to $CD11b^{+}CD11c^{-}$ cells, which increased dramatically within hours of zymosan treatment and remained elevated for weeks (Fig. 3*A*). Consistent with our findings of PD-L1–dependent diabetes inhibition, zymosan treatment markedly upregulated PD-L1 expression on $CD11b^{+}$ APCs recovered from the pancreas during the same period (Fig. 3*B*, 3*C*). Interestingly, zymosan treatment also induced surface expression of the LAP of TGF-β, and using fluorescently conjugated zymosan, we were able to determine that LAP expression was largely dependent on internalization of zymosan (Fig. 3*D*, 3*E*). Upon in vitro restimulation, mononuclear cells from the pancreas of zymosan-treated, but not untreated, NOD mice secreted substantial quantities of biologically active TGF-β (Fig. 3*F*).

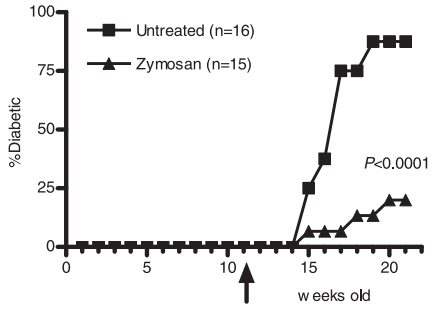

FIGURE 1. A single injection of zymosan significantly delays the onset of type 1 diabetes in NOD mice. Nondiabetic female NOD mice were injected once i.p. with 2 mg of zymosan at 11 wk of age (indicated by arrow), and monitored for the appearance of glucosuria. Statistical analysis was by log-rank test of Kaplan-Meier survival curves. Representative of at least three independent experiments. The experiment depicted was terminated when the mice reached 21 wk of age.

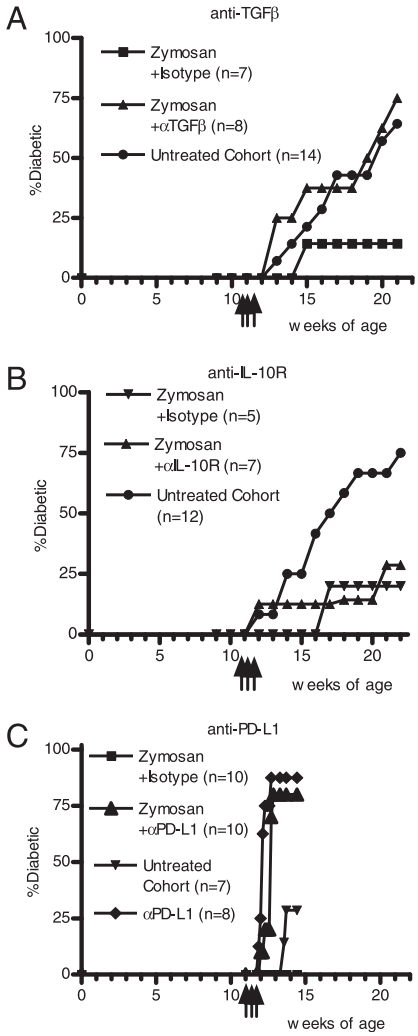

FIGURE 2. Delay of diabetes onset by zymosan requires TGF-β and PD-L1. *A*, Neutralization of TGF-β restores normal diabetes progression in zymosan-treated mice. *B*, Blockade of the IL-10R does not alter the ability of zymosan to delay diabetes. Nondiabetic female NOD mice were injected i.p. with 2 mg of zymosan at 10–11 wk of age and received 5 mg of Abs on days 0, 2, and 5 following zymosan treatment (indicated by arrows). *C*, Anti–PD-L1 triggers rapid onset of diabetes in both untreated and zymosan-treated NOD mice. Anti–PD-L1 (2 mg) was injected i.p. into 11-wk-old female NOD mice that had received zymosan 5 d previously or into untreated mice. For *C*, the Ab treatment was discontinued when mice became diabetic, and the experiment was halted when the mice reached 15 wk of age. The onset of diabetes was assessed by the appearance of glucose in the urine.

Zymosan drives bioactive TGF-β secretion from Mϕs via TLR2, ERK, and p38 MAPKs

Our findings of biologically active TGF-β secretion by pancreatic mononuclear cells from zymosan-treated mice are intriguing in view of the potent ability of TGF-β to inhibit diabetes development in NOD mice (19–21), as well as in view of our findings of a requirement for TGF-β in the delay of diabetes by zymosan (Fig. 2*A*). Relatively little is known about the pathways driving biologically active TGF-β production in response to fungal Ags. Transcription and translation of TGF-β are under complex regulation, and although latent TGF-β is expressed at high levels, little active TGF-β is available to act on cellular receptors (17). Post-translational processing of TGF-β involves proteolytic cleavage

Downloaded from http://www.jimmunol.org/ by guest on March 4, 2022

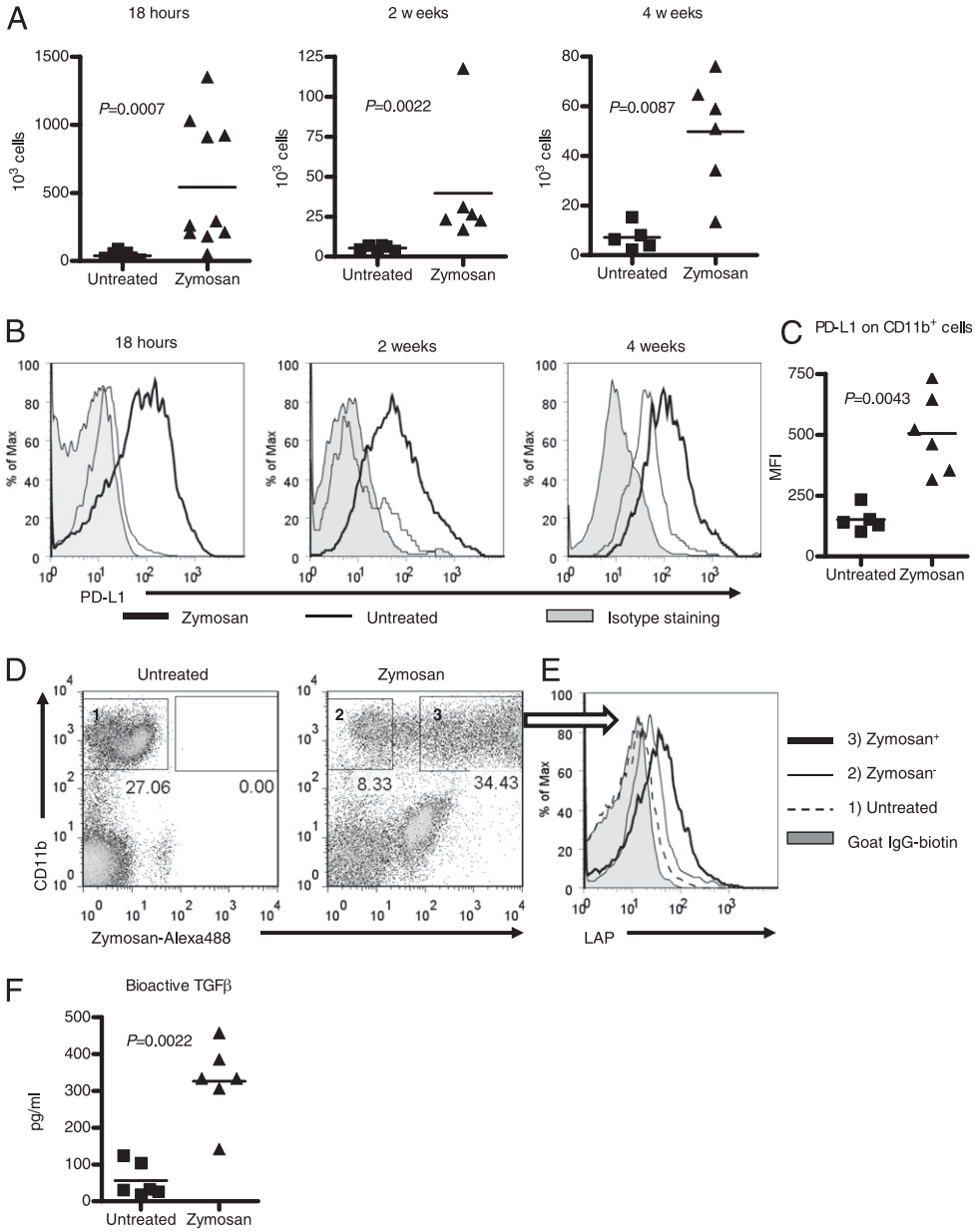

FIGURE 3. *A*, Zymosan provokes increases in CD11b$^+$ cells in the pancreas of NOD mice. Nondiabetic female NOD mice were injected i.p. with 2 mg zymosan at 10-11 wk of age, and leukocytes were extracted from the pancreas at 18 h, 2 wk or 4 wk posttreatment. Expression of CD11b, PD-L1 and LAP were assessed by flow cytometry. *B*, Pancreatic CD11b$^+$ Mϕs from zymosan-treated mice exhibit long-term upregulation of PD-L1. Representative FACS plots from 18 h, 2 wk, or 4 wk posttreatment. *C*, Summary data from six mice per condition at 2 wk posttreatment. *D*, Pancreatic Mϕs internalize zymosan-Alexa 488. Representative FACS plots are shown at 18 h after injection of 500 μg of zymosan-Alexa 488 i.p., gating on viable (7-aminoactinomycin D$^-$) cells. *E*, Pancreatic Mϕs that internalize zymosan-Alexa 488 express surface LAP. Data are shown gating on the CD11b$^+$ zymosan null or bright populations depicted in *D*: (*1*) CD11b$^+$ pancreatic Mϕs from an untreated mouse, (*2*) zymosan$^-$ Mϕs from a zymosan-treated mouse, and (*3*) zymosan$^+$ Mϕs. Goat IgG-biotin is a background staining control for the LAP Ab. Representative of two independent experiments. *F*, Upon in vitro culture, pancreatic leukocytes from zymosan-treated mice secrete substantial amounts of biologically active TGF-β. Mice ($n = 6$) were treated as in *A*, and after 18 h pancreatic leukocytes were extracted and 1×10^5 cells were cultured for 48 h. Pancreatic cell culture supernatants were analyzed using the MLE/PAI-L cell line. Statistical analysis by Mann-Whitney *U* test, two-tailed.

and extraction of active TGF-β from the latent complex with LAP (17), which we found expressed on CD11b$^+$ Mϕs in the pancreas of zymosan-treated mice (Fig. 3*D*). Consistent with our ex vivo findings of bioactive TGF-β production by pancreatic mononuclear cells from zymosan-treated mice (Fig. 3*E*), Mϕs stimulated in vitro with zymosan secreted robust amounts of biologically active TGF-β, which was not seen in response to the TLR2/1 ligand Pam_3CysK_4, the TLR4 agonist LPS, or the TLR2/6 agonist FSL-1 (Fig. 4*A*).

Many of the immune mediators produced by APCs in response to zymosan are induced following ligation of both TLR2 and dectin-1 (54, 55). SIGNR1 has also been implicated in the production of TNF (56), and, recently, dectin-2 has been shown to play a partially redundant role with dectin-1 in driving Syk-dependent cytokine secretion (46). Both the TLR2/MyD88 and dectin/Syk pathways trigger downstream activation of p38 and ERK MAPK, among other signaling molecules, leading to transcription of cytokine genes (43, 57). Using flow cytometric analysis of NOD peritoneal Mϕs (pMϕs), we confirmed that zymosan stimulation resulted in strong phosphorylation of both p38 and ERK MAPK, as assessed by flow cytometry (Fig. 4*B–D*). Phosphorylation of p38

Downloaded from http://www.jimmunol.org/ by guest on March 4, 2022

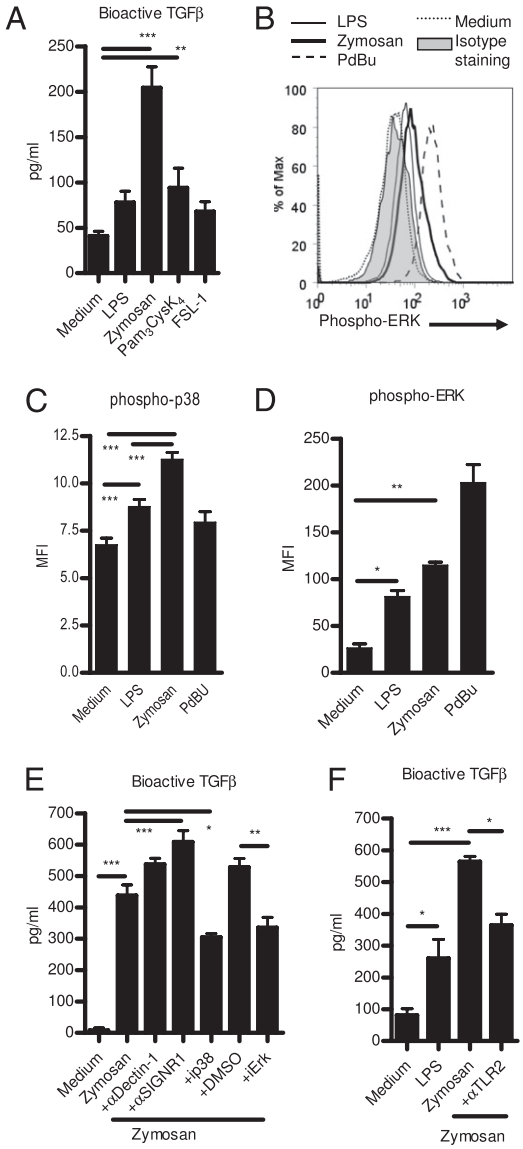

FIGURE 4. Secretion of bioactive TGF-β by pMϕs in response to zymosan depends on TLR2 and on p38 and ERK MAPKs. *A*, Zymosan drives the secretion of robust amounts of bioactive TGF-β from Mϕs. pMϕs (1×10^5) were cultured for 48 h in the presence of TLR agonists. *B*, Representative FACS plot showing phospho-ERK MAPK in zymosan-stimulated Mϕs. Greater p38 (*C*) and ERK (*D*) MAPK phosphorylation in response to zymosan than LPS stimulation. Phorbol 12,13-dibutyrate was used as a positive control to strongly activate ERK. *E*, Signaling through p38 and ERK MAPKs is required for maximal secretion of TGF-β by zymosan-stimulated pMϕs, whereas dectin-1 and SIGNR1 inhibit TGF-β production. *F*, Anti-TLR2 reduces production of bioactive TGF-β. Data presented as means ± SEM of three to four individual mice. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ between indicated groups. All *p* values were calculated using repeated measures ANOVA with Bonferroni's multiple comparison test. PdBu, phorbol 12,13-dibutyrate.

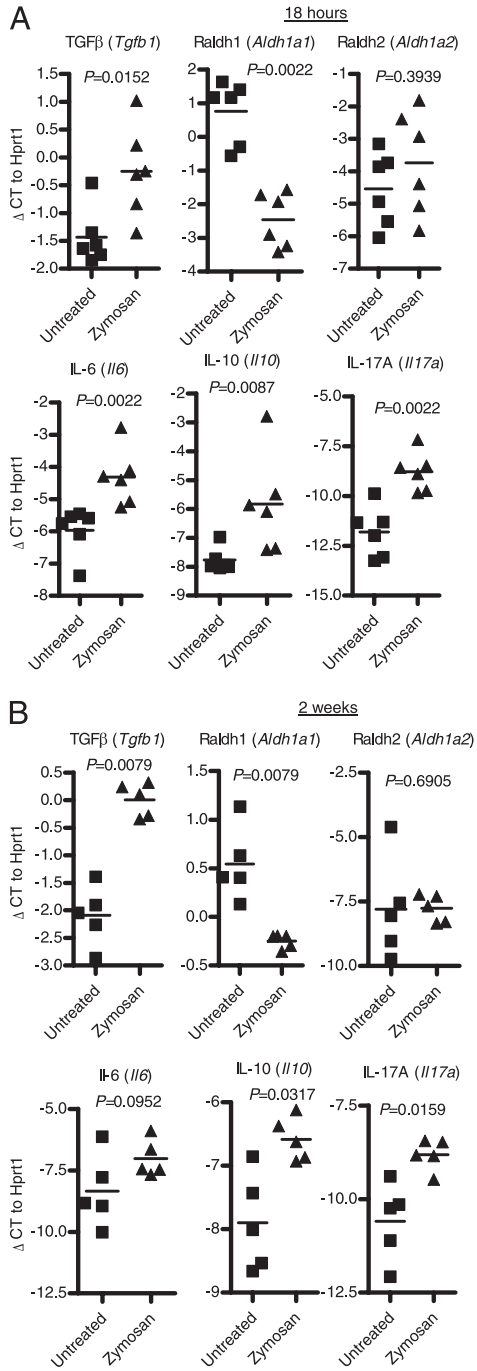

FIGURE 5. Zymosan upregulates TGF-β and other cytokines in the pancreas of prediabetic NOD mice. Nondiabetic female NOD mice were injected i.p. with 2 mg of zymosan at 10–11 wk of age, and leukocytes were isolated from the pancreas at (*A*) 18 h or (*B*) 2 wk posttreatment. Real-time RT-PCR was performed on mRNA-extracted pancreatic leukocytes from five to six individual mice. Data shown are the mean change in C_T values normalized to the housekeeping gene *Hprt1*. Statistical analysis by Mann-Whitney *U* test, two-tailed.

MAPK was greater than seen with the TLR4 agonist LPS (Fig. 4*C*). TLR2 ligation has been shown to activate both p38 and ERK MAPK (57), and inhibition of either kinase reduced the production of TGF-β (Fig. 4*E*).

A recent report detailing the ability of zymosan to suppress autoimmunity found that this effect was due to interactions with TLR2, whereas proinflammatory cytokines could be induced through dectin-1 (41). Therefore, we investigated the requirements for PRR ligation in Mϕ secretion of bioactive TGF-β. Ab blockade of TLR2 significantly reduced the production of TGF-β (Fig. 4*F*), whereas blockade of SIGNR1 or dectin-1 modestly increased the secretion of TGF-β (Fig. 4*E*).

Altered cytokine profiles in the pancreas of zymosan-treated NOD mice

Zymosan has been shown to promote Th17 responses (44, 50), which are dependent on TGF-β and IL-6 (58), as well as driving Foxp3$^+$ Tregs through a combination of TGF-β, IL-10, and retinoic

Downloaded from http://www.jimmunol.org/ by guest on March 4, 2022

acid (41). Because activation of Tregs or immune deviation to Th17 might forestall a Th1-driven pathology, we examined the expression of relevant genes in the cellular infiltrates recovered from the pancreas of zymosan-treated NOD mice. Along with TGF-β, expression of IL-6, IL-10, and IL-17 were increased at 18 h and 2 wk after injection (Fig. 5). Ab neutralization of IL-6 and IL-10 (Fig. 2*B* and data not shown) indicated little impact of these cytokines or a Th17 response on the progression of diabetes. The induction of TGF-β at both time points supports the critical role for this cytokine in suppression of diabetes by zymosan (Fig. 2*A*), and it is consistent with our findings of LAP expression by CD11b$^+$ Mϕs at 18 h after treatment (Fig. 3*E*) as well as the T cell secretion of TGF-β found by Karumuthil-Melethil et al. (40) in the adaptive phase.

Conversion of retinoic acid from retinaldehyde is controlled by multiple enzymes, including retinaldehyde dehydrogenases 1 and 2 (*Aldh1a1* and *Aldh1a2*), which have been recently found to be inducible by TLR ligands (41, 59) as well as being constitutively expressed in APCs from GALTs (28, 60). Although zymosan has been shown to upregulate expression of both *Aldh1a1* and *Aldh1a2* in splenic DCs from C57BL/6 mice (41), we did not find increased mRNA for either enzyme in the pancreatic infiltrating cells of zymosan-treated NOD mice, and in fact *Aldh1a1* levels diminished (Fig. 5), suggesting that retinoic acid induction may not be involved in the establishment of a benign pancreatic infiltrate by zymosan.

APCs from the pancreas of zymosan-treated mice expand Foxp3$^+$ Tregs in vitro

After in vivo exposure to zymosan, DCs have been shown to expand Foxp3$^+$ T cells through a combination of TGF-β, IL-10, and retinoic acid (41). PD-L1 expression on specialized DC subsets has also been suggested to enhance the conversion of naive T cells into Foxp3$^+$ Tregs (31, 32) and possibly maintain higher levels of Foxp3 expression in Tregs (31).

To examine the ability of infiltrating APCs to expand Tregs, we cocultured CFSE-labeled NOD splenocytes with the infiltrating monocytes in the presence of anti-CD3 and initially monitored the ability of these cells to influence division of Foxp3-expressing cells. We found that these infiltrating cells significantly enhanced the ratio of Foxp3$^+$-to-Foxp3$^-$ T cells in vitro (Fig. 6*A*). This increase in Foxp3$^+$ Tregs was reduced by neutralization of TGF-β (Fig. 6*B*), but not by anti–PD-L1 (Fig. 6*C*). In contrast to the data obtained by others using zymosan-exposed DCs, expansion of Foxp3$^+$ T cells by Mϕs from the pancreas of zymosan-treated NOD mice was not affected by inhibition of the retinoic acid receptor using LE540 (Fig. 6*D*). Although expression of PD-L1 and production of retinoic acid have been shown to contribute to DC-mediated induction of Foxp3$^+$ Tregs (28, 29, 32), less is known about the function of these molecules in Mϕs (60). To determine whether the CD11b$^+$TGF-β$^+$ PD-L1$^+$ APCs were the cells responsible for expanding Tregs, we fractionated the cells recovered from the pancreas of zymosan-treated mice by adherence, isolating a 95% pure population of

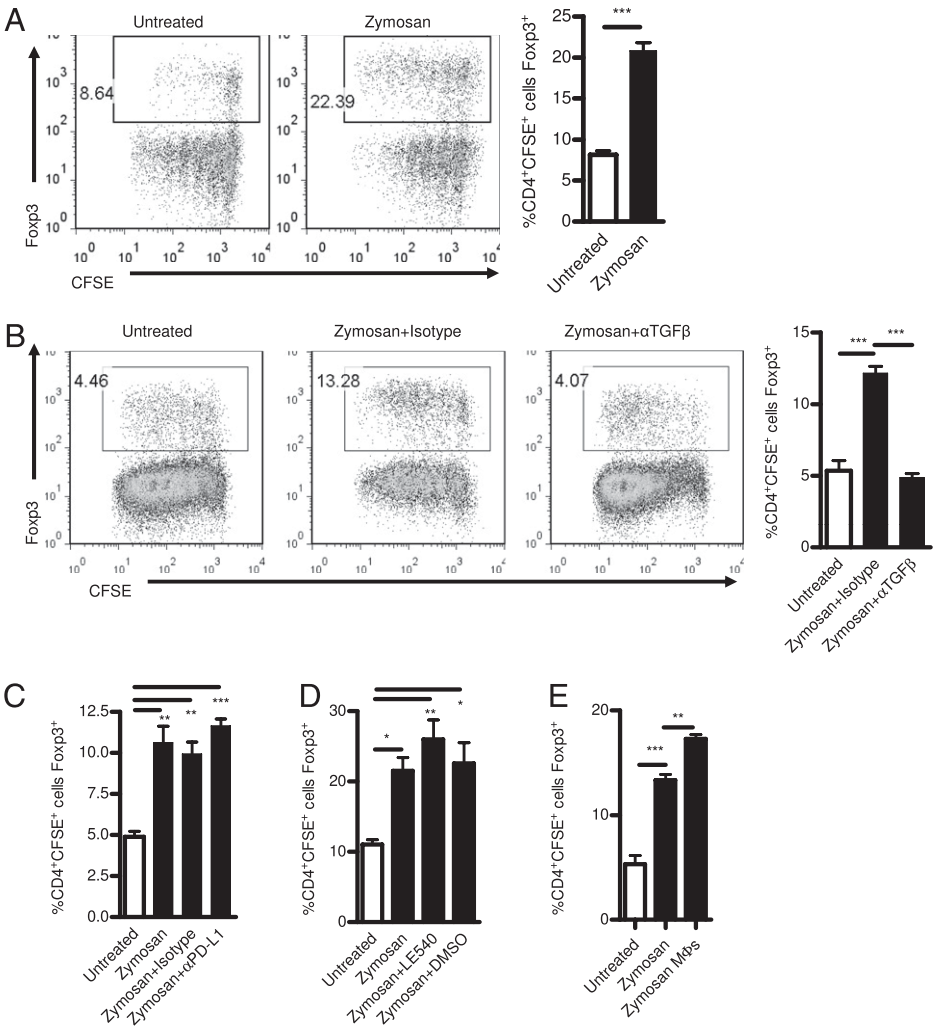

FIGURE 6. CD11b$^+$ Mϕs from the pancreas of zymosan-treated mice expand Foxp3$^+$ Tregs via TGF-β. *A*, The pancreatic infiltrate from zymosan-treated NOD mice expands Foxp3$^+$ Tregs in vitro. ***$p < 0.001$ by two-tailed unpaired *t* test. *B*, Zymosan-induced Treg expansion requires TGF-β. Foxp3$^+$ T cells expand independently of (*C*) PD-L1 or (*D*) retinoic acid. *E*, Expansion of Foxp3$^+$ T cells is mediated by adherent CD11b$^+$ cells. Pancreatic infiltrating leukocytes were isolated from untreated mice or mice that had received zymosan 18 h previously. Total pancreatic mononuclear cells or adherent Mϕs (1×10^5) were used to condition CFSE-labeled responding splenocytes (5×10^5) from untreated NOD mice in the presence of anti-CD3 (2 μg/ml). Representative FACS plots and summary data (n = 3-4 with means ± SEM) are shown analyzing CD4$^+$CFSE$^+$ cells, with numbers indicating the percentage of cells expressing Foxp3. Data shown are representative of at least two independent experiments each. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ between indicated groups by one-way ANOVA with Bonferroni's multiple comparison test.

Downloaded from http://www.jimmunol.org/ by guest on March 4, 2022

adherent CD11b$^+$CD11c$^-$ cells. These adherent cells displayed a significantly greater ability to expand Foxp3$^+$ T cells (Fig. 6*E*).

Zymosan promotes Foxp3$^+$ Tregs in the pancreas of NOD mice

A previous report on zymosan treatment of NOD mice showed activation of Tregs without altered proportional representation of Foxp3$^+$ Tregs in the spleen or pancreatic draining lymph nodes, but it did not examine the cells within the pancreatic lesion (40). We found that a single injection of zymosan dramatically increased both proportional representation and the absolute number of Foxp3$^+$ CD4$^+$ T cells in the pancreatic infiltrate as compared with untreated mice (Fig. 7). Despite increases in mRNA for IL-17 and IL-6 (Fig. 5), we did not observe significant changes in the frequencies of Th17, Th1, or Th2 cells in the pancreas following zymosan treatment (Supplemental Fig. 1 and data not shown).

Discussion

Previously it was shown that NOD mice could be protected from diabetes past 30 wk of age with a long course of repeated injections of zymosan (40). In this study, we have found that a single zymosan treatment evokes a significant delay, although diabetes does eventually occur (Fig. 1 and data not shown). We have used this treatment to understand how zymosan modifies the immune system and slows the autoimmune attack. Studies from the EAE model of multiple sclerosis suggested that effects of zymosan on DCs, particularly splenic DCs, suppressed the onset of autoimmunity via TLR2-dependent activation of ERK, leading to production of TGF-β, retinoic acid, and IL-10 (41, 42). We found important effects on pancreatic Mϕ populations in NOD mice, whereas the effects on CD11c$^+$ DCs were far less marked (data not shown). Considerable increases in CD11b$^+$ APCs expressing LAP and PD-L1 correlated with enhanced TGF-β mRNA and protein levels in the mononuclear cell infiltrates of zymosan-treated mice and suggested that these cells contributed to the TGF-β–dependent delay of diabetes onset. We therefore explored the mechanisms by which zymosan alters Mϕ phenotype and the onset of autoimmunity.

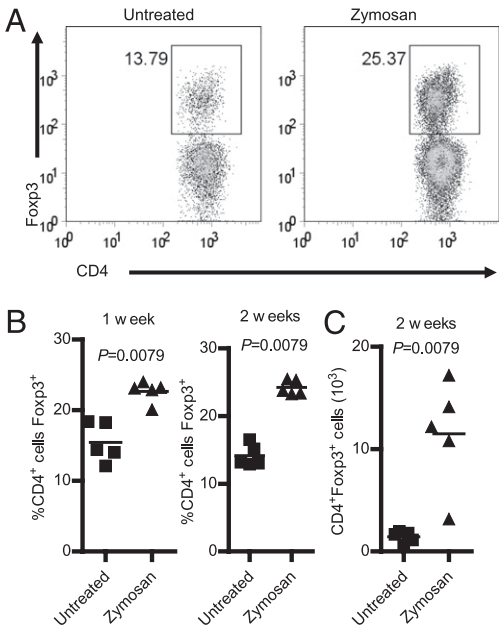

FIGURE 7. CD4$^+$Foxp3$^+$ Tregs increase in the pancreatic infiltrate following zymosan treatment. *A*, Representative FACS plots of Foxp3 expression in pancreatic CD4$^+$ T cells 2 wk after zymosan treatment. *B*, Summary data from five mice per group showing increased proportional representation of Foxp3$^+$ Tregs in the pancreas of zymosan-treated NOD mice at 1 or 2 wk posttreatment. *C*, Absolute numbers of pancreatic CD4$^+$ Foxp3$^+$ cells are also increased by zymosan treatment. Female NOD mice (10–11 wk old) were injected with 2 mg of zymosan i.p. Statistical analysis by two-tailed Mann-Whitney *U* test.

Zymosan is a complex Ag capable of interacting with multiple pattern recognition receptors, and we importantly identify TLR2 as a major receptor for driving bioactive TGF-β production by Mϕs. Zymosan induced Mϕ production of bioactive TGF-β through TLR2-involved activation of p38 and ERK MAPK. Strong activation of ERK in APCs has been suggested to induce cytokines that inhibit Th1 responses, and in some cases Th17 responses (41), while activation of p38 MAPK is known to be essential to TLR-driven activation of IL-12p70 (61). ERK-dependent cytokine induction by zymosan has been widely investigated, whereas p38 MAPK has been assumed to contribute to a lesser degree (41, 62). We found that bioactive TGF-β secretion by pMϕs was equally dependent on ERK and p38 MAPK, and it required TLR2 without assistance from dectin-1. However, Ab blockade of TLR2 did not completely abrogate TGF-β production, which may reflect incomplete receptor blockade or contributions of other receptors.

Different APC subsets regulate progression of diabetes in NOD mice (8), and treatments that enhance the frequency of immature or tolerogenic APC subsets, particularly those that promote Foxp3$^+$ Tregs, can suppress diabetes onset (63–65). In this study we found that TGF-β and PD-L1–expresssing APCs appeared in the pancreas in response to zymosan, and that delay of diabetes required TGF-β and PD-L1. We demonstrated that Mϕs isolated from the pancreas of zymosan-injected mice were able to expand Foxp3-expressing cells in vitro and that TGF-β was required for this expansion. As TGF-β is also known to inhibit Th1 responses and T cell proliferation (17), and to mediate aspects of Treg suppression (24), its effects may not be limited to Treg expansion in vivo.

In addition to shaping potentially tolerogenic T cell and APC phenotypes, TGF-β promotes the repair of damaged tissues through restructuring of the extracellular matrix (66), and it is conceivable that TGF-β induced by zymosan treatment may also act directly or indirectly to enhance the survival or function of residual β cells. During development, TGF-β promotes the development and differentiation of the endocrine pancreas over exocrine tissue (21, 67–69). In the adult pancreas, however, TGF-β signals have been shown to repress insulin transcription in β cells via Smad3 (70), suggesting that the direct effects of TGF-β on the islets would disfavor glucose regulation. In contrast, the immunological roles of TGF-β in suppressing type 1 diabetes are well established (18–27).

Interactions of PD-L1 with its receptor PD-1 are required to maintain peripheral tolerance at all stages in the development of diabetes in NOD mice (35). It has been suggested that the critical site of PD-L1 expression for suppression of diabetes development is in fact on the islets of Langerhans cells, and that the contributions of PD-L1 on hematopoietic cells are negligible (37). Epithelial cells not of hematopoietic origin can also function as APCs in tissues, however (71), and not all of the CD11b$^+$PD-L1^{high} APCs present in the pancreas following zymosan treatment express CD45, the common leukocyte Ag (data not shown). Furthermore, in zymosan-treated NOD mice, PD-L1 expression on CD11b$^+$ APCs is induced to far higher than unstimulated levels, and T cell expression of PD-1 also increases (Supplemental Fig. 2). Since a relatively benign pancreatic infiltrate exists in zymosan-treated NOD mice, it is possible that PD-L1 is actively required to reinforce diabetes prevention. We did not find any role for PD-L1 in the induction of Foxp3 expression by zymosan, but it has been shown that PD-L1 can potentiate and maintain Foxp3$^+$ Treg

Downloaded from http://www.jimmunol.org/ by guest on March 4, 2022

suppressive function (31) as well as inducing Foxp3 expression (31, 32). We cannot rule out a link between PD-L1 and Foxp3$^+$ Tregs in NOD mice in vivo following zymosan treatment.

In this study we provide insight into the innate and adaptive immune modulation that occurs in NOD mice treated with a fungal Ag, and we demonstrate the importance of effects on pancreatic Mϕs. We provide further evidence that innate activation through TLR2 can have tolerogenic effects, and that induction of TGF-β and PD-L1 significantly delays the onset of overt diabetes in mice with advanced insulitis.

Acknowledgments

We thank Dr. Nick Holmes and Dr. Stephen Newland for helpful discussions and Yvonne Sawyer for technical assistance with the project.

Disclosures

The authors have no financial conflicts of interest.

References

1. Mora, C., F. S. Wong, C. H. Chang, and R. A. Flavell. 1999. Pancreatic infiltration but not diabetes occurs in the relative absence of MHC class II-restricted CD4 T cells: studies using NOD/CIITA-deficient mice. *J. Immunol.* 162: 4576–4588.
2. Miller, B. J., M. C. Appel, J. J. O'Neil, and L. S. Wicker. 1988. Both the Lyt-2$^+$ and L3T4$^+$ T cell subsets are required for the transfer of diabetes in nonobese diabetic mice. *J. Immunol.* 140: 52–58.
3. Wicker, L. S., E. H. Leiter, J. A. Todd, R. J. Renjilian, E. Peterson, P. A. Fischer, P. L. Podolin, M. Zijlstra, R. Jaenisch, and L. B. Peterson. 1994. β_2-microglobulin-deficient NOD mice do not develop insulitis or diabetes. *Diabetes* 43: 500–504.
4. Phillips, J. M., N. M. Parish, C. Bland, Y. Sawyer, H. De La Peña, and A. Cooke. 2009. Type 1 diabetes development requires both CD4$^+$ and CD8$^+$ T cells and can be reversed by non-depleting antibodies targeting both T cell populations. *Rev. Diabet. Stud.* 6: 97–103.
5. Serreze, D. V., H. D. Chapman, D. S. Varnum, M. S. Hanson, P. C. Reifsnyder, S. D. Richard, S. A. Fleming, E. H. Leiter, and L. D. Shultz. 1996. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Igμ null mice. *J. Exp. Med.* 184: 2049–2053.
6. Serreze, D. V., S. A. Fleming, H. D. Chapman, S. D. Richard, E. H. Leiter, and R. M. Tisch. 1998. B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J. Immunol.* 161: 3912–3918.
7. Hutchings, P., H. Rosen, L. O'Reilly, E. Simpson, S. Gordon, and A. Cooke. 1990. Transfer of diabetes in mice prevented by blockade of adhesion-promoting receptor on macrophages. *Nature* 348: 639–642.
8. Saxena, V., J. K. Ondr, A. F. Magnusen, D. H. Munn, and J. D. Katz. 2007. The countervailing actions of myeloid and plasmacytoid dendritic cells control autoimmune diabetes in the nonobese diabetic mouse. *J. Immunol.* 179: 5041–5053.
9. Jun, H. S., C. S. Yoon, L. Zbytnuik, N. van Rooijen, and J. W. Yoon. 1999. The role of macrophages in T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J. Exp. Med.* 189: 347–358.
10. Höglund, P., J. Mintern, C. Waltzinger, W. Heath, C. Benoist, and D. Mathis. 1999. Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. *J. Exp. Med.* 189: 331–339.
11. Gagnerault, M. C., J. J. Luan, C. Lotton, and F. Lepault. 2002. Pancreatic lymph nodes are required for priming of β cell reactive T cells in NOD mice. *J. Exp. Med.* 196: 369–377.
12. Cantor, J., and K. Haskins. 2007. Recruitment and activation of macrophages by pathogenic CD4 T cells in type 1 diabetes: evidence for involvement of CCR8 and CCL1. *J. Immunol.* 179: 5760–5767.
13. Tang, Q., J. Y. Adams, C. Penaranda, K. Melli, E. Piaggio, E. Sgouroudis, C. A. Piccirillo, B. L. Salomon, and J. A. Bluestone. 2008. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity* 28: 687–697.
14. Gallichan, W. S., B. Balasa, J. D. Davies, and N. Sarvetnick. 1999. Pancreatic IL-4 expression results in islet-reactive Th2 cells that inhibit diabetogenic lymphocytes in the nonobese diabetic mouse. *J. Immunol.* 163: 1696–1703.
15. Goudy, K. S., B. R. Burkhardt, C. Wasserfall, S. Song, M. L. Campbell-Thompson, T. Brusko, M. A. Powers, M. J. Clare-Salzler, E. S. Sobel, T. M. Ellis, et al. 2003. Systemic overexpression of IL-10 induces CD4$^+$CD25$^+$ cell populations in vivo and ameliorates type 1 diabetes in nonobese diabetic mice in a dose-dependent fashion. *J. Immunol.* 171: 2270–2278.
16. Pennline, K. J., E. Roque-Gaffney, and M. Monahan. 1994. Recombinant human IL-10 prevents the onset of diabetes in the nonobese diabetic mouse. *Clin. Immunol. Immunopathol.* 71: 169–175.
17. Li, M. O., Y. Y. Wan, S. Sanjabi, A. K. Robertson, and R. A. Flavell. 2006. Transforming growth factor-β regulation of immune responses. *Annu. Rev. Immunol.* 24: 99–146.
18. Peng, Y., Y. Laouar, M. O. Li, E. A. Green, and R. A. Flavell. 2004. TGF-β regulates in vivo expansion of Foxp3-expressing CD4$^+$CD25$^+$ regulatory T cells responsible for protection against diabetes. *Proc. Natl. Acad. Sci. USA* 101: 4572–4577.
19. Moritani, M., K. Yoshimoto, S. F. Wong, C. Tanaka, T. Yamaoka, T. Sano, Y. Komagata, J. Miyazaki, H. Kikutani, and M. Itakura. 1998. Abrogation of autoimmune diabetes in nonobese diabetic mice and protection against effector lymphocytes by transgenic paracrine TGF-β1. *J. Clin. Invest.* 102: 499–506.
20. Piccirillo, C. A., Y. Chang, and G. J. Prud'homme. 1998. TGF-β1 somatic gene therapy prevents autoimmune disease in nonobese diabetic mice. *J. Immunol.* 161: 3950–3956.
21. Grewal, I. S., K. D. Grewal, F. S. Wong, H. Wang, D. E. Picarella, C. A. Janeway, Jr., and R. A. Flavell. 2002. Expression of transgene encoded TGF-β in islets prevents autoimmune diabetes in NOD mice by a local mechanism. *J. Autoimmun.* 19: 9–22.
22. Luo, X., K. V. Tarbell, H. Yang, K. Pothoven, S. L. Bailey, R. Ding, R. M. Steinman, and M. Suthanthiran. 2007. Dendritic cells with TGF-β1 differentiate naive CD4$^+$CD25$^-$ T cells into islet-protective Foxp3$^+$ regulatory T cells. *Proc. Natl. Acad. Sci. USA* 104: 2821–2826.
23. Luo, X., H. Yang, I. S. Kim, F. Saint-Hilaire, D. A. Thomas, B. P. De, E. Ozkaynak, T. Muthukumar, W. W. Hancock, R. G. Crystal, and M. Suthanthiran. 2005. Systemic transforming growth factor-β1 gene therapy induces Foxp3$^+$ regulatory cells, restores self-tolerance, and facilitates regeneration of β cell function in overtly diabetic nonobese diabetic mice. *Transplantation* 79: 1091–1096.
24. Green, E. A., L. Gorelik, C. M. McGregor, E. H. Tran, and R. A. Flavell. 2003. CD4$^+$CD25$^+$ T regulatory cells control anti-islet CD8$^+$ T cells through TGF-β-TGF-β receptor interactions in type 1 diabetes. *Proc. Natl. Acad. Sci. USA* 100: 10878–10883.
25. Tonkin, D. R., and K. Haskins. 2009. Regulatory T cells enter the pancreas during suppression of type 1 diabetes and inhibit effector T cells and macrophages in a TGF-β-dependent manner. *Eur. J. Immunol.* 39: 1313–1322.
26. Belghith, M., J. A. Bluestone, S. Barriot, J. Mégret, J. F. Bach, and L. Chatenoud. 2003. TGF-β-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat. Med.* 9: 1202–1208.
27. You, S., B. Leforban, C. Garcia, J. F. Bach, J. A. Bluestone, and L. Chatenoud. 2007. Adaptive TGF-β-dependent regulatory T cells control autoimmune diabetes and are a privileged target of anti-CD3 antibody treatment. *Proc. Natl. Acad. Sci. USA* 104: 6335–6340.
28. Coombes, J. L., K. R. Siddiqui, C. V. Arancibia-Cárcamo, J. Hall, C. M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103$^+$ DCs induces Foxp3$^+$ regulatory T cells via a TGF-β and retinoic acid-dependent mechanism. *J. Exp. Med.* 204: 1757–1764.
29. Sun, C. M., J. A. Hall, R. B. Blank, N. Bouladoux, M. Oukka, J. R. Mora, and Y. Belkaid. 2007. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J. Exp. Med.* 204: 1775–1785.
30. Yamazaki, S., D. Dudziak, G. F. Heidkamp, C. Fiorese, A. J. Bonito, K. Inaba, M. C. Nussenzweig, and R. M. Steinman. 2008. CD8$^+$CD205$^+$ splenic dendritic cells are specialized to induce Foxp3$^+$ regulatory T cells. *J. Immunol.* 181: 6923–6933.
31. Francisco, L. M., V. H. Salinas, K. E. Brown, V. K. Vanguri, G. J. Freeman, V. K. Kuchroo, and A. H. Sharpe. 2009. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J. Exp. Med.* 206: 3015–3029.
32. Wang, L., K. Pino-Lagos, V. C. de Vries, I. Guleria, M. H. Sayegh, and R. J. Noelle. 2008. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3$^+$CD4$^+$ regulatory T cells. *Proc. Natl. Acad. Sci. USA* 105: 9331–9336.
33. Fife, B. T., K. E. Pauken, T. N. Eagar, T. Obu, J. Wu, Q. Tang, M. Azuma, M. F. Krummel, and J. A. Bluestone. 2009. Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. *Nat. Immunol.* 10: 1185–1192.
34. Fife, B. T., and J. A. Bluestone. 2008. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunol. Rev.* 224: 166–182.
35. Ansari, M. J., A. D. Salama, T. Chitnis, R. N. Smith, H. Yagita, H. Akiba, T. Yamazaki, M. Azuma, H. Iwai, S. J. Khoury, et al. 2003. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J. Exp. Med.* 198: 63–69.
36. Guleria, I., M. Gubbels Bupp, S. Dada, B. Fife, Q. Tang, M. J. Ansari, S. Trikudanathan, N. Vadivel, P. Fiorina, H. Yagita, et al. 2007. Mechanisms of PDL1-mediated regulation of autoimmune diabetes. *Clin. Immunol.* 125: 16–25.
37. Keir, M. E., S. C. Liang, I. Guleria, Y. E. Latchman, A. Qipo, L. A. Albacker, M. Koulmanda, G. J. Freeman, M. H. Sayegh, and A. H. Sharpe. 2006. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J. Exp. Med.* 203: 883–895.
38. Wang, J., T. Yoshida, F. Nakaki, H. Hiai, T. Okazaki, and T. Honjo. 2005. Establishment of NOD-Pdcd1$^{-/-}$ mice as an efficient animal model of type I diabetes. *Proc. Natl. Acad. Sci. USA* 102: 11823–11828.
39. Dunne, D. W., and A. Cooke. 2005. A worm's eye view of the immune system: consequences for evolution of human autoimmune disease. *Nat. Rev. Immunol.* 5: 420–426.
40. Karumuthil-Melethil, S., N. Perez, R. Li, and C. Vasu. 2008. Induction of innate immune response through TLR2 and dectin 1 prevents type 1 diabetes. *J. Immunol.* 181: 8323–8334.

Downloaded from http://www.jimmunol.org/ by guest on March 4, 2022

41. Manicassamy, S., R. Ravindran, J. Deng, H. Oluoch, T. L. Denning, S. P. Kasturi, K. M. Rosenthal, B. D. Evavold, and B. Pulendran. 2009. Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. *Nat. Med.* 15: 401–409.
42. Dillon, S., S. Agrawal, K. Banerjee, J. Letterio, T. L. Denning, K. Oswald-Richter, D. J. Kasprowicz, K. Kellar, J. Pare, T. van Dyke, et al. 2006. Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. *J. Clin. Invest.* 116: 916–928.
43. LeibundGut-Landmann, S., O. Gross, M. J. Robinson, F. Osorio, E. C. Slack, S. V. Tsoni, E. Schweighoffer, V. Tybulewicz, G. D. Brown, J. Ruland, et al. 2007. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat. Immunol.* 8: 630–638.
44. Veldhoen, M., R. J. Hocking, R. A. Flavell, and B. Stockinger. 2006. Signals mediated by transforming growth factor-β initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease. *Nat. Immunol.* 7: 1151–1156.
45. De Luca, A., C. Montagnoli, T. Zelante, P. Bonifazi, S. Bozza, S. Moretti, C. D'Angelo, C. Vacca, L. Boon, F. Bistoni, et al. 2007. Functional yet balanced reactivity to *Candida albicans* requires TRIF, MyD88, and IDO-dependent inhibition of Rorc. *J. Immunol.* 179: 5999–6008.
46. Robinson, M. J., F. Osorio, M. Rosas, R. P. Freitas, E. Schweighoffer, O. Gross, J. S. Verbeek, J. Ruland, V. Tybulewicz, G. D. Brown, et al. 2009. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J. Exp. Med.* 206: 2037–2051.
47. Lin, L., A. S. Ibrahim, X. Xu, J. M. Farber, V. Avanesian, B. Baquir, Y. Fu, S. W. French, J. E. Edwards, Jr., and B. Spellberg. 2009. Th1-Th17 cells mediate protective adaptive immunity against *Staphylococcus aureus* and *Candida albicans* infection in mice. *PLoS Pathog.* 5: e1000703.
48. Netea, M. G., R. Sutmuller, C. Hermann, C. A. Van der Graaf, J. W. Van der Meer, J. H. van Krieken, T. Hartung, G. Adema, and B. J. Kullberg. 2004. Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. *J. Immunol.* 172: 3712–3718.
49. Sutmuller, R. P., M. H. den Brok, M. Kramer, E. J. Bennink, L. W. Toonen, B. J. Kullberg, L. A. Joosten, S. Akira, M. G. Netea, and G. J. Adema. 2006. Toll-like receptor 2 controls expansion and function of regulatory T cells. *J. Clin. Invest.* 116: 485–494.
50. Yoshitomi, H., N. Sakaguchi, K. Kobayashi, G. D. Brown, T. Tagami, T. Sakihama, K. Hirota, S. Tanaka, T. Nomura, I. Miki, et al. 2005. A role for fungal β-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. *J. Exp. Med.* 201: 949–960.
51. Abe, M., J. G. Harpel, C. N. Metz, I. Nunes, D. J. Loskutoff, and D. B. Rifkin. 1994. An assay for transforming growth factor-β using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal. Biochem.* 216: 276–284.
52. Krutzik, P. O., M. R. Clutter, and G. P. Nolan. 2005. Coordinate analysis of murine immune cell surface markers and intracellular phosphoproteins by flow cytometry. *J. Immunol.* 175: 2357–2365.
53. Anderson, M. S., and J. A. Bluestone. 2005. The NOD mouse: a model of immune dysregulation. *Annu. Rev. Immunol.* 23: 447–485.
54. Brown, G. D., J. Herre, D. L. Williams, J. A. Willment, A. S. Marshall, and S. Gordon. 2003. Dectin-1 mediates the biological effects of β-glucans. *J. Exp. Med.* 197: 1119–1124.
55. Gantner, B. N., R. M. Simmons, S. J. Canavera, S. Akira, and D. M. Underhill. 2003. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* 197: 1107–1117.
56. Taylor, P. R., G. D. Brown, J. Herre, D. L. Williams, J. A. Willment, and S. Gordon. 2004. The role of SIGNR1 and the β-glucan receptor (dectin-1) in the nonopsonic recognition of yeast by specific macrophages. *J. Immunol.* 172: 1157–1162.
57. Agrawal, S., A. Agrawal, B. Doughty, A. Gerwitz, J. Blenis, T. Van Dyke, and B. Pulendran. 2003. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J. Immunol.* 171: 4984–4989.
58. Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24: 179–189.
59. Yokota, A., H. Takeuchi, N. Maeda, Y. Ohoka, C. Kato, S. Y. Song, and M. Iwata. 2009. GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. *Int. Immunol.* 21: 361–377.
60. Kamada, N., T. Hisamatsu, H. Honda, T. Kobayashi, H. Chinen, M. T. Kitazume, T. Takayama, S. Okamoto, K. Koganei, A. Sugita, et al. 2009. Human $CD14^+$ macrophages in intestinal lamina propria exhibit potent antigen-presenting ability. *J. Immunol.* 183: 1724–1731.
61. Kang, Y. J., J. Chen, M. Otsuka, J. Mols, S. Ren, Y. Wang, and J. Han. 2008. Macrophage deletion of p38α partially impairs lipopolysaccharide-induced cellular activation. *J. Immunol.* 180: 5075–5082.
62. Alvarez, Y., C. Municio, S. Alonso, M. Sánchez Crespo, and N. Fernández. 2009. The induction of IL-10 by zymosan in dendritic cells depends on CREB activation by the coactivators CREB-binding protein and TORC2 and autocrine PGE_2. *J. Immunol.* 183: 1471–1479.
63. Feili-Hariri, M., X. Dong, S. M. Alber, S. C. Watkins, R. D. Salter, and P. A. Morel. 1999. Immunotherapy of NOD mice with bone marrow-derived dendritic cells. *Diabetes* 48: 2300–2308.
64. Gaudreau, S., C. Guindi, M. Ménard, G. Besin, G. Dupuis, and A. Amrani. 2007. Granulocyte-macrophage colony-stimulating factor prevents diabetes development in NOD mice by inducing tolerogenic dendritic cells that sustain the suppressive function of $CD4^+CD25^+$ regulatory T cells. *J. Immunol.* 179: 3638–3647.
65. Kared, H., A. Masson, H. Adle-Biassette, J. F. Bach, L. Chatenoud, and F. Zavala. 2005. Treatment with granulocyte colony-stimulating factor prevents diabetes in NOD mice by recruiting plasmacytoid dendritic cells and functional $CD4^+CD25^+$ regulatory T-cells. *Diabetes* 54: 78–84.
66. Schultz, G. S., and A. Wysocki. 2009. Interactions between extracellular matrix and growth factors in wound healing. *Wound Repair Regen.* 17: 153–162.
67. Lee, M. S., D. Gu, L. Feng, S. Curriden, M. Arnush, T. Krahl, D. Gurushanthaiah, C. Wilson, D. L. Loskutoff, H. Fox, et al. 1995. Accumulation of extracellular matrix and developmental dysregulation in the pancreas by transgenic production of transforming growth factor-β 1. *Am. J. Pathol.* 147: 42–52.
68. Sanvito, F., P. L. Herrera, J. Huarte, A. Nichols, R. Montesano, L. Orci, and J. D. Vassalli. 1994. TGF-β1 influences the relative development of the exocrine and endocrine pancreas in vitro. *Development* 120: 3451–3462.
69. Miralles, F., T. Battelino, P. Czernichow, and R. Scharfmann. 1998. TGF-β plays a key role in morphogenesis of the pancreatic islets of Langerhans by controlling the activity of the matrix metalloproteinase MMP-2. *J. Cell Biol.* 143: 827–836.
70. Lin, H. M., J. H. Lee, H. Yadav, A. K. Kamaraju, E. Liu, D. Zhigang, A. Vieira, S. J. Kim, H. Collins, F. Matschinsky, et al. 2009. Transforming growth factor-β/Smad3 signaling regulates insulin gene transcription and pancreatic islet β-cell function. *J. Biol. Chem.* 284: 12246–12257.
71. Rodewald, H. R. 2008. Thymus organogenesis. *Annu. Rev. Immunol.* 26: 355–388.

Downloaded from http://www.jimmunol.org/ by guest on March 4, 2022