Inhibition of hyaluronan synthesis restores immune tolerance during autoimmune insulitis

Nadine Nagy,1 Gernot Kaber,1 Pamela Y. Johnson,2 John A. Gebe,2 Anton Preisinger,2 Ben A. Falk,2 Vivekananda G. Sunkari,1 Michel D. Gooden,2 Robert B. Vernon,2 Marika Bogdani,2 Hedwich F. Kuipers,1 Anthony J. Day,3 Daniel J. Campbell,4 Thomas N. Wight,2 and Paul L. Bollyky1

1Division of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford University School of Medicine, Stanford, California, USA. 2Matrix Biology Program, Benaroya Research Institute, Seattle, Washington, USA. 3Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom. 4Immunology Program, Benaroya Research Institute, Seattle, Washington, USA.

We recently reported that abundant deposits of the extracellular matrix polysaccharide hyaluronan (HA) are characteristic of autoimmune insulitis in patients with type 1 diabetes (T1D), but the relevance of these deposits to disease was unclear. Here, we have demonstrated that HA is critical for the pathogenesis of autoimmune diabetes. Using the DO11.10xRIPmOVA mouse model of T1D, we determined that HA deposits are temporally and anatomically associated with the development of insulitis. Moreover, treatment with an inhibitor of HA synthesis, 4-methylumbelliferone (4-MU), halted progression to diabetes even after the onset of insulitis. Similar effects were seen in the NOD mouse model, and in these mice, 1 week of treatment was sufficient to prevent subsequent diabetes. 4-MU reduced HA accumulation, constrained effector T cells to nondestructive insulitis, and increased numbers of intraislet FOXP3+ Tregs. Consistent with the observed effects of 4-MU treatment, Treg differentiation was inhibited by HA and anti-CD44 antibodies and rescued by 4-MU in an ERK1/2-dependent manner. These data may explain how peripheral immune tolerance is impaired in tissues under autoimmune attack, including islets in T1D. We propose that 4-MU, already an approved drug used to treat biliary spasm, could be repurposed to prevent, and possibly treat, T1D in at-risk individuals.

Introduction
Autoimmune type 1 diabetes (T1D) is characterized by progressive, immune cell-mediated destruction of pancreatic β cells and the failure of regulatory mechanisms that normally prevent destructive insulitis, including FOXP3+ Tregs (1, 2). The local tissue environment is thought to contribute to immune regulation and the development of T1D (3–5), but the relevant mechanisms are unclear.

Recently, we reported (6) that autoimmune insulitis in T1D was associated with islet-specific deposition of hyaluronan (HA), an extracellular matrix (ECM) polysaccharide thought to contribute to chronic inflammation in a variety of settings (7–9). Using human T1D tissue samples from cadaveric organ donors obtained through the Juvenile Diabetes Research Foundation (JDRF) Network for Pancreatic Organ Donors with Diabetes (nPOD) program, we discovered that HA deposits were present in islets from donors with recent-onset T1D but not in those with longstanding T1D or type 2 diabetes or nondiabetic controls.

These T1D-associated HA deposits were also linked with local alterations in molecules that bind to HA, including TNF-stimulated gene-6 (TSG6), and inter-a-inhibitor (IaI). There is increasing evidence that HA/IaI/TSG6 complexes have powerful tissue-protecting effects and that the precise organization of the HA matrix in vivo dictates its functional effect (10–12). Together, these data implicated HA and the islet ECM in the onset of T1D.

However, it was unclear from these previous studies whether HA deposition preceded or merely followed autoimmune insulitis or whether HA contributed to diabetes pathogenesis.

To address these questions, we turned to a highly predictable and synchronous model of T1D, the DO11.10xRIPmOVA (DORmO) mouse model. These mice are the offspring of DO11.10 and RIP-mOVA transgenic mice. They carry a T cell receptor transgene specific for OVA (emulating autoreactive CD4+ T cells), while simultaneously expressing OVA in conjunction with the insulin gene promoter on pancreatic β cells (emulating the autoantigen). DORmO mice spontaneously develop autoimmune insulitis starting at 4 weeks of age, with nearly 100% becoming diabetic by 20 weeks of age (13).

To define the contributions of HA to insulitis, we treated these animals with 4-methylumbelliferone (4-MU), a pharmacologic inhibitor of HA synthesis (14). Treating DORmO mice with 4-MU provided us with a synchronous model of T1D in which disease progression could be manipulated and monitored. 4-MU treatment was also assessed in NOD mice. Along with providing us with another mouse model of T1D, autoimmune insulitis in these animals is thought to share similarities with that seen in human T1D (15).

Using these models, we tested the hypotheses that HA is fundamentally required for progression of autoimmune insulitis and that pharmacologic inhibition of HA synthesis may prevent progression of autoimmune diabetes.

Results
DORmO mice develop progressive, HA-associated insulitis. DORmO mice developed autoimmune insulitis at approximately 4 weeks
enzymes. Analyses of islet mRNA from 8-week-old mice showed that HA synthase 3 (Has3) was upregulated, while hyaluronidase 1 (Hyal1) was downregulated. However, hyaluronidase 2 (Hyal2) expression was increased, while that of Rhamm and layilin, two HA receptors that contribute to HA clearance, was increased (Supplemental Figure 2, A–G). This complexity may reflect the multiple cell types present within inflamed islets, with potentially disparate patterns of HA synthesis and catabolism.

We asked whether HA was systemically increased in DORmO mice. Levels of circulating HA were unchanged (Figure 2C), and we did not see heightened HA deposition in the heart, lung, or liver (data not shown).

We also considered whether islet HA deposition was driven by hyperglycemia. Arguing against this, islet HA was increased at 4 weeks (Figure 2A), well before the onset of hyperglycemia (Supplemental Figure 1A). In hyperglycemic mice in which the etiology of diabetes was not autoimmune, namely mice treated with the β cell toxin streptozotocin (STZ) (Figure 2D), and db/db mice (Figure 2E), less intraislet HA was seen, and this was not associ-
Hyaladherins are altered during T1D progression. Along with changes in HA, we observed progressive alterations in both the amount and the distribution of hyaladherins in DORMo insulin. Both TSG6 (Supplemental Figure 3, A–F, and S) and Iai (Supplemental Figure 3, G–L, and U) decreased in the DORMo pancreatic islets during disease progression. Tsg6 mRNA, however, was increased (Supplemental Figure 3T), while Iai-encoding mRNA expression decreased (Supplemental Figure 3V). Staining for versican was largely unchanged (Supplemental Figure 3, M–R, and W), but mRNA expression increased (Supplemental Figure 3X). These data indicate that insulin was associated with extensive changes in the islet ECM.

4-MU treatment prevents progression to diabetes. While our data suggested that HA deposits are temporally and anatomically associated with insulin, it was unclear whether HA contributed to disease pathogenesis. We therefore administered 4-MU to DORMo mice to test whether inhibition of HA synthesis could prevent autoimmune diabetes.

Adding 4-MU to chow significantly reduced HA content in DORMo islets (Figure 4, A–E). HA content was likewise reduced in isolated islets that were cultured overnight in 4-MU (Figure 4F). These data are consistent with the established role of 4-MU as an inhibitor of HA synthesis in other tissues (19, 20).

Together with this effect on HA, 4-MU treatment starting at 8 weeks of age prevented progression to hyperglycemia in DORMo mice (Figure 4G). Indeed, DORMo mice were normoglycemic during 4-MU treatment for up to a year, indicating that 4-MU-mediated prevention of diabetes was sustainable and efficacious in this model over long periods of time.

Further, treatment of NOD mice with 4-MU for 1 week prevented the subsequent progression to hyperglycemia in these animals (Figure 4H). Of note, insulinitis is typically already well established in this model by the age at which mice were treated with 4-MU (5 weeks of age) (Supplemental Figure 4 and ref. 21). Moreover, HA deposition is also a feature of insulitis in NOD mice as well (16). These data indicate that 4-MU can prevent diabetes in multiple mouse models of T1D.

To evaluate whether 4-MU eliminated autoreactive T cells, we took DORMo mice made normoglycemic by 4-MU off of this drug. These mice rapidly became hyperglycemic (Figure 5A), suggesting that 4-MU suspends autoimmune destruction without eliminating the potential for autoimmunity.

To test whether 4-MU promoted regeneration of β cells, we initiated 4-MU treatment at 12 weeks of age, by which time DORMo mice are typically borderline hyperglycemic and have substantially diminished β cell mass. 4-MU treatment did not prevent the onset of diabetes in these animals (Figure 5B) or improve glycemic control, as...
Figure 3. HA deposits characterize sites of insulitis in DORmO and human T1D. (A and B) HA staining in pancreatic tissue isolated from (A) a DORmO mouse and (B) a human cadaveric donor with T1D. Infiltrated islets are circled in red; unaffected islets are circled in black. (C and D) HA staining of representative BALB/c and DORmO islets, demonstrating interstitial (orange arrowhead) and peri-islet (blue arrowheads) patterns of HA distribution. HA associated with lymphocytic infiltrates (red arrows) was only seen in DORmO mice. (E and F) Costaining of HA and DAPI, demonstrating HA accumulation in association with insulitis. (G and H) HA deposits in human insulitis (red arrows) from two cadaveric donors with T1D. Original magnification, ×40.

measured by intraperitoneal glucose tolerance testing (IPGTT) (Figure 5, C and D). Further, we asked whether mice rendered diabetic via STZ treatment had improved glycemic control on 4-MU, and they did not (Figure 5, E and F). Thus, these data did not support the hypothesis that 4-MU promoted regeneration of β cells or improved metabolic control of hyperglycemia. Instead, these data suggested that 4-MU treatment suspended the progressive deterioration of insulin production otherwise observed in DORmO mice and that this effect was predicated upon the presence of viable β cells.

4-MU treatment establishes regulatory checkpoints in insulitis. By 15 weeks of age, insulin-producing β cells were characteristically lost in DORmO mice (Figure 6, A and B). However, in DORmO mice fed 4-MU, insulin staining was preserved (Figure 6, C–E), consistent with these mice remaining normoglycemic (Supplemental Figure 5A).

Robust insulitis was nonetheless still evident in 4-MU-treated DORmO mice (Figure 6, F and G). This effect was uniform across nearly all the islets that we examined. This nondestructive, “respectful” insulitis persisted while mice were maintained on 4-MU.

These effects were not associated with generalized immunosuppression. We observed a reduction in total splenocyte counts but unchanged lymphocyte counts in pancreatic lymph nodes (PLNs), mesenteric lymph nodes (MLNs), or inguinal lymph nodes (ILNs) (Supplemental Figure 5B). Proliferation of splenocytes in response to OVA peptide ex vivo was intact (Supplemental Figure 5C), and 4-MU-treated mice did not have reduced percentages of CD3+ T cells (Supplemental Figure 5D) or CD19+ B cells (Supplemental Figure 5E). Moreover, lymphocytes remained primed to destroy β cells, as evidenced by the persistent insulitis (Figure 6, F and G) and the rapid (<2 week) progression to diabetes that we observed after cessation of 4-MU treatment. Overall, the evidence was not consistent with generalized immune suppression.

We considered whether 4-MU treatment prevented autoimmunity through impaired leukocyte trafficking. However, the histologic data from 4-week-old mice (Figure 1P) indicated that lymphocytosis was typically already established prior to initiation of 4-MU at 8 weeks of age.

4-MU treatment promotes FOXP3 induction in vitro and in vivo. The reestablishment of the “respectful” insulitis that we observed upon histologic staining led us to wonder whether 4-MU treatment might promote peripheral immune tolerance. One major source of immune tolerance is FOXP3+ Tregs.

We indeed observed that the percentage of T cells expressing the Treg marker FOXP3 was increased in islets of DORmO mice after treatment with 4-MU (Figure 7). Along with this increase in Tregs in insulitis, we observed a nonsignificant increase in FOXP3+ Tregs in the spleens, ILNs, PLNs, and MLNs (Supplemental Figure 5F). Together, these data suggested that 4-MU treatment promoted an increase in Tregs in insulitis.

To evaluate this possibility in the absence of hyperglycemia or other complicating factors, we examined Treg induction upon 4-MU treatment in a pair of in vivo mouse models. First, we assessed the impact of 4-MU on the percentage of GFP/FOXP3+ Tregs in total CD4+ T cells in BALB/c mice fed 4-MU or control chow for 2 weeks. We observed increased proportions of Tregs among CD4+ T cells in the spleens, ILNs, MLNs, and PLNs of the 4-MU-treated animals (Figure 8A) but no change in the total percentage of T cells (Supplemental Figure 6A). Similarly, the percentage of CD19+ B cells (Supplemental Figure 6B), CD86+CD19+MHC-II+ antigen-presenting cells (Supplemental Figure 6C), and activated CD44hiCD4+ T cells (Supplemental Figure 6D) was not altered.

To test whether 4-MU could alter the peripheral development of Tregs, we then examined the induction of Tregs upon transfer of purified CD4+GFP+FOXP3+ T cells into RAG-deficient animals in the setting of 4-MU or control chow. We found that 4-MU treatment enhanced the fraction of GFP/FOXP3+ Tregs in the spleens, ILNs, MLNs, and PLNs of the 4-MU-treated animals (Figure 8B). Together, these results indicated that 4-MU enhances the peripheral differentiation of Tregs.

HA and CD44 suppress FOXP3 induction. Because 4-MU, an inhibitor of HA synthesis, promotes FOXP3 levels, we asked whether HA and the HA receptor CD44 inhibit FOXP3 induction. Indeed, we observed that plate-coated HA and anti-CD44 antibody both diminished FOXP3 induction from CD4+GFP+FOXP3+ precursors (Figure 8, C and D).

To better assess the contribution of CD44 to Treg induction, we then performed Treg induction using CD4+GFP+FOXP3+ T
anti-CD3/28 antibody activation (Supplemental Figure 7C). Both of these findings have been reported previously and attributed to defects in activation-induced cell death pathways (22). The absolute numbers of splenic CD4+GFP/FOXP3+ Tregs were actually higher on average in Cd44–/– mice, although this trend did not reach statistical significance (Supplemental Figure 7D). Together, these data are consistent with an increased generation of Tregs in Cd44–/– mice but suggest that this effect was obscured by heightened proliferation of effector T cells in these same animals.

Inhibition of ERK1/2 signaling partially overcomes CD44-mediated inhibition of FOXP3. We observed that the inhibitory effects of anti-CD44 antibody on FOXP3 induction enhanced anti-CD28 antibody-mediated FOXP3 inhibition (Figure 9, A and B), an effect known to proceed through AKT signaling (23). These data suggested that CD44 signaling might inhibit FOXP3 induction via pathways in addition to or other than AKT.

One signaling pathway known to inhibit FOXP3 induction is ERK1/2 (24). We previously reported that, when using human T cells isolated from Cd44+/+, Cd44+/−, or Cd44−/− mice as precursors, T cells from Cd44−/− mice had the greatest FOXP3 induction, with cells from heterozygous Cd44+/− mice and homozygous Cd44+/+ mice exhibiting less FOXP3 induction in inverse proportion to the number of CD44+ alleles they possessed (Figure 8, E and F).

To assess the impact of CD44 on Treg induction in vivo, we then performed a cotransfer of equivalent numbers of GFP/FOXP3−CD4+GFP/FOXP3−CD45.1 and GFP/FOXP3+CD4+GFP/FOXP3+CD45.2 T cells into Rag−/− hosts. After 4 days, the numbers of CD3+GFP/FOXP3+ cells and the ratio of Cd44−/− (CD45.2) vs. Cd44+/− (CD45.1) Tregs were assessed. We found that Cd44+/− (CD45.2) Tregs represent the majority of these cells in vivo (Figure 8G).

In light of these data, we were initially surprised when we observed that Cd44+/− mice did not naturally have increased numbers of CD4+GFP/FOXP3+ Tregs (Supplemental Figure 7A). However, spleens from Cd44+/− mice had significantly greater total numbers of CD4+ T cells (Supplemental Figure 7B), and CD4+ T cells from these animals had a hyperproliferative response to anti-CD3/28 antibody activation (Supplemental Figure 7C). Both of these findings have been reported previously and attributed to defects in activation-induced cell death pathways (22). The absolute numbers of splenic CD4+GFP/FOXP3+ Tregs were actually higher on average in Cd44−/− mice, although this trend did not reach statistical significance (Supplemental Figure 7D). Together, these data are consistent with an increased generation of Tregs in Cd44−/− mice but suggest that this effect was obscured by heightened proliferation of effector T cells in these same animals.

Inhibition of ERK1/2 signaling partially overcomes CD44-mediated inhibition of FOXP3. We observed that the inhibitory effects of anti-CD44 antibody on FOXP3 induction enhanced anti-CD28 antibody-mediated FOXP3 inhibition (Figure 9, A and B), an effect known to proceed through AKT signaling (23). These data suggested that CD44 signaling might inhibit FOXP3 induction via pathways in addition to or other than AKT.

One signaling pathway known to inhibit FOXP3 induction is ERK1/2 (24). We previously reported that, when using human...
T cells, CD44 signaling promotes phosphorylation of ERK1/2 (pERK1/2) (25). In mouse CD4+ T cells we likewise found that CD44 crosslinking induced pERK1/2 (Figure 9C).

We then asked whether inhibition of pERK1/2 could restore the loss of FOXP3 induction we observed upon anti-CD44 antibody treatment. Using CD4+GFP/FOXP3– T cells activated for 72 hours in the setting of TGF-β and IL-2, we found that SUO126, an ERK1/2 inhibitor, could overcome the inhibition of FOXP3 induction with or without CD44 costimulation (Figure 9, D and E).

Finally, we asked whether infiltrating lymphocytes express CD44. We indeed observed an increase in CD44+ staining during insulitis progression (Figure 9, F–L), suggesting that cells present in insulitis may be responsive to local HA.

Together, these data implicate a role for CD44 and HA in inhibition of Treg differentiation and support the hypothesis that 4-MU treatment relieves this inhibition by reducing HA-mediated CD44 signaling.

**Discussion**

We have identified a critical role for HA in the pathogenesis of autoimmune diabetes. We observed that HA deposits are both temporally and anatomically associated with autoimmune insulitis in both T1D and in the DORMO mouse model of the disease. Both the amount and distribution of HA closely tracked with the infiltration of CD3+ T cells and the disappearance of insulin staining. HA was not increased within neighboring islets without active insulitis.
Further, inhibition of HA synthesis using 4-MU prevented diabetes and preserved insulin content within islets in DORmO mice, despite ongoing, robust lymphocytic infiltrates. These effects were stable while mice were on continuous treatment for over a year. Of note, this treatment was initiated when the mice were 8 weeks of age, at which time insulitis is typically well established in DORmO mice. This is a strong, protective phenotype in a model in which the incidence of autoimmune diabetes is typically 100% (13). Furthermore, a short 1-week course of 4-MU treatment was sufficient to prevent diabetes progression in the canonical mouse model of T1D, the NOD mouse. We conclude from these data that HA production is necessary for destructive insulitis and that 4-MU treatment can forestall progression to diabetes in multiple mouse models of T1D.

Our data indicate that HA suppressed FOXP3+ Treg induction. Both HA and antibodies directed at the HA receptor, CD44, inhibited induction of FOXP3+ Tregs from CD4+GFP/FOXP3- precursors. Conversely, this inhibition was relieved by inhibition of HA synthesis with 4-MU. Together with our earlier work (26, 27), these data suggest that CD44 restricts the expansion of Tregs but promotes their phenotypic stability. This is the inverse of TLR2 agonists, which are known to promote Treg induction but inhibit suppression (28, 29).

Further, inhibition of HA synthesis using 4-MU prevented diabetes and preserved insulin content within islets in DORmO mice, despite ongoing, robust lymphocytic infiltrates. These effects were stable while mice were on continuous treatment for over a year. Of note, this treatment was initiated when the mice were 8 weeks of age, at which time insulitis is typically well established in DORmO mice. This is a strong, protective phenotype in a model in which the incidence of autoimmune diabetes is typically 100% (13). Furthermore, a short 1-week course of 4-MU treatment was sufficient to prevent diabetes progression in the canonical mouse model of T1D, the NOD mouse. We conclude from these data that HA production is necessary for destructive insulitis and that 4-MU treatment can forestall progression to diabetes in multiple mouse models of T1D.

Our data indicate that HA suppressed FOXP3+ Treg induction. Both HA and antibodies directed at the HA receptor, CD44, inhibited induction of FOXP3+ Tregs from CD4+GFP/FOXP3- precursors. Conversely, this inhibition was relieved by inhibition of HA synthesis with 4-MU. Together with our earlier work (26, 27), these data suggest that CD44 restricts the expansion of Tregs but promotes their phenotypic stability. This is the inverse of TLR2 agonists, which are known to promote Treg induction but inhibit suppression (28, 29).

The impact of HA on local immune regulation may also be influenced by the local organization of HA in vivo. We observed decreases in the hyaladherins TSG6 and IαI during the progression to T1D. Because these molecules regulate HA binding to CD44, they may affect the phenotypes associated with HA (32). Other components of the ECM are also reported to contribute to the development and progression of insulitis, either through HA-dependent or -independent mechanisms (6, 33–37). Taken together with our recent work on HA in human T1D (6), the progression to autoimmune diabetes is associated with profound changes in the ECM architecture.

It is notable that substantial intraislet HA deposits persisted, despite 4-MU treatment. Since 4-MU works at the level of HA synthesis (rather than clearance or catabolism), we suspect that it may take time for HA at sites of inflammation to disappear. The fact that β cell destruction is nonetheless forestalled suggests that 4-MU may also induce qualitative changes in resulting ECM that influence local immune function. Histologic assessments may also understate the HA decrease because they measure the area within which HA is deposited and not the quantity of HA there. This is perhaps evident in the difference between the decrease in HA area measured histologically (>25%; Figure 6. 4-MU treatment promotes nondestructive insulitis. (A–D) Insulin staining of representative pancreatic tissue sections from DORmO and BALB/c mice fed either 4-MU or control chow. (E) Average insulin+ area of islets for these mice. 25 islets were visualized per mouse, and staining and data are for 6 mice per condition. (F and G) Representative images of insulin staining of pancreatic islets from 15-week-old DORmO mice treated with 4-MU for 7 weeks. Original magnification, ×40. Data represent mean ± SEM; *P < 0.05 vs. respective control by unpaired t-test.
4E) and the drop in HA content in 4-MU-treated islets measured biochemically (>60%; Figure 4F), although these data are from different experiments.

These data may also help explain reports that 4-MU reduces inflammation. 4-MU has been mostly used in animal models to prevent tumor metastasis, in which HA is thought to drive angiogenesis and tumor proliferation (38–40). A handful of reports suggest that 4-MU may also have utility in other inflammatory settings (9, 41). However, to our knowledge, insight into the relevant immunologic mechanisms is limited to two studies. One study reported that 4-MU treatment reduced MMP expression in a mouse model of collagen-induced arthritis (42). Another recent report suggested that 4-MU increased spinal cord expression of CXCL12 and decreased Th1 responses, while increasing numbers of Tregs (43). Here, we established that HA inhibits FOXP3 expression via a CD44- and ERK1/2-dependent pathway, and that these Tregs constrained effector T cells at the site of autoimmunity. Further, we have demonstrated that 4-MU works in the setting of established autoimmune diabetes (analogous to how it would actually be used in people), that it can be used as a chronic therapy, and that it can be used to treat autoimmune diabetes. These data should greatly facilitate the clinical translation of 4-MU.

These data are particularly exciting because 4-MU is already an approved drug. Called “hymecromone,” it is used throughout Europe and Asia to treat biliary spasm (44, 45). It is an orally available agent that has been used for over 30 years in both adults and children. Because of this established track record and favorable side effect profile, 4-MU may be uniquely appropriate as a therapy for T1D.

It is noteworthy that 4-MU treatment did not restore normoglycemia to STZ-treated mice or to DORmO mice when it was initiated at 12 weeks of age or later. Additionally, 4-MU treatment did not appear to promote islet regeneration. This suggests that residual populations of viable β cells are required for 4-MU effects. Moreover, autoimmune diabetes developed rapidly upon cessation of the drug, suggesting that long-term therapy may be necessary.

In summary, our data indicate that the local accumulation of HA follows the temporal and geographic progression of autoimmune insulitis in this model. Moreover, inhibition of HA synthesis promotes FOXP3+ Treg induction via a CD44- and ERK1/2-dependent pathway and prevents progression of β cell destruction. In light of these data, we propose that hymecromone (4-MU) treatment, alone or following a tolerance induction regimen (46), may have great potential as a maintenance agent to prevent TID progression in at-risk, autoantibody-positive individuals who still retain some level of endogenous insulin production.

Methods

Mice. All animals were bred and maintained under specific pathogen-free conditions, with free access to food and water, in the vivarium at the Benaroya Research Institute and the animal facilities at Stanford University Medical School (Stanford, California, USA). DO11.10 transgenic mice were purchased from The Jackson Laboratory (JAX) and bred with BALB/c mice expressing RIPmOva (available at the Benaroya Research Institute) to generate the DORmO double-transgenic mice. NOD mice were purchased from JAX. Foxp3-3-GFP C57BL/6 mice were a gift from Alexander Rudensky (Memorial Sloan Kettering, New York, New York, USA). CD44-deficient C57BL/6 (Cd44−/−) mice were purchased from JAX and intercrossed with Foxp3-GFP mice to generate Foxp3-GFP Cd44−/− mice.

Human tissues. We used cadaveric pancreatic tissue sections from donors with T1D. These were obtained through the JDRF-sponsored nPod program. Case numbers cited herein were assigned by nPod unless otherwise noted. Demographic attributes of these donors and detailed histopathological characterization of their tissue samples have been previously described (6). All the tissues showed well-preserved morphology without any evidence of autolysis.

4-MU treatment. The 4-MU (Alfa Aesar) was pressed into the mouse chow by TestDiet and irradiated before shipment, as previously described (20). We previously determined that this chow formulation delivers 250 mg/mouse/d, yielding a plasma drug concentration of 640.3 ± 17.2 nmol/l in mice, as measured by HPLC-MS.
Unless otherwise noted, mice were initiated on the 4-MU chow at 5, 8, or 12 weeks of age and were maintained on this diet until they were euthanized.

**Weight and diabetes monitoring.** Beginning at 4 weeks of age, mice were weighed weekly as well as bled via the saphenous vein for the determination of their blood glucose level using a Contour blood glucose meter and blood glucose monitoring strips (Bayer Healthcare). When two consecutive blood glucose readings of 300 mg/dl were recorded, animals were considered diabetic. When two consecutive blood glucose readings of 250 mg/dl were recorded, animals were euthanized. For IPGTTs, mice were fasted (given water only) for 8 to 12 hours. Mice were then injected intraperitoneally with d-glucose (stock solution in PBS) at a dose of 1 g/kg body weight. Saphenous blood glucose readings were taken at 0, 15, 30, 60, and 120 minutes after injection.

**Flow cytometry and phenotyping.** Mouse leukocyte populations were isolated from ILNs, MLNs, PLNs, and spleens as previously described (26). Mouse flow cytometry experiments used the following fluorochrome-labeled antibodies from BD Biosciences: CD3e (145-2C11), CD4 (RM4-5), CD25 (PC61.5), and CD44 (IM7). FOXP3 (FJK.16a) antibody and staining reagents from eBioscience were used as per the manufacturer’s instructions. FACS samples were stained in media on ice for 45 minutes, washed once, resuspended in FACS stain buffer (PBS containing 1% FBS, 0.1% Na-azide), and run on a FACS caliber flow cytometer (BD). Analysis was performed using CELLQuest (BD) and FlowJo (Treestar Inc.) software.

**Isolation of leukocyte populations and Treg induction.** CD4+ cells were isolated from splenocytes and lymphocytes using MACS Kits (Miltenyi Inc.), and the GFP/FOXP3+ fraction was isolated from the CD4+ population using a FACS Vantage cell sorter (BD Biosciences). CD4+/GFP– FOXP3– T cells (2 × 105 per plate) were cultured in DMEM-10 (Invitrogen) supplemented with 10% FBS for 96 hours on these plates, followed by collection of the cells and culture supernatants for analysis. For Treg induction, CD4+/GFP– FOXP3– T cells were used to ensure that any FOXP3 induction we observed would be from conventional T cell progenitors. Cells were cultured in DMEM-10 (Invitrogen) supplemented with 10% FBS (Hyclone, GE Healthcare), penicillin/streptomycin, 50 μg/ml anti-CD44 antibody (IM7, BD Biosciences), and 2.5 g/ml anti-CD3 antibody (145-2C11, BD Biosciences) or 10% BSA. CD4+/GFP– FOXP3– T cells (2 × 105 per plate) were cultured for 96 hours on these plates, followed by collection of the cells and culture supernatants for analysis. For Treg induction, CD4+/GFP–

For intracellular phospho staining, freshly isolated CD4+CD25– T cells were incubated with anti-CD44 antibody (10 μg/ml) or an isotype control antibody for 20 minutes on ice. Following one wash, goat antimouse F(ab’)2 fragment (20 μg/ml) was added. Following incubation at 37°C for the indicated times, cells were fixed and stained for FACS analysis per the manufacturer’s protocols using anti-phospho-ERK1/2 (pT202/pY204) (BD Biosciences). For intracellular phospho staining, freshly isolated CD4+CD25– T cells were incubated with anti-CD44 antibody (10 μg/ml) or an isotype control antibody for 20 minutes on ice. Following one wash, goat antimouse F(ab’)2 fragment (20 μg/ml) was added. Following incubation at 37°C for the indicated times, cells were fixed and stained for FACS analysis per the manufacturer’s protocols using anti-phospho-ERK1/2 (pT202/pY204) (BD Biosciences).

**Figure 8. 4-MU treatment relieves CD44-mediated inhibition of FOXP3 induction.** (A) Percentage of GFP/FOXp3 Tregs of total CD4+ T cells in BALB/c mice fed 4-MU or control chow for 2 weeks (n = 5–6 mice per group). (B) In vivo induction of FOXP3+ Tregs assessed 4 days after transfer of GFP/FOXp3 CD4+ T cells into Rag–/– hosts given 4-MU or control chow (n = 3 Rag–/– recipient animals). Data are from the spleens of recipient animals. (C) CD25 and FOXP3 expression by CD4-GFP/FOXp3 T cells activated for 72 hours with or without plate-bound HA or anti-CD44 antibody. (D) Pooled data for 3 independent experimental replicates for the representative data in C. (E) FOXP3 induction using CD44–/–, CD44+–, or CD44++ precursors. (F) Pooled data for 3 independent experimental replicates for the representative data in E. (G) In vivo induction of FOXP3 assessed using cotransfer of equivalent numbers of GFP/FOXp3 CD4+ CD44–/– CD45.1 and GFP/FOXp3 CD4+ CD44–/– CD45.2 T cells into Rag–/– hosts. After 4 days, the numbers of induced CD3+GFP/FOXp3+ Tregs in the spleens of recipient animals were assessed and the ratio of CD44–/– Tregs versus CD44++ Tregs was determined (n = 3 Rag–/– recipient animals). Data represent mean ± SEM; *P < 0.05 vs. respective control by unpaired t test.
medium was supplemented with 0.8 mg/ml collagenase P (11-249-002-001, Roche) and filtered at 0.22 μm prior to injection. Subsequently, 2–3 excised pancreata were placed in separate 50 ml conical centrifuge tubes and incubated in 5 ml islet medium for 13 minutes at 37°C. The medium was then decanted, fresh 4°C islet medium was added, and the tubes were shaken vigorously to disrupt the pancreata. The tissue suspensions were filtered through a 30-mesh metal screen to remove large debris, the filtrates were pelleted by centrifugation, and the pellets resuspended in 4°C islet medium. The resuspended material was centrifuged through Histopaque 1077 to isolate the islets, which were washed, resuspended in islet medium, and placed in a tissue culture incubator. After all pancreata were processed, the isolated islets were hand picked.

Isolated islet in vitro experiment. Islets were cultured in RPMI (Invitrogen) supplemented with 10% pooled human serum, 100 μg/ml penicillin, 100 U/ml streptomycin, and 1 mM Na-pyruvate (Invitrogen) and 10% pooled human serum, 100 μg/ml penicillin, 100 U/ml streptomycin, and 1 mM Na-pyruvate (Invitrogen) supplemented with 10% pooled human serum, 100 μg/ml penicillin, 100 U/ml streptomycin, and 1 mM Na-pyruvate (Invitrogen). FOXP3+ T cells were cultured in the setting of 50 ng/ml TGF-β and 100 IU/ml IL-2, both added at the inception of the experiment. GFP/FOXP3 expression was assessed after 72 to 96 hours.

For the in vivo FOXP3+ Treg induction experiments, CD4+/GFP/FOXP3+ T cells were isolated as described above. 1 × 10^6 of these cells were transferred into 6- to 8-week-old Rag2−/− mice purchased from JAX. The percentage of GFP/FOXP3+ Tregs among all CD4+ splenocytes was assessed after 72 to 96 hours.

Islet isolation. Islets were isolated as described previously (47). Briefly, mice at 8 weeks of age were anesthetized with 2,2,2 tribromoethanol in PBS. The descending aorta of each anesthetized mouse was transected, the bile duct was clamped at its distal (intestinal) end, and a 30-gauge needle was used to inflate each pancreas through the common bile duct with 4 ml 4°C islet medium comprised of RPMI 1640 containing 1.0 g NaHCO₃, 10% FBS (S12450H, Atlanta Biologicals), 1 mM Na-pyruvate, and penicillin/streptomycin. The islet medium was supplemented with 0.8 mg/ml collagenase P (11-249-002-001, Roche) and filtered at 0.22 μm prior to injection. Subsequently, 2–3 excised pancreata were placed in separate 50 ml conical centrifuge tubes and incubated in 5 ml islet medium for 13 minutes at 37°C. The medium was then decanted, fresh 4°C islet medium was added, and the tubes were shaken vigorously to disrupt the pancreata. The tissue suspensions were filtered through a 30-mesh metal screen to remove large debris, the filtrates were pelleted by centrifugation, and the pellets resuspended in 4°C islet medium. The resuspended material was centrifuged through Histopaque 1077 to isolate the islets, which were washed, resuspended in islet medium, and placed in a tissue culture incubator. After all pancreata were processed, the isolated islets were hand picked.

Isolated islet in vivo experiment. Islets were cultured in RPMI (Invitrogen) supplemented with 10% pooled human serum, 100 μg/ml penicillin, 100 U/ml streptomycin, and 1 mM Na-pyruvate (Invitrogen).
rorgen), with or without 100 μg/ml of 4-MU (Sigma-Aldrich) for 24 hours. At the same time, [H3]-glucosamine was added at a concentration of 40 μCi/ml to islet cultures. After 24 hours, the islets were digested with pronase (100 μg/ml) in 0.5 M Tris, pH 6.5, overnight at 37°C. Following digestion, the pronase was inactivated by heating to 100°C for 20 minutes. Radiolabeled macromolecules were then recovered and separated from unincorporated precursor by precipitation on nitrocellulose membranes using slot blot analysis as described previously (48). Briefly, 200 μl sample was added to an equal volume of 2% cetylpyridinium chloride (CPC) and 50 mM NaCl buffer, and the solution blotted onto a 0.45-μm nitrocellulose membrane (Schleicher and Schuell). The membrane was washed 6 times in 2% CPC and 50 mM NaCl buffer and once in deionized water before air drying at room temperature overnight. Incorporation of [3H]-glucosamine into HA was measured by digesting an equivalent radiolabeled aliquot with Streptomyces hyaluronidase (2 U/ml) for 24 hours at 37°C before slot blotting. HA was measured as the amount of hyalurondase-sensitive material precipitated to the nitrocellulose membrane. To determine the amount of chondroitin sulfate and dermatan sulfate present in the sample, an equal aliquot of sample was adjusted to pH 8.0 before digesting with chondroitin ABC lyase (0.03 U/ml) (North Star BioProducts). All scintillation counting was done on Beckman LS 6500 (Beckman Instruments).

**Tissue processing and imaging.** Tissues for histochrome were taken from animals and immediately transferred into 10% neutral buffered formalin or methyl Carnoy's fixative. The tissue was processed to paraffin on a Leica ASP300 Tissue Processor (Leica Microsystems Inc.). Then, 5-μm thick sections were cut on a Leica RM 2255 Microtome (Leica Microsystems Inc.).

All staining steps were performed on a Leica Bond Max automated immune histochemistry (IHC) stainer (Leica Microsystems Inc.). For HA affinity histochemistry, the Bond Intense R Detection Kit, a streptavidin-HRP system (Leica Microsystems Inc.), was used with 4 μg/ml biotinylated-HABP in 0.1% BSA-PBS as the primary antibody. The Bond Polymer Detection Kit was used for all other immunohistochemistry. This detection kit contains a goat anti-rabbit reagent conjugated to polymeric HRP and a rabbit anti-mouse post-primary reagent for use with mouse primary antibodies.

TSG6, CD44, and Iod IHC required pretreatment using heat-mediated antigen retrieval with EDTA at high pH (Bond Epitope Retrieval Solution 2) for 10 minutes. For TSG6 IHC, sections were incubated for 1 hour with rabbit anti-mouse TSG6 (RAM-1, generated in-house) at 1:1,000 in Bond Antibody Diluent. For CD44 IHC, sections were incubated for 1 hour with rabbit anti-mouse versican (GAG β-domain) (AB1033, Millipore) in Bond Antibody Diluent, and detection was performed using the Bond Polymer Detection Kit.

The paraffin slides for the immune fluorescence HA staining were deparaffinized in xylene and were diluted into PBS in descending concentrations of ethanol. The slides were then rinsed several times in PBS and blocked in 4% BSA for 5 hours. The tissues were probed with 4 μg/ml HABP in blocking medium, overnight at room temperature. The slides were rinsed in PBS for 30 minutes, before the secondary Streptavidin (S32354, Molecular Probes) was used at 1:400 for 2 hours. The slides were rinsed in PBS, and the nuclei were stained with Propidium Iodide (P3566, Molecular Probes) at 1:200. The Propidium Iodide was mixed into the mounting medium with Prolong Gold Antifade (P36930, Molecular Probes).

All images were visualized using a Leica DMRB inverted fluorescence microscope equipped with a Pursuit 4-megapixel cooled color/monochrome charge-coupled device camera (Diagnostic Instruments). Images were acquired using the Spot Pursuit camera and Spot Advance Software (SPOT Imaging Solutions; Diagnostic Instruments). Image analysis was performed using ImageJ (NIH), as described previously (6).

**Real-time quantitative PCR.** Islets were harvested for total RNA isolation using the High Pure RNA Isolation Kit (Roche Applied Science) and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For real-time quantitative PCR, all reagents were supplied by Applied Biosystems, unless otherwise noted. Relative quantification of Has1, Has2, Has3, Hyal1, Hyal2, IaI, Rheam, and versican gene expression was performed using TaqMan Gene Expression Assays (Applied Biosystems): Has1, Mm00468496_m1; Has2, Mm00515089_m1; Has3, Mm00515092_m1; Hyal1, Mm00476206_m1; Hyal2, Mm01230689_g1; IaI, Mm01277164_m1; Rheam, Mm00469183_m1; and versican, Mm01283063_m1. Briefly, 100 ng cDNA was amplified in 1X TaqMan Gene Expression Master Mix (Applied Biosystems) with a 250 nM TaqMan probe (Applied Biosystems) in a 20 μl reaction. Amplification of TSG6, insulin, and layilin was performed using 100 ng cDNA in 1X Power SYBR Green PCR Master Mix (Applied Biosystems) and 1 μM primer. Melting curve analysis confirmed that only one product was amplified. Expression was normalized to eukaryotic 18S rRNA Endogenous Control (4333760, Life Technologies). All reactions were run using the standard program for 50 cycles on an ABI7900HT thermocycler. All samples were performed in duplicate, and copy number estimates were generated from a standard curve created by using a selected reference cDNA template and TaqMan probe. Primers for TSG6 and insulin were designed with NCBI Primer-BLAST, synthesized by Sigma-Aldrich, and are as follows: TSG6F: 5′-ATTTGAAGGGTGTCGTCTCG-3′, TSG6R: 5′-GTTTGCAATGGGTTACCCG-3′, insulinF: 5′-GTTGGGCACTCTCCCATCCC-3′, insulinR: 5′-CACCAACACGCTTACCAGCC-3′, layilinF: 5′-ATGACCGGTGCAACATGAAGA-3′, and layilinR: 5′-GCTTACCTCCAGGCCAAAT-3′. Data for mRNA
expression are provided as mean ± SEM of the estimated copy number, normalized to 18S rRNA, and differences between enriched primary islet cell populations were analyzed.

**Measurement of HA plasma levels.** Samples were thawed and then assayed for HA levels in a single batch using a modified HA-ELISA (49). Each sample was analyzed in triplicate, with a mean value obtained for each individual.

**HA quantification.** Tissues were first lyophilized and weighed and then were digested with proteinase K (250 μg/ml) in 100 mM ammonium acetate, pH 7.0, overnight at 60°C. After digestion, the enzyme was inactivated by heating to 100°C for 20 minutes. The total amount of HA was determined by a modified competitive ELISA in which the samples to be assayed were first mixed with biotinylated HA-binding protein and then added to HA-coated microtiter plates, the final signal being inversely proportional to the level of HA added to the bFG (50).

**Statistics.** Data are expressed as mean ± SEM of n independent measurements. Comparison between 2 groups was performed with unpaired 2-tailed t tests. A P value of less than 0.05 was considered statistically significant. Data analysis was performed with the use of GraphPad Prism 5.0 software.

**Study approval.** All animal experiments and procedures were approved by the Animal Care and Use Committee at the Benaroya Research Institute and/or at the Stanford University Medical School.

4. Glisic S, Ehlenbach S, Jailwala P, Waukau J, Jana S, Ghosh S. Inducible regulatory T cells (iTregs) from recent-onset type 1 diabetes subjects show increased in vitro suppression and higher ITCH expression are provided as mean ± SEM of the estimated copy number, normalized to 18S rRNA, and differences between enriched primary islet cell populations were analyzed.

**Measurement of HA plasma levels.** Samples were thawed and then assayed for HA levels in a single batch using a modified HA-ELISA (49). Each sample was analyzed in triplicate, with a mean value obtained for each individual.

**HA quantification.** Tissues were first lyophilized and weighed and then were digested with proteinase K (250 μg/ml) in 100 mM ammonium acetate, pH 7.0, overnight at 60°C. After digestion, the enzyme was inactivated by heating to 100°C for 20 minutes. The total amount of HA was determined by a modified competitive ELISA in which the samples to be assayed were first mixed with biotinylated HA-binding protein and then added to HA-coated microtiter plates, the final signal being inversely proportional to the level of HA added to the bFG (50).

**Statistics.** Data are expressed as mean ± SEM of n independent measurements. Comparison between 2 groups was performed with unpaired 2-tailed t tests. A P value of less than 0.05 was considered statistically significant. Data analysis was performed with the use of GraphPad Prism 5.0 software.

**Study approval.** All animal experiments and procedures were approved by the Animal Care and Use Committee at the Benaroya Research Institute and/or at the Stanford University Medical School.

4. Glisic S, Ehlenbach S, Jailwala P, Waukau J, Jana S, Ghosh S. Inducible regulatory T cells (iTregs) from recent-onset type 1 diabetes subjects show increased in vitro suppression and higher ITCH expression are provided as mean ± SEM of the estimated copy number, normalized to 18S rRNA, and differences between enriched primary islet cell populations were analyzed.

**Measurement of HA plasma levels.** Samples were thawed and then assayed for HA levels in a single batch using a modified HA-ELISA (49). Each sample was analyzed in triplicate, with a mean value obtained for each individual.

**HA quantification.** Tissues were first lyophilized and weighed and then were digested with proteinase K (250 μg/ml) in 100 mM ammonium acetate, pH 7.0, overnight at 60°C. After digestion, the enzyme was inactivated by heating to 100°C for 20 minutes. The total amount of HA was determined by a modified competitive ELISA in which the samples to be assayed were first mixed with biotinylated HA-binding protein and then added to HA-coated microtiter plates, the final signal being inversely proportional to the level of HA added to the bFG (50).

**Statistics.** Data are expressed as mean ± SEM of n independent measurements. Comparison between 2 groups was performed with unpaired 2-tailed t tests. A P value of less than 0.05 was considered statistically significant. Data analysis was performed with the use of GraphPad Prism 5.0 software.

**Study approval.** All animal experiments and procedures were approved by the Animal Care and Use Committee at the Benaroya Research Institute and/or at the Stanford University Medical School.