Chromatin remodeling resets the immune system to protect against autoimmune diabetes in mice

Running title: Epigenetic control of diabetes

Tejas Patel[1], Vasu Patel[1], Rajvir Singh[1], and Sundararajan Jayaraman[1,2]

[1]Department of Surgery, [2]Department of Medicine, College of Medicine, University of Illinois at Chicago, Chicago, IL 60612

Correspondence to: Sundararajan Jayaraman, Dept. of Medicine, COMRB 3168, 909 South Wolcott Avenue, M/C 719, University of Illinois at Chicago, College of Medicine, Chicago, IL 60612, USA. Phone: 312-355-5133; Fax: 312-996-2242;

e-mail: anue2468@uic.edu

ABSTRACT

Epigenetic alteration of the genome has been shown to provide palliative effects in mouse models of certain human autoimmune diseases. We have investigated whether chromatin remodeling could provide protection against autoimmune diabetes in NOD mice. Treatment of female mice during the transition from prediabetic to diabetic stage (18-24 weeks of age) with the well-characterized histone deacetylase inhibitor, Trichostatin A effectively reduced the incidence of diabetes. However, similar treatment of overtly diabetic mice during the same time period failed to reverse the disease. Protection against diabetes was accompanied by histone hyperacetylation in pancreas and spleen, enhanced frequency of $CD4^+$ $CD62L^+$ cells in the spleen, reduction in cellular infiltration of islets, restoration of normoglycemia and glucose-induced insulin release by beta cells. Activation of splenic T lymphocytes derived from protected mice *in vitro* with pharmacological agents that bypass the antigen receptor or immobilized anti-CD3 antibody resulted in enhanced expression of *Ifng* mRNA and protein without altering the expression of *Il4*, *Il17*, *Il18*, *Inos*, and *Tnfa* genes nor the secretion of IL-2, IL-4, IL-17, and TNF-α proteins. Consistently, expression of the transcription factor involved in *Ifng* transcription, *Tbet/Tbx21* but not *Gata3* and *Rorgt,* respectively required for the transcription of *Il4* and *Il17,* was upregulated in activated splenocytes of protected mice. These results indicate that chromatin remodeling can lead to amelioration of diabetes by employing multiple mechanisms including differential gene transcription. Thus, epigenetic modulation could be a novel therapeutic approach to block the transition from benign to frank diabetes.

KEY WORDS: Epigenetics, type 1 diabetes, interferon gamma gene, T regulatory cells.

INTRODUCTION

Epigenetics, heritable changes in gene expression without altering the DNA sequence, involves DNA methylation and chromatin remodeling by phosphorylation, methylation, sumoylation, ubiquitination, and acetylation of histones[1]. Reversible acetylation of the ε-amino group of lysine in the histone tails by histone acetyl transferases and deacetylation by histone deacetylases (HDAC) are the best-characterized post-translational modifications of histones. Hyperacetylated histones are correlated with transcriptional permissiveness whereas hypoacetylated histones mediate gene repression[1]. Small molecule HDAC inhibitors are major tools for studying the correlation between overall chromatin modifications and cellular functions[2]. The HDAC inhibitors are often used to specifically inhibit the HDAC activity. Inhibition of HDAC activity *in vitro* increases histone acetylation within 2-4 h of treatment with HDAC inhibitors including Trichostatin A (TSA), and consequently induces up- and down-regulation of genes[3-4]. The HDAC inhibitor mediated regulation of a small portion of genes (5-20%) is dependent on the cell type, dose and type of inhibitors used, suggesting that chromatin remodelling by HDAC inhibitors is a gene-specific event with a variable transcriptional outcome[2-4].

TSA, an antibiotic derived from *Streptomyces sp.* is one of the most potent and best-characterized HDAC inhibitors and it inhibits both class I (HDAC 1-3, and -8) and class II (HDAC 4-7, and -9) HDAC family members[2]. Treatment of immune cells *in vitro* with TSA has been shown to suppress the expression of genes involved in immune responses, such as interleukin-2 (IL-2)[5], IL-6, IL-10, and IL-12[6], CD25[7], and CD154[7-8]. In addition, TSA has been shown to upregulate the expression of interferon-gamma (IFN-

γ) in human lupus T cells *in vitro*[8]. While these studies indicate that HDAC inhibition can modulate the expression of cytokines in T lymphocytes stimulated *in vitro*, it not clear whether HDAC inhibitors can reprogram the T-cells to express altered cytokine profile *in vivo*. Recent studies indicate that treatment of normal[9] and autoimmune prone mice[10] intensely with TSA could increase both the number and function of $CD4^+CD25^+FoxP3^+$ T regulatory cells. However, it remains to be determined whether hyperacetylation can influence other T regulatory subsets also. The utility of the HDAC inhibitors including TSA to ameliorate autoimmune lupus[6], experimental colitis[11], airway hyperreactivity[12], and experimental autoimmune encephalomyelitis[13] in mouse models has been demonstrated. A preliminary investigation suggested the usefulness of the HDAC inhibitor FR901228 in a very small number (n=3) of newly diagnosed diabetic mice[7]. However, it is not clear whether epigenetic modulation could be useful in preventing the onset of autoimmune diabetes. Here, we investigated this possibility and unraveled some of the underlying mechanisms involved in protection against autoimmune diabetes by chromatin remodeling.

RESULTS

Amelioration of autoimmune diabetes by TSA treatment

The efficacy of HDAC inhibition to protect female NOD mice against the naturally occurring autoimmune diabetes was determined by administering 500 μg of TSA/kg body weight s.c. at weekly intervals. Cumulative data obtained from five experiments indicate that TSA treatment between 18 and 24 weeks (n=68) but not at earlier time points provided long-term protection against diabetes (Figure 1a). Protection was exerted by TSA since administration of the vehicle, dimethyl sulfoxide (DMSO) failed to provide similar palliative effect. Further analysis revealed that a majority (61%) of mice (n=31) that were not diabetic at the initiation of drug treatment (18 weeks of age) remained diabetes-free till the end of the experiment, 34 weeks of age (Figure 1b). However, TSA treatment during the same time interval in a considerable number of overtly diabetic mice (n=17) failed to reverse the full-blown disease (Figure 1c). Overtly diabetic mice lost >20% of the body weight within 2 to 4 weeks of diagnosis and therefore killed. Protected mice did not display any abnormality in overall body condition and intake of food and water. At the time of sacrifice, 32 to 36 weeks of age, the body weight of TSA-treated-non-diabetic mice was 25 +/- 1.5 g in contrast to 17 +/-1.6 g in diabetic mice. Post-mortem examination of TSA-treated mice revealed no lesion at the site of drug injection or any gross abnormality of major internal organs, such as heart, lung, liver, stomach, intestine, kidney, and spleen.

It was next investigated whether restoration of blood glucose homeostasis was associated with TSA-mediated long-term protection against diabetes. To this end, 32-36 weeks old mice (8-12 weeks after TSA treatment) were given an i.p. challenge of glucose

following 4 h of starvation. Data shown in Figure 2a indicate clearance of high blood glucose levels in protected mice with kinetics similar to that of DMSO-treated-non-diabetic and untreated-non-diabetic mice. In contrast, diabetic mice, regardless of treatment, failed to clear excess glucose in the peripheral blood. Consistent with normoglycemia, a sharp increase in insulin level was observed in the blood of TSA-treated-non-diabetic mice within 15 min of glucose challenge (Figure 2b). In contrast, only a meager raise in serum insulin level was observed in TSA-treated-diabetic mice as long as 60 min after glucose challenge. In data not shown, we observed insulin release in response to high glucose challenge in non-diabetic but not in diabetic mice that were either treated with DMSO or left untreated. Thus, prevention of diabetes by TSA treatment is associated with the preservation of the pancreatic β cell function. Whereas most intervention strategies are effective at the prediabetic stage[14], TSA treatment was effective when administered during the period corresponding to the putative transition from peri-insulitis to overt diabetes[15], indicating the clinical relevance of TSA treatment to intervene diabetes manifestation at a comparatively later stage.

TSA treatment induces histone hyperacetylation

Since HDAC inhibitors facilitate hyperacetylation of histones and subsequent modification of gene expression[2-3], we next ascertained whether TSA treatment could increase histone acetylation in the target organ pancreas and in the spleen, which contains T lymphocytes capable of transferring diabetes into histocompatible, immunodeficient NOD.*scid* mice. Mice were killed after 2 or 12 weeks of the last injection of TSA given between 18-24 weeks of age and the acidic nuclear extracts from spleens and pancreata were immunoblotted by using antibodies against acetylated H3 and native H3 histone.

Representative data from 3 experiments, each containing 3-5 mice per treatment group, indicate prominent acetylation of histone H3 in both spleens and pancreata of mice that were treated with TSA 2 weeks earlier (Fig. 3a). Substantial acetylation of H3 was also found in splenocytes and pancreata of 12 weeks old prediabetic mice treated for 24 h with TSA *in vitro*. Equivalent loading of nuclear extracts was verified by immunoblotting with an antibody against native H3. Increases in H3 acetylation in spleens and pancreata after *in vivo* and *in vitro* treatment with TSA were verified by densitometry (Figure 3c). When analyzed 12 weeks after TSA treatment (36 weeks of age), discernible increase in the level of H3 histone acetylation was not found in both spleens and pancreata regardless of whether they were derived from nondiabetic or diabetic mice (Figure 3b and d). These results indicate that the HDAC inhibitor therapy led to robust histone hyperacetylation in spleens and pancreata of NOD mice shortly after treatment that subsided when long-term protection was stabilized, asserting the anticipated reversible effect of HDAC inhibitors.

Differential effects of TSA on T cell subsets

The effect of HDAC inhibitor treatment on various T cell subsets was then determined by flow cytometry. To this end, spleens were obtained from mice when protection was stably established, between 32 and 36 weeks of age. Results shown in Figure 4a indicate that TSA treatment did not significantly alter the frequency of $CD4^+$ or $CD8^+$ cells. Interestingly, the frequency of the $CD4^+CD62L^+$ subset was significantly increased in protected mice (Figure 4b). However, neither $CD4^+CD25^+$ nor $CD4^+FoxP3^+$ cells were influenced by TSA treatment. These data suggest that the long-term effects of HDAC inhibitor treatment include the upregulation of the $CD4^+CD62L^+$ cells implicated in the regulation of autoimmune diabetes[16]. Although the frequency of $CD4^+CD25^+$ and

$CD4^+FoxP3^+$ T regulatory cells did not significantly increase after the stabilization of protection against diabetes (32-36 weeks of age), a transient increase in the numbers and function of these cells shortly after the drug treatment, as observed in other model systems[9-10], remains a possibility.

TSA treatment reduces inflammation of the islets

Since cellular inflammation is the primary mechanism of beta cell destruction leading to hyperglycemia, we next ascertained the long-term effects of TSA treatment on the inflammatory response in the pancreata of mice in which protection was stably established, between 32 and 36 weeks of age. Histological examination revealed that the islets of untreated and DMSO-treated diabetic mice had severe inflammation (Grade 3, Figure 5a and c). On the other hand, the islets in the pancreata of TSA-treated and protected mice had substantially less severe cellular infiltration (Figure 5b) and the majority of them displayed no or minimal inflammation (Grade 1). Interestingly, most of the islets from cured mice were smaller and juxtaposed to pancreatic ducts (Figure 5b), consistent with the possible neogeneration of islets. Confocal analysis confirmed the abundance of insulin-positive β cells without invasive cellular infiltration in the islets of protected mice (Figure 5b). These data indicate that TSA-mediated amelioration of diabetes is associated with the blockade of progression from peri-insulitis to invasive and destructive insulitis.

Selective upregulation of IFN-γ associates with protection against diabetes

Although alteration of gene expression will be evident shortly (1-2 weeks) after TSA treatment when hyperacetylation of histones was robust (Figure 3a), we wanted to examine whether long-term protection correlates with changes in gene expression. Even

though HDAC inhibitors have been shown to suppress a number of cytokines *in vitro*[4-8], we concentrated only on the cytokines that have been directly implicated in diabetes manifestation such as *Il4*[17], *Il17*[18], *Il18*[19], *Ifng*[20-22] *Tnfa*[23], and *Inos*[24]. Since our preliminary data indicated no significant difference in the expression of intracellular IL-10 in splenoctyes of drug treated mice as assessed by flow cytometry, we did not further study *Il10* gene expression. Since the role of IL-6 in autoimmune diabetes remains obscure[25] and IL-12 acts predominantly through IFN-γ[22], these genes were also not investigated. In addition to cytokines, the transcription factors *Tbet* also known as *Tbx21*[26], *Gata3*[27], and *Rorgt*[28], respectively involved in the transcription of *Ifng*, *Il4*, and *Il17* were also examined. Total RNA was extracted from splenocytes of 32 to 36 weeks old mice (after 8 to 12 weeks of the last injection of TSA) and converted into cDNA and used for gene amplification by real-time quantitative RT-PCR as described[29]. The relative mRNA expression was calculated by the $2^{-\Delta\Delta C}{}_{T}$ method[29-30] using the housekeeping gene, *Gapdh* as reference. The relative abundance of the steady state levels of *Il4*, *Il17*, *Il18*, *Ifng*, *Tnfa*, *Inos, Tbx21*, *Gata3*, and *Rorgt* mRNA was comparable between unstimulated splenocytes of untreated-diabetic and untreated-non-diabetic mice (Figure 6a). Similarly, the steady state level expression of the cytokine genes and the transcription factors analyzed did not differ between TSA treated diabetic and non-diabetic mice (Figure 6b).

To determine the influence of histone hyperacetylation on the inducible expression of genes in T lymphocytes that are relevant to diabetogenesis, splenocytes were stimulated with PMA and ionomycin that bypass the antigen receptor and engage the downstream signalling pathways or exposed to immobilized anti-CD3 antibody. The data are presented as the fold change in gene expression normalized to the housekeeping

gene *Gapdh* and relative to the untreated control, following the $2^{-\Delta\Delta C}{}_{T}$ method[29-30]. Substantially higher levels of *Ifng* mRNA were expressed in splenocytes of TSA-treated-non-diabetic mice in comparison to similarly treated diabetic mice whether they were activated with PMA and ionomycin (Figure 7a) or immobilized anti-CD3 antibody (Figure 7b). In contrast, the expression of other cytokine genes implicated in diabetes, such as *Il4*, *Il17*, *Il18*, *Tnfa,* and *Inos* were comparable in spleens of TSA-treated, non-diabetic and diabetic mice. While a majority (>80%) of DMSO treated mice routinely develop diabetes, a small fraction of these mice remains free of diabetes. Data shown in Figure 7c indicate that resistance to diabetes in DMSO-treated mice was also associated with increased *Ifn* expression in activated splenocytes. Similarly, in comparison to untreated-diabetic mice, the inducible expression of *Ifng* was enhanced in splenocytes of a small number (n=3) of untreated mice that did not become overtly diabetic during the entire observation period (36 weeks of age) (data not shown). Thus, the ability of T lymphocytes to express *Ifng* upon stimulation is consistently enhanced in a small number of untreated-non-diabetic mice and in a large number of TSA-treated-non-diabetic mice.

Determination of cytokines by ELISA revealed that the splenocytes of TSA-treated-non-diabetic mice secreted more IFN-γ than those of diabetic mice, irrespective of whether they were stimulated with PMA + ionomycin (Figure 8a) or immobilized anti-CD3 antibody (Figure 8b). However, the levels of IL-2 or Th2- and Th17-restricted lymphokines respectively, IL-4 and IL-17, and TNF-α produced by splenocytes of diabetic and protected mice were comparable. Taken together, the data indicate that TSA-mediated long-term protection against diabetes is associated with increased ability of T

lymphocytes to express IFN-γ both at the gene and protein levels upon specific stimulation.

Upregulation of *Tbet/Tbx21* expression in mice protected from diabetes

The ability to express *Tbet/Tbx21,* crucial for IFN-γ transactivation[26] was significantly enhanced in splenocytes of non-diabetic mice that were left untreated or treated with TSA or DMSO. This was seen regardless of whether splenocytes were stimulated with PMA + ionomycin (Figure 9a) or immobilized anti-CD3 antibody (Figure 9b). However, the levels of *Gata3* and *Rorgt,* respectively involved in the transcription of *Il4* and *Il17*[27-28] did not differ between TSA-treated-non-diabetic and -diabetic mice (Figure 9c). Similar data were obtained in untreated and DMSO-treated mice (not shown). Collectively, these data indicate that the attenuation of diabetes manifestation in untreated mice and in those treated with DMSO or TSA is consistently associated with the coordinated upregulation of *Ifng* and its corresponding transcription factor *Tbx21* without influencing other genes implicated in diabetogenesis.

DISCUSSION

Our data demonstrate that treatment with the well-characterized HDAC inhibitor TSA can attenuate spontaneous autoimmune diabetes in female NOD mice. This was associated with reduced inflammation of the pancreatic islets, restoration of glucose-induced insulin release response in β cells, and selective increase in a subset of T regulatory cells. Drug treatment induced histone hyperacetylation and selective enhancement of the inducible expression of *Ifng* and its transcription factor *Tbx21*. Thus, epigenetic alterations of the genome may help to reduce the incidence of spontaneous autoimmune diabetes by multiple mechanisms.

Whereas our data demonstrate a strong correlation between the upregulation of IFN-γ and resistance against diabetes, previous studies reported conflicting results, primarily owing to the differences inherent to the model systems employed. In acute models of diabetes such as involving cyclophosphamide treatment[20-21] or adoptive transfer of diabetogenic T cells[20, 31], diabetes was blocked by treatment with neutralizing anti-IFN-γ antibodies. On the other hand, ablation of genes encoding IFN-γ and its receptor reduced but did not prevent spontaneous diabetes[22, 32-36]. Interestingly, injection of recombinant derived IFN-γ decreased the incidence of naturally occurring diabetes, accompanied by reduction in IL-12, insulitis and anti-islet effector reactivity[37]. Although IL-12 engaged an IFN-γ-independent default pathway in IFN-γ receptor ablated mice, under normal circumstances, recombinant IL-12 prevented diabetes by IFN-γ-mediated reduction in pancreatic inflammation and T cell activation[22]. A protective role of IFN-γ was also implicated in the attenuation of spontaneous diabetes in NOD mice vaccinated with Bacillus Calmette´ Guerin[38] or injected with Ig-glutamic acid decarboxylase[39].

Although neutralization of IFN-γ reduced IL-17-producing cells and abolished protection in this model[39], TSA treatment did not suppress IL-4 or IL-17 both at the mRNA and protein levels. Furthermore, *Rorgt* and *Gata3,* transcription factors respectively crucial for *IL4*[27] and *Il17*[28] expression were also not repressed in these mice. Thus, attenuation of naturally occurring diabetes by epigenetic modulation of the genome is associated with upregulation of IFN-γ-producing T cells and not suppression of Th2 or Th17 cells.

Although IFN-γ is predominantly produced by the Th1 subset of $CD4^+$ T-cells, $CD8^+$ and NK-T cells also produce this lymphokine that can influence both the innate and adaptive immune system. Exogenous administration of IL-12[22] or Bacillus Calmette´ Guerin vaccination[38] increased apoptosis of $CD4^+$ cells in a manner dependent on IFN-γ. It will be important to determine whether administration of the neutralizing antibody against IFN-γ could abrogate protection against diabetes by suppressing T regulatory population or nullifying deletion of diabetogenic T lymphocytes in TSA-treated mice. Since IFN-γ cross-regulates other signal transduction pathways in a number of cell types including islet cells[40-41], exaggerated IFN-γ expression may produce beneficial effects by employing other mechanisms as well.

In addition to *Ifng*, TSA treatment also upregulated the expression of the transcription factor *Tbx21*. Interestingly, similar coordinated upregulation of these genes was evident in T lymphocytes of non-diabetic mice that were either treated with the vehicle DMSO or left untreated. We have recently shown that transfusion with allogeneic newborn blood protected NOD mice from spontaneous diabetes associated with increased expression of *Tbx21*[29]. Since *Tbx21* is required not only for the transcription of *Ifng*[26] but also for a number of immune functions including priming of T cells by dendritic cells and

expression of CD122 and CxCR3[42-43], upregulation of *Tbx21* may provide protection against diabetes via multiple mechanisms. It is interesting to note that the deletion of *Tbx21* in both the innate and adaptive immune system prevented the development of diabetes in NOD mice[44]. In contrast, *Tbx21* deletion did not prevent insulitis in Balb/c mice[45], suggesting that insulitis, an obligate component of autoimmune diabetes is not influenced by *Tbx21* deletion. Since a number of immune functions require *Tbx21*[26, 42-43], further work is necessary to delineate the mechanisms by which *Tbx21* contributes to protection against autoimmune diabetes. Histone acetylation marks have been revealed at the *Ifng* locus of poralized Th1 cells driven to produce IFN-γ, distinct from those found in IL-4-producing Th2 cells by chromatin immunoprecipitation[46]. Similar studies may unravel unique histone marks at the *Ifng* and *Tbet* loci of $CD4^+$ T lymphocytes implicated in diabetes[47]. These data will also shed light on the epigenetic memory that renders female NOD mice highly susceptible to diabetes and how TSA treatment can erase this epigenetic memory resulting in the cure of the disease.

Induction of *Ifng* and *Tbx21* but not seven other genes tested was upregulated in T cells derived from TSA-treated mice that were protected from diabetes. Since the role of IL-6[25] in autoimmune diabetes remains unclear, it was not examined whether TSA treatment could alter its expression. It is well documented that IL-2 has dual roles in immune responses. In addition to promoting the proliferation and differentiation of T-cells, B-cells and NK cells, IL-2 is also essential for the development, homeostasis and function of $CD4^+CD25^+FoxP3^+$ T regulatory cells, which in turn control the immune responses including autoimmune pathologies[48]. Engineered haplodeficiency of *Il2* gene expression reduced IL-2 production by twofold, diminished the function of $CD4^+CD25^+$

T regulatory cells and exacerbated diabetes[48]. However, protection against diabetes induced by TSA treatment in wild type NOD mice was not associated with dramatic alteration in the secretion of IL-2 by T lymphocytes or the frequency of IL-2-dependent $CD4^+CD25^+FoxP3^+$ T regulatory cells. Thus, amelioration of diabetes by chromatin remodeling does not appear to be due to IL-2 mediated effects on the immune system including the $CD4^+CD25^+FoxP3^+$ subset of T regulatory cells.

Our data showing selective upregulation of *Ifng* are consistent with the notion that the alteration of the 'histone code' is a powerful way of modulating a small set of inducible genes (2-20%) in a tissue-specific and context-dependent manner[2-3]. Importantly, we also observed an association between the coordinated upregulation of *Ifng* and *Tbx21* and resistance to diabetes in a small number of untreated and DMSO-treated mice. This unexpected and surprising finding suggests that protection against diabetes and enhanced ability of T lymphocytes to express *Ifng* and *Tbx21* in response to T cell receptor-mediated activation may be causatively linked. Further, this intriguing finding suggests that erasing the epigenetic memory by TSA treatment may facilitate the expression of the gene profile similar to that of naturally protected mice. Since all of the untreated mice develop peri-insulitis and only some of them will never progress to become overtly diabetic, mechanisms must exist in these mice to retard full-blown diabetes. Epigenetic modulation appears to amplify these mechanisms leading to the blockade of overt diabetes in a large proportion of diabetes-prone mice. While exaggeration of *Ifng* and *Tbx21* may play a pivotal role, regulation of other genes at earlier time points may also contribute to protection against diabetes due to epigenetic changes of the genome. Further work is necessary to address this issue.

Treatment with TSA induced prominent hypercetylation of H3 histone in splenocytes shortly after treatment (1-2 weeks), as observed in other models[11, 13]. In addition to the spleen, histone acetylation was also found in the target organ pancreas, which may lead to altered gene expression. Epigenetic changes of the pancreas are likely to complement the epigenetic changes in T lymphocytes to bring about protection against diabetes. When protection was fully stabilized (32-36 weeks of age), H3 acetylation detected by western blotting was undetectable, indicating the anticipated reversible nature of histone acetylation[1-3]. This indicates that the disease intervention is largely dependent on events that occur during the treatment period (18-24 weeks of age). Although this period corresponds to check point 2, thought to include a second wave of immune response culminating in full-blown diabetes[15], some mice proceeded to become diabetic even after continuous drug treatment. Whereas weekly administration of TSA (500 µg/kg body weight) failed to reverse the end stage disease in a large number of mice, treatment with a comparable dose (8 µg per ~20 g mouse equals to 400 µg/Kg body weight) of FR901228 twice a week for 8 weeks was shown to reverse overt diabetes in a very small number (n=3) of NOD mice[7]. Whereas the cyclic tetrapeptide FR901228 inhibits only the class I HDACs, the hydroxamic acid TSA is the most potent inhibitor of class I, II and IV HDACs[2]. Although the differences in the selectivity of the HDAC inhibitors cannot explain the discrepancy between these results, it is possible that more frequent injections of FR901228 may curtail the ongoing inflammatory response better than weekly injections with TSA. Nevertheless, it is important to recognize the major obstacles in reversing the end stage diabetes. By the time diabetes is diagnosed, >80% of islets are already destroyed and the remaining β-cells are under severe attack by inflammatory

responses. Any intervention strategy should not only curtail the ongoing autoimmune attack on β-cells effectively but also facilitate the replenishment of islets to restore normoglycemia rapidly. Our data are congruent with the necessity of intervention preferably during the transition from prediabetic to diabetic stage, 18-24 weeks of age, in most female NOD mice. This time frame is adequate to quell the damage caused by autoimmune attack and allow the replenishment of islets. However, the transition from prediabetes to overt diabetes may not occur synchronously as evidenced by the occurrence of overt diabetes in a small fraction of mice that were <18 weeks old. Heterogeneity in response to HDAC inhibitor treatment may explain why some mice succumb to diabetes even when the drug treatment began at 18 weeks of age and continued for another 6 weeks. It is possible that earlier therapeutic intervention (<18 weeks of age) may provide benefit to those mice that become overtly diabetic much earlier than most NOD mice. Nevertheless, the observation that TSA can prevent the transition from insulitis to overt diabetes in a majority of mice has clinical relevance since most intervention therapies reported so far are effective at the prediabetic period but not at an advanced stage of the disease[14].

In addition to histones, HDAC inhibitors can also facilitate acetylation of more than 50 transcription factors [2-3]. Treatment of mice with higher concentrations of TSA than used in our studies resulted in FoxP3 acetylation and upregulation of $CD4^+FoxP3^+$ T regulatory cell number and function[9-10]. In contrast, only the frequency of $CD4^+CD62L^+$ but not $CD4^+FoxP3^+$ T regulatory cells was increased when analyzed 8 to 12 weeks after the last injection of TSA in contrast to typically 1-2 weeks after drug treatment in other reports. Although we analyzed the frequency of the T regulatory cells in the spleen as

most previous studies, it will be more informative to examine the changes in the numbers and function of the T regulatory cells present in the pancreatic lymph nodes and in the target organ pancreas of TSA treated mice. Since the $CD4^+CD62L^+$ but not $CD4^+CD25^+$ T regulatory cells had been shown to protect mice from autoimmune diabetes[16], our data indicating a specific increase in $CD4^+CD62L^+$ cells by epigenetic modulation may have implications for the treatment of diabetes. Since most of the islets in cured mice appeared smaller and juxtaposed to pancreatic ducts, it is possible that TSA treatment can facilitate the neogeneration of islets *in vivo* as observed in fetal islets *in vitro*[49]. Thus, treatment with HDAC inhibitors may provide protection against spontaneous autoimmune diabetes by multiple mechanisms. A thorough understanding of these mechanisms is a prerequisite for the application of this novel approach to treat patients with type 1 diabetes. Although genetic and environmental factors have been implicated in the manifestation of autoimmune diabetes, our data indicate that epigenetics is involved in fine-tuning of the immune system to deviate the autoimmune process.

METHODS

Mice

Female NOD/Ltj (H-2^{g7}) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). The University of Illinois at Chicago approved the animal protocol. Experiments were conducted according to the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

Treatment of mice and diabetes monitoring

A stock solution of TSA (Biomol International, Plymouth Meeting, PA) was prepared at 1 mg/ml concentration in DMSO and stored at -20^{o} C. TSA or equivalent amount of DMSO was diluted in phosphate buffered saline and injected s.c. into female NOD/Ltj mice at weekly intervals. Tail vein blood was tested weekly for glucose levels using a glucometer and >250 mg/dL of glucose were considered diabetic[29]. For glucose tolerance test, food was withdrawn for 4 h before an i.p. injection of dextrose (2 g/kg body weight) and blood glucose levels were tested at different time intervals as described[29]. Insulin levels in the sera were measured using an Ultra Sensitive Mouse Insulin ELISA kit obtained from Crystal Chem (Downers Grove, IL, range: 0.1-12.8 ng/ml).

Flow cytometry

Splenocytes were stained with FITC-coupled anti-CD4, PE-conjugated anti-CD8, or a combination of PE-conjugated anti-CD4 and FITC-labeled anti-CD25 (eBioscience, San Diego, CA) and analyzed by flow cytometry as described[29]. Intracellular FoxP3 staining was performed using a kit from eBioscience. Briefly, splenocytes were stained

with CD4-FITC, permeabilized and stained with PE-conjugated anti-FoxP3 antibody. Values were subtracted from those stained with isotype matched PE-conjugated antibody.

Assessment of gene expression by real-time RT-PCR

Splenocytes (5 x 10^6/ml) from individual mice were stimulated with 100 ng of phorbol 12-myristate 13-acetate (PMA) and 1 μg of ionomycin (Sigma-Aldrich, St. Louis, MO) or cultured in tissue culture plates previously coated with 5 μg/ml of anti-CD3 antibody (2C11, eBioscience, San Diego, CA) in complete RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with antibiotics and 10% FBS[29, 50]. After overnight culture, splenocytes from 3 to 5 mice from the same experimental group were pooled and lysed in TRIzol (Invitrogen). Total RNA was isolated, treated with DNase and reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosytems, Carlsbad, CA). Real-time quantitative RT-PCR was performed on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems) using 1 μl of cDNA equivalent to 100 ng of RNA and 2X SYBR Premix Ex Taq (Perfect Real Time) reagent (Takara-Clontech, Mountain View, CA). Primer sets for mouse *Gapdh, Il4*, *Il17*, *Il18*, *Ifng*, *Tnfa*, *Inos*, *Tbet/Tbx21*, *Gata3*, and *Rorgt* were designed as described[29] and synthesized at Integrated DNA Technologies (Coralville, IA). Sizes of amplicons and the absence of primer-dimer were verified by electrophoresis of PCR products as described[29]. Each sample was analyzed in triplicate. Since normalized C_T values are not recommended for statistical evaluation[30] , the level of expression of any given gene in each sample was determined using the endogenous reference gene *Gapdh* as the normalizer using the $2^{-\Delta\Delta C}{}_T$ method[29-30] . Differences in the expression of genes

between unstimulated and stimulated cultures were calculated as the fold change in gene expression normalized to *Gapdh* and relative to the untreated control, using the $2^{-\Delta\Delta C_T}$ method[29-30]. Statistical significance was calculated only between the groups that were analyzed simultaneously.

ELISA

Culture supernatant collected after overnight incubation of splenocytes was assessed for cytokine levels using commercially available ELISA kits (eBioscience).

Immunoblotting

Acidic nuclear extracts were prepared from spleens and pancreata of untreated and TSA-treated mice as described[6]. As positive controls, extracts were also prepared from splenocytes (5×10^6 cells) and small pieces (1-2 cm) of pancreata obtained from prediabetic (12 weeks old) female NOD mice after overnight activation with 100 ng of TSA/ml of media. Proteins (10µg) were separated on a SDS-PAGE gel, transferred onto a polyvinylidene fluoride membrane and probed with rabbit polyclonal pan specific anti-histone H3 and anti-acetyl histone H3 antibodies separately (Upstate Biotech, Billerica, MA). After incubation with horseradish peroxidase conjugated anti-rabbit antibody, proteins were visualized using ECL plus (GE Healthcare, Piscataway, NJ). The integrated density of bands were measured using the ImageJ 1.34s software. The integrated density of acetylated H3 was divided by that of total H3 to obtain the fold change in acetylation of H3 in individual samples.

Histology and confocal microscopy

Formalin fixed pancreatic sections were stained with H & E and at least 3 sections per pancreas were analyzed by light microscopy and a score was assigned as follows[29]: 1, No to mild peri-insulitis, 2, Moderate infiltration (<50%) and 3, Severe (>50%) and destructive infiltration. Sections were stained with 1:100 diluted guinea pig anti-insulin antiserum (Zymed Laboratories, South San Francisco, CA) followed by 1:1000 diluted tetramethyl rhodamine iso-thiocyanate labeled rabbit-anti-guinea pig Ig (Sigma-Aldrich). Nuclei were counterstained with Hoechst and confocal images were acquired on a Zeiss LSM510 laser-scanning microscope and processed using the Zeiss LSM Image browser (4.0 version, Zeiss, Oberkochen, Germany).

Statistics

Statistical analysis was performed using an unpaired two-tailed Student's *t* test (GraphPad Prism, San Diego, CA).

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FIGURE LEGENDS

Figure 1. Amelioration of diabetes by TSA treatment.

(a). Female NOD mice were injected s.c. with TSA or DMSO and blood glucose levels monitored weekly. Mice were considered diabetic when blood glucose levels reached >250 mg/dL. Numbers of mice tested per group are shown in parentheses. The statistical significance between untreated and those injected with TSA between 18-24 weeks (wk) is indicated. (**b)**. Mice treated with TSA between 18-24 weeks were further analyzed. Mice that were non-diabetic at the beginning of drug treatment were followed for diabetes incidence. Blood glucose levels of individual mice that became diabetic during the course of treatment and those remained non-diabetic are shown. (**c).** Blood glucose levels of overtly diabetic mice at the initiation of TSA treatment (18 weeks) are shown. All of these mice received weekly injections of TSA till 24 weeks of age unless mice died of overt diabetes.

Figure 2. Normoglycemia in protected mice.

(a). Mice were left untreated or treated with TSA or DMSO between 18-24 weeks of age and challenged with glucose when they reached 32 to 36 weeks of age. Blood glucose levels in different groups of mice are shown (mean +/- SD). Numbers of mice are shown in parentheses (**b).** Insulin levels (mean +/-SD) in TSA-treated-non-diabetic and TSA-treated-diabetic mice challenged with high levels of glucose are shown.

Figure 3. Transient H3 histone acetylation by TSA treatment.

(a). Mice were killed 2 weeks after the last injection of TSA given between 18 and 24 weeks of age. Representative data from 3 independent experiments are shown. In each

experiment, spleens and pancreata were harvested and pooled from 3-5 mice per treatment group and acidic nuclear extracts derived from them were immunoblotted with antibodies against acetylated and native H3 histone. Splenocytes and pancreata were also obtained from 12 weeks old 3 prediabetic mice, pooled and treated overnight with TSA *in vitro*. (**b).** Acetylation of H3 histone was analyzed in tissues obtained from 36 weeks old mice that received the last injection of TSA 12 weeks earlier. Splenocytes and pancreata were obtained from 3-5 mice per group in each experiment and the data were shown from a representative of two different experiments. (**c**) The intensity of the individual bands shown in (**a**) was determined using the ImageJ software. Fold change in H3 acetylation was calculated by dividing the intensity of acetylated H3 by total H3 levels in each sample. Shown is the mean +/- standard deviation. The levels of significance between groups are also indicated. (**d**). The fold change in H3 histone acetylation in samples shown in (**b**) is indicated and the differences between groups were not significant.

Figure 4. Increase in a subset of T regulatory cells by TSA treatment.

(a). The frequency of $CD4^+$ and $CD8^+$ cells in splenocytes of diabetic mice (n=17) and those treated with TSA between 18 and 24 weeks of age and remained diabetes free (n=17) is shown. Mice were killed between 32-36 weeks of age and splenocytes were analyzed by flow cytometry. Shown is mean +/- SD. (**b).** Splenocytes from untreated-diabetic (n=12) and TSA-treated, non-diabetic mice (n=7) were also analyzed between 32 and 36 wks of age by flow cytometry. The frequency of indicated subsets in splenocytes is shown as mean +/- SD. The statistical significance between groups is shown.

Figure 5. Reduced insulitis in pancreata of TSA treated mice.

Histological sections of pancreata obtained from diabetic (**a**) and TSA-treated-non-diabetic mice (**b**) were stained with H & E (upper panels, x200 magnification). Lower panels represent confocal images of pancreatic sections stained with an antibody to insulin (red) and Hoechst to visualize nuclei (blue). Bar size = 20 μM. (**c).** Pancreatic sections were scored for cellular infiltration as follows: 1, no or little infiltration; 2. peri-insulitis; and 3. heavy and invasive cellular infiltration. A total of 45-48 islets were scored from 8 to 15 mice per group.

Figure 6. Steady state levels of gene expression.

Splenocytes from diabetic and non-diabetic mice that were untreated (**a**) or treated with TSA (**b**) were analyzed for the steady state levels of gene expression by real-time RT-PCR. Relative mRNA abundance was analyzed simultaneously in diabetic and non-diabetic mice from untreated mice and those treated with TSA. The expression level of the gene of interest was normalized against *Gapdh* individually and determined by using the $2^{-\Delta\Delta C}{}_{T}$ method[30]. Data shown are mean +/- SD of triplicate determinations from samples pooled from 3 to 5 mice per group. The experiment was repeated 3 to 5 times with separate pools of samples with similar results. The differences in gene expression between diabetic and non-diabetic mice were not statistically different.

Figure 7. Modulation of gene expression in splenocytes of TSA-treated mice.

Spleens were harvested from 28 to 36 weeks old mice that were treated with TSA during 18-24 weeks of age. Splenocytes were cultured in media alone or with PMA + ionomycin (**a**). Cells were also incubated in plates coated with anti-CD3 antibody (**b**). Spleen cells

from DMSO-treated mice were incubated with PMA + ionomycin (**c**). Expression of each gene was calculated in cells cultured in media (control) alone and after specific stimulation. The fold change in specific mRNA expression was calculated by comparing the relative gene expression normalized to *Gapdh* in control and stimulated samples using the $2^{-\Delta\Delta C}{}_{T}$ method[30]. Data shown are mean +/- SD of triplicate determinations from samples pooled from 3 to 5 mice per group and the experiment was repeated 3 to 5 times with similar results. Only the *Ifng* mRNA expression between diabetic and non-diabetic mice regardless of treatment was statistically significant.

Figure 8. Upregulation of IFN-γ secretion by TSA treatment.

Splenocytes from diabetic mice and TSA-treated-non-diabetic mice were stimulated with PMA + ionomycin (**a**) or immobilized anti-CD3 antibody (**b**). Controls were cultured in media alone. After overnight culture, supernatant was collected and assayed for the levels of indicated cytokines by ELISA. Data shown are mean +/- SD of duplicate samples pooled from 2-3 experiments. Each experiment contained spleens from 3-5 mice per group. Data shown are those obtained in stimulated cultures subtracted from media controls. Only the amounts of IFN-γ between control and TSA-treated mice were statistically different.

Figure 9. Upregulation of *Tbx21* but not *Gata3* or *Rorgt* in non-diabetic mice regardless of treatment.

Diabetic and non-diabetic mice from untreated, DMSO-treated and TSA-treated mice were killed at 22 to 36 weeks of age. Splenocytes were stimulated either with PMA + ionomycin (**a**) or immobilized anti-CD3 antibody (**b**). The increase in the expression of *Tbx21* gene in activated and unstimulated cells from each group was normalized to the

endogenous reference *Gapdh* and determined using the $2^{-\Delta\Delta C}{}_{T}$ method[30]. The bars indicate the increase in the expression of *Tbx21* in stimulated cells compared to those incubated with media alone. The statistical differences between diabetic and non-diabetic mice are indicated. Inducible expression of *Gata3* and *Rorgt* was also analyzed in splenocytes of TSA-treated-diabetic and drug treated-non-diabetic mice after activation with PMA + ionnomycin (**c**). The differences in the expression of genes between the groups were not significant. Representative data from 3 experiments, each with 3 to 5 mice are shown. Each bar represents mean +/- SD of triplicate determinations per sample.

Fig. 1

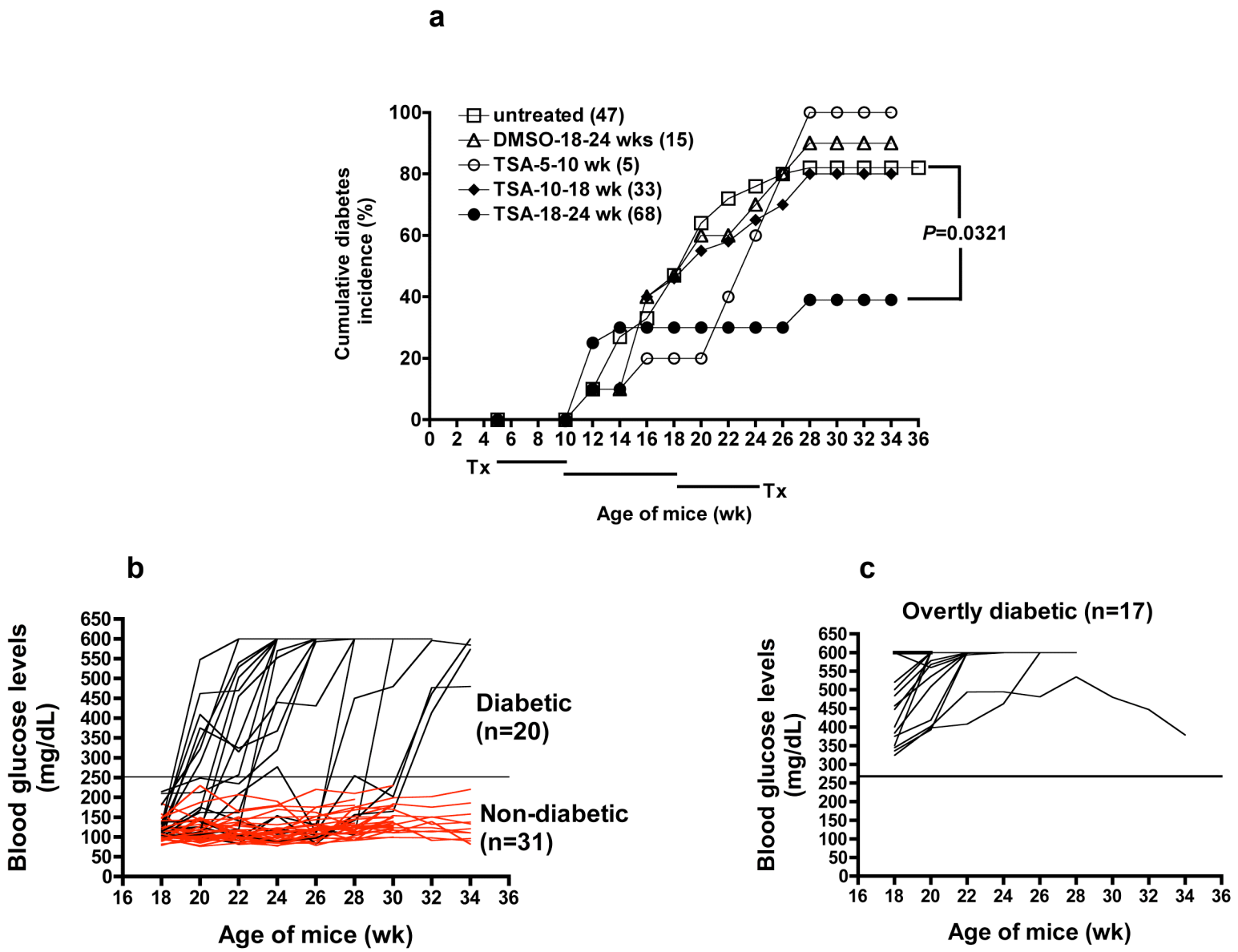
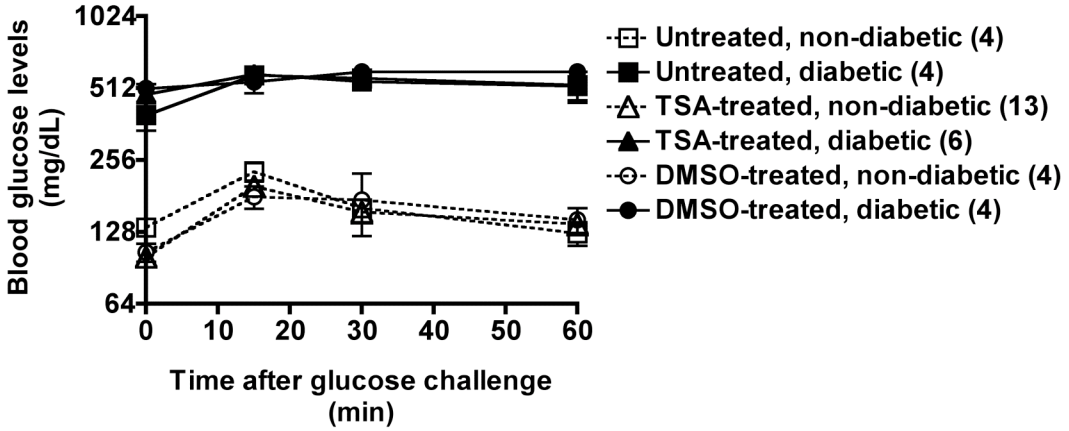

a
untreated (47)
DMSO-18-24 wks (15)
TSA-5-10 wk (5)
TSA-10-18 wk (33)
TSA-18-24 wk (68)
Cumulative diabetes incidence (%)
P=0.0321
Tx
Tx
Age of mice (wk)
b
Blood glucose levels (mg/dL)
Diabetic (n=20)
Non-diabetic (n=31)
Age of mice (wk)
c
Overtly diabetic (n=17)
Blood glucose levels (mg/dL)
Age of mice (wk)

Fig. 2

a


Blood glucose levels (mg/dL)
1024
512
256
128
64
0
10
20
30
40
50
60
Time after glucose challenge (min)
Untreated, non-diabetic (4)
Untreated, diabetic (4)
TSA-treated, non-diabetic (13)
TSA-treated, diabetic (6)
DMSO-treated, non-diabetic (4)
DMSO-treated, diabetic (4)

b

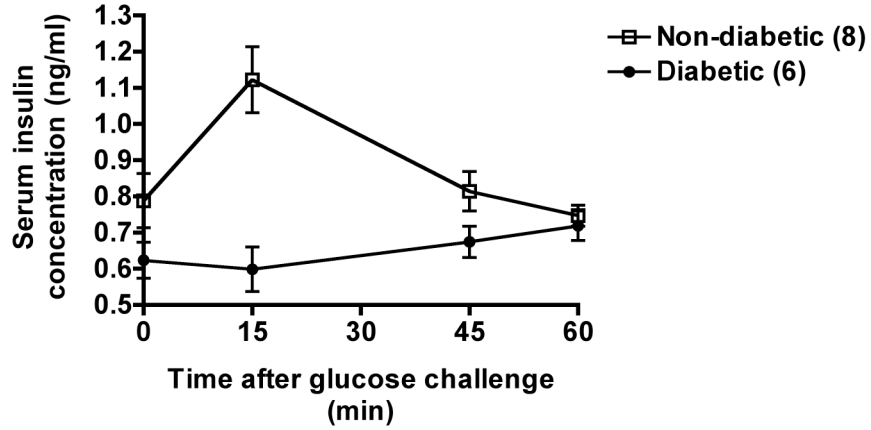

Serum insulin concentration (ng/ml)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0
15
30
45
60
Time after glucose challenge (min)
Non-diabetic (8)
Diabetic (6)

Fig. 3

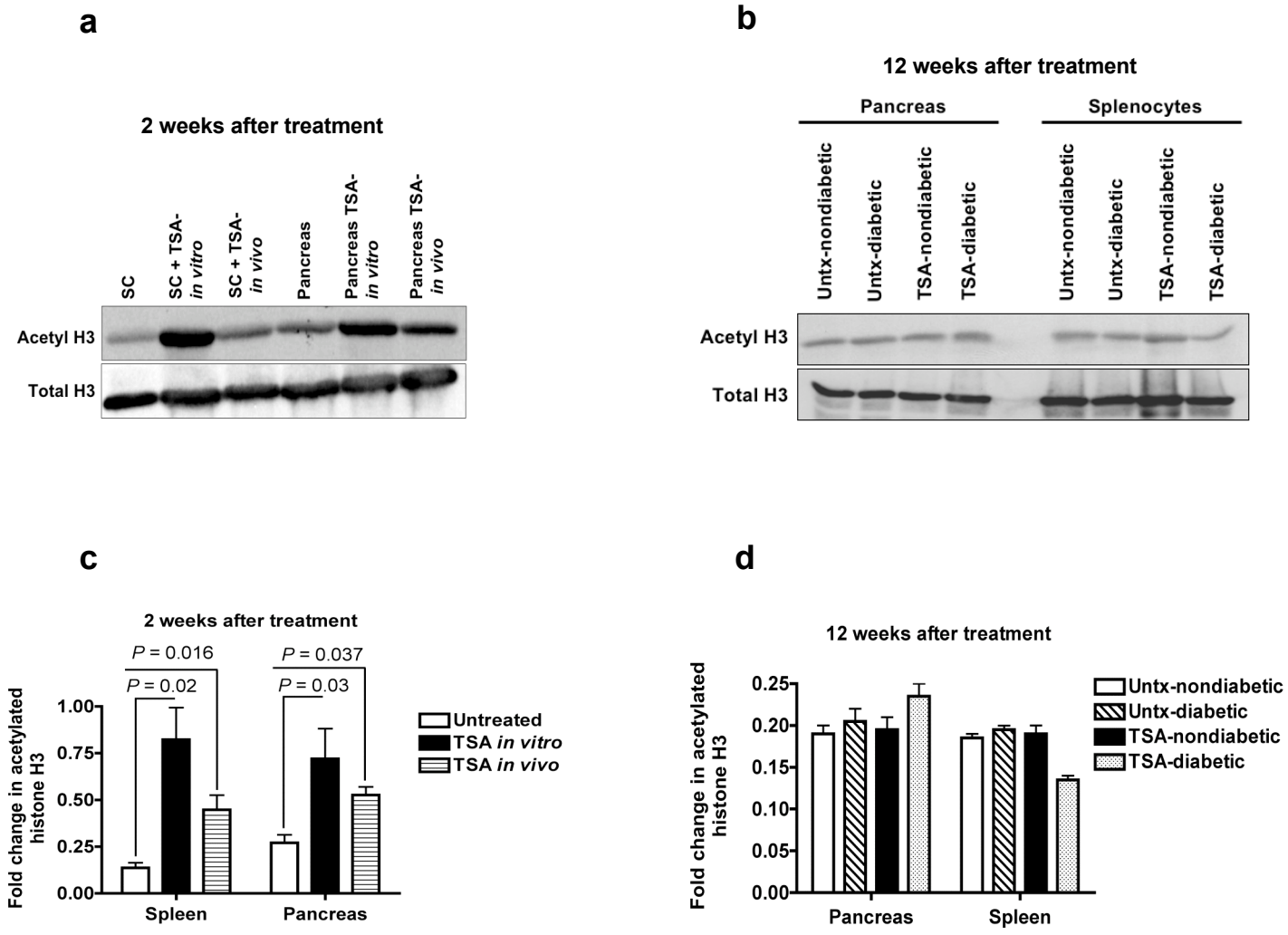

a
2 weeks after treatment
SC
SC + TSA- in vitro
SC + TSA- in vivo
Pancreas
Pancreas TSA- in vitro
Pancreas TSA- in vivo
Acetyl H3
Total H3
b
12 weeks after treatment
Pancreas
Splenocytes
Untx-nondiabetic
Untx-diabetic
TSA-nondiabetic
TSA-diabetic
Acetyl H3
Total H3
c
2 weeks after treatment
P = 0.016
P = 0.02
P = 0.037
P = 0.03
Fold change in acetylated histone H3
1.00
0.75
0.50
0.25
0.00
Spleen
Pancreas
Untreated
TSA in vitro
TSA in vivo
d
12 weeks after treatment
Fold change in acetylated histone H3
0.25
0.20
0.15
0.10
0.05
0.00
Pancreas
Spleen
Untx-nondiabetic
Untx-diabetic
TSA-nondiabetic
TSA-diabetic

Fig. 4

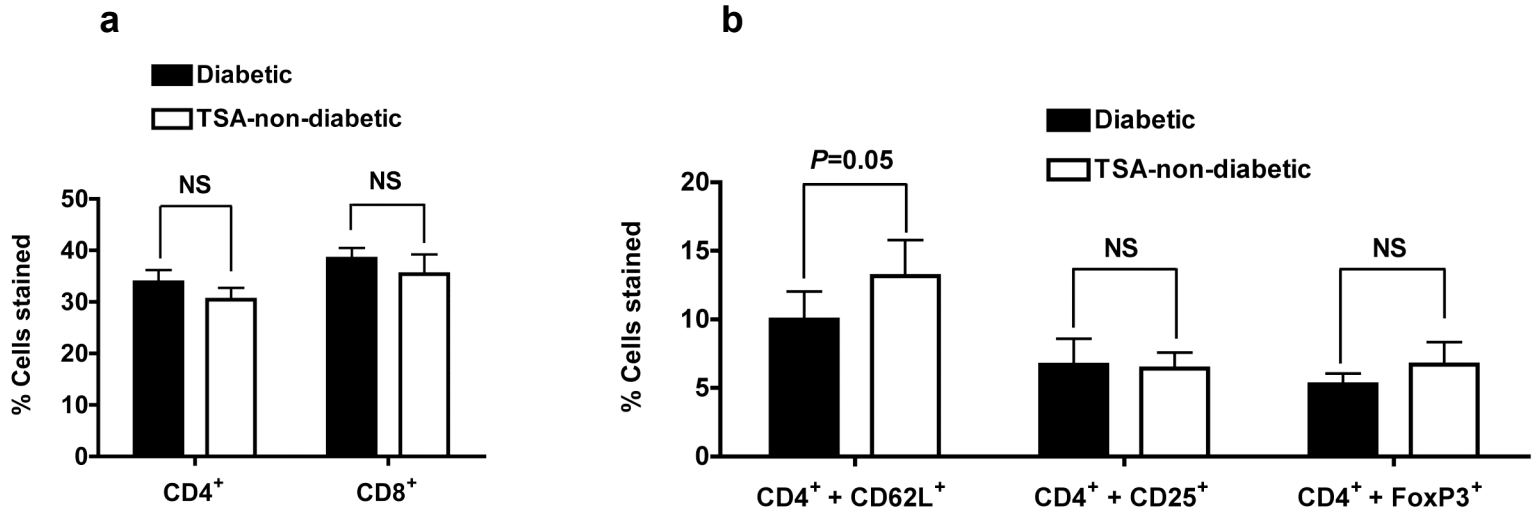

a
Diabetic
TSA-non-diabetic
NS
NS
% Cells stained
50
40
30
20
10
0
CD4+
CD8+
b
Diabetic
TSA-non-diabetic
P=0.05
NS
NS
% Cells stained
20
15
10
5
0
CD4+ + CD62L+
CD4+ + CD25+
CD4+ + FoxP3+

Fig. 5

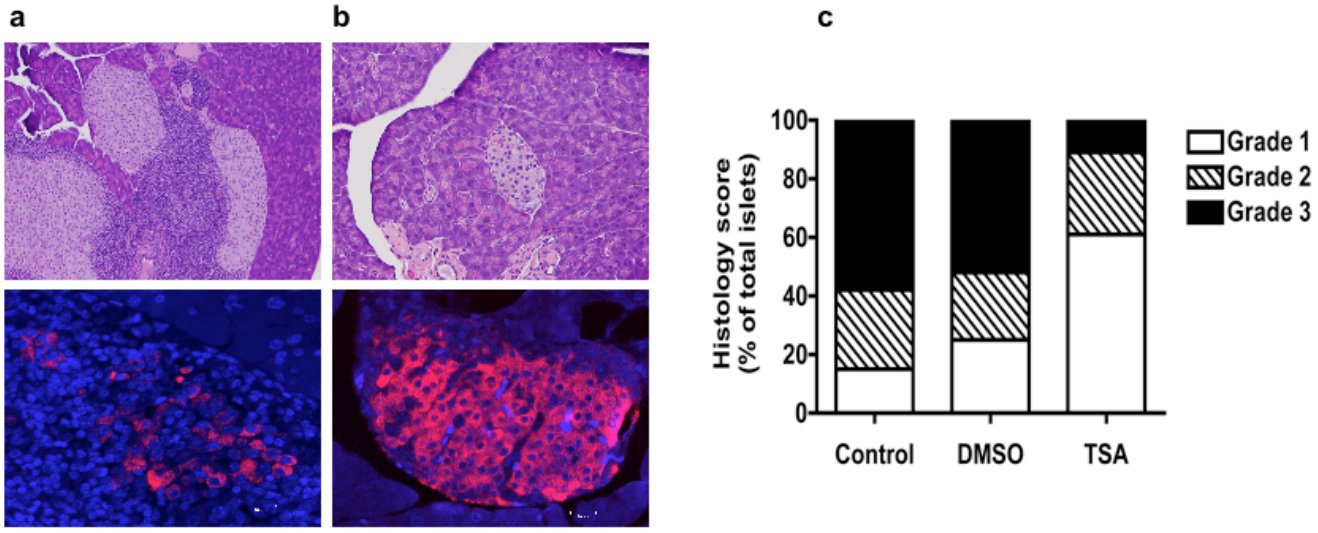

a
b
c
Histology score
(% of total islets)
100
80
60
40
20
0
Control
DMSO
TSA
Grade 1
Grade 2
Grade 3

Fig. 6

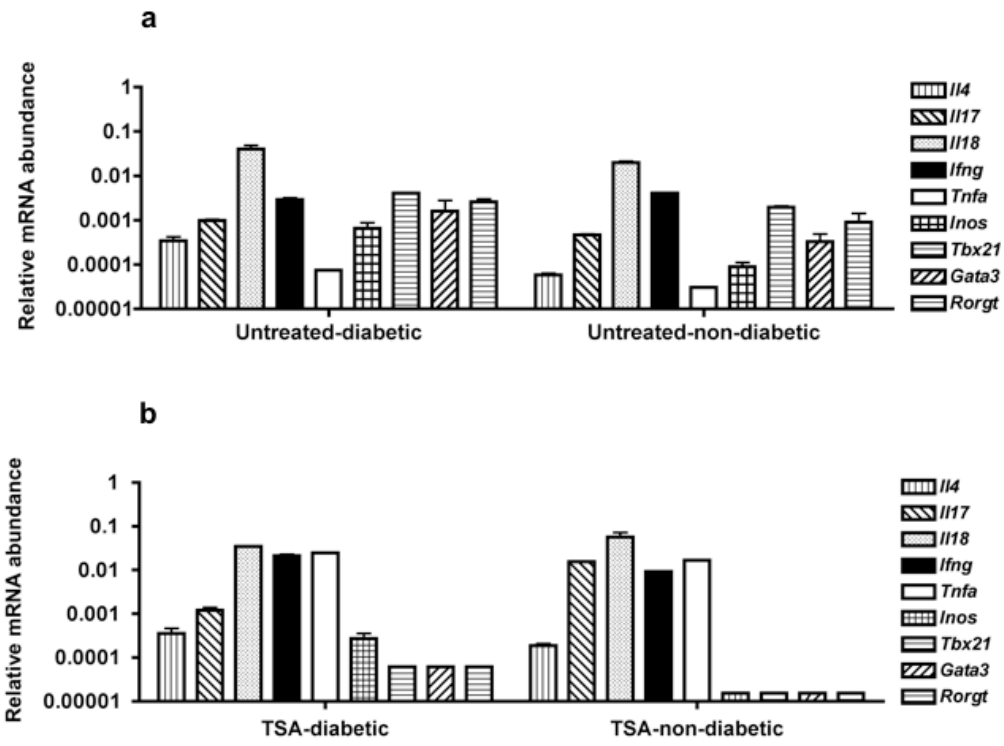

a
Relative mRNA abundance
1
0.1
0.01
0.001
0.0001
0.00001
Untreated-diabetic
Untreated-non-diabetic
Il4
Il17
Il18
Ifng
Tnfa
Inos
Tbx21
Gata3
Rorgt
b
Relative mRNA abundance
1
0.1
0.01
0.001
0.0001
0.00001
TSA-diabetic
TSA-non-diabetic
Il4
Il17
Il18
Ifng
Tnfa
Inos
Tbx21
Gata3
Rorgt

Fig. 7

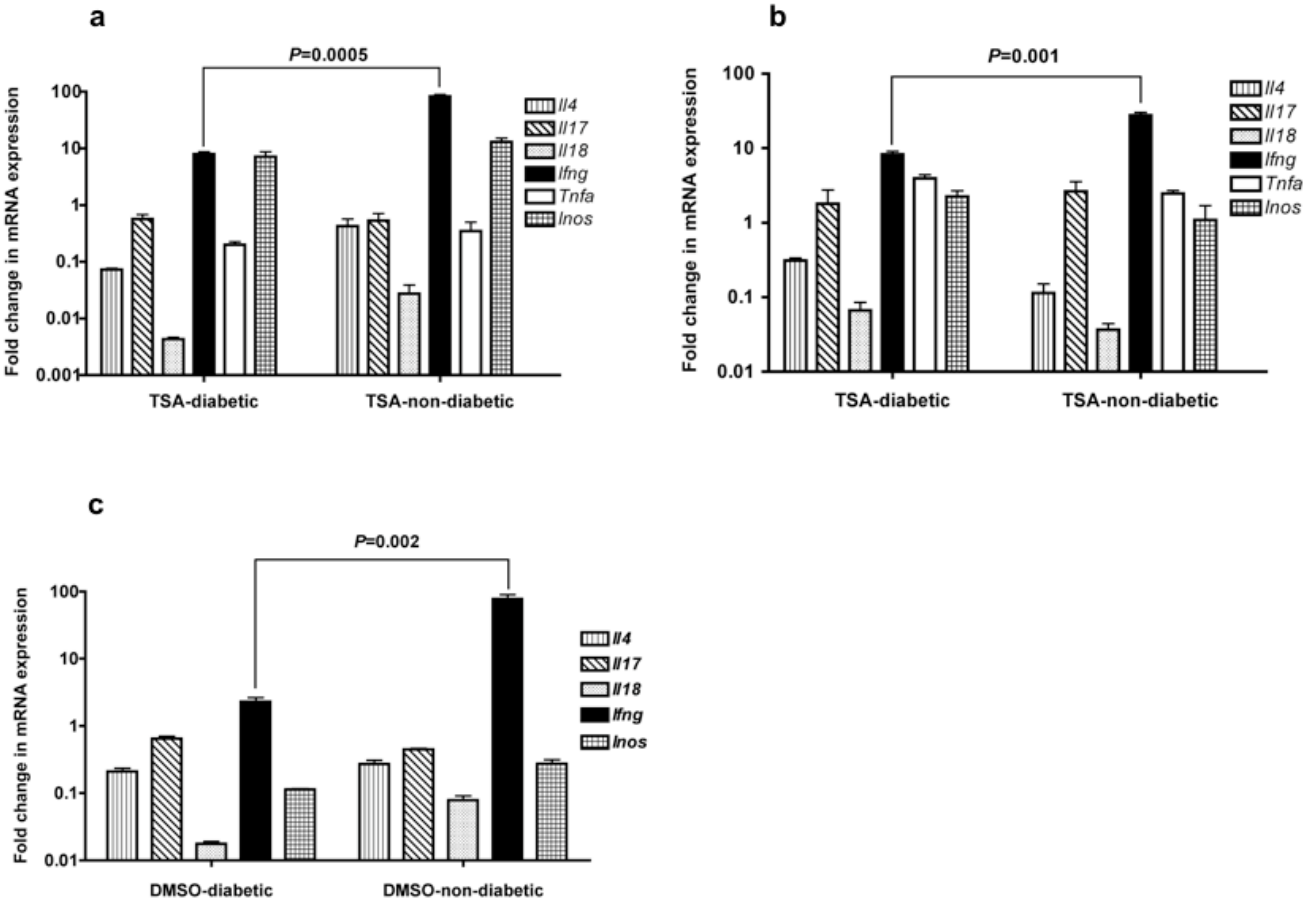

a
P=0.0005
Fold change in mRNA expression
100
10
1
0.1
0.01
0.001
Il4
Il17
Il18
Ifng
Tnfa
Inos
TSA-diabetic
TSA-non-diabetic
b
P=0.001
Fold change in mRNA expression
100
10
1
0.1
0.01
Il4
Il17
Il18
Ifng
Tnfa
Inos
TSA-diabetic
TSA-non-diabetic
c
P=0.002
Fold change in mRNA expression
100
10
1
0.1
0.01
Il4
Il17
Il18
Ifng
Inos
DMSO-diabetic
DMSO-non-diabetic

Fig. 8

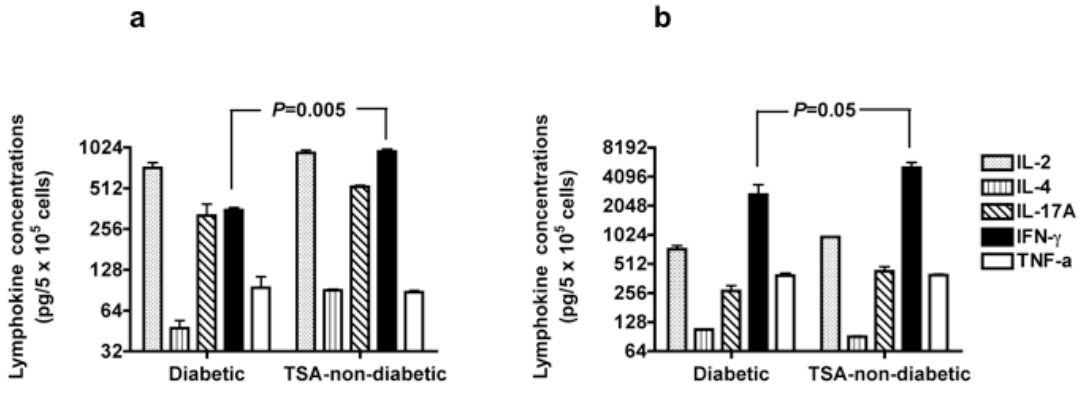
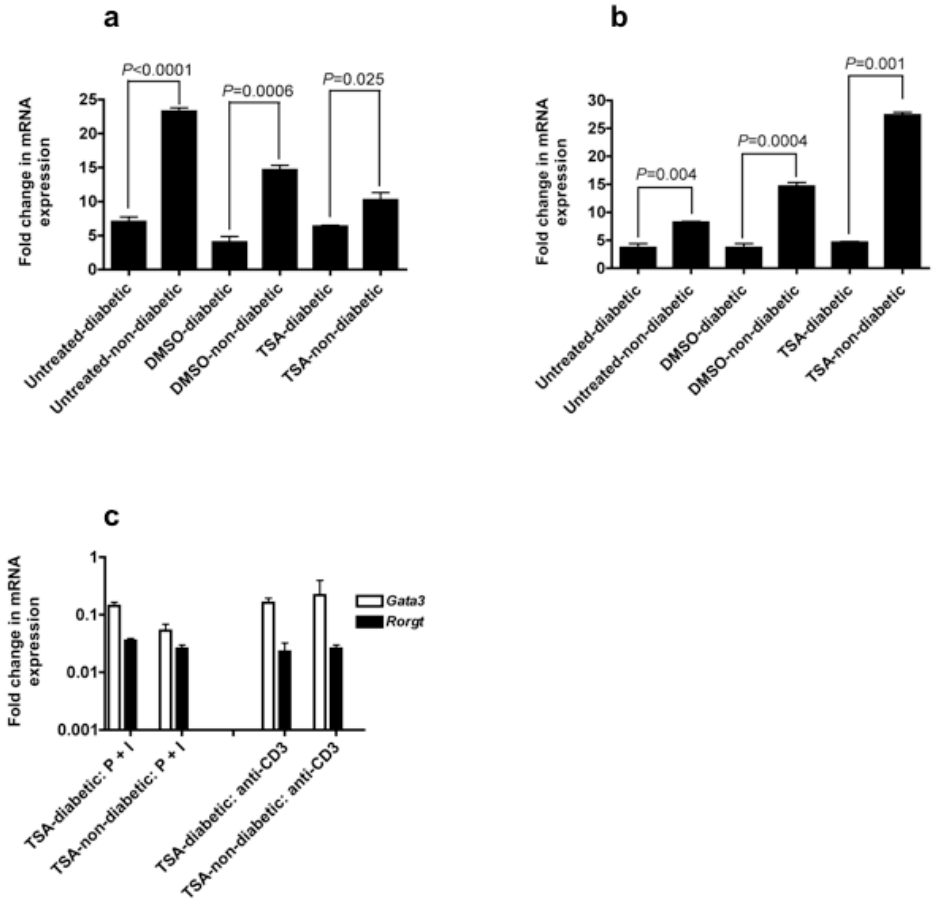

a
b
P=0.005
P=0.05
Lymphokine concentrations (pg/5 x 10^5 cells)
1024
512
256
128
64
32
8192
4096
2048
1024
512
256
128
64
Diabetic
TSA-non-diabetic
IL-2
IL-4
IL-17A
IFN-γ
TNF-a

Fig. 9


a
P<0.0001
P=0.0006
P=0.025
Fold change in mRNA expression
25
20
15
10
5
0
Untreated-diabetic
Untreated-non-diabetic
DMSO-diabetic
DMSO-non-diabetic
TSA-diabetic
TSA-non-diabetic
b
P=0.004
P=0.0004
P=0.001
Fold change in mRNA expression
30
25
20
15
10
5
0
Untreated-diabetic
Untreated-non-diabetic
DMSO-diabetic
DMSO-non-diabetic
TSA-diabetic
TSA-non-diabetic
c
Fold change in mRNA expression
1
0.1
0.01
0.001
Gata3
Rorgt
TSA-diabetic: P + I
TSA-non-diabetic: P + I
TSA-diabetic: anti-CD3
TSA-non-diabetic: anti-CD3