Epigenetic modulation of selected immune response genes and altered functions of T lymphocytes and macrophages collectively contribute to autoimmune diabetes protection

Sundararajan Jayaraman[1,2*], Maria Arianas[1], Arathi Jayaraman[1],

[1]Dept. of Surgery, the University of Illinois at Chicago, Chicago, IL 60612

[2]Current address: Dept. of Surgery, University of Illinois College of Medicine at Peoria, IL 61603

*Correspondence: anue2468@uic.edu

SUMMARY

We have previously demonstrated that treatment of female NOD mice with the histone deacetylase inhibitor Trichostatin A (TSA) bestowed irreversible protection against diabetes. Herein we show that drug treatment diminished the infiltration of the pancreas with $CD4^+$ and $CD8^+$ T cells and Ly-$6C^+$ monocytes. Significantly, TSA administration selectively repressed the expression of a set of genes exaggerated during diabetes and constitutively expressed primarily in the spleen and rarely in the pancreas. These genes encode lymphokines, macrophage-associated determinants, and transcription factors. Although the copy numbers of many histone deacetylases increased during diabetes in the spleen and pancreas, only those upregulated in the spleen were rendered sensitive to repression by TSA treatment. The T lymphocytes derived from drug-treated donors displayed diminished diabetogenic potential following transfer into immunodeficient NOD.*scid* mice. In the immunocompromised recipients, diabetes caused by the transfer of activated T lymphocytes from untreated diabetic mice was hampered by the co-transfer of highly purified splenic Ly-$6C^+$ macrophages from drug-treated mice. However, the transfer of Ly-$6C^+$ macrophages from drug-treated mice failed to block ongoing diabetes in wild-type NOD mice. These data demonstrate that the modified gene expression and functional alteration of T lymphocytes and macrophages collectively contribute to diabetes protection afforded by the histone modifier in female NOD mice.

Keywords: Epigenetics, Genes, Histone deacetylases, Lymphocytes, Macrophages, Trichostatin A, Type 1 Diabetes.

INTRODUCTION

Type 1 diabetes (T1D), a T cell-mediated autoimmune disease, occurs at an alarming rate in genetically susceptible individuals [1-3]. Although genome-wide association studies implicated more than 40 different genetic loci, the causal genes of T1D remain unknown [4]. Lack of familial history and robust concordance among identical twins in developing T1D implied the involvement of environmental factors. Epigenetics provides a mechanism by which external factors can produce a variety of phenotypic variations with identical genotypes. Epigenetic mechanisms include DNA methylation, post-translational modifications of histones, and microRNA-mediated gene regulation [5]. Methylation of cytosine residues in gene promoters results in transcriptional silencing of some [6] but not all genes [7]. Reversible acetylation of the e-amino group of lysine in the histone tails by histone acetyltransferases and deacetylation by histone deacetylases (HDACs) are the best-characterized post-translational modifications of histones [8]. Transcriptional permissiveness correlates with histone acetylation in contrast to deacetylation resulting in gene repression. Non-selective, small molecule HDAC inhibitors can induce chromatin modifications and thereby affect gene expression. Trichostatin A (TSA) derived from *Streptomyces platensis* is the most potent inhibitor of class I, class IIa, class IIb, and class IV HDACs, which increases histone acetylation resulting in up- and down-regulation of genes *in vitro* [9] and *in vivo* [10-16].

Previously, we have demonstrated the utility of TSA to mitigate autoimmune diseases such as T1D [10-13] and experimental autoimmune encephalomyelitis [14-16]. The protection afforded by the histone modifier was accompanied by reduced inflammation of the pancreatic islets and regeneration of insulin-producing β-cells [10]. Significantly, treatment with TSA abrogated the ability of splenocytes to adoptively transfer T1D into immunodeficient NOD.*scid*

mice, indicating the effect of the epigenetic drug on diabetogenic T lymphocytes [11]. Transcriptome analysis of *uninduced* splenocytes unraveled the exaggerated expression of inflammatory genes, including the macrophage selective *Cela3b* (elastase 3) in diabetic mice repressed by TSA. These results are consistent with the possibility that chromatin remodeling by TSA can retard the induction and progression of T1D by altering the transcriptional programs of multiple cell types. In the current investigation, we analyzed the effects of the epigenetic drug specifically on T lymphocytes and macrophages, respectively implicated in induction and manifestation of T1D [17-19], by quantitative reverse transcriptase-mediated polymerase chain reaction (qRT-PCR) and an adoptive transfer model. Our study unraveled that a select set of both exaggerated and constitutively expressed genes encoding cytokines, cell surface determinants, transcription factors, and HDACs was modulated by the histone modifier more predominantly in the spleen. Our data also demonstrate the distinct effects of TSA on the function of T-cells and macrophages. These data lend novel insights into the mechanisms of T1D and its possible manipulation by epigenetic reprogramming.

MATERIALS AND METHODS

Diabetes treatment

The Office of Animal Care and Institutional Biosafety of the University of Illinois at Chicago approved the animal protocol. Experiments were conducted following the NIH guide for the care and use of laboratory animals.

Six to eight weeks old female NOD/ShiLtj (H-2^{g7}) mice purchased from The Jackson Laboratory (Bar Harbor, ME) were maintained under specific pathogen-free conditions and provided with animal chow and water *ad libitum*. Mice were injected s.c. with 500 µg/Kg body

weight of TSA or DMSO at weekly intervals between 16 and 24 wks of age [10-11]. Non-fasting blood glucose level exceeding 250 mg/dL on two consecutive weekly determinations was considered as diabetic.

Confocal microscopy

The following antibodies were used for staining pancreatic sections: guinea pig anti-insulin Ab (Zymed Laboratories, South San Francisco, CA), tetramethylrhodamine iso-thiocyanate–rabbit antisera raised against guinea pig Ig (Sigma Aldrich, St. Louis, MO), anti-Ly-6C-FITC (clone HK1.4, eBiosciences, San Diego, CA), anti-Ly-6G-FITC (clone 1AB, BD Pharmingen, San Diego, CA), rat monoclonal anti-CD4 (clone RM4-5, eBiosciences-ThermoFisher Scientific, Waltham, MA), DyLight™ 488 goat anti-rat IgG (catalog # 405409, BioLegend, San Diego, CA) and anti-CD8-FITC (clone 53-6.7, eBiosciences). Confocal images were acquired using a Zeiss LSM510 laser scanning microscope and processed using the Zeiss LSM Image browser (4.0 version; Zeiss, Oberkochen, Germany) and Adobe Photoshop Elements version 9.0, as described [20].

Gene expression analysis

Spleens were harvested from 20-24-wks old diabetic mice and 26-28 wks old mice treated with TSA from 16 to 24-wks of age. Total RNA was extracted from the spleen and pancreas of five *individual mice* per group/experiment, treated with Turbo DNase, and converted to cDNA using High-capacity cDNA Reverse Transcription kit (Applied Biosystems), as described [10-11, 14-16, 20]. Real-time qRT-PCR was performed in *triplicate* on Applied Biosystems ViiA7 Real-time PCR system using 1 µl of cDNA equivalent to 100 ng of total RNA and 2X SYBR Green master mix. The primers used in this study were designed and validated

previously [10-11, 14-16, 20] and synthesized at the Integrated DNA Technologies (Coralville, IA). The level of specific gene expression in each sample was ascertained using *Gapdh* as the normalizer and the $2^{-\Delta\Delta C}$T method.

Adoptive transfer of diabetes

Spleens were harvested from 20-24-wks old diabetic mice and from 26-28 wks old mice treated with TSA between 16 to 24-wks of age. Splenocytes (10 x 10^6/ml) were cultured with five µg of Concanavalin A (Sigma Aldrich) for two days as described [19]. More than 95% of these cells were $CD3^+$, as determined by flow cytometry. Splenocytes were also stained with PE-Cyanine5 conjugated anti-CD11b (clone M1/70, eBiosciences) and anti-Ly-6C-FITC antibodies and sorted on a MoFlo Astrios cell sorter, which yielded 2.27 % of the input with > 98% $CD11b^+$Ly-$6C^+$ cells. Both male and female NOD.*scid* mice were injected i.v. with activated 2 x 10^6 T lymphocytes with or without sorted 2 x 10^5 $CD11b^+$Ly-$6C^+$ cells or purified monocytes alone. Splenocytes were also stained with anti-Ly-6C-FITC and incubated with anti-FITC antibody and purified on a magnetic column (Miltenyi Biotec Inc., Auburn, CA). This procedure yielded 2 % of the input with >95% Ly-$6C^+$ cells, as determined by flow cytometry. Female wild-type NOD mice received two injections of purified 2 x 10^5 Ly-$6C^+$ cells at 13 and 21-wk of age. Numbers of mice tested are given in the figure legends.

Statistics

The difference in diabetes incidence between controls (untreated and DMSO-treated mice) and those treated with TSA was analyzed for statistical significance using the Wilcoxon Signed Rank Test. Gene expression data were analyzed for statistical significance between indicated groups by two-way ANOVA or two-tailed unpaired *t*-test as indicated in individual

figure legends using GraphPad Prism (6.0) software. A *P*-value of <0.05 was considered significant.

RESULTS

Protection from T1D is associated with constrained infiltration of inflammatory cells into the islets.

We have consistently observed that 80-100% of female NOD mice procured from The Jackson Laboratory and housed in our animal facility developed T1D by 18 weeks of age [10-13]. Weekly treatment with TSA starting at 16 wk and until 24 wks of age substantially attenuated T1D (Supplementary Fig. 1). Injection of the vehicle DMSO failed to alter the course or intensity of the disease. We also documented that DMSO treatment did not change gene expression compared to untreated diabetic mice. Since DMSO-treated mice did not display gross changes compared to untreated diabetic mice, we compared the untreated diabetic mice with TSA-treated mice in all experiments. Treatment with TSA reduced the invasive cellular infiltration of the islets and reversed moderate to a severe loss of insulin-producing beta cells [10]. However, the nature of cellular infiltration was not characterized. To this end, we performed confocal analysis, which indicated the distribution of Ly-6C$^+$ macrophages, Ly-6G$^+$ neutrophils, CD4$^+$, and CD8$^+$ T-cells across the entire area of the diabetic pancreas (Fig. 1). Treatment with TSA significantly reduced the infiltration of primarily CD4$^+$ cells and, to some extent, CD8$^+$ T-cells implicated in T1D [17]. The influx of Ly-6C$^+$ macrophages but not neutrophils was also reduced by TSA treatment, consistent with a role for macrophages in diabetes [18-19]. Flow cytometric analysis confirmed that the numbers of CD11b$^+$Ly-6C$^+$ monocytes but not CD11b$^+$F4/80$^+$ macrophages or CD11c$^+$ dendritic cells were also diminished

in the spleen of TSA-treated mice (Supplementary Fig. 2). These data indicated that reduced influx of $CD4^+$ and $CD8^+$ T cell subsets and Ly-$6C^+$ macrophages into the islets serves as a mechanism of TSA-mediated amelioration of autoimmune diabetes.

TSA treatment selectively repressed the expression of Th1 and Th17 lymphokines, primarily in the spleen.

Our previous transcriptome analysis of uninduced splenocytes indicated the downregulation of a few inflammatory genes, including *Cela3b* (elastase) by TSA treatment [11]. In the current investigation, we analyzed a total of 43 genes selected for their putative roles in T1D. These included genes encoding 11 lymphokines, 13 accessory cell-associated determinants, seven transcription factors, a chemokine, and 11 genes encoding HDACs. Total RNA was extracted from diabetic and TSA-treated animals, converted to cDNA, and used as the template in the qRT-PCR assay as described before [10-16]. The genes critical for Th17 cell development, namely *Il17a* and *Il23* [21-22], and the Th1-specific *Ifng* [23] were significantly increased in the spleen of diabetic mice compared to the prediabetic mice displaying normal levels of blood glucose (Fig. 2a). Significantly, TSA treatment diminished the levels of these genes. Although *Il4*, *Il18*, and *Il27p28* were not exaggerated during diabetes, they were nevertheless repressed by treatment with the histone modifier. However, other genes such as *Il22*, *Il27ebi3*, *Il10*, *Il12*, and *Tgfb* were neither upregulated during diabetes nor modulated by the histone modifier, indicating that they are unlikely to contribute to diabetogenesis.

Preliminary data indicated that the expression of the genes analyzed herein remained comparable in the pancreas of prediabetic and diabetic mice. Therefore, the gene expression levels in the pancreas of diabetic and TSA-treated mice were compared. Unlike the spleen, TSA

treatment increased the level of *Il4* but repressed the transcription of *Il22* and *Il23* in the pancreas (Fig. 2b). Collectively, these results suggest that TSA treatment differentially regulated the expression of both inducible and constitutively expressed immune response-related genes in the spleen and pancreas.

Histone modifier regulates constitutive expression of macrophage-associated cytokines primarily in the spleen.

The chemokine CCL2, implicated in the mobilization of myeloid cells from the bone marrow causing insulitis [24], was upregulated in the spleen of diabetic mice, which was repressed by TSA treatment (Fig. 3a). Although macrophage-selective genes such as *Csf2*, *Mmp12*, and *Ym1* were not exaggerated during diabetes and yet were reduced by the histone modifier. Significantly, *Cela3b* expression was lower in TSA-treated spleens [11], which was replicated herein. Only the steady-state level of *Cd39* was subject to regulation by the HDAC inhibitor in the pancreas (Fig. 3b). These data suggest that TSA treatment predominantly diminished the constitutive expression of many macrophage-selective genes in the spleen.

Epigenetic drug impacts the expression of transcription factors sparingly in the spleen.

Notably, the transcription factor *Ahr* involved in nitric oxide and arginine production, and alteration of M1/M2 macrophage polarization [25] was transcriptionally increased in the spleen of diabetic mice, which was reduced by TSA treatment (Fig. 4a). Only the constitutive expression of *Rorgt*, crucial for the transcription of IL-17A [26], was repressed by TSA treatment. Surprisingly, *Tbet* and *Gata3*, respectively responsible for the transcription of IFN-γ and IL-4, and other transcription factors such as *Eomes*, *Dec1*, and *Foxp3* were neither transcriptionally upregulated during diabetogenesis nor subject to epigenetic regulation.

Interestingly, the *Foxp3* transcript level was lower in the TSA-treated pancreas (Fig. 4b). These data suggest that TSA-mediated protection from diabetes is associated with the transcriptional repression of a few transcription factors in the lymphoid organ and pancreas.

Epigenetic modifier differentially modulated HDACs in the spleen and pancreas.

Although in vivo treatment of NOD mice with TSA induced the hyperacetylation of histone H3 in the spleen and pancreas of NOD mice [11], it is unclear whether it can differentially affect the expression of class I (*Hdac1*, *Hdac2*, *Hdac3*, and *Hdac8*), class IIa (*Hdac4*, *Hdac5*, *Hdac7*, and *Hdac9*), class IIb (*Hdac6* and *Hdac10*), and class IV (*Hdac11*) HDAC genes in the lymphoid system and the target organ pancreas. In the spleen of prediabetic, normoglycemic, 12-wk old female NOD mice, the basal levels of the *Hdac5* and *Hdac8* were lower (Fig. 5a). During diabetes manifestation, the transcription of *Hdac4*, *Hdac8*, and *Hdac9* genes was increased, which was repressed by TSA treatment. Interestingly, *Hdac1*, *Hdac3*, *Hdac4*, *Hdac8*, and *Hdac11* were upregulated in the pancreas of diabetic mice (Fig. 5b). Whereas most of these genes were not repressed by the histone modifier, *Hdac4* was upregulated in drug-treated mice. It is noteworthy that *Hdac2*, *Hdac5*, *Hdac7*, *Hdac9*, and *Hdac10* genes could not be reliably detected in the pancreas of prediabetic, overtly diabetic, or TSA-treated mice by qRT-PCR in repeated experiments (n=25-30 mice). Thus, TSA-mediated protection against T1D was accompanied by repression of the exaggerated expression of *Hdac4*, *Hdac8*, and *Hdac9* genes in the spleen, while *Hdac4* was upregulated in the pancreas.

TSA treatment differentially affected T lymphocytes and macrophages.

We next determined whether epigenetically modulated genes could impact the diabetogenic potential of T lymphocytes and the presumptive effector functions of macrophages

in diabetes [18-19]. The data shown in Fig. 6a indicate that the transfer of activated T lymphocytes from diabetic mice mediated the disease in 100% of the immunodeficient NOD.*scid* mice by nine wks. However, similarly activated T lymphocytes derived from TSA-treated mice failed to induce the disease in NOD.*scid* recipients, consistent with the data obtained using whole splenocytes [11]. Interestingly, the adoptive transfer of sorted $CD11b^{+}Ly\text{-}6C^{+}$ macrophages from diabetic or TSA-treated mice did not cause diabetes in the immunodeficient mice, indicating that the macrophages are not the effectors of T1D in contrast to a previous observation of macrophage-mediated beta-cell cytotoxicity *in vitro* [19]. Surprisingly, the co-transfer of $CD11b^{+}Ly\text{-}6C^{+}$ macrophages purified from the TSA-treated but not diabetic mice suppressed the T-cell mediated diabetes in NOD.*scid* recipients. In contrast to the data obtained in an adoptive transfer model, two consecutive injections of splenic $Ly6\text{-}C^{+}$ macrophages purified from TSA-treated or diabetic mice failed to influence the ongoing disease in wild-type female NOD mice (Fig. 6b), inconsistent with diabetes suppression mediated by polarized M2-type macrophages reported previously [27]. These data suggest that the epigenetic modulation of macrophages resulted in distinct outcomes dictated by the recipients' immune status.

DISCUSSION

Although many intervention strategies have been previously reported to delay the diabetes onset in female NOD mice, the success of translation of these studies into T1D treatment remains extremely slim [28]. We have reported that TSA treatment provided irreversible protection against diabetes in female NOD mice [10-13]. Protection was associated with increased histone H3 hyperacetylation in both the spleen and pancreas [10]. We now show that diabetes protection is associated with reduced infiltration of $CD4^{+}$, $CD8^{+}$, and $Ly6\text{-}C^{+}$ cells in the pancreas (Fig. 1), indicating the action of the chromatin modifier on specific immune cell

types. This is consistent with reduced transcription and expression of IFN-γ and IL-17A in the peripheral lymphoid organ following TSA treatment.* These data are congruent with the notion that the protective effects of the histone modifier against autoimmune diabetes involved the reduction of T helper subsets and compromised homing of the macrophages and T-cell subsets to the islets of Langerhans.

For the first time, we show herein that diabetogenesis involves exaggerated expression of *Hdac4*, *Hdac8*, and *Hdac9* genes in the secondary lymphoid organ, which were repressed by the histone modifier (Fig. 5a). Although many *Hdac* genes were also exaggerated in the pancreas of diabetic mice, surprisingly, they were resistant to TSA treatment except that the *Hdac4* gene was instead upregulated (Fig. 5b). Thus, the *Hdac* genes are differentially regulated by histone hyperacetylation in the lymphoid tissue *vs.* the target organ, consistent with the significant role of T-cells in diabetes induction. Our result indicated that the *Hdac3* gene was not repressed either in the spleen or pancreas by TSA treatment (Fig. 5a-b), which is at odds with the finding that administering substantial amounts of the HDAC3 selective inhibitor BRD3308 conferred marginal protection against diabetes in NOD mice [29]. It is unclear whether the BRD3308 inhibitor can exert immunosuppressive effects as TSA [10-11, Fig. 1-6]. However, BRD3308 reduced cytokine-induced apoptosis of dispersed primary islets *in vitro* and improved glycemia and insulin secretion in obese diabetic rats [30-32]. However, evidence for suppressing autoimmune responses by BRD3308 is not apparent. Interestingly, TSA blocked the inflammatory cytokine-mediated beta-cell toxicity *in vitro* more effectively than the BRD3308 inhibitor [32]. Since the non-selective HDAC inhibitor TSA is endowed with both immunomodulatory [10-13, Fig. 1-6] and beta-cell protective effects [32], it seems ideal for

treating autoimmune diabetes with an extremely low dose regimen compared to large amount of the HDAC3 selective BRD3308 inhibitor required to exert marginal protection [29].

An important finding is that out of 32 immune response-related genes investigated, only a few were exaggerated during diabetes and regulated by TSA treatment. Notably, *Ifng* and *Il17a*, the Th1 and Th17 subsets' signature genes, were magnified during diabetes (Fig. 2a). Although IFN-γ-producing Th1 cells were shown to mediate diabetes in neonatal NOD mice [33], the genetic absence of IFN-γ delayed but did not prevent diabetes [34]. Paradoxically, a protective role of IFN-γ in diabetes was also reported [35-36]. Whereas IL-17A was shown to contribute to diabetes [37], others raised questions about the independence of Th17 cells to induce diabetes [38-39]. Interestingly, T1D onset was shown to depend on both IL-17 and IFN-γ signaling [40]. Our data showing the repression of both I*l17a* and *Ifng* in TSA-treated mice that attained normoglycemia supports this contention. A closer analysis unraveled that the TSA treatment reduced the numbers of cells expressing *Ifng* and *Il17a*, indicating the numeric reduction of Th1 and Th17 cells in the secondary lymphoid organ*. In addition, TSA treatment prevented the upregulation of *Il23*, required for the generation of Th17 cells [22] (Fig. 2) and *Ccl2* implicated in diabetes [24] (Fig. 3). A novel finding is that the gene encoding the transcription factor *Ahr* [25] was accentuated in the spleen during diabetes and regulated by the histone modifier (Fig. 4), indicating a crucial for this transcription factor in autoimmune diabetes manifestation. In addition to these upregulated genes, the steady-state levels of several genes implicated in T1D such as *Il4* [41], *Il18* [42], and *Il27p28* [43] were also diminished in TSA-treated spleen (Fig. 2). Similarly, the basal level expression of the transcription factor *Rorgt*, crucial for IL-17A transcription [26], was suppressed in the spleen of drug-treated mice. Interestingly, *Il22*, *Il23*, and *Cd39* implicated in diabetes [44] were similarly repressed in the pancreas of mice cured of

diabetes. Interestingly, *Foxp3*, crucial for immunoregulation [45], was decreased in the pancreas after TSA treatment (Fig. 4). These findings support the contention that the regulation of T1D is independent of $CD4^+FoxP3^+$ cells, as we reported earlier [11], similar to a model of T1D delay mediated by the administration of the migration inhibitory factor antagonist [46]. Overall, our data provide supporting evidence for the contribution of previously known genes such as *Ifng*, *Il17a*, and *Ccl2*, and novel evidence for *Il23* and *Ahr* in diabetes manifestation, more so in lymphoid tissue than in the pancreas. Some of these genes upregulated during diabetogenesis may be the effect rather than the cause of diabetes.

The diminished diabetogenic potential of whole splenocytes [10] and the enriched T lymphocytes (Fig. 6a) by epigenetic reprogramming reiterates the importance of T-cells in diabetes*. In addition to T lymphocytes, macrophages had been proposed to play a crucial role in the effector phase of T1D [18-19]. Both antigen presentation to cytotoxic $CD8^+$ T cells [18] and direct cytotoxicity on dispersed islet cells *in vitro* [19] have been attributed to macrophages. However, direct evidence for the role of macrophages in diabetogenesis is lacking. We observed that the $CD11b^+$Ly-$6C^+$ macrophages from diabetic mice failed to transfer diabetes to immunodeficient mice, indicating that the macrophages do not serve as effectors of T1D (Fig. 6a), unlike proposed [19]. Unexpectedly, we found that TSA treatment endowed the $CD11b^+$Ly-$6C^+$ macrophages with the ability to suppress disease induction upon co-transfer with diabetogenic T cells into NOD.*scid* recipients (Fig. 6a). However, the transfer of Ly-$6C^+$ macrophages derived from TSA-treated mice failed to suppress ongoing diabetes in wild-type NOD mice (Fig. 6b). This abnormal finding appears similar to the failure of polarized Th2 [41] and Th17 cells [38] derived from the BDC2.5 TCR transgenic mice to transfer diabetes into neonatal wild-type NOD mice. Still, they readily transferred the disease to immunodeficient

NOD.*scid* mice. These intriguing findings were attributed to the lack of endogenous immunoregulatory mechanisms in immunodeficient mice facilitating the action of transferred cells without opposition, unlike the wild-type NOD mice. Our data are also different from diabetes suppression in NOD mice mediated by the injection of M2-type macrophages polarized *in vitro* from bone-marrow-derived monocytes using a cocktail of IL-4/IL-10/TGF-β [27]. It is possible that epigenetic modulation may not convert macrophages into M2-type macrophages and therefore will have less clinical value. Although histone hyperacetylation has been proposed to impact monocytes and macrophages central to tissue homeostasis and immune responses [47-48], little evidence exists about the functional modulation of macrophages under diabetic conditions. Previously, we observed that NOD mice treated with TSA and cured of T1D displayed a lower level of the *Cela3b* (elastase 3) transcript [10], which was validated herein (Fig. 3a). Moreover, TSA treatment downregulated macrophage-specific genes such as *Csf2*, *Mmp12*, and *Ym1*. Further studies are required to determine whether these changes can contribute to the suppressive function of macrophages observed in a lymphopenic environment and not in immunocompetent wild-type NOD mice.

In conclusion, the data presented herein and elsewhere [10-13*] indicate that the histone modifier mediated protection against T1D involves the modified expression of a select set of immune response-related genes such as *Il17a*, *Ifng*, *Ccl2*, and *Ahr* in the spleen and altered functions of T cells and macrophages. However, changes in the expression of 32 immune response genes in the pancreas of TSA-treated mice remained minimal compared to the lymphoid tissue. This indicates that the lymphoid system takes a more active role in the induction and manifestation of autoimmune diabetes. Although we have analyzed substantial numbers of immune response-related genes in the pancreas of diabetic mice and their

manipulation by TSA treatment, genes pertinent to the exocrine and endocrine tissues remain to be scrutinized. Since T1D induction is dependent on T lymphocytes, our epigenetic analysis has focused on the Th1 and Th17 subsets, allowing the parsing of the roles of, respectively, *Ifng* and *Il17a*.* The disease protection afforded by TSA treatment is inversely correlated with the levels of these small numbers of genes mainly in the lymphoid tissue, suggesting that they could serve as biomarkers of T1D and targets for manipulation of the disease. We believe that similar analysis of multiple immune response-related genes and inflammatory genes in T1D patients may provide significant insights into disease pathogenesis.

ACKNOWLEDGEMENTS

Mark Holterman is acknowledged for the support of this work. The expert help of the Imaging Core and Flow Cytometry Facility at the University of Illinois at Chicago in acquiring confocal images and sorting macrophages, respectively is acknowledged.

AUTHOR CONTRIBUTIONS

SJ designed and performed animal experiments, flow cytometry, and qRT-PCR, supervised others, secured support, analyzed the data, and wrote the paper. MA conducted qRT-PCR experiments and edited the manuscript. AJ performed confocal microscopy and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

FIGURE LEGENDS

Fig. 1 Cellular infiltration in the pancreas was retarded by TSA treatment

Diabetic mice and those treated with TSA during 16 and 24-wk of age were sacrificed when they were 26-28-wks old. Sections of the pancreas were stained with guinea pig antisera against insulin followed by TRITC labeled rabbit anti-guinea pig antibody (red), along with FITC-conjugated anti-Ly-6C, anti-Ly-6G, anti-CD4 or anti-CD8 antibodies (green). After counterstaining with Hoechst (blue), the sections were and analyzed on a confocal microscope. Representative images from multiple experiments are shown.

Fig. 2 Differential modulation of lymphokine genes by the histone modifier

Spleen and pancreas were harvested from 12-wk old prediabetic mice and 25-26-wks old diabetic mice. Mice treated with TSA between 16 and 24-wks of age were sacrificed when they were 25-26-wks old. Five mice per group were tested in three different experiments. Total RNA was extracted from the spleen (**a**) and pancreas (**b**) of individual mice. RNA was pooled from the same group (n=5), converted to cDNA, and used as the template in qRT-PCR. Data indicate the mean +/- SD of three determinations. Representative data from three independent experiments are depicted. Asterisks indicate statistical significance in the spleen samples from prediabetic, diabetic, and TSA-treated mice, as assessed by Two-way ANOVA (**a**) and by two-tailed unpaired t-test between diabetic and TSA-treated pancreas (**b)** (P<0.05). Increased values between prediabetic and diabetic mice were indicated by double asterisks whereas a single asterisk denoted the decrease in values in TSA-treated mice compared to diabetic mice.

Fig. 3 Differential regulation of accessory cell-associated determinants following TSA treatment

The expression levels of genes associated with accessory cells were analyzed in (**a**) spleen and (**b**) pancreas. Total RNA was extracted and gene expression was analyzed by qRT-PCR using the same cDNA preparations analyzed in Fig. 2. Data indicate arithmetic mean +/- SD of three determinations. Representative data from three independent experiments are depicted (*n*=5 mice per group/experiment). Asterisks indicate statistical significance in the spleen samples from prediabetic, diabetic, and TSA-treated mice, as assessed by Two-way ANOVA (**a**) and by two-tailed unpaired *t*-test between diabetic and TSA-treated pancreas **(b)** ($P<0.05$). Increased values between prediabetic and diabetic mice were indicated by double asterisks, whereas the decrease in values in TSA-treated mice compared to diabetic mice was denoted by a single asterisk.

Fig. 4 Selective regulation of the transcription factor genes by TSA

The expression levels of genes encoding various transcription factors were analyzed in (**a**) spleen and (**b**) pancreas by qRT-PCR using the same cDNA preparations analyzed in Fig. 2 and 3. Data indicate the mean +/- SD of three determinations. Representative data from three independent experiments are depicted (*n*=5 mice per group/experiment). Double asterisks indicate a statistically significant increase between prediabetic and diabetic mice (*$P<0.05$), whereas a single asterisk denotes a decrease in the value between diabetic and TSA-treated groups (**$P<0.01$). Statistical significance was assessed respectively by Two-way ANOVA (**a**) and two-tailed unpaired *t*-test between diabetic and TSA-treated pancreas **(b)**.

Fig. 5 Differential modulation of *Hdac* genes by the epigenetic drug

The expression levels of *Hdac* genes in the spleen (**a**) and pancreas (**b**) were determined in cDNA samples illustrated in Fig. 2-4. Data indicate the mean +/- SD of three determinations. Representative data from three independent experiments are depicted (n=5 mice per group/experiment). An increase in the values between prediabetic and diabetic mice was indicated by double asterisks. The decrease in values in TSA-treated mice compared to diabetic mice was denoted by a single asterisk. Statistical significance was assessed using Two-way ANOVA (**a**) and by a two-tailed unpaired t-test between diabetic and TSA-treated pancreas (**b).** *P<0.05 and **P<0.01.

Fig. 6 Epigenetic regulation of T cells and macrophages

(**a**) Diabetes was monitored in NOD.*scid* mice (10-12-wks old) injected i.v. with mitogen-activated 2 x 10^6 T lymphocytes from diabetic or TSA-treated NOD mice (26-28-wks of age) (P=0.0027). Mice were also co-transferred with diabetic T-cells and 2 x 10^5 sorted $CD11b^+Ly\text{-}6C^+$ macrophages from the spleens of diabetic mice or those treated with TSA and diabetes monitored (P=0.003). Some mice received only macrophages isolated from diabetic mice or TSA-treated mice. Four mice per group were tested. Representative data from two independent experiments are shown. Statistical significance between specified groups was assessed using a two-tailed, unpaired t-test. (**b**) Wild-type female NOD mice were injected with purified 2 x 10^5 $Ly6\text{-}C^+$ macrophages from diabetic mice or those treated with TSA at 13 and 21-wks of age, as indicated by the arrows. Diabetes was monitored weekly. Five mice per group were tested. Data shown are from two independent experiments.

REFERENCES

1. American Diabetes Association (2007) Diagnosis and classification of diabetes mellitus. Diabetes Care 30: Suppl 1:S42-47.
2. Hyttinen V, Kaprio J, Kinnunen L, Koskenvuo M, Tuomilehto J (2003) Genetic liability of type 1 diabetes and the onset age among 22,650 young Finnish twin pairs: a nationwide follow-up study. Diabetes 52**:**1052-1055.
3. Shaw JE, Sicree RA, Zimmet PZ (2010) Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract 87:4-14.
4. Todd JA (2010) Etiology of type 1 diabetes. Immunity 32:457–467.
5. Allis CD, Jenuwein T (2016) The molecular hallmarks of epigenetic control. Nat Rev Genet 17:487-500.
6. Bird A (2017) Genetic determinants of the epigenome in development and cancer. Swiss Med Wkly 147:w14523.
7. Jayaraman A, Jayaraman S (2019) DNA hypermethylation does not negatively impact the transcription of the TNF-α gene in an acute T-cell leukemia. Epigenomics 11:1753-1763.
8. Micelli C, Rastelli G (2015) Histone deacetylases: structural determinants of inhibitor selectivity. Drug Discov Today 20:718-735.
9. de Ruijter J, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J 370:737–749.
10. Patel T, Patel V, Singh R, Jayaraman S (2011) Chromatin remodeling resets the immune system to protect against autoimmune diabetes in mice. Immunol Cell Biol 89:640–649.

11. Jayaraman S, Patel A, Jayaraman A, Patel V, Holterman M, Prabhakar B (2013) Transcriptome analysis of epigenetically modulated genome indicates signature genes in manifestation of type 1 diabetes and its prevention in NOD mice. PLoS One 8:e55074.

12. Jayaraman S (2014) Novel methods of type 1 diabetes treatment. Discov Med 17:347-355.

13. Jayaraman S (2018) Type 1 diabetes and epigenetics. In: Zhang R (ed). The epigenetics of autoimmunity. Translational Epigenetics Series, Volume 5. Elsevier Inc. Amsterdam, The Netherlands, pp 188-205.

14. Jayaraman A, Soni A, Prabhakar BS, Holterman M, Jayaraman S (2017) The epigenetic drug Trichostatin A ameliorates experimental autoimmune encephalomyelitis via T cell tolerance induction and impaired influx of T cells into the spinal cord. Neurobiol Dis 108:1-12.

15. Jayaraman A, Sharma M, Prabhakar B, Holterman M, Jayaraman S (2018) Amelioration of progressive autoimmune encephalomyelitis by epigenetic regulation involves selective repression of mature neutrophils during the preclinical phase. Exp Neurol 304:14-20.

16. Jayaraman A, Avgush K, Kulam R, Soni A, Khan A, Kerdjoudj M, Jayaraman S (2020) Treatment of autoimmune encephalomyelitis with a histone deacetylase inhibitor. Analyzing the role of immune-response genes. Free Neuropathology 1:19.

17. Christianson SW, Shultz LD, Leiter EH (1993) Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD.NON-Thy-1a donors. Diabetes 42:44-55.

18. Jun HS, Yoon CS, Zbytnuik L, van Rooijen N, Yoon JW (1999) The role of macrophages in T cell-mediated autoimmune diabetes in nonobese diabetic mice. J Exp Med 189:347-358.

19. Calderon B, Suri A, Unanue ER (2006) In CD4+ T-cell-induced diabetes, macrophages are the final effector cells that mediate islet beta-cell killing: studies from an acute model. Am J Pathol 169:2137–2147.

20. Jayaraman S, Patel T, Patel V, Ajani S, Garza R, Jayaraman A, Kwon S, Singh R, Rondelli D, Prabhakar BS, Holterman M (2010) Transfusion of nonobese diabetic mice with allogeneic newborn blood ameliorates autoimmune diabetes and modifies the expression of selected immune response genes. J Immunol 184:3008-3015.

21. Gaffen SL (2011) Recent advances in the IL-17 cytokine family. Curr Opin Immunol 23:613-619.

22. McGeachy MJ, Cua DJ (2007) The link between IL-23 and Th17 cell-mediated immune pathologies. Semin Immunol 19:372-376.

23. Skapenko A, Schulze-Koops H (2007) Analysis of Th1/Th2 T-cell subsets. Methods Mol Med 136:87-96.

24. Martin AP, Rankin S, Pitchford S, Charo IF, Furtado GC, Lira SA (2008) Increased expression of CCL2 in insulin-producing cells of transgenic mice promotes mobilization of myeloid cells from the bone marrow, marked insulitis, and diabetes. Diabetes 57:3025-3033.

25. Climaco-Arvizu S, Domínguez-Acosta O, Cabañas-Cortés MA, Rodríguez-Sosa M, Gonzalez FJ, Vega L, Elizondo G (2016) Aryl hydrocarbon receptor influences nitric

oxide and arginine production and alters M1/M2 macrophage polarization. Life Sci 155:76–84.

26. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 126:1121–1133.

27. Parsa R, Andresen P, Gillett A, Mia S, Zhang XM, Mayans S, Holmberg D, Harris RA (2012) Adoptive transfer of immunomodulatory M2 macrophages prevents type 1 diabetes in NOD mice. Diabetes 61:2881–2892.

28. Shoda LK, Young DL, Ramanujan S, Whiting CC, Atkinson MA, Bluestone JA, Eisenbarth GS, Mathis D, Rossini AA, Campbell SE, Kahn R, Kreuwel HT (2005) A comprehensive review of inventions in the NOD mouse and implications for translation. Immunity 23:115–126.

29. Dirice E, Ng RWS, Martinez R, Hu J, Wagner FF, Holson EB, Wagner BK, Kulkarni RN (2017) Isoform-selective inhibitor of histone deacetylase 3 (HDAC3) limits pancreatic islet infiltration and protects female nonobese diabetic mice from diabetes. J Biol Chem 292:17598–17608.

30. Lundh M, Christensen DP, Damgaard Nielsen M et al (2012) Histone deacetylases 1 and 3 but not 2 mediate cytokine-induced beta cell apoptosis in INS-1 cells and dispersed primary islets from rats and are differentially regulated in the islets of type 1 diabetic children. Diabetologia 55:2421–2431.

31. Lundh M, Galbo T, Poulsen SS, Mandrup-Poulsen T (2015) Histone deacetylase 3 inhibition improves glycaemia and insulin secretion in obese diabetic rats. Diabetes Obes Metab 17:703-707.

32. Wagner FF, Lundh M, Kaya T, McCarren P, Zhang YL, Chattopadhyay S, Gale JP, Galbo T, Fisher SL, Meier BC, Vetere A, Richardson S, Morgan NG, Christensen DP, Gilbert TJ, Hooker JM, Leroy M, Walpita D, Mandrup-Poulsen T, Wagner BK, Holson EB (2016) An isochemogenic set of inhibitors to define the therapeutic potential of histone deacetylases in β-cell protection. ACS Chem Biol 11:363-374.

33. Katz JD, Benoist C, Mathis D (1995). T helper cell subsets in insulin-dependent diabetes. Science 268:1185-1188.

34. Hultgren B, Huang X, Dybdal N, Stewart TA (1996). Genetic absence of gamma-interferon delays but does not prevent diabetes in NOD mice. Diabetes 45, 812-817.

35. Trembleau S, Penna G, Gregori S, Giarratana N, Adorini L (2003). IL-12 administration accelerates autoimmune diabetes in both wild-type and IFN-gamma-deficient nonobese diabetic mice, revealing pathogenic and protective effects of IL-12-induced IFN-gamma. J Immunol 170, 5491-5501.

36. Zhang J, Huang Z, Sun R, Tian Z, Wei H (2012). IFN-γ induced by IL-12 administration prevents diabetes by inhibiting pathogenic IL-17 production in NOD mice. J Autoimmun 38:20-28.

37. Bellemore SM, Nikoopour E, Krougly O, Lee-Chan E, Fouser LA, Singh B (2016) Pathogenic T helper type 17 cells contribute to type 1 diabetes independently of interleukin-22. Clin Exp Immunol 183:380-388.

38. Martin-Orozco N, Chung Y, Chang SH, Wang YH, Dong C (2009). Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. Eur J Immunol 39:216-224.

39. Bending D, De la Peña H, Veldhoen M, Phillips JM, Uyttenhove C, Stockinger B, Cooke A (2009). Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. J Clin Invest 119:565-572.

40. Kuriya G, Uchida T, Akazawa S, Kobayashi M, Nakamura K, Satoh T, Horie I, Kawasaki E, Yamasaki H, Yu L, Iwakura Y, Sasaki H, Nagayama Y, Kawakami A, Abiru N (2013) Double deficiency in IL-17 and IFN-γ signalling significantly suppresses the development of diabetes in the NOD mouse. Diabetologia 56:1773-1780.

41. Pakala SV, Kurrer MO, Katz JD (1997) T helper 2 (Th2) T cells induce acute pancreatitis and diabetes in immune-compromised nonobese diabetic (NOD) mice. J Exp Med 186:299-306.

42. Marleau AM, Sarvetnick NE (2011) IL-18 is required for self-reactive T cell expansion in NOD mice. J Autoimmun 36:263–277.

43. Ciecko AE, Foda B, Barr JY, Ramanathan S, Atkinson MA, Serreze DV, Geurts AM, Lieberman SM, Chen YG (2019) Interleukin-27 is essential for type 1 diabetes development and Sjögren syndrome-like Inflammation. Cell Rep 29:3073–3086.e5.

44. Chia JS, McRae JL, Cowan PJ, Dwyer KM (2012) The CD39-adenosinergic axis in the pathogenesis of immune and nonimmune diabetes. J Biomed Biotechnol 2012:320495.

45. Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. Science 299**:**1057-1061.

46. Korf H, Breser L, Van Hoeck J, Godoy J, Cook DP, Stijlemans B, De Smidt E, Moyson C, Monteiro Carvalho Mori Cunha JP, Rivero V, Gysemans C, Mathieu C (2017) MIF inhibition interferes with the inflammatory and T cell-stimulatory capacity of NOD macrophages and delays autoimmune diabetes onset. PLoS One 12:e0187455.

47. Das Gupta K, Shakespear MR, Iyer A, Fairlie DP, Sweet MJ (2016) Histone deacetylases in monocyte/macrophage development, activation and metabolism: refining HDAC targets for inflammatory and infectious diseases. Clin Transl Immunology 5:e62.

48. Roger T, Lugrin J, Le Roy D, Goy G, Mombelli M, Koessler T, Ding XC, Chanson AL, Reymond MK, Miconnet I, Schrenzel J, François P, Calandra T (2011) Histone deacetylase inhibitors impair innate immune responses to 43 Toll-like receptor agonists and to infection**.** Blood 117:1205–1217.

*Patel V, Jayaraman A, Jayaraman S. Epigenetic reprogramming ameliorates type 1 diabetes by decreasing the generation of Th1 and Th17 subsets and restoring self-tolerance in $CD4^+$ T cells. Under consideration of publication. 2021.

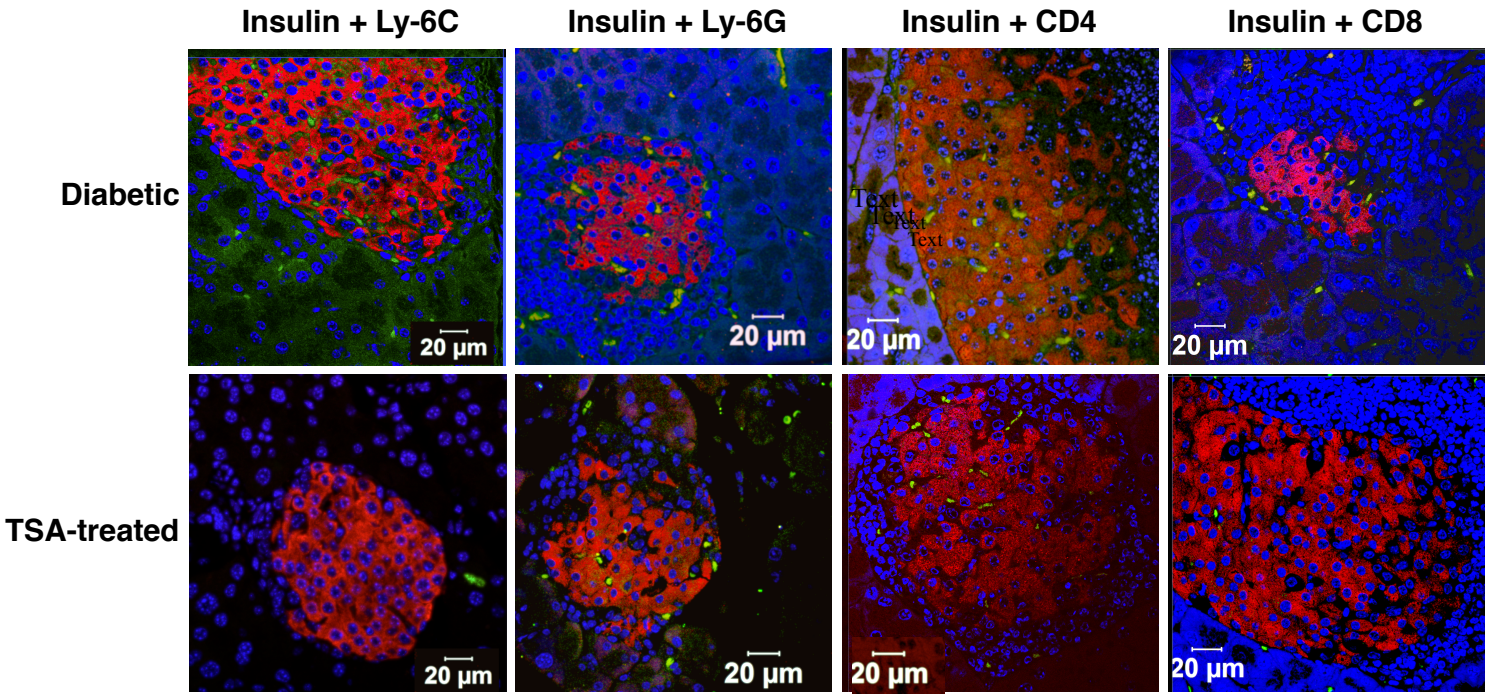
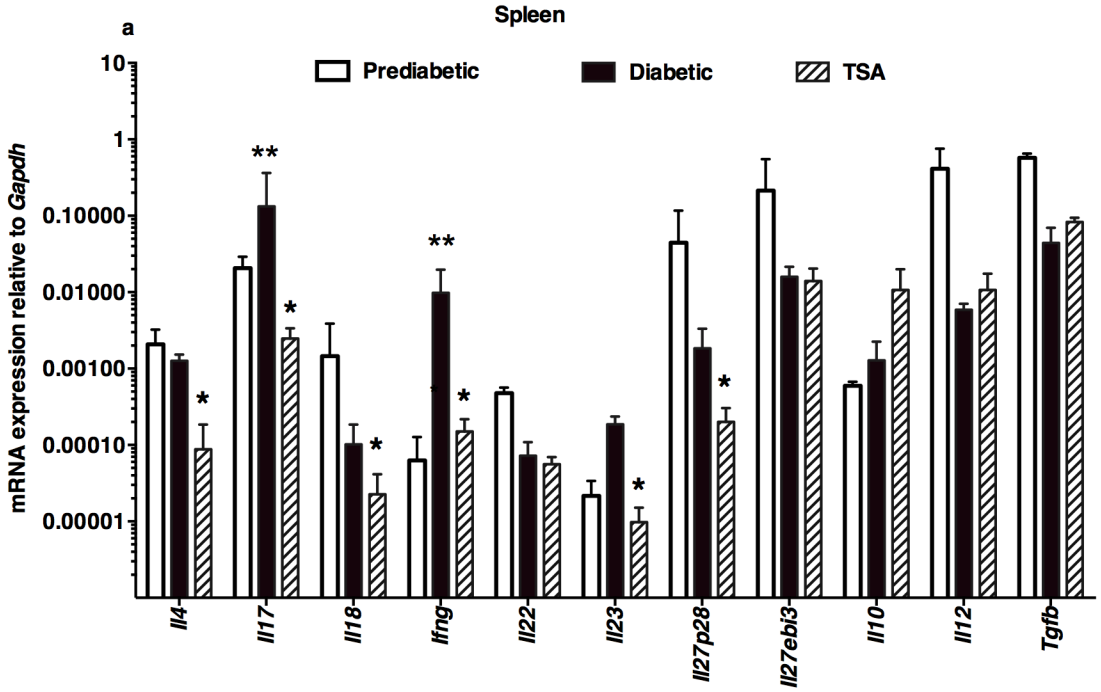

Insulin + Ly-6C
Insulin + Ly-6G
Insulin + CD4
Insulin + CD8
Diabetic
TSA-treated
20 µm
Text
Text
Text
Text

Fig. 1


a
Spleen
Prediabetic
Diabetic
TSA
mRNA expression relative to Gapdh
10
1
0.10000
0.01000
0.00100
0.00010
0.00001
**
*
Il4
Il17
Il18
Ifng
Il22
Il23
Il27p28
Il27ebi3
Il10
Il12
Tgfb

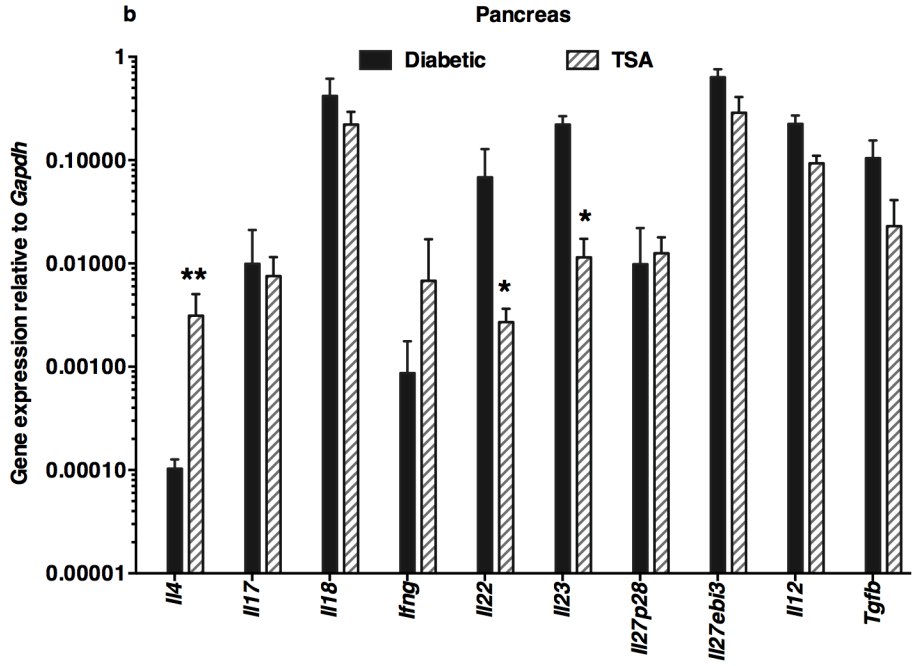
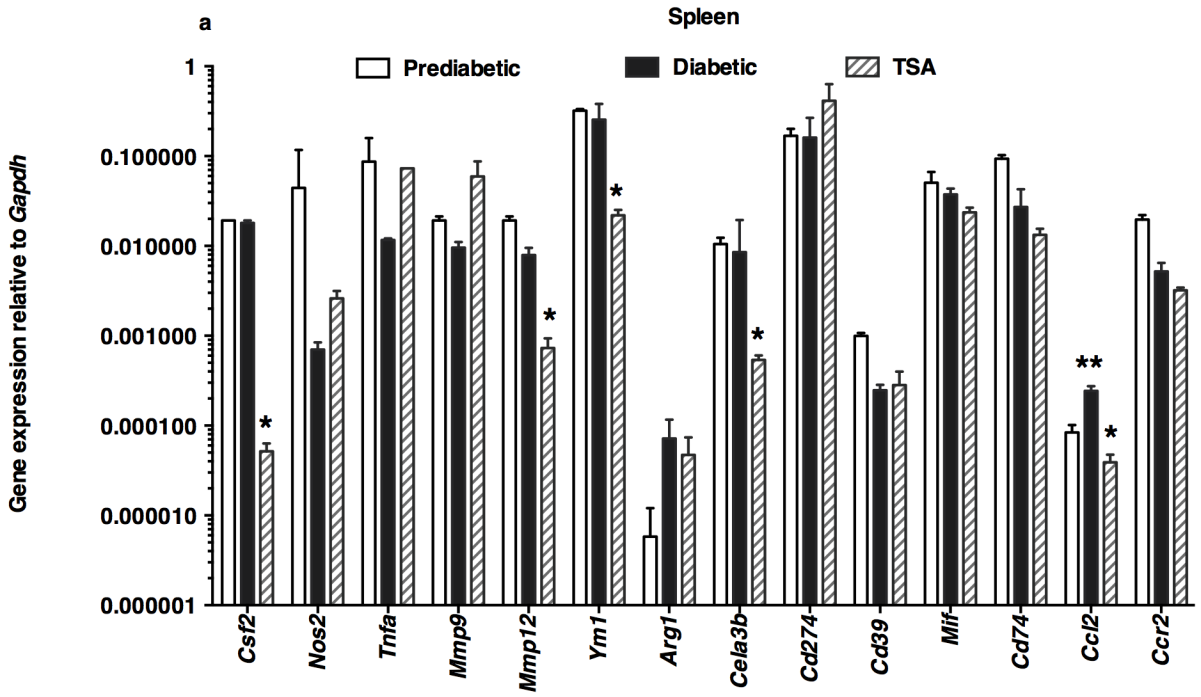

b
Pancreas
Diabetic
TSA
Gene expression relative to Gapdh
1
0.10000
0.01000
0.00100
0.00010
0.00001
**
*
Il4
Il17
Il18
Ifng
Il22
Il23
Il27p28
Il27ebi3
Il12
Tgfb

Fig. 2


a
Spleen
Prediabetic
Diabetic
TSA
Gene expression relative to Gapdh
1
0.100000
0.010000
0.001000
0.000100
0.000010
0.000001
*
*
*
*
**
*
Csf2
Nos2
Tnfa
Mmp9
Mmp12
Ym1
Arg1
Cela3b
Cd274
Cd39
Mif
Cd74
Ccl2
Ccr2

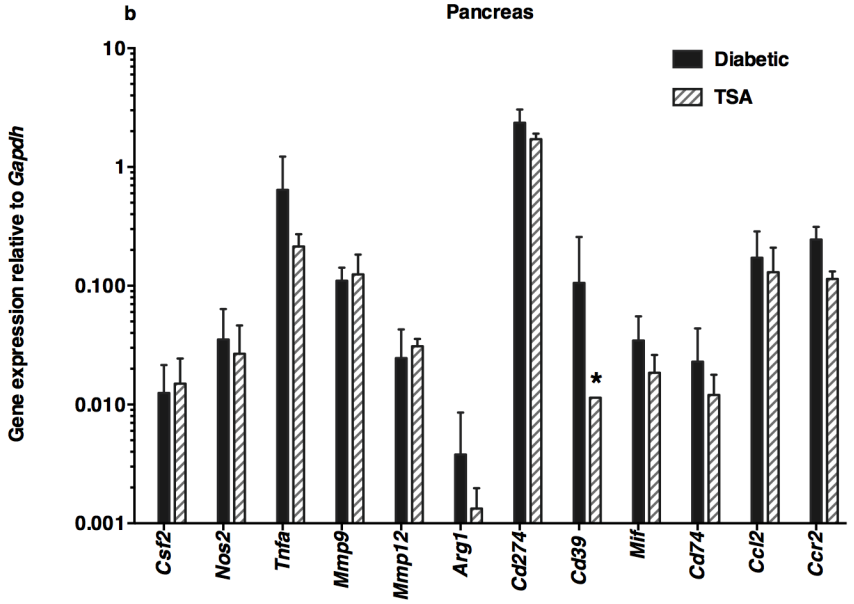
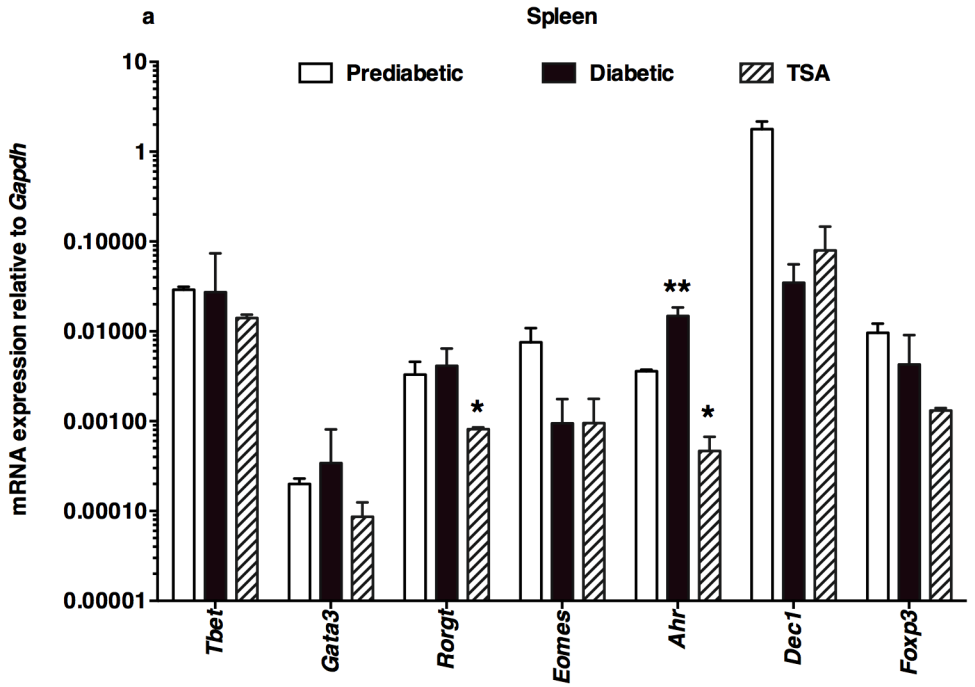

b
Pancreas
Diabetic
TSA
Gene expression relative to Gapdh
10
1
0.100
0.010
0.001
*
Csf2
Nos2
Tnfa
Mmp9
Mmp12
Arg1
Cd274
Cd39
Mif
Cd74
Ccl2
Ccr2

Fig. 3


a
Spleen
Prediabetic
Diabetic
TSA
mRNA expression relative to Gapdh
10
1
0.10000
0.01000
0.00100
0.00010
0.00001
**
*
*
Tbet
Gata3
Rorgt
Eomes
Ahr
Dec1
Foxp3

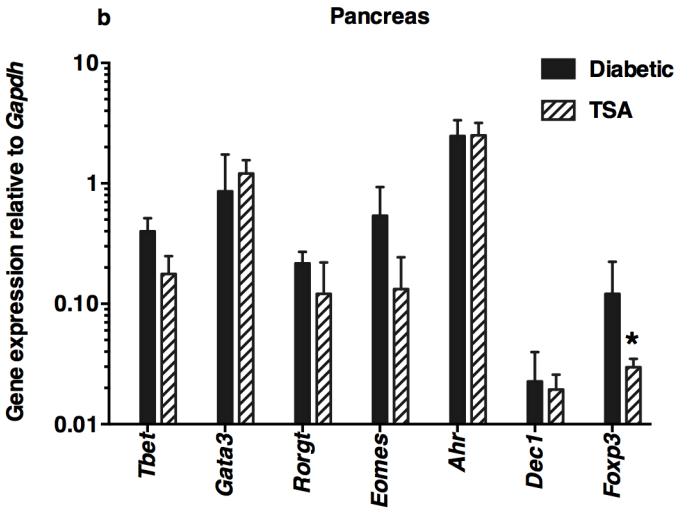
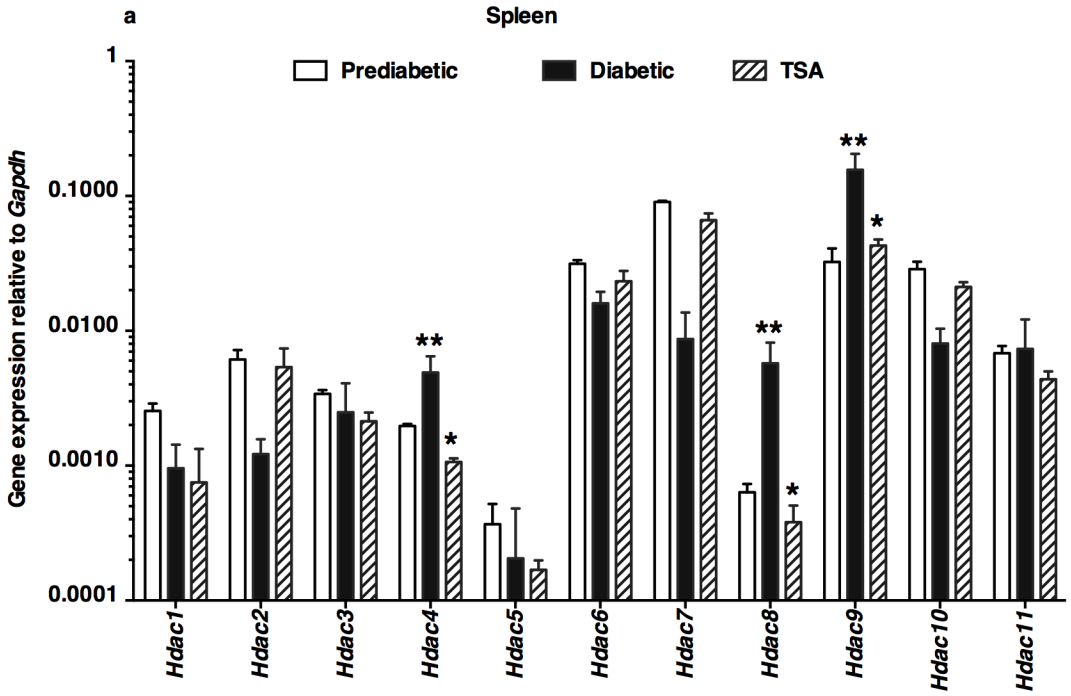

b
Pancreas
Diabetic
TSA
Gene expression relative to Gapdh
10
1
0.10
0.01
*
Tbet
Gata3
Rorgt
Eomes
Ahr
Dec1
Foxp3

Fig. 4


a
Spleen
Prediabetic
Diabetic
TSA
Gene expression relative to Gapdh
1
0.1000
0.0100
0.0010
0.0001
**
*
Hdac1
Hdac2
Hdac3
Hdac4
Hdac5
Hdac6
Hdac7
Hdac8
Hdac9
Hdac10
Hdac11

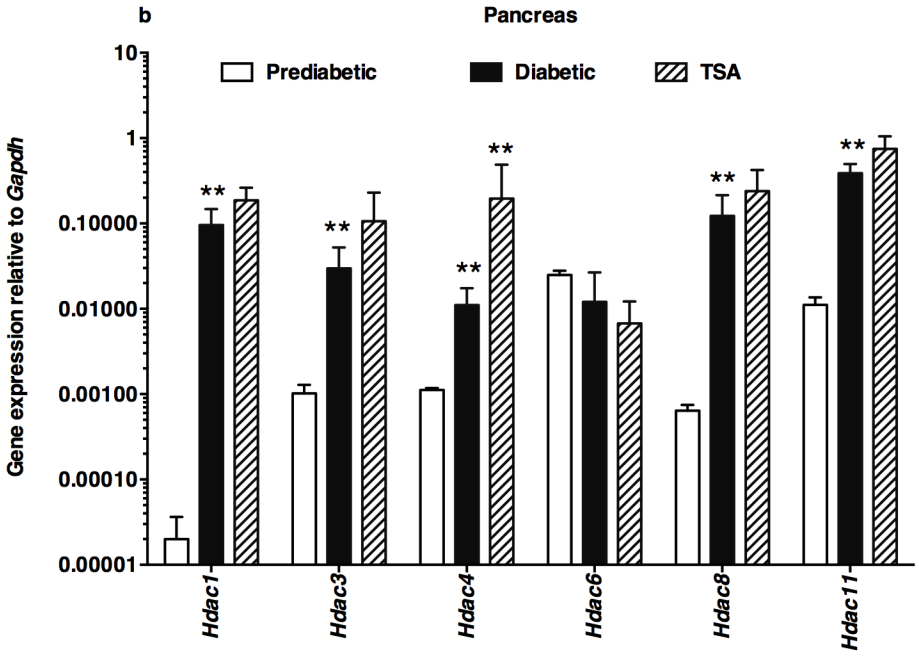

b
Pancreas
Prediabetic
Diabetic
TSA
Gene expression relative to Gapdh
10
1
0.10000
0.01000
0.00100
0.00010
0.00001
**
Hdac1
Hdac3
Hdac4
Hdac6
Hdac8
Hdac11

Fig. 5

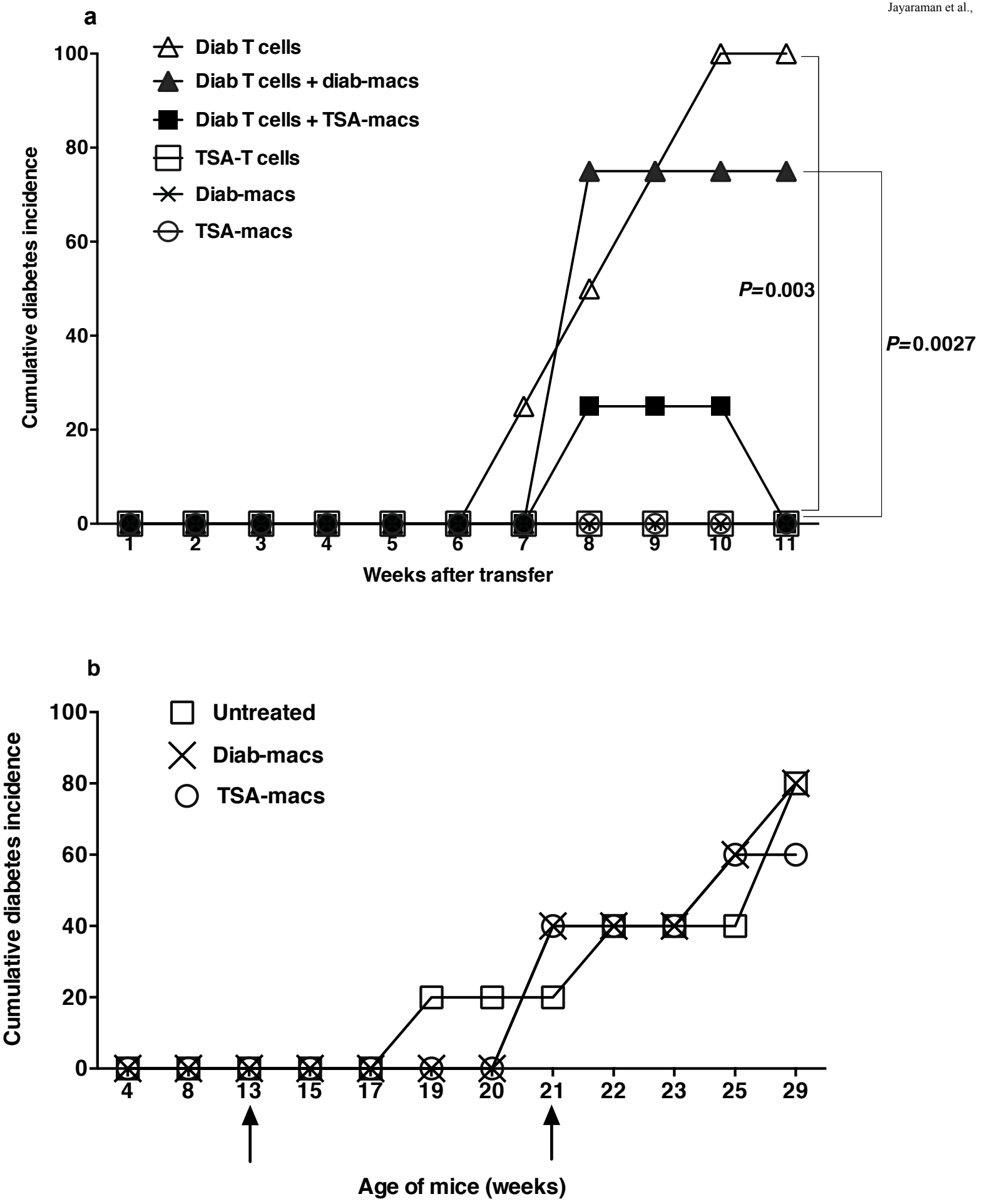

a
Diab T cells
Diab T cells + diab-macs
Diab T cells + TSA-macs
TSA-T cells
Diab-macs
TSA-macs
Cumulative diabetes incidence
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
Weeks after transfer
P=0.003
P=0.0027
b
Untreated
Diab-macs
TSA-macs
Cumulative diabetes incidence
100
80
60
40
20
0
4
8
13
15
17
19
20
21
22
23
25
29
Age of mice (weeks)

Fig. 6