D-mannose induces regulatory T cells and suppresses immunopathology

DOI:
10.1038/nm.4375

Document Version
Accepted author manuscript

D-mannose induces regulatory T cells and suppresses immunopathology

Dunfang Zhang[1,2], Cheryl Chia[1], Xue Jiao[1,3], Wenwen Jin[1], Shimpei, Kasagi[1], Ruiqing Wu[1,2], Joanne E. Konkel[1], Hiroko Nakatsukasa[1], Peter Zanvit[1], Nathan Goldberg[1], Qianming Chen[2], Lingyun Sun[4], Zi-Jiang Chen[3] and WanJun Chen[1*]

[1]Mucosal Immunology Section, NIDCR, National Institutes of Health, Bethesda, MD 20892, USA; [2]State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, Sichuan 610041, China; [3]Center for Reproductive Medicine, Shandong University, Jinan 250001, China; [4] Department of Rheumatology and Immunology, The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, China.

*Correspondence and requests for materials should be addressed to WanJun Chen, Mucosal Immunology Section, NIDCR, NIH, Bethesda, MD 20892, USA
Tel (301) 435-7168, Email: wchen@mail.nih.gov

Abstract

D-Mannose, a C-2 epimer of glucose, exists naturally in many plants and fruits, and is found in human blood at concentrations less than one-fiftieth that of glucose. However, while the roles of glucose in T cell metabolism, diabetes and obesity are well characterized, the function of D-mannose in T cell immune responses remains unknown. Here, we show that supra-physiological levels of D-mannose safely achievable by drinking water supplementation suppress immunopathology in mouse models of autoimmune diabetes and airway inflammationand increase the proportion of Foxp3$^+$ regulatory T cells (Treg) in mice. In vitro, D-mannose stimulates T_{reg} differentiation of human and mouse cells by promoting TGF-β activation from the latent form, which in turn is mediated by upregulation of integrin $\alpha_v\beta_8$ and reactive oxygen species generated by increased fatty acid oxidation. This previously unrecognized immunoregulatory function of D-mannose may have clinical applications for immunopathology.

Introduction

D-Mannose is a C-2 epimer of glucose and occurs naturally in many plants and fruits, especially cranberries. However, in contrast to glucose, which has a central role in energy generation, storage, and regulation in the cell and plays a pathogenic role in diabetes and obesity, physiological concentration of D-mannose in the blood is less than one-fiftieth those of glucose[1], and has not received much attention. Nevertheless, D-mannose is important in the glycosylation of certain proteins[2,3]. D-mannose has been reported to be mainly beneficial in human diseases. In this regard, a D-mannose supplement has been shown to be an effective therapeutic

means for congenital disorders of glycosylation type Ib (CDG Ib)[2,4]. It has also been utilized as a non-antibiotic treatment for bacterial urinary tract infection in animals[5] and humans[6] with a proposed mechanism of binding to the type 1 pili of enteric bacteria and thereby blocking their adhesion to uroepithelial cells[7]. It is well-established that glucose has a vital role in immune cells, especially in T cell activation and differentiation[8-10]. However, it is unknown whether D-mannose has any effect on T cells and immune responses. Here we show that oral administration of D-mannose suppresses immunopathology in models of autoimmune diabetes and airway inflammation. D-mannose can induce Treg generation from naïve $CD4^+$ T cells by promoting the activation of the latent form of TGF-β. D-mannose-mediated TGF-β activation requires integrin $\alpha_v\beta_8$ and reactive oxygen species (ROS) in T cells. Moreover, D-mannose decreases glycolysis but increases fatty acid oxidation in T cells. Collectively, this drives immune responses toward an immunoregulatory phenotype and subsequent tolerance.

Results

D-Mannose induces Treg

To determine whether D-mannose plays a role in T cell activation, we cultured naïve murine $CD4^+CD25^-$ T cells in medium supplemented with mannose or other sugars in the presence of T cell receptor (TCR) stimulation. D-mannose neither affected the T cell-activation associated markers nor apoptosis (**Supplementary Fig. 1a,b**). However, D-mannose suppressed T cell proliferation (**Fig. 1a**), resulting in lower absolute cell numbers after 48-72 h of culture. In D-mannose-treated T cells, levels of mRNAs associated with Th1 (*Ifng* and *Il2*), Th2 (*Il4* and *Il13*) and *Il6* were higher, yet *Il17a* and *Il10* mRNA remained unchanged(**Supplementary Fig. 2a-g**). Uniquely, however, only D-mannose generated significantly more $Foxp3^+$ Treg and higher *Foxp3* mRNA levels at 24h from the naïve $CD4^+CD25^-$ T cells compared to those in control medium (**Fig. 1b -d**), while the absolute number of $CD4^+Foxp3^-$ non-Treg was lower (**Supplementary Fig. 2h**). TregTregSimilarly, Tregnumbers were higher in D-mannose-treated TCR-stimulated cultures of naive $CD4^+CD25^-GFP^-$ ($Foxp3^-$) T

cells isolated from Foxp3-GFP transgenic mice (**Fig. 1e**). The induction of Treg was D-mannose dose dependent, whereas similar doses of glucose or fructose failed to increase T_{reg} generation (**Supplementary Fig. 3a**). Similarly, stimulation of naïve $CD4^+$ T cells with D-mannose in cultures of soluble CD3 antibody and splenic antigen-presenting cells (APCs) also significantly increased Treg generation (**Fig. 1f, Supplementary Fig. 3b**). Thus, D-mannose specifically induces *Foxp3* expression and the T_{reg} fate in naïve T cells *in vitro*.

Extending this finding to antigen-specific stimulation, D-mannose converted naïve $CD4^+CD25^-$ KJ1-TCR transgenic T cells into $Foxp3^+$ Tregin response to their cognate antigen $OVAp_{323-339}$ (**Fig. 1g, Supplementary Fig. 3c**). Importantly, D-mannose induced Treg *in vivo*. BALB/cJ mice received D-mannose in drinking water for two weeks followed by adoptive transfer of the $KJ1\text{-}26^+CD4^+CD25^-$ naïve T cells and ovalbumin to induce Treg generation in a mucosal T_{reg} induction model[11,12] (**Supplementary Fig. 4a**). D-mannose significantly increased the frequency of Tregin transferred TCR transgenic T cells in response to ovalbumin in Peyer's patches, mesenteric lymph nodes , lamina propria and spleen (**Fig. 1h)** without affecting the host Treg frequency(**Supplementary Fig. 4b**). However, there was no significant decrease in IFN-γ^+ and IL-$17A^+$ T cells in transferred DO11.10 TCR transgenic T cells (**Supplementary Fig. 4c, d**). Taken together, our findings reveal that D-mannose promotes Treg differentiation.

D-mannose-induced Treg exhibit suppressive capacity

We next investigated the function of Treginduced by D-mannose. *In vitro,* D-mannose-induced Treg(Man iT_{reg}) potently suppressed naïve $CD4^+$ T cell proliferation similar to TGF-β1-induced T_{reg} (TGF-β iT_{reg}) and freshly isolated $CD4^+CD25^+GFP^+$ Treg (tTreg) from the spleen of Foxp3-GFP transgenic mice *in vitro*[13] (**Supplementary Fig. 5a**). To investigate the immunosuppressive function of mannose-induced Treg *in vivo*, we used a T cell-transfer model of colitis[14]. $CD4^+CD45RB^{hi}$ T cells were transferred into *Rag1*$^{-/-}$ mice, with or without co-transfer of Man iT_{reg}, TGF-β1 iT_{reg} or tTregfrom Foxp3-GFP mice. All three types of

Tregeffectively prevented the weight loss and suppressed colon inflammation (**Supplementary Fig. 5b, c**). In addition, D-mannose-induced Treg exhibited a DNA methylation pattern similar to that of TGF-β1-induced Treg(data not shown)[15]. Thus, mannose-induced Tregpossess suppressive capacity.

D-Mannose induces T_{reg} differentiation by activation of TGF-β

TregAs TGF-β signaling is critical for Treg generation[16-18], we investigated the role of TGF-β in this process. We first determined that D-mannose treatment enhanced TGF-β signal transduction, as demonstrated by increased expression of the TGF-β-inducible genes[19,20] *Smad7* and *Fos* mRNAs (**Supplementary Fig. 6a, b**). Pharmacological or antibody-mediated blockade of TGF-β-signaling completely abrogated the increase in *Foxp3* mRNA and the consequent increase in Treg induced by D-mannose in wild-type naïve $CD4^+$ T cells (**Fig 2a,b, Supplementary Fig. 6c**) and in naïve KJ1-26^+CD4^+ T cells cultured with $OVAp_{323-339}$ (**Supplementary Fig. 6d**). Interestingly, naïve $CD4^+CD25^-$ T cells from TGF-β receptor I- (*Tgfbr1*$^{f/f}$CD4-Cre^+)[18,21] or II- (*Tgfbr2*$^{f/f}$-ER-Cre^+ plus tamoxifen treatment) deficient mice[22] completely failed to differentiate into Treg in response to D-mannose stimulation (**Fig. 2 c, d**). Consistently, $CD4^+$ naïve T cells deficient in Smad3 (*Smad3*$^{-/-}$)[23], a critical mediator downstream of TGF-β[24,25], showed a significant reduction in D-mannose-induced Treg (**Supplementary Fig. 6e**). TGF-β signaling was also required for D-mannose-induced Treg generation *in vivo* (**Supplementary Fig. 7a**), as co-injection with anti-TGF-β antibody abolished the increase of Treg in adoptively transferred KJ1-26^+CD4^+ T cells induced by D-mannose in BALB/cJ mice (**Fig. 2e**), without affecting the frequency of host TregTreg (**Supplementary Fig. 7b**). Thus, TGF-β signaling is required for D-mannose induction of Treg in culture and *in vivo*.

We also investigated whether D-mannose promoted generation of human Treg. D-mannose treatment indeed significantly up-regulated the frequency of human $CD4^+CD25^{+hi}Foxp3^+$ Treg [26] (**Fig. 2f**). Blockade of TGF-β signaling significantly reduced the effect (**Fig. 2f**). D-mannose-induced human Treg exhibited suppressive

activity towards normal human $CD4^+$ T cell proliferation similar to TGF-β1-induced Treg in the standard suppression assays *in vitro*[13] (**Supplementary Fig. 8a,b**). Thus, as in mouse T cells, D-mannose also drives T_{reg} generation in human naïve $CD4^+$ T cells in a TGF-β-dependent mechanism.

We next determined which aspects of TGF-β-signaling [19,27,28] were influenced by D-mannose. D-mannose did not change the levels of TGF-β1 mRNA or total protein suggesting no effect on TGF-β transcription or protein synthesis (**Supplementary Fig. 9a**). D-mannose treatment, in the presence of TCR stimulation, slightly upregulated both TGF-β receptor I (TβRI) and II (TβRII) mRNA expression compared to TCR stimulation alone (**Supplementary Fig. 9b-c**).

TGF-β is produced as a latent form complexed with latent-associated protein (LAP-TGF-β)[28,29]. D-mannose increased T_{reg} numbers only slightly in presence of bioactive TGF-β1, (**Supplementary Fig. 9d**), suggesting that increased expression of the TGFβ receptors is not the main mechanism of D-mannose action. In contrast, D-mannose significantly potentiated Treg generation in cultures supplemented with LAP-TGF-β. (**Fig. 2b-d, g**). This was further shown by the significant increases in *Foxp3* mRNA (**Fig. 2a**) in T cells at 24 h and by the dose-dependence of LAP-TGF-β1 in D-mannose-treated cells (**Fig. 2h**). Similarly, stimulation of WT naïve $CD4^+$ T cells by anti-CD3 and APCs or TCR transgenic KJ1-$26^+CD4^+CD25^-$ naïve T cells by $OVAp_{323-339}$ and APCs, together with D-mannose and LAP-TGF-β1 treatment also significantly increased Treg compared to T cells cultured without D-mannose (**Supplementary Fig. 6c, d**). Moreover, when naïve $CD4^+$ $CD25^-$ T cells were cultured in X-vivo-20 medium, which importantly does not contain TGF-β as it is serum-free, D-mannose promoted T_{reg} generation only in presence of LAP-TGF-β1, but not alone nor in presence of active TGF-β1 (**Supplementary Fig. 9e,f**).

To confirm that D-mannose-mediated activation of LAP-TGF-β1 enhanced TGF-β signal transduction, we determined that phospho-Smad3 was increased in T cells in response to D-mannose and LAP-TGF-β1 (**Supplementary Fig. 9g**). Importantly, the D-mannose- and LAP-TGF-β1-mediated increases in Treg was completely abolished when TGFβ-signaling was blocked (**Fig. 2g**), or when naïve $CD4^+$ T cells lacked

either TβRI or TβRII Tregexpression (**Fig. 2c, d**). Naïve *Smad3*$^{-/-}$ CD4^{+} T cells also showed a 2.5-fold decrease in D-mannose/LAP-TGFβ1 induced Treg (**Supplementary Fig. 6e**). Finally, we investigated if D-mannose could induce significant numbers of Treg at physiologically relevant concentrations (~1mM) by culturing naïve CD4^{+} T cells with LAP-TGF-β1 *in vitro* in the presence of different doses of D-mannose (**Supplementary Fig. 9h**). Taken together, our findings suggest that D-mannose induced Treg generation by augmenting TGF-β signaling via activation of latent TGF-β.

Integrin $\alpha_v\beta_8$ and ROS are required for D-mannose-mediated TGF-β1 activation and T_{reg} generation

Several molecules have been suggested to be involved in TGF-β activation in immune cells including integrin $\alpha_v\beta_8$ (encoded by *Itgav* and *Itgb8* subunits),[30-32] reactive oxygen species (ROS).,[33,34] D-mannose stimulation increased *Itgav* and *Itgb8* mRNA in naïve CD4^{+} T cells (**Fig. 3a, b**) compared to T cells without D-mannose. We then activated naïve CD4^{+}CD25^{-} ITGβ$_8$-deficient T cells [32] *in vitro* in the presence of D-mannose and demonstarted that loss of ITGβ$_8$in T cells significantly reduced D-mannose-induced Treg generation even in the absence of exogenous LAP-TGF-β1 (**Fig. 3c**). When exogenous LAP-TGF-β1 was added to the cultures, ITGβ$_8$ KO T cells exhibited a significant defect in Treg generation (~50% decrease) in response to D-mannose (**Fig. 3c**), suggesting that ITGβ$_8$ plays an important role in mediating TGF-β1 activation induced by D-mannose.

As the action of ITGβ$_8$ could not totally account for all TGF-β1 activation, we also determined that ROS participated in TGF-β1-activation by D-mannose. ROS can be produced in T cells upon TCR activation[36,37]. We found that D-mannose increased ROS production in T cells compared to TCR stimulation alone (**Fig. 3d**). Importantly, blockade of ROS activity with N-acetyl-L-cysteine (NAC)[38] also significantly reduced D-mannose-induced TregTreg in the presence of LAP-TGF-β1 (**Fig. 3c**). Notably, neutralization of ROS with NAC in ITGβ$_8$ KO naïve CD4^{+} T cells further decreased Treg generation induced by D-mannose by 70-80% (**Fig. 3c**), suggesting a

combinatorial function involving the integrin and ROS pathways. Thus, the ITG$\alpha_v\beta_8$ and ROS pathways play independent yet complementary roles in D-mannose-mediated TGF-β1 activation and consequent Treg generation *in vitro*.

D-mannose increases fatty acid oxidation

We next investigated how D-mannose induced ROS. Following TCR activation, naïve T cells switch from oxidative phosphorylation (OXPHOS) to aerobic glycolysis[8]. As D-mannose and D-glucose increased ROS production to similar levels (**Supplementary Fig. 10a**), we hypothesized that D-mannose might also up-regulate glycolysis in T cells. To test this, we cultured naïve T cells in sugarless media supplemented with pyruvate and L-glutamine, and measured their Extracellular Acidification Rate (ECAR) in response to D-glucose or D-mannose. Unexpectedly, cells fed with D-mannose displayed a markedly lower ECAR at maximal respiration compared to cells cultured in D-glucose (**Supplementary Fig. 10b**). This indicated a reduced capacity to utilize glycolysis in response to stress.

As fatty acid oxidation (FAO) can generate ROS[39,40] we hypothesized that D-mannose might induce ROS via this pathway. To this end, we measured the oxygen consumption rate (OCR) of naïve $CD4^+$ T cells cultured in D-mannose or D-glucose. Although both processes of OXPHOS and FAO occur in the mitochondria and contribute to OCR, only FAO is affected by Etomoxir, an inhibitor of CPT1, the enzyme responsible for the transport of fatty acids into mitochondria. Cells cultured in D-mannose displayed significantly higher OCR (~25%) at maximal respiration compared to cells cultured in D-glucose (**Supplementary Fig. 10c**). However, simultaneous treatment with Etomoxir led to a decrease in OCR of mannose-cultured cells, with no such decrease observed in Etomoxir-treated glucose-cultured cells, indicating that a portion of the OCR in mannose-treated cells can be attributed to active FAO, especially where spare capacity is required (i.e., during proliferation) (**Supplementary Fig. 10c**). The greater utilization of FAO in mannose-cultured T cells might explain the higher ROS levels observed in these cells, although how D-mannose-induced FAO drives more ROS remains unknown. Nonetheless, the

increased level of ROS contributes to the activation of latent TGF-β during the generation of TregTreg. Thus, we suggest that D-mannose cultured T cells preferentially use FAO to which can result in higher levels of ROS that results in greater TGF-β activation.

D-mannose suppresses type I diabetes in NOD mice

We next determined whether D-mannose had beneficial effects in autoimmunity, as modeled by type I diabetes in NOD mice, where pathology involves Treg defects [41]. NOD mice received D-mannose in the drinking water starting at 7.5 weeks of age, when the mice are considered pre-diabetic, and at which point the inflammatory process has just been initiated but blood glucose levels are still within the normal range[41] (**Supplementary Fig. 11a**). As expected, control NOD mice started to develop diabetes at about 12-13 weeks of age, and 80-90% became diabetic by the age of 23 weeks (**Fig. 4a**). However, most of the NOD mice fed with D-mannose were diabetes-free through 23 weeks of age (**Fig. 4a**). Consistent with the protection of mice from diabetes, D-mannose-treated mice showed considerably less insulitis and more preserved islets compared to controls (**Fig. 4b-c**).

We examined T cell responses of these mice at 13-14 weeks of age, when the untreated NOD mice were expected to start to develop hyperglycemia. The frequency of $CD4^+CD25^+Foxp3^+$ Treg in the spleen and pancreatic draining lymph nodes (DLN) was significantly higher in D-mannose-treated mice than in untreated mice (**Fig. 4d**). In contrast, the frequencies of $CD4^+$ IFN-γ^+ and $CD8^+$ IFN-γ^+ T cells were lower in the spleen of the NOD mice fed with D-mannose (**Fig. 4e,f**). In addition, $CD4^+$IL-4^+ (Th2) cells were also lower in the spleen of D-mannose-treated mice (**Supplementary Fig. 11b**), whereas the frequencies of $CD4^+$IL-$17A^+$ (Th17) and $CD4^+$IL-10^+ T cell were unchanged (**Supplementary Fig. 11b**). In the pancreas, the frequency of $CD4^+Foxp3^+$ Treg was increased and that of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells was decreased in D-mannose-treated mice (**Supplementary Fig. 11c**). In addition to the *ex vivo* analysis of T cells, we also examined the autoantigen-specific T cell cytokine production in splenic T cells in response to restimulation

with a pancreas-derived peptide, GAD65. GAD65-specific T cell IFN-γ production was significantly reduced in the spleen of D-mannose-treated NOD mice (**Fig. 4g**), whereas GAD65-specific IL-17 and IL-10 production did not change (**Supplementary Fig. 11d**). Moreover, treatment of NOD mice with D-mannose when they reached prediabetic blood glucose levels of 140-160 mg/dL (**Supplementary Fig. 11e**) or the new onset diabetic levels of 200-230 mg/dL [42,43] (**Supplementary Fig. 12a**) suppressed the progression of diabetes(**Fig. 4h-k, Supplementary Fig. 12b-d**). Similar to the treated pre-diabetic NOD mice, D-mannose treatment at these stages also significantly increased the frequency of Treg and decreased the frequencies of Th1 and $CD8^+$ IFN-γ^+ T cells in the pancreases (**Fig. 4h-k, Supplementary Fig. 12**).

To study whether the increase of Treg is involved in D-mannose treatment-mediated suppression of diabetes, we depleted $CD4^+CD25^+$ TregTreg with anti-CD25 antibody in D-mannose-treated as well as untreated NOD mice as described in **Supplementary Fig. 13a**. We first determined that anti-CD25 antibody sufficiently depleted Treg in NOD mice (~50% depletion of total $CD4^+Foxp3^+$ Treg; 70-80% depletion of $CD4^+CD25^+Foxp3^+$ Treg). In NOD mice, anti-CD25 antibody injection did not significantly change the frequency of $CD25^+Foxp3^-$ effector cells. We found that depletion of $CD4^+CD25^+$ Treg abolished the protective effects of D-mannose on diabetes development (**Fig. 5a**), whereas the same anti-CD25 antibody treatment at this stage slightly affected the development of diabetes in untreated NOD mice (**Fig. 5a**). Consistently, anti-CD25 antibody injection reversed the D-mannose-mediated decrease in insulitis and preservation of total number of islets in the pancreas (**Fig. 5b,c**). In the spleen and DLN of D-mannose treated mice, the increase in the frequency of $CD4^+CD25^+Foxp3^+$ Treg was abolished by anti-CD25 antibody treatment (**Fig. 5d**). Consequently, the increased ratios between $CD25^+Foxp3^+$ Treg and $CD4^+$ IFN-γ^+ or $CD8^+$ IFN-γ^+ T cells in D-mannose-treated mice were eliminated Tregfollowing anti-CD25 antibody treatment (**Fig. 5e,f**). Furthermore, we validated a role of Treg in D-mannose-mediated suppression of diabetes in NOD.$Foxp3^{DTR}$ mice treated with diphtheria toxin (data not shown). These data suggest that the

increase of Treg is involved in D-mannose treatment-mediated suppression of diabetes. Lastly, neutralization of endogenous TGF-β with anti-TGF-β antibody (**Supplementary Fig. 13b)** in D-mannose-treated NOD mice also abrogated the suppressive effects of D-mannose on diabetes with abolishment of the increased Treg (**Fig. 5g,h, Supplementary Fig. 13c,d**). These data altogether indicate that D-mannose suppresses immunopathology of autoimmune diabetes in NOD mice, and that Treg and TGF-β are involved in this process.

D-mannose prevents and suppresses ovalbumin-induced airway inflammation

The success of D-mannose supplementation in the suppression of diabeties development in NOD mice encouraged us to investigate whether mannose-mediated Treg generation has a broader impact in immunopathologies. We next tested D-mannose function in a model of lung airway inflammation. We utilized an ovalbumin-induced airway inflammation model in which KJ1-26$^+$CD4$^+$CD25$^-$ naïve T cells isolated from DO11.10 TCR-transgenic Rag2$^{-/-}$ mice were adoptively transferred into BALB/cJ mice[44-46]. This system allowed us to assess the conversion of KJ1-26$^+$CD4$^+$CD25$^-$ naïve T cells to Treg *in vivo* in the airway inflammation model in response to D-mannose administration (**Supplementary Fig. 14a**). Indeed, we found that D-mannose treatment prevented the development of airway inflammation in the lungs, demonstrated by considerably less infiltration of inflammatory cells and reduced mucus production in the airways (**Fig. 6a,b**). The bronchoalveolar lavage (BAL) fluid of D-mannose-treated mice had significantly fewer inflammatory leukocytes, particularly eosinophils (**Fig. 6c**). In accordance with the diminished inflammation in their lungs, D-mannose-treated mice showed significantly lower frequencies of IL-13$^+$ and substantially reduced frequencies of IL-4$^+$ KJ1-26$^+$ T cells in the lungs and in the peripheral lymphoid tissues (**Figure 6 d,e**). Intriguingly, there were also lower frequencies of IL-13$^+$ and IL-4$^+$ Th2 cells in the non-transgenic CD4$^+$ host T cells in the lungs of mice treated with D-mannose, although there were no changes in their peripheral lymphoid tissues (**Supplementary Fig. 14b,c**). Importantly, D-mannose treatment resulted in a

significant increase in KJ1-26$^+$CD4$^+$Foxp3$^+$ Treg in the lungs as well as in the spleen and DLN (**Fig. 6f**). In contrast, there was a decrease in the frequency of non-transgenic CD4$^+$Foxp3$^+$ host Treg in the lungs and peripheral lymphoid tissues in the same D-mannose-treated mice (**Supplementary Fig. 14d**).

To investigate whether D-mannose is effective in a clinically relevant setting, we first induced airway inflammation, followed by treatment of the mice with D-mannose in drinking water (**Supplementary Fig. 15a**). We found that D-mannose treatment significantly ameliorated airway inflammation (**Fig. 6g-i**). This was accompanied by decreased frequencies of IL-4$^+$ and IL-13$^+$ T cells and increased frequencies of Foxp3$^+$ Treg within the transferred KJ1-26$^+$ CD4$^+$ T cells in the lungs, DLNs and spleen (**Fig. 6j-l**). The frequencies of IL-4$^+$ and IL-13$^+$ cells within the non-transgenic host CD4$^+$ T cells in the lungs were also significantly decreased but the frequency of the host Treg was not changed in the same D-mannose treated mice (**Supplementary Fig. 15b-d**). These results collectively show that mannose supplementation induces antigen-specific Treg and suppresses immunopathology of airway inflammation in mice, indicating a broad function of mannose-mediated immunoregulation.

Discussion

Here we have outlined a previously unrecognized ability of D-mannose, a hexose sugar, to suppress experimental type I diabetes and lung airway inflammation. We have also demonstrated that D-mannose induces Treg from naïve CD4$^+$ T cells by enhancing TGF-β signaling via activation of TGFβ from its latent form. ITG$\alpha_v\beta_8$ and ROS pathways were required for T_{reg} induction by D-mannose in T cells. Molecular studies revealed that D-mannose induced ITG$\alpha_v\beta_8$ expression and utilized fatty acid oxidation to increase ROS in T cells. Importantly, we determined that D-mannose enhances Treg generation in human T cells.

D-mannose induces Treg from naïve CD4$^+$ T cells. Supporting this conclusion are our *in vitro* experiments whereby naïve CD4$^+$ T cells were stimulated in the culture medium containing different hexose sugars. Only D-mannose was able to induce

significant numbers of Foxp3$^+$ Treg. This Treg generation was dose-dependent for D-mannose (0-50 mM). D-mannose induction of Treg requires TCR signaling, as D-mannose treatment without TCR stimulation cannot induce Treg. This was further shown in an *in vivo* DO11.10 TCR-transgenic naïve T cell adoptive transfer system. D-mannose induced more DO11.10 T_{reg} cells *in vivo*, but there was no detectable increase in Treg in host BALB/cJ CD4$^+$ T cells. This finding is important for potential future application in human autoimmune diseases, because it would be preferable if D-mannose were to induce only autoantigen-specific Treg without affecting other irrelevant Treg populations. However, it should be noted that the reasons for the lack of increase in the frequency of Treg among the polyclonal non-transgenic CD4$^+$ T cells remain unknown and require further investigation. Of note, under these culture conditions D-mannose also decreases mRNA levels of multiple effector T cell cytokines including *Ifng*, *Il4*, *Il6* and *Il13* without significant change in the *Il10* and *Il17a* mRNAs.Treg.

TGF-β is essential in the generation of Foxp3$^+$ Treg from naïve CD4$^+$ T cells[16-18], and we show that an enhancement of TGF-β signaling is an underlying mechanism promoting D-mannose Treg generation. Indeed, blockade of TGF-β signaling *in vitro* and *in vivo* abolished D-mannose-induced Treg generation. Further mechanistic studies revealed that the activation of latent TGF-β, rather than TGF-β protein synthesis, was key to the increased TGF-β signaling by D-mannose in T cells. Moreover, the findings that addition of exogenous LAP-TGF-β1 but not active TGFβ1 enhanced Treg generation in D-mannose-treated T cells further support this conclusion, as LAP-TGFβ1 cannot signal without activation. A previous publication has shown that a high concentration of glucose (e.g., >25 mM) is able to activate TGF-β and may be involved in the growth of epithelial and mesenchymal cells[35]. Our results here show that 5-50 mM glucose failed to induce significant T_{reg} generation in T cells in the absence of exogenous LAP-TGF-β1. The detailed mechanism remains unknown, but it might be possible that a high concentration of glucose induces more T cell activation through glycolysis and higher production of inflammatory cytokines such as IFN-γ, IL-6, and IL-4[8,47], which may antagonize *Foxp3* induction by TGF-β.

Indeed, we noticed that glucose treatment of TCR-stimulated naïve $CD4^+$ T cells in the presence of anti-IL-4, -IL-6, and -IFN-γ antibodies increased the frequency of Treg, although the increase is still far less than that of D-mannose-induced Treg. .

We then determined that D-mannose-mediated TGF-β activation and Treg generation involved $ITG\alpha_v\beta_8$ and ROS[48,30-32]. Supporting this conclusion is the findings that D-mannose increased $ITG\alpha_v\beta_8$ expression and ROS production in T cells, and that deletion of $ITG\beta_8$ gene and/or blockade of ROS activity abolished the majority of D-mannose Treg generation. The exact mechanisms underlying D-mannose-driven ROS production remain unknown, but may involve increased fatty acid oxidation that also produces more ROS, as D-mannose increases fatty acid oxidation in T cells. The findings that D-mannose suppresses glycolysis in T cells may provide an explanation for decreased T cell proliferation and IFN-γ production under D-mannose treatment, which are canonical features of glycolysis induced by TCR stimulation. Notably, while both glucose and mannose can upregulate ROS, only D-mannose but not the corresponding amount of glucose, can induce *Foxp3*. Nevertheless, further molecular details on this pathway are required. However, one possibility might be that D-mannose but not glucose up-regulates $ITG\alpha_v\beta_8$ expression. Although we cannot conclude that this is the case, our data strongly suggest that D-mannose up-regulation of $ITG\alpha_v\beta_8$ is an important factor in the difference.

Importantly, D-mannose effectively suppresses autoimmune type I diabetes and airway inflammation in mice. Oral supplementation of D-mannose to NOD mice before they develop hyperglycemia affects the development of diabetes in the NOD mice. In addition, oral administration of D-mannose was able to block the progress of diabetes even in newly onset diabetic NOD mice. Similarly, oral administration of D-mannose also prevented and suppressed established airway inflammation in the lungs. Of note, long-term supplementation of D-mannose had no obvious side-effects on the NOD mice, a finding that might have implications for developing a similar clinical therapy for human type I diabetes[49]. Although a systemic increase in active TGF-β might have potential effects on the fibrotic response[50], the fact that D-

mannose is well-tolerated in mice here suggests that TGF-β activation might have local effects on surrounding T cells, which are less likely to cause a significant fibrotic response. Nevertheless, more long-term studies could help eliminate this possibility. Moreover, since D-mannose can activate TGF-β and promote Treg generation, whether it affects other disease conditions such as cancer also remains to be investigated.

D-mannose treatment increased Treg frequencies, and decreased IFN-γ-producing T effector cells in the NOD mice. Although the beneficial effects on D-mannose in NOD mice are abrogated in the absence of T_{reg} cells and TGF-β, the central role of this pathway to the disease development and the limitations of the NOD experimental model do not allow to dissect the extent to which T_{reg} and TGF-β mediate D-mannose effects on diabetes in this study, and it is possible that D-mannose may act through additional mechanisms to suppress diabetes *in vivo.*

Notably, this immunoregulatory effect by D-mannose was replicated and confirmed in an ovalbumin-induced airway inflammation model, indicating a broader efficacy of mannose-mediated therapeutic effects on immunopathology. The notion that D-mannose treatment induces antigen-specific Treg and suppression of immunopathology can be supported in this ovalbumin-induced airway inflammation model.

The physiological level of D-mannose in the blood is approximately 100 μM. However, it has been reported that circulating D-mannose increases up to 9-fold (from 100 to 900 μM) in mice receiving D-mannose in drinking water, with no adverse consequences [49]. Here we employed used the same amount of D-mannose to supplement the drinking water in our *in vivo* experiments. In humans, stable serum mannose levels up to 2mM could be reached and were well tolerated without signs of liver or renal toxicity[51]. We found that even amounts as low as 1 mM D-mannose could induce a considerable level of Treg, adding credence to the physiological significance of the findings. Moreover, studying the possible connection between consumption of fruits rich in D-mannose (e.g. cranberries) and autoimmunity may be an interesting and important issue.

In sum, we have discovered a previously unrecognized immunoregulatory effect of D-mannose on T cells in both preventive and therapeutic models of type I diabetes and lung airway inflammation. This study warrants further exploration of the basic immunological mechanisms and potential clinical applications of hexose sugars.

Acknowledgment. This research was supported by the Intramural Research Program of NIH, NIDCR. We thank Dr. E. Shevach (NIAID, NIH) for providing the *Itgb8*$^{f/f}$*Cd4*-Cre^{+} mice and Drs. C. Benoist and D. Mathis (Harvard Medical School) for providing the NOD.Foxp3^{DTR} mice. We also thank the NIDCR flow cytometry core for support.

Author Contributions: D.Z. designed and did most of the experiments, analyzed and interpreted the data and contributed to the writing of the manuscript. C.C and X.J designed and did experiments, analyzed data and contributed to the writing of the manuscript. S.K, R.W, J.E.K, H.N, P.Z, N.G, and W.J performed experiments; Q.C, L.S, Z-J.C, provided support and/or critical scientific input. W.J.C conceived of and directed the research, designed the experiments and wrote the paper.

Competing Financial Interests Statement: The authors declare no competing financial interests.

References:

1. Etchison, J.R. & Freeze, H.H. Enzymatic assay of D-mannose in serum. *Clin Chem* **43**, 533-538 (1997).
2. Schneider, A.*, et al.* Successful prenatal mannose treatment for congenital disorder of glycosylation-Ia in mice. *Nat Med* **18**, 71-73 (2012).
3. Alton, G.*, et al.* Direct utilization of mannose for mammalian glycoprotein biosynthesis. *Glycobiology* **8**, 285-295 (1998).
4. de Lonlay, P. & Seta, N. The clinical spectrum of phosphomannose isomerase deficiency, with an evaluation of mannose treatment for CDG-Ib. *Biochimica et biophysica acta* **1792**, 841-843 (2009).

5. Michaels, E.K., Chmiel, J.S., Plotkin, B.J. & Schaeffer, A.J. Effect of D-mannose and D-glucose on Escherichia coli bacteriuria in rats. *Urol Res* **11**, 97-102 (1983).
6. Kranjcec, B., Papes, D. & Altarac, S. D-mannose powder for prophylaxis of recurrent urinary tract infections in women: a randomized clinical trial. *World J Urol* **32**, 79-84 (2014).
7. Schaeffer, A.J., Chmiel, J.S., Duncan, J.L. & Falkowski, W.S. Mannose-sensitive adherence of Escherichia coli to epithelial cells from women with recurrent urinary tract infections. *J Urol* **131**, 906-910 (1984).
8. Wang, R. & Green, D.R. Metabolic checkpoints in activated T cells. *Nat Immunol* **13**, 907-915 (2012).
9. MacIver, N.J., Michalek, R.D. & Rathmell, J.C. Metabolic regulation of T lymphocytes. *Annu Rev Immunol* **31**, 259-283 (2013).
10. Vander Heiden, M.G., Cantley, L.C. & Thompson, C.B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324**, 1029-1033 (2009).
11. Coombes, J.L.*, et al.* A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* **204**, 1757-1764 (2007).
12. Sun, C.M.*, et al.* Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* **204**, 1775-1785 (2007).
13. Thornton, A.M. & Shevach, E.M. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* **188**, 287-296 (1998).
14. Read, S., Malmstrom, V. & Powrie, F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* **192**, 295-302 (2000).
15. Huehn, J., Polansky, J.K. & Hamann, A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nature reviews. Immunology* **9**, 83-89 (2009).
16. Chen, W.*, et al.* Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* **198**, 1875-1886 (2003).
17. Chen, W. & Konkel, J.E. Development of thymic Foxp3(+) regulatory T cells: TGF-beta matters. *Eur J Immunol* **45**, 958-965 (2015).
18. Konkel, J.E., Jin, W., Abbatiello, B., Grainger, J.R. & Chen, W. Thymocyte apoptosis drives the intrathymic generation of regulatory T cells. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E465-473 (2014).
19. Derynck, R. & Zhang, Y.E. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**, 577-584 (2003).
20. Zhang, Y., Feng, X.H. & Derynck, R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. *Nature* **394**, 909-913 (1998).

21. Liu, Y., *et al.* A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat Immunol* **9**, 632-640 (2008).
22. Nakatsukasa, H., *et al.* The DNA-binding inhibitor Id3 regulates IL-9 production in CD4(+) T cells. *Nat Immunol* **16**, 1077-1084 (2015).
23. Yang, X., *et al.* Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *EMBO J* **18**, 1280-1291 (1999).
24. Tone, Y., *et al.* Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat Immunol* **9**, 194-202 (2008).
25. Chen, W. & Konkel, J.E. TGF-beta and 'adaptive' Foxp3(+) regulatory T cells. *J Mol Cell Biol* **2**, 30-36 (2010).
26. Baecher-Allan, C., Brown, J.A., Freeman, G.J. & Hafler, D.A. CD4+CD25high regulatory cells in human peripheral blood. *J Immunol* **167**, 1245-1253 (2001).
27. Chen, W. & Wahl, S.M. TGF-beta: receptors, signaling pathways and autoimmunity. *Curr Dir Autoimmun* **5**, 62-91 (2002).
28. Massague, J. & Chen, Y.G. Controlling TGF-beta signaling. *Genes Dev* **14**, 627-644 (2000).
29. Shi, M., *et al.* Latent TGF-beta structure and activation. *Nature* **474**, 343-349 (2011).
30. Worthington, J.J., *et al.* Integrin alphavbeta8-Mediated TGF-beta Activation by Effector Regulatory T Cells Is Essential for Suppression of T-Cell-Mediated Inflammation. *Immunity* **42**, 903-915 (2015).
31. Travis, M.A., *et al.* Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice. *Nature* **449**, 361-365 (2007).
32. Edwards, J.P., Thornton, A.M. & Shevach, E.M. Release of active TGF-beta1 from the latent TGF-beta1/GARP complex on T regulatory cells is mediated by integrin beta8. *J Immunol* **193**, 2843-2849 (2014).
33. Chen, W., Frank, M.E., Jin, W. & Wahl, S.M. TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. *Immunity* **14**, 715-725 (2001).
34. Amarnath, S., Dong, L., Li, J., Wu, Y. & Chen, W. Endogenous TGF-beta activation by reactive oxygen species is key to Foxp3 induction in TCR-stimulated and HIV-1-infected human CD4+CD25- T cells. *Retrovirology* **4**, 57 (2007).
35. Wu, L. & Derynck, R. Essential role of TGF-beta signaling in glucose-induced cell hypertrophy. *Dev Cell* **17**, 35-48 (2009).
36. Hildeman, D.A., Mitchell, T., Kappler, J. & Marrack, P. T cell apoptosis and reactive oxygen species. *J Clin Invest* **111**, 575-581 (2003).
37. Sena, L.A., *et al.* Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling. *Immunity* **38**, 225-236 (2013).
38. Bulua, A.C., *et al.* Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). *J Exp Med* **208**, 519-533 (2011).

39. Rosca, M.G.*, et al.* Oxidation of fatty acids is the source of increased mitochondrial reactive oxygen species production in kidney cortical tubules in early diabetes. *Diabetes* **61**, 2074-2083 (2012).
40. Seifert, E.L., Estey, C., Xuan, J.Y. & Harper, M.E. Electron transport chain-dependent and -independent mechanisms of mitochondrial H2O2 emission during long-chain fatty acid oxidation. *J Biol Chem* **285**, 5748-5758 (2010).
41. Anderson, M.S. & Bluestone, J.A. The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol* **23**, 447-485 (2005).
42. Akirav, E.M.*, et al.* Detection of beta cell death in diabetes using differentially methylated circulating DNA. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 19018-19023 (2011).
43. American Diabetes, A. Diagnosis and classification of diabetes mellitus. *Diabetes care* **37 Suppl 1**, S81-90 (2014).
44. Walter, D.M.*, et al.* Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. *J Immunol* **167**, 4668-4675 (2001).
45. Takaoka, A.*, et al.* A critical role for mouse CXC chemokine(s) in pulmonary neutrophilia during Th type 1-dependent airway inflammation. *J Immunol* **167**, 2349-2353 (2001).
46. Staudt, V.*, et al.* Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* **33**, 192-202 (2010).
47. Chang, C.H.*, et al.* Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* **153**, 1239-1251 (2013).
48. Travis, M.A. & Sheppard, D. TGF-beta activation and function in immunity. *Annu Rev Immunol* **32**, 51-82 (2014).
49. Davis, J.A. & Freeze, H.H. Studies of mannose metabolism and effects of long-term mannose ingestion in the mouse. *Biochimica et biophysica acta* **1528**, 116-126 (2001).
50. Derynck, R. & Akhurst, R.J. Differentiation plasticity regulated by TGF-beta family proteins in development and disease. *Nat Cell Biol* **9**, 1000-1004 (2007).
51. Mayatepek, E., Schroder, M., Kohlmuller, D., Bieger, W.P. & Nutzenadel, W. Continuous mannose infusion in carbohydrate-deficient glycoprotein syndrome type I. *Acta paediatrica* **86**, 1138-1140 (1997).

Figure legends

Figure 1. D-mannose induces Treg differentiation *in vitro* and *in vivo*. (**a-d**) $CD4^+CD25^-$ (naïve) T cells from spleen and periphery lymph nodes (LNs) of *C57BL/6* mice were cultured with αCD3 and αCD28 up to 3 days in 10% FBS glucose-free

DMEM complete medium (CTRL) or with addition of 25 mM mannose (Man) or other sugars. Proliferation was measured by CFSE labeling. (**a**) Representative FACS plots gated on un-proliferated cells in $CD4^+$ T cells cultured for 24, 48 and 72 hrs. (**b**) Representative FACS plots and (**c** upper panel) frequency of $CD25^+Foxp3^+$ Treg in $CD4^+$ T cells after 3 days culture. (**c** bottom panel) Absolute numbers of $CD25^+Foxp3^+$ T_{reg} cells. (**d**) mRNA expression of *Foxp3* at 24 h. (**e**) $CD4^+CD25^+GFP^+$ ($Foxp3^+$) Treg ratio after $CD4^+CD25^-GFP^-$ ($Foxp3^-$) T cells from spleen and LNs of Foxp3-GFP transgenic mice were cultured with αCD3 and αCD28 in 25mM mannose DMEM compared with CTRL medium for 3 days. (**f**) Naive T cells from spleen and LNs of *C57BL/6* mice were cultured with soluble αCD3 plus APCs and frequency of $CD25^+Foxp3^+$ Treg was determined after 3 days. (**g**) KJ1-$26^+CD4^+CD25^-$ naïve T cells from spleen and LNs of DO11.10 TCR transgenic *Rag2*$^{-/-}$ mice were cultured with $OVAp_{323-339}$ plus APCs and frequency of KJ1-$26^+CD25^+Foxp3^+$ Treg was determined after 4 days. (**h**) KJ1-$26^+CD4^+CD25^-$ naïve T cells from spleen and LNs of DO11.10 TCR transgenic *Rag2*$^{-/-}$ mice were adoptively transferred into BALB/cJ mice and frequency of KJ1-$26^+CD4^+Foxp3^+$ Treg in adoptive transferred cells in indicated organs were determined after 5 days ovalbumin gavage (n=3 or 4). Summary data are presented as mean ± s.e.m. *$p<0.05$, **$p<0.01$; by a one-way analysis of variance (ANOVA) with Tukey's *post hoc* test (**a-c**) or unpaired two-tailed Student's *t*-tests (**d-h**).Data are pooled from three (**e, g**) or five (**c, d, f**) experiments or are representative of three (**a, h**) or five (b) independent experiments..

Figure 2. D-mannose induces Treg differentiation via activation of TGF-β. (**a, b**) $CD4^+CD25^-$ (naïve) T cells from spleen and LNs of *C57BL/6* mice were cultured with αCD3 and αCD28, with or without latent TGF-β1, αTGF-β or SB431542 and frequency of $CD25^+Foxp3^+$ Treg was determined after 3 days. (**a**) mRNA expression of *Foxp3* at 24 h. (**b**) Frequency of $CD25^+Foxp3^+$ Treg in $CD4^+$ T cells. (**c, d**) Naïve $CD4^+$ T cells deficient in TβRI (**c**) or TβRII (**d**) from spleen and LNs of *Tgfbr1*$^{f/f}$CD4-Cre^+ or tamoxifen treated *Tgfbr2*$^{f/f}$-ER-Cre^+ mice were cultured with αCD3 and αCD28, with or without latent TGF-β1, and frequency of $CD25^+Foxp3^+$ Treg was determined after 3 days.(**e**) KJ1-$26^+CD4^+CD25^-$ naïve T cells from spleen and LNs of

DO11.10 TCR transgenic *Rag2*$^{-/-}$ mice were adoptively transferred into BALB/cJ mice and frequency of KJ1-26^{+}CD4^{+}Foxp3^{+} Treg in adoptive transferred cells in indicated organs were determined after 5 days ovalbumin gavage (n=5). (**f**) Naïve CD4^{+} T cells from spleen and LNs of *C57BL/6* mice were cultured with αCD3 and αCD28, with or without latent TGF-β1, αTGF-β plus SB431542, and frequency of CD25^{+}Foxp3^{+} Treg was determined after 3 days. (**g**) Naïve CD4^{+} T cells from spleen and LNs of *C57BL/6* mice were cultured with αCD3 and αCD28 plus different doses of latent TGF-β1 and frequency of CD25^{+}Foxp3^{+} Treg was determined after 3 days. Summary data are presented as mean ± s.e.m. *p<0.05, **p<0.01; by a one-way analysis of variance (ANOVA) with Tukey's *post hoc* test (**a-f**) or unpaired two-tailed Student's *t*-tests (**g**).Data are pooled from five (**a-d, f**) experiments or are representative of two (**e**) or three (**g**) independent experiments.

Figure 3. ITG$\alpha_v\beta_8$ and ROS are required for D-mannose mediated TGF-β1 activation and Treg generation. (**a, b**) CD4^{+}CD25^{-} (naïve) T cells from spleen and LNs of *C57BL/6* mice were cultured with αCD3 and αCD28, and mRNA expression of *Itgb8* (**a**) and *Itgav* (**b**) were determined at 24 h. (**c, d**) CD4^{+}CD25^{-} (naïve) T cells from spleen and LNs of control or *Itgb8*$^{f/f}$ CD4-Cre^{+} mice were cultured with or without latent TGF-β1 or NAC, and frequency of CD25^{+}Foxp3^{+} Treg was determined after 3 days. Representative FACS plots (**c**) and frequency (**d**) of CD25^{+}Foxp3^{+} Treg (**e**) CD4^{+}CD25^{-} (naïve) T cells from spleen and LNs of *C57BL/6* mice were cultured with αCD3 and αCD28, and expression of ROS in CD4^{+} T cells culture was determined after 24 h. (**f**) Naïve CD4^{+} T cells purified from human PBMC were cultured with αCD3 and αCD28, IL-2, with or without αTGF-β plus SB431542, and frequency of CD25^{+hi} Foxp3^{+} Treg were checked after 4 days. Summary data are presented as mean ± s.e.m. *p<0.05, **p<0.01; by a one-way analysis of variance (ANOVA) with Tukey's *post hoc* test (**d,f**) or unpaired two-tailed Student's *t*-tests (**a,b**). Data are pooled from three to five (**a, b, d, f**) experiments or are representative of three (**c, e**) independent experiments.

Figure 4. D-mannose suppresses type I diabetes in NOD mice.

(**a-c**) Female NOD mice were fed with mannose water from 7.5 weeks of age, and checked for development of T1D. (**a**) Frequency of T1D-free mice in indicated groups over time (n=20). Representative histology sections (**b**) were shown and frequency (**c**) of islets with grade X insulitis in indicated groups was graded. (**d-h**) Female NOD mice were treated with D-mannose dissolved in water daily from the 7.5 week of age, and euthanized when the mice were 14 weeks old. Frequencies of $CD25^+Foxp3^+$Treg (**d**), IFN-γ^+ $CD4^+$ T cells (**e**), IFN-γ^+ $CD8^+$ T cells (**f**) in the spleen and DLN of female NOD mice were determined. (**g**) Frequencies of $CD4^+Foxp3^+$ Treg, IFN-γ^+ $CD4^+$ T cells, and IFN-γ^+ $CD8^+$ T cells in the pancreases of female NOD mice were determined. (**h**) Splenocytes of the NOD mice were cultured with GAD65 peptide (1 μg/ml) and IFN-γ in the culture medium was determined by Enzyme-linked immunosorbent assay (ELISA) after 3 days. Summary data are presented as mean ± s.e.m. *p<0.05, **p<0.01; by a Mantel–Cox log-rank test (**a**) or unpaired two-tailed Student's *t*-tests (**d-f**,**b**). Data are pooled from four (**a, c**) experiments or are representative of two (**h**) or four (**b, d-g**) independent experiments.

Figure 5. Treg and TGF-β are involved in D-mannose–mediated suppression of autoimmune diabetes in NOD mice.

(**a-f**) Female NOD mice were fed with mannose water from 7.5 weeks of age, injected with αCD25/ isotype control antibodies twice when the mice were 13-14 weeks old, and then checked for development of T1D followed by euthanization at end. (**a**) Frequency of T1D-free mice in indicated groups over time (n=10). (**b, c**) Representative histology sections (**b**) were shown and frequency (**c**) of islets with grade X insulitis in indicated groups was graded. Frequency of $CD25^+Foxp3^+$Treg (**d**), ratio of $CD25^+Foxp3^+$Treg / IFN-γ^+ $CD4^+$ T cells (**e**) and ratio of $CD25^+Foxp3^+$Treg / IFN-γ^+ $CD8^+$ T cells (**f**) in the spleen and DLN of female NOD mice were determinted. (**g, h**) Female NOD mice were fed with mannose water from 7.5 weeks of age, injected with αTGF-β/isotype control antibody once a week for 6 weeks, and checked for development of T1D followed by euthanization at end. (**g**) Frequency of

T1D-free mice in indicated groups over time (n=10). (**h**) Frequency of islets with grade X insulitis in indicated groups. Summary data are presented as mean ± s.e.m. *p<0.05, **p<0.01; by a Mantel–Cox log-rank test (**a,g**) or one-way analysis of variance (ANOVA) with Tukey's *post hoc* test (**d-f**). Data are pooled from two (**a, c, g, h**) experiments or are representative of two (**b, d-f**) independent experiments.

Figure 6. D-mannose induces antigen-specific Treg and suppresses ovalbumin-induced airway inflammation in BALB/cJ mice. (**a-f**) Disease suppression by D-mannose treatment in ovalbumin-induced airway inflammation model in BALB/cJ mice. Representative histology sections (**a**) and collated scores (**b**) showing airway inflammation by periodic acid Schiff staining. (**c**) Absolute numbers of polymorphonuclear neutrophils (PMN), basophils (Bas), eosinophils (Eos), macrophages (Mac) and lymphocytes (Lymph) in BAL fluid of BALB/cJ mice after intranasal challenges with ovalbumin.. (**d-f**) Frequencies of IL-4$^+$ T cells (**d**), IL-13$^+$ T cells (**e**) and CD25$^+$Foxp3$^+$ Treg (**f**) in KJ1-26$^+$CD4$^+$ T cells in BALB/cJ mice were determined after airway inflammation induction. (**g-l**) Disease amelioration by D-mannose treatment in ovalbumin-induced airway inflammation model in BALB/cJ mice. Representative histology sections (**g**) and collated scores (**h**) showing airway inflammation by periodic acid Schiff staining. (**i**) Absolute numbers of PMN, Bas, Eos, Mac and Lymph in BAL fluid of BALB/cJ mice after intranasal challenges with ovalbumin. (**j-l**) Frequencies of IL-4$^+$ T cells (**j**), IL-13$^+$ T cells (**k**) and CD25$^+$Foxp3$^+$ Treg (**l**) in KJ1-26$^+$CD4$^+$ T cells in BALB/cJ mice were determined. n=4 mice per group. Summary data are presented as mean ± s.e.m. *p<0.05, **p<0.01; by an unpaired two-tailed Student's *t*-tests. Data are representative of two independent experiments.

Online Materials and Methods:

Mice

C57BL/6, BALB/cJ, Rag1$^{-/-}$, CD45.1 (on a C57BL/6 background) and NOD/ShiLtJ mice were obtained from The Jackson Laboratory. DO11.10 TCR-transgenic Rag2$^{-/-}$ (on a BALB/cJ background) mice were purchased from Taconic. *Tgfbr1$^{f/f}$Cd4*-Cre^{+18}, *Tgfbr2$^{f/f}$ER*-Cre^{+22}, *Smad3*$^{-/-}$[23](on a C57BL/6 background), and Foxp3-GFP

reporter[16] mice (C57BL/6 background) were previously described and bred in our facility under specific pathogen-free conditions. *Itgb8*$^{f/f}$*Cd4*-Cre^{+} mice[32] were obtained from Dr. EM. Shevach (NIAID, National Institutes of Health, Bethesda, MD), and NOD.Foxp3^{DTR} mice[52] were obtained from Drs. C. Benoist/D. Mathis (Harvard Medical School, Boston, MA). All mice used for experiments were aged 5-12 weeks. All animal studies were performed according to National Institutes of Health (NIH) guidelines for use and care of live animals and approved by the Animal Care and Use Committees of National Institute of Dental and Craniofacial Research (NIDCR).

Antibodies and reagents

Purified anti-mouse CD3 (no azide and low endotoxin; 145-2C11), purified anti-mouse CD28 (no azide and low endotoxin; 37.51), purified anti-human CD3 (OKT3), purified anti-human CD28 (CD28.2), and the following fluorochrome-conjugated antibodies: anti-mouse CD4 (RM4-5), anti-mouse CD8α (53-6.7), anti-mouse TCR-β (H57-597), anti-mouse CD45 (30-F11), anti-mouse CD25 (PC61.5 and eBio7D4), anti-mouse DO11.10 TCR (KJ1-26), anti-mouse/rat-Foxp3 (FJK-16a), anti-mouse IL-4 (11B11), anti-mouse IL-13 (eBio13A), anti-human CD4 (RPA-T4), anti-human CD45RA (HI100), anti-human CD25 (BC96) and anti-human Foxp3 (PCH101) were purchased from eBioscience. Fluorochrome-conjugated anti-mouse IL-17A (TC11-18H10.1), anti-mouse IFN-γ (XMG1.2), anti-mouse IL-10 (JES5-16E3) were purchased from BioLegend. Fluorochrome-conjugated anti-human Ki67 (Cat# 556027) was purchased from BD Pharmingen. Recombinant human IL-2 (202-IL), human latent TGF-β1 (299-LT) and human TGF-β1 (240-B) were purchased from R&D Systems. Anti-TGF-β antibody (1D11.16.8) and isotype control (MOPC-21), anti-CD25 antibody (PC-61.5.3) and isotype control (HRPN) were purchased from Bio X Cell. SB431542 (TGF-βR inhibitor) was purchased from Selleckchem (Cat# S1067), and was used at 5 μM, NAC (ROS scavenger) was purchased from Calbiochem (Cat# 106425), and was used at 10 mM.

Flow Cytometry Analysis

Intranuclear staining was carried out using the Fixation/Permeabilization buffer solution (eBioscience) according to the manufacturer's instructions. For intracellular cytokine staining, cells were stimulated with PMA (10 ng/ml), Ionomycin (250 ng/ml) and Golgi-Plug (1:1000 dilution, BD Pharmingen) at 37 ℃ for 4 h, followed by fixation with the Fixation/Permeabilization buffer solution (BD Biosciences) according to manufacturer's instructions. Stained cells were analyzed on a FACS-Calibur or LSRFortessa (BD Biosciences) and data was analyzed with FlowJo software.

***In vitro* differentiation of mouse Treg**

$CD4^+CD25^-$ (Naïve) T cells or $CD4^+CD25^-GFP^-$(Foxp3$^-$) T cells were purified by magnetic cell sorting (Miltenyi Biotec) or FACS sorting (BD FACSAria II) and cultured at 0.4 × 10^6 cells/well in 24-well plates with plate-bound anti-CD3 (1.5μg/mL) and soluble anti-CD28 (1.5μg/mL), with or without latent TGF-β1 (10 ng/mL) or TGF-β1 (2 ng/mL) at 37℃. Cells were cultured in "complete" glucose-free Dulbecco's Modified Eagle Medium (DMEM) without adding exogenous glucose (called Control medium (CTRL), this medium contains low levels of endogenous glucose (~0.5-0.6 mM) derived from 10% fetal bovine serum) or in the same medium supplemented with a high concentration (25 mM) of D-mannose or other sugar monomers including glucose, fructose, galactose, or mannitol (a mannose derivative) in the presence of T cell receptor (TCR) stimulation. Three days later, cells were analyzed by FACS staining.

Real-time RT-PCR

Total RNA was derived from cultured cells with an RNeasy Mini kit (Qiagen) and cDNA was synthesized using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative real-time PCR was performed according to the protocol of TaqMan gene expression assay kits (Applied Biosystems). Results were normalized to the expression of *Hprt* mRNA.

In vivo induction of ovalbumin-specific Treg

BALB/cJ mice were fed with mannose water (1.1M) or plain control water for 10 days prior to cell transfer (and were fed with mannose/control water until point of sacrifice). BALB/cJ mice were then injected with 1×10^6 KJ1-26$^+$CD4$^+$CD25$^-$ naïve T cells isolated from DO11.10 TCR-transgenic Rag2$^{-/-}$ mice, and were fed with ovalbumin by gavage (160 mg/day) for another 5 days. Mice were then euthanized and Treg in the small intestine lamina propria (LPLs), spleen, mesenteric lymph nodes (MLN) and Peyer's patches were analyzed by FACS staining.

Non-obese diabetic (NOD) mouse model

Female NOD/ShiLtJ mice were fed with D-mannose water (1.1M) from 7.5 weeks of age, and the blood sugar level of mice was measured every three days. NOD/ShiLtJ mice were reared as described[53]. Once the concentration of blood sugar reached over 200 mg/dL for 2 consecutive weeks, the mouse was euthanized; all mice were euthanized when more than half of the mice in the control group developed disease. For therapeutic experiments on T1D, newly onset diabetic NOD mice[42,53] (blood glucose levels were 200-230mg/dl) were fed with D-mannose water till the termination of the experiment as indicated. The spleen, DLN and pancreas of mice were harvested for FACS and histology.

ovalbumin-induced airway inflammation model

Ovalbumin-induced airway inflammation induction was performed as described previously[46] with some modifications. Briefly, BALB/cJ mice were fed with D-mannose water (1.1M) followed by adoptive transfer of 1×10^6 KJ1-26$^+$CD4$^+$CD25$^-$ naïve T cells isolated from DO11.10 TCR-transgenic Rag2$^{-/-}$ mice and injection of ovalbumin to induce ovalbumin-specific Treg. The mice were then challenged with ovalbumin by intranasal injection (i.n.) for continuous 4 days (100 μg/day/mouse). For therapeutic experiments on airway inflammation, BALB/cJ mice were induced to develop airway inflammation, followed by supplementation with D-mannose in water, following which, the mice were challenged again with ovalbumin. One day

after the last challenge, mice were euthanized and the spleen, DLN and lung were harvested for analyses.

In vitro T_{reg} suppression assays

$CD4^+CD25^-$ (Naïve) T cells were isolated from spleen and LN of congenic CD45.1 mice. $CD4^+CD25^+GFP^+$(Foxp3$^+$) Treg (CD45.2$^+$) were isolated via FACS sorting (BD FACSAria II) from the spleen of Foxp3-GFP mice or from cultured $CD4^+CD25^-GFP^-$ (Foxp3$^-$) T cells with plate-bound anti-CD3 (1.5μg/mL) and soluble anti-CD28 (1.5μg/mL) in the presence of mannose or TGF-β1 for 3 days (All were CD45.2$^+$). Treg were cultured at the indicating ratios with CFSE-labeled $CD45.1^+CD4^+CD25^-$ T cells in the presence of γ-irradiated spleen cells and 0.5μg/ml anti-CD3. CFSE-dilution of CD45.1$^+$ effector T cells was analyzed by FACS after 3 days of culture.

T cell transfer colitis

$CD4^+CD25^-CD45RB^{hi}$ T cells were isolated from spleen and LN of congenic CD45.1 mice via FACS sorting (BD FACSAria II) and injected into $Rag1^{-/-}$ mice (0.4×10^6 per mouse) to induce colitis as described[14]. Some $Rag1^{-/-}$ mice were co-transferred with indicated different populations of Treg (0.1×10^6 per mouse) by intravenous injection (i.v.). The weight of the mice was measured every other day. The tissues were harvested at the end of the experiments for histopathological and immunological analyses.

Cell isolation from small intestine and pancreas.

To obtain LPLs from the small intestine, intraepithelial lymphocytes (IELs) were depleted by mechanical separation from the small intestine as previously described[54,55]. LPLs were then separated from gut tissue after vigorous shaking in RPMI medium supplemented with Liberase TL (0.25mg/ml) and DNase (0.2mg/ml). After isolation, cell suspensions were passed through 70-μm and 40-μm cell strainers and cell populations used for flow cytometry. To obtain pancreatic lymphocytes, pancreatic tissue was treated with collagenase IV (4 mg/ml) and DNase (4 mg/ml) for 30 minutes, after which cell suspensions were passed

consecutively through 70-μm and 40-μm cell strainers, and analyzed by flow cytometry.

In vitro culture of human T cells

Human peripheral blood mononuclear cells were provided by healthy volunteers and obtained from the NIH Department of Transfusion Medicine (DTM) through their approved protocol number: NCT000001846. Blood samples were provided by the DTM on a de-identified basis. A signed informed consent was obtained from all donors. Naïve $CD4^+$ T cells were purified by naïve T cell isolation kit II (Miltenyi Biotec) or FACS sorting, and cultured with plate-bound anti-human CD3 (5 μg /mL) and soluble anti-human CD28 (2.5 μg/mL) plus IL-2 (10 ng/ml). Cells were analyzed by FACS staining four days later.

Statistics

Unless otherwise noted, comparison between two different groups was performed using unpaired two-tailed Student's *t*-tests or one-way analysis of variance analysis (with Tukey multiple comparison posttests) for more than two group comparisons. Kaplan–Meier method was used and Mantel–Cox log-rank test was performed for evaluation of T1D development in NOD mice. All *P* values less than 0.05 were considered significant. Statistical analysis was performed with GraphPad Prism 6.

52. Feuerer, M., Shen, Y., Littman, D.R., Benoist, C. & Mathis, D. How punctual ablation of regulatory T cells unleashes an autoimmune lesion within the pancreatic islets. *Immunity* **31**, 654-664 (2009).
53. Kasagi, S.*, et al.* In vivo-generated antigen-specific regulatory T cells treat autoimmunity without compromising antibacterial immune response. *Sci Transl Med* **6**, 241ra278 (2014).
54. Zanvit, P.*, et al.* Antibiotics in neonatal life increase murine susceptibility to experimental psoriasis. *Nat Commun* **6**, 8424 (2015).
55. Konkel, J.E.*, et al.* Control of the development of CD8alphaalpha+ intestinal intraepithelial lymphocytes by TGF-beta. *Nat Immunol* **12**, 312-319 (2011).