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ARTICLE

Methylation of insulin DNA in response to proinflammatory cytokines during the progression of autoimmune diabetes in NOD mice

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Abstract
Aims/hypothesis Type 1 diabetes is caused by the immunological destruction of pancreatic beta cells. Preclinical and clinical data indicate that there are changes in beta cell function at different stages of the disease, but the fate of beta cells has not been closely studied. We studied how immune factors affect the function and epigenetics of beta cells during disease progression and identified possible triggers of these changes.
Methods We studied FACS sorted beta cells and infiltrating lymphocytes from NOD mouse and human islets. Gene expression was measured by quantitative real-time RT-PCR (qRT-PCR) and methylation of the insulin genes was investigated by high-throughput and Sanger sequencing. To understand the role of DNA methyltransferases, *Dnmt3a* was knocked down with small interfering RNA (siRNA). The effects of cytokines on methylation and expression of the insulin gene were studied in humans and mice.
Results During disease progression in NOD mice, there was an inverse relationship between the proportion of infiltrating lymphocytes and the beta cell mass. In beta cells, methylation marks in the *Ins1* and *Ins2* genes changed over time. Insulin gene expression appears to be most closely regulated by the methylation of *Ins1* exon 2 and *Ins2* exon 1. Cytokine transcription increased with age in NOD mice, and these cytokines could induce methylation marks in the insulin DNA by inducing methyltransferases. Similar changes were induced by cytokines in human beta cells in vitro.
Conclusions/interpretation Epigenetic modification of DNA by methylation in response to immunological stressors may be a mechanism that affects insulin gene expression during the progression of type 1 diabetes.

Keywords Beta cell · Cytokines · DNA methylation · DNA methyltransferases · *Dnmt3a* · Infiltrating lymphocytes · *Ins1* · *Ins2* · Type 1 diabetes

Abbreviations

DNMT	DNA methyltransferase
MAB	Monoclonal antibody
qRT-PCR	Quantitative real-time RT-PCR
siRNA	Small interfering RNA
TMRE	Tetramethylrhodamine ethyl ester perchlorate

Introduction

Clinical experience and preclinical studies indicate that immune cell regulation of beta cells is important for the progression of type 1 diabetes. After the clinical presentation of hyperglycaemia, there is some recovery of beta cell function, indicating that a functional component may impair insulin secretion at disease onset (in addition to loss of total beta cell mass as a consequence of autoimmune destruction) [1–3]. At the time of presentation with hyperglycaemia, degranulated beta cells may be found in the islets of NOD mice, although insulin granules can be recovered after anti-CD3 monoclonal

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antibody (mAb) immune therapy [4]. Crosstalk between immune and beta cells may also control disease progression. Despite the fact that autoantigens are present for several years after the initial sensitisation of beta cells, the disease progresses over a short period of time, suggesting that changes in beta cell function may be involved.

DNA methylation is an important regulatory mechanism in pancreatic organogenesis and for maintaining beta cell differentiation capacity. For example, the ability of beta cells to express insulin in response to glucose is acquired during postnatal life [5]. DNA methyltransferase 3A (DNMT3A) appears to play a central role in the acquisition of glucose stimulated insulin secretion by beta cells [6]. It is likely that DNA methylation is crucial for both beta cell maturation and function. DNMT3A initiates a metabolic programme that enables the coupling of insulin secretion to glucose levels.

Epigenetic changes may occur in response to environmental stressors. For example, the genes encoding superoxide dismutase and matrix metalloproteinase are epigenetically modified in a diabetic environment, and are thought to be responsible for regulating nuclear transcription factor and vascular endothelial growth factor (VEGF) in diabetic retinopathy [7]. The 'metabolic memory' that persists following glucose control of diabetes is also postulated to be due to epigenetic modifications in endothelial cells [8]. Ishikawa et al reported that long-term, high levels of glucose induce DNA methylation in INS-1 cells [9]. Several other studies have also shown epigenetic modifications in diabetes and after glucose treatment [10–13].

We postulated that epigenetic changes might occur in beta cells during the development of autoimmune diabetes, and that these changes might affect the function or other characteristics of beta cells. We focused on DNA methylation, which is a frequently biological method of fine-tuning gene expression [14]. Interestingly, recent studies into the dynamics of DNA methylation have revealed that this is a reversible modification, suggesting that it may contribute to the plasticity of genomic responses to seasonal cues in natural environments [15]. The contribution of epigenetic mechanisms such as DNA methylation to maintaining correct cellular function is highlighted by observations that their deregulation together with genetic alterations lead to the development of various diseases and to the establishment and progression of tumours [16].

We studied the cellular and molecular changes in islets and beta cells during the progression of diabetes in NOD mice. Autoimmunity is initiated in NOD mice between 3 and 4 weeks of age, followed by adaptive immune responses that are responsible for killing beta cells [17]. In a quantitative analysis in NOD mice, we showed a decline in beta cell numbers with increased infiltration of T cells into the islets of Langerhans with ageing. We found that CpG sites within the *Ins1* and *Ins2* genes, which appear to be related to insulin gene transcription, undergo methylation in the prediabetic period. We identified the expression of cytokines in the islets of prediabetic NOD mice that are able to induce DNMTs and insulin gene methylation associated with the control of insulin transcription. Thus, epigenetic modifications in response to immunological stressors may represent a mechanism that affects insulin gene expression during progression of autoimmune diabetes.

Methods

Mice Female NOD, NOD/*scid* and B6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained under pathogen-free conditions. All protocols were approved by the Yale Institutional Animal Care and Use Committee.

Human islets Human islet samples were obtained from adult, non-diabetic organ donors from the Integrated Islet Distribution Program, as previously described [6].

Mouse islet isolation and beta cell staining and purification Islets were hand-picked from NOD, NOD/*scid* and B6 mice of different ages ($n = 4$–6/group), and single cell suspensions were prepared after collagenase digestion. Cells were stained with FluoZin-3 (Invitrogen, Waltham, MA, USA) and tetramethylrhodamine ethyl ester perchlorate (TMRE; Life Technologies, Carlsbad, CA, USA) as previously described [18] for beta cell purification, and with an anti-CD45 mAb (eBioscience, San Diego, CA, USA) for islet-infiltrating lymphocytes identification using a FACSAria II (BD, Franklin Lakes, NJ, USA). In some experiments, single islet cells were also stained with ant-insulin (R&D, Minneapolis, MN, USA) and anti-glucagon (Abcam, Cambridge, UK) antibodies labelled with FITC.

Bisulphite sequencing Genomic DNA (≥100 ng) was recovered from sorted beta cells, lymphocytes and serum, spleen, liver, kidney and lung tissues. High-throughput targeted methylation sequencing of *Ins1* and *Ins2* DNA was performed by Zymo Research (Irvine, CA, USA. www.zymoresearch.com).

Sanger sequencing of a 200 bp region in *Ins1* exon 2 was used to sequence cell clones. This region includes seven CpG sites and site-specific methylation patterns can be used to discriminate beta and non-beta cells (electronic supplementary material [ESM] Fig. 3). Bisulphite primers (ESM Table 1) and the pGEM-T vector (Promega, Fitchburg, WI, USA) were used for PCR amplification and cloning. Sanger sequencing was used to determine the DNA methylation status of approximately 80 positive clones (Keck Laboratory, Yale School of Medicine, http://medicine.yale.edu/keck).

Islet culture and siRNA transfection A total of 200 human islets or 150 handpicked islets isolated from 4-week-old NOD mice were cultured with or without cytokine cocktails in DMEM (2.2 g/l glucose) (Gibco; ThermoFisher, Waltham, MA, USA) containing 10% FBS for 48 h. In some experiments, cells were transfected with small interfering (si)RNA targeting *Dnmt3a* (SMARTpool; Dharmacon, Lafayette, CO, USA) or control siRNA using Lipofectamine 2000 (Invitrogen) for 24 h prior to cytokine treatment for 48 h [6]. Islets were then digested to obtain single cells and stained for beta cell sorting. DNA and RNA were purified for DNA methylation and gene transcription analysis, respectively.

Probes, primers and quantitative real-time RT-PCR TaqMan probes for mouse *Ins1*, *Ins2* and *Actb* were used to perform quantitative real-time RT-PCR (qRT-PCR) using a TaqMan Gene Expression Master Mix (ThermoFisher), and qRT-PCR was performed using a QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany). Primer pairs used in this study are listed in ESM Table 1. The *Actb* housekeeping gene was used to normalise the input RNA in all qRT-PCR assays. In some cases, insulin gene transcription was presented as $\Delta C_t = C_t$ *Actb* $- C_t$ *Ins1* or *Ins2*.

Statistical analysis Unless otherwise indicated, data are expressed as means ± SEM. The differences between experimental groups and time points were compared using unpaired and paired Student's *t* tests (normally distributed data) and Mann–Whitney *U* tests (non-normally distributed data). Multiple groups were compared using one- or two-way ANOVA with Tukey post hoc analysis using GraphPad Prism 6 (La Jolla, CA, USA). Differences with a p value of <0.05 were considered statistically significant.

Results

Changes in beta cell number and function during diabetes progression in NOD mice To identify changes in the cellular composition of islets over time, we analysed islets from NOD mice by FACS. We stained islet cells with FluoZin-3 (a zinc-selective, cell-permeable dye) to identify insulin-producing beta cells and with the TMRE mitochondrial membrane potential indicator to distinguish viable beta cells [18]. We also used anti-CD45 mAb staining to identify immune cells infiltrating the islets. Representative data from 12-week-old NOD mice are shown in Fig. 1a. Islet-infiltrating lymphocytes were detectable as early as 4 weeks of age and increased with increasing age. Over the same period, the number of beta cells declined. By 12 weeks of age, approximately 60% of the total cells present in the islets were infiltrating lymphocytes and beta cells comprised approximately 30% of islet cells. As the proportion of lymphocytes increased, the proportion of beta cells declined (Fig. 1b). To determine whether there were also changes in alpha cells due to lymphocyte infiltration, we stained islet cells with anti-glucagon and anti-insulin antibodies. Representative FACS profiles from 12-week-old NOD and B6 mice are shown in Fig. 1c. With increasing age, in addition to a decrease in the total fraction of insulin-positive beta cells, we noticed an increase of islet cells that were negative for both insulin and glucagon in NOD mouse islets (Fig. 1c). The presence of $CD45^+$ islet-infiltrating lymphocytes negatively correlated with the percentage of insulin-positive but not with the percentage of glucagon-positive cells (Pearson's coefficient: insulin $r^2 = 0.5480$, $p = 0.0016$; glucagon, NS; Fig. 1d).

We measured *Ins1* and *Ins2* gene transcription by qRT-PCR in beta cells sorted from NOD mice at different ages. The *Ins1* and *Ins2* genes were transcribed at a ratio of approximately 1:2 at all time points measured, but transcription of both genes changed over time (one-way ANOVA: *Ins1* $p < 0.001$; *Ins2* $p < 0.01$; Fig. 2). Compared with 3 weeks of age, there was an increase in the transcription of both insulin genes in beta cells at 4 weeks of age ($p < 0.0001$), most likely due to the rapid gain in body mass after weaning (Fig. 2). The transcription of both insulin genes fluctuated at between 6 and 7 weeks of age ($p < 0.0001$), but increased to levels comparable with those at 4 weeks at 9 weeks, and then declined at 12 weeks ($p < 0.0001$; Fig. 2), corresponding to the appearance of glucose intolerance [19]. These data indicate that, in addition to the anatomical loss of beta cells, there is decreased insulin gene transcription in the remaining beta cells.

The dynamics of insulin gene methylation in NOD mouse beta cells during diabetes progression DNA was isolated from sorted beta cells (as shown in Fig. 1) and high-throughput targeted methylation sequencing of *Ins1* and *Ins2* DNA was performed. We first grouped CpG sites by gene regions in *Ins1* and *Ins2* (ESM Fig. 1; www.ensembl.org/index.html) and determined the methylation ratio of individual CpG site by age (Fig. 3). (NB There are no CpG sites in the *Ins1* promoter and only one CpG site in the *Ins1* intron.) There was a large variation in the frequency of methylated sites in both insulin genes over time. Moreover, the methylation status of individual sites within the same region appeared to be random, except for sites within *Ins1* exon 2 (Fig. 3a) and *Ins2* exon 1 (Fig. 3d). Therefore, we focused our studies on these regions. We first determined whether methylation changes at any site or gene region were associated with insulin gene transcription (Fig. 4). Methylation of *Ins1* exon 2 showed a modest inverse association with the *Ins1* mRNA level (Pearson's $r^2 = 0.43$, $p = 0.1564$; Fig. 4a, b). The methylation level of *Ins2* exon 1 was significantly inversely related to *Ins2* mRNA levels ($r^2 = 0.85$, $p = 0.0089$; Fig. 4c, d). Interestingly we did not

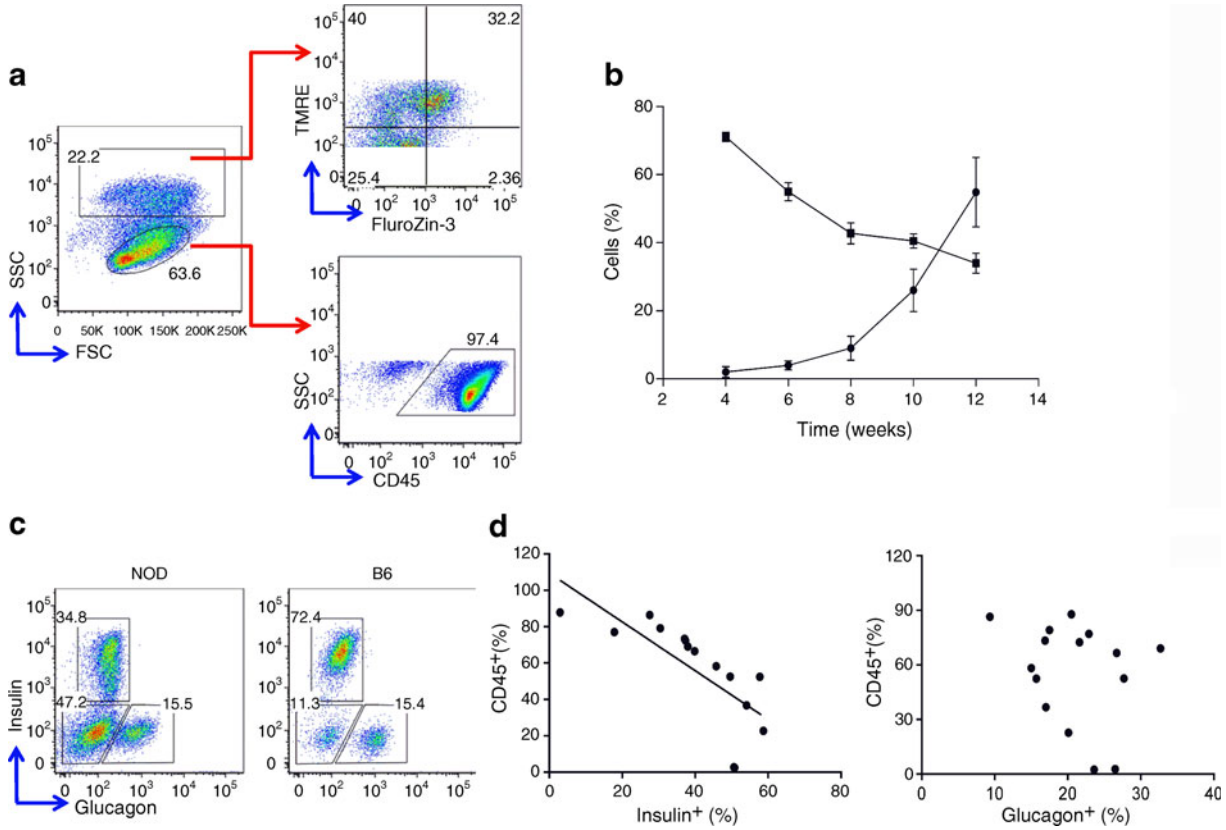

Fig. 1 Analysis of beta cells and lymphocytes in islets from NOD mice. (**a**) Representative gating strategy for islet cells from 12-week-old NOD mice (see Methods). Data represent three experiments (n=9). FSC: forward scatter; SSC: side scatter. (**b**) The percentage of lymphocytes (circles) and beta cells (squares) at different time points (mean ± SEM, n=4–5 mice each). (**c**) FACS analysis of insulin and glucagon expression in $CD45^-$ cells in islets from 12-week-old NOD and B6 mice. Data represent three experiments (n=9). (**d**) Correlation between insulin- or glucagon-producing islet cells and $CD45^+$ lymphocytes. Pearson's r^2: insulin, $r^2 = 0.5480$, p=0.0016; glucagon, NS

identify a relationship between *Ins*2 promoter methylation and *Ins*2 mRNA levels (Fig. 3c and ESM Fig. 2).

We also compared the methylation ratio of CpG sites in *Ins1* exon 2 from beta cells with those of other tissues. We found significantly less methylation in beta cells isolated from 3-week-old NOD mice compared with the other tissues (ESM Fig. 3).

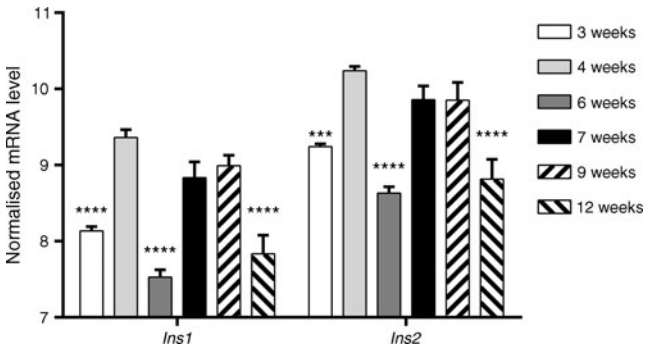

Fig. 2 Insulin gene transcription in beta cells from NOD mice over time. Beta cells from NOD mice of different ages were sorted. *Ins1* and *Ins2* gene transcription was analysed by qRT-PCR and normalised to *Actb* mRNA levels (ΔC_t=*Actb*−*Ins1/2*). Data show the mean ± SEM of three experiments, each with 4–5 mice. One-way ANOVA: *Ins1* p<0.001, *Ins2* p<0.01. Post hoc comparison with 4-week-old mice: ***p<0.001, ****p<0.0001

Induction of insulin gene methylation by proinflammatory cytokines in vitro During the progression of type 1 diabetes in NOD mice, proinflammatory cytokines are expressed in the islets by infiltrating immune cells [3, 20, 21]. We hypothesised that soluble inflammatory mediators may contribute to the observed epigenetic changes. We first analysed the expression of proinflammatory cytokines by qRT-PCR in whole islets from NOD mice of various ages. Compared with islets from 3-week-old NOD mice, there were significant increases in *Cxc110* mRNA (p<0.01) as early as 5 weeks of age and in *Tnf* mRNA (p<0.05) and *Ifng* mRNA (p<0.0001) at 8 weeks. By 11 weeks of age, *Tnf* mRNA (p<0.001) and *I16* (p<0.0001) levels had increased further, and *I11b* mRNA (p<0.05) also showed a significant increase (Fig. 5a).

To determine whether these islet-infiltrating cytokines directly affect insulin gene transcription and DNA methylation, we cultured islets from 4-week-old NOD mice with cytokines, and analysed insulin gene transcription and insulin DNA methylation in sorted beta cells. Previous studies have highlighted the pathological effects of IL-1β, IL-6 and IFN-γ in the destruction of

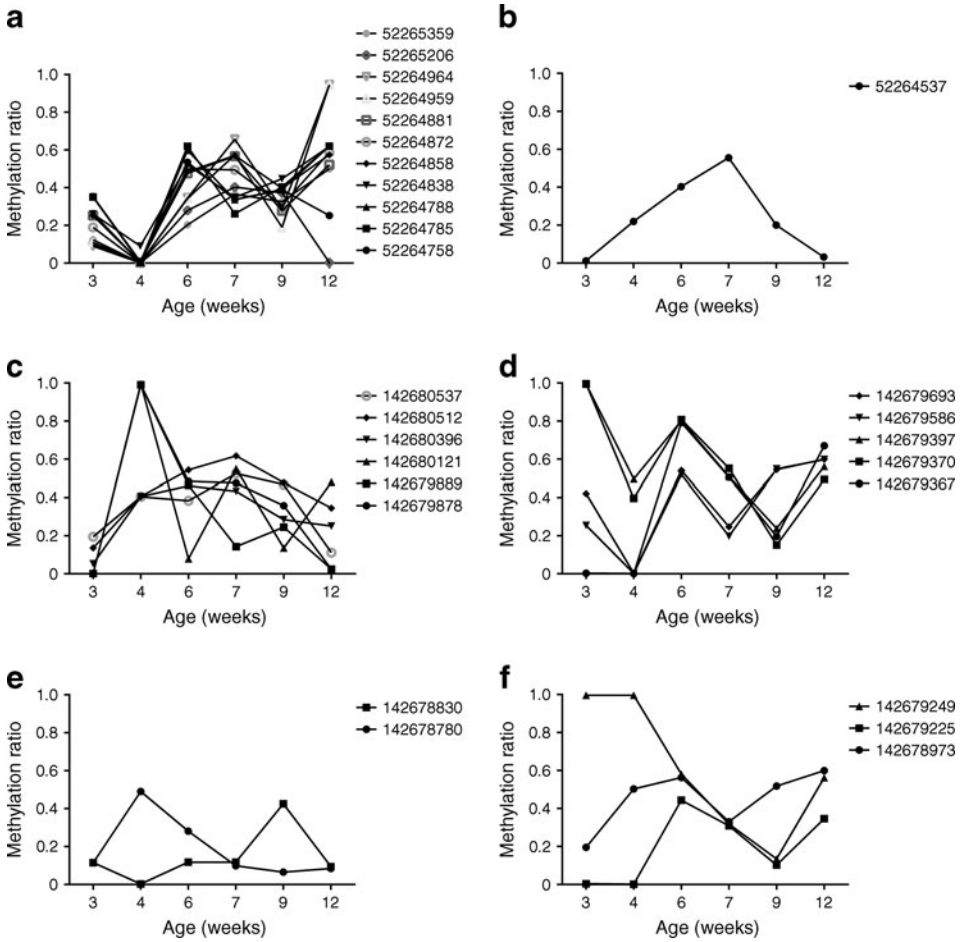

Fig. 3 Methylation of insulin genes in beta cells during diabetes progression in NOD mice. The methylation ratios for CpG sites (shown in key) in the DNA for *Ins1* (**a**) exon 2 and (**b**) intron 1–2 and of *Ins2* (**c**) promoter, (**d**) exon 1, (**e**) exon 2 and (**f**) intron 1–2. Data were obtained from targeted high-throughput methylation sequencing of beta cell DNA pooled from 4 to 6 NOD mice of each age group. The average reads for each CpG site were 30,000–50,000

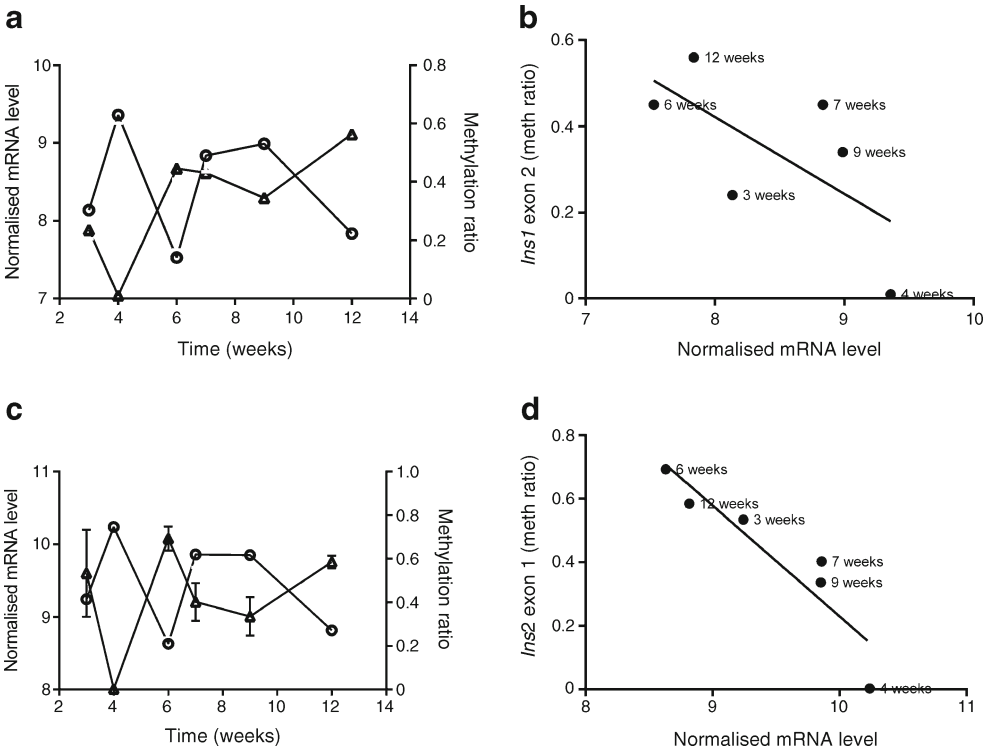

Fig. 4 Correlation between methylation of CpG sites within the insulin gene DNA and transcription in beta cells from NOD mice over time. (**a**, **c**) The average methylation status of CpG sites within *Ins1* exon 2 (**a**, triangles) and *Ins2* exon 1 (**c**, triangles) in beta cells (as described in Fig. 3), together with *Ins1* (**a**, circles) and *Ins2* (**c**, circles) mRNA levels in the same cells (as described in Fig. 2). (**b**, **d**) Correlations between the average methylation (meth) levels of *Ins1* exon 2 and *Ins2* exon 1 and the respective gene transcription Pearson's correlation: (**b**) r^2=0.43, p=0.1564; (**d**) r^2=0.85, p=0.0089. Transcription data are the means ± SEM of 3 experiments with at least 4 mice in each age group

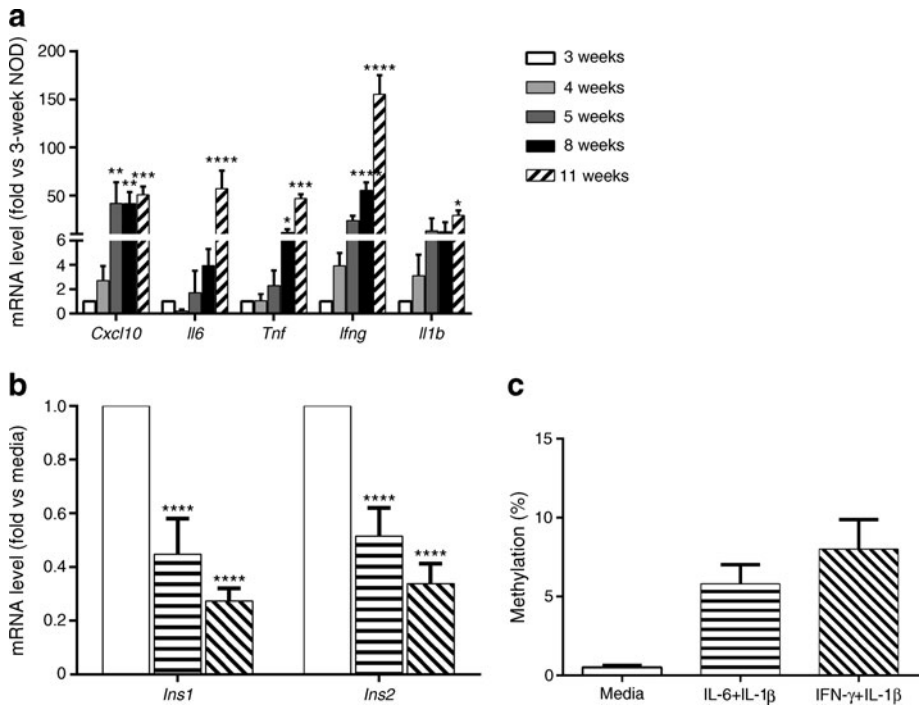

Fig. 5 Cytokine expression in islets and their effects on insulin DNA methylation. (**a**) Cytokine gene transcription in islets from NOD mice was determined by qRT-PCR and presented as fold changes relative to levels in 3-week-old NOD mice. (**b**, **c**) Islets from 4-week-old NOD mice were cultured in medium without (white bars) or with either IL-6 plus IL-1β (horizontal stripes) or IFN-γ plus IL-1β (diagonal stripes) for 48 h. (**b**) Insulin gene transcription was analysed in sorted beta cells by qRT-PCR and normalised to transcription levels in beta cells from islets cultured in medium alone. (**c**) Methylation status of *Ins1* was determined by cloning and sequencing *Ins1* exon 2 (χ^2 test, $p<0.0001$). Data are the means ± SEM of 3–4 experiments, with (**a**) 3–4 mice per time point or (**b**, **c**) 6 mice per group. (**a**, **b**) ANOVA: *$p<0.01$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$

human beta cells [22]. Our results showed reduced expression of *Ins1* and *Ins2* mRNA in beta cells cultured with IL-6 plus IL-1β or with IFN-γ plus IL-1β (Fig. 5b). Methylation of *Ins1* exon 2 was significantly higher in beta cells from islets cultured with these cytokines (χ^2 test, $p<0.0001$; Fig. 5c).

DNA methyltransferases are induced in beta cells during NOD mouse diabetes progression and by expressed cytokines To determine whether cytokines induce methyltransferases that may methylate the insulin genes, we first analysed the transcription levels of *Dnmt* genes in islets from NOD mice of different ages, in beta cells and in islet infiltrating lymphocytes sorted from 6-week-old NOD mice. The expression of *Dnmt1* increased over time in islets from NOD mice aged 3 to 11 weeks ($p<0.05$). *Dnmt3a* and *Dnmt3b* mRNA were significantly increased at 8 weeks of age and increased further at 11 weeks ($p<0.0001$; Fig. 6a). In contrast, there was no significant increase in the levels of *Dnmt3a* or *Dnmt3b* transcripts in NOD/*scid* mice over time (ESM Fig. 4).

Beta cells were responsible for changes in *Dnmt3a* and *Dnmt3b* mRNA levels but not for changes in *Dnmt1* mRNA levels because, the levels of these methyltransferase transcripts were much higher in sorted beta cells than in infiltrating lymphocytes ($p<0.0001$; *Dnmt1*, NS; *Dnmt3a*, $p<0.0001$; *Dnmt3b*, $p<0.0001$; Fig. 6b).

We also assessed the effects of cytokines on methyltransferase expression in vitro. Whole islets were cultured with cytokines for 48 h and then methyltransferase expression in sorted beta cells was measured by qRT-PCR: *Dnmt3a* expression was increased ($p<0.0001$) and there was a 1.58-fold induction of *Dnmt3b* mRNA when islets were cultured with IFN-γ plus IL-1β, but this difference was not statistically significant ($p=0.13$; Fig. 6c).

To confirm the effect of cytokines on methyltransferases induction, we silenced *Dnmt3a* in beta cells with siRNA (Fig. 6d) and compared the effects of cytokines (IFN-γ plus IL-1β) on *Ins2* exon 1 methylation and insulin gene expression to those of beta cells transfected with control siRNA. SiRNA targeting *Dnmt3a* reversed the effects of cytokines (IFN-γ plus IL-1β) on insulin DNA methylation (χ^2 test, $p<0.0001$) and gene expression ($p<0.0001$; Fig. 6d, e), indicating that islet-infiltrating cytokines could induce DNA methyltransferases and thus insulin DNA methylation, and decrease insulin gene transcription.

Cytokine induction of *DNMT* mRNA in human beta cell in vitro We next performed similar experiments to test the

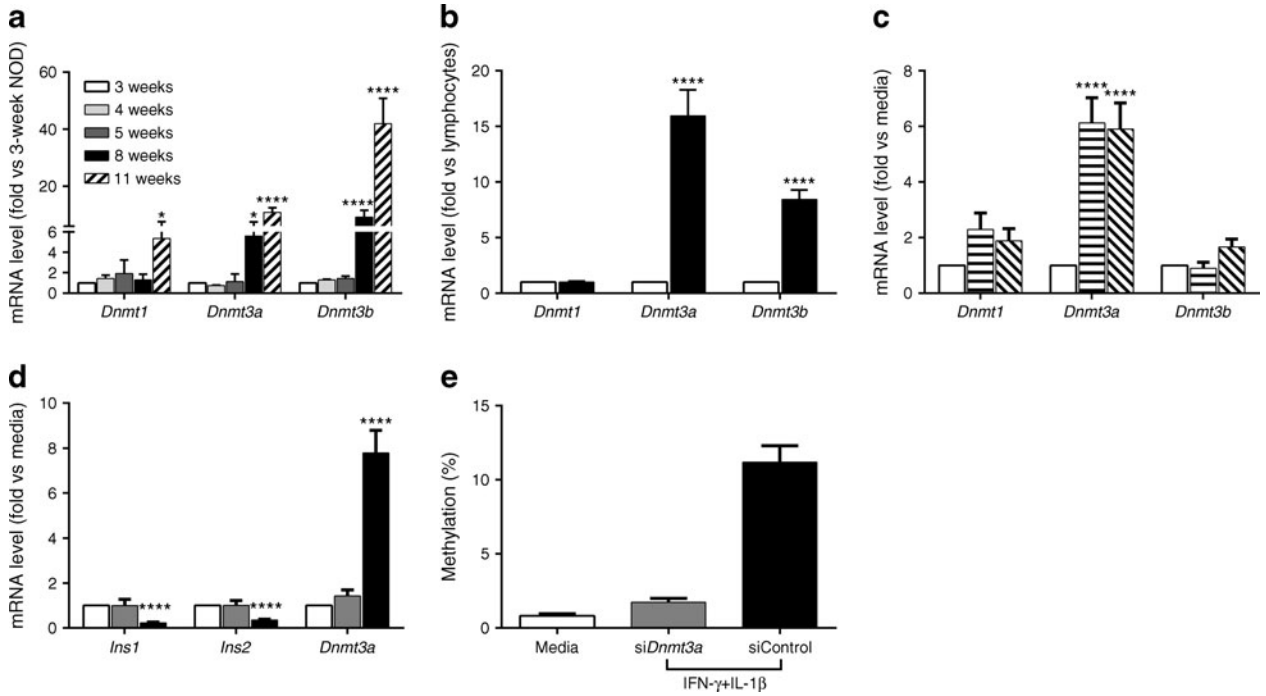

Fig. 6 Expression of *Dnmt* genes in islets and beta cells. (**a**) The transcription of several *Dnmt* genes was measured in islets from NOD mice at the indicated ages and normalised to levels in islets from 3-week-old mice. (**b**) Beta cells and islet-infiltrating lymphocytes were sorted from islets of 6-week-old NOD mice. The levels of *Dnmt* gene transcription in beta cells (black bars) was normalised to levels in islet-infiltrating lymphocytes (white bars). Data are the means ± SEM of 3 experiments, each with 4 mice. (**c**) The expression of *Dnmt* genes was determined by qRT-PCR in beta cells from islets cultured in IL-6 plus IL-1β (horizontal stripes) or IFN-γ plus IL-1β (diagonal stripes). (**d**, **e**) Islets from 4-week-old NOD mice were transfected with siRNA against *Dnmt3a* (grey bars) or scrambled control siRNA (siControl; black bars) 24 h prior to cytokine treatment with IFN-γ plus IL-1β for 48 h. Levels of *Ins1* and *Ins2* expression and *Dnmt3a* mRNA in beta cells were then analysed by qRT-PCR. (**d**) Methylation of CpG sites in *Ins2* exon 1 was determined by Sanger sequencing. (**e**) χ^2 test, $p<0.0001$. (**a**–**d**) ANOVA with Tukey's multiple comparison tests: *$p<0.05$, ****$p<0.0001$. (**c**, **d**) Data were normalised to beta cells from islets cultured in control medium. (**d**, **e**) Data are the means ± SEM from three experiments, each with eight mice

effects of cytokines on the expression of *DNMT* genes in human islets. We cultured islets from a non-diabetic donor with a cytokine cocktail (TNF-α, IFN-γ plus IL-1β) and measured the expression of *DNMT* genes by qRT-PCR. Both *DNMT1* ($p<0.0001$) and *DNMT3A* ($p<0.0001$) mRNA levels were increased, while *INS* gene transcription ($p<0.01$) was decreased with this treatment (Fig. 7).

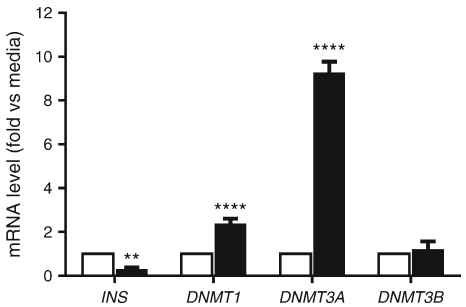

Fig. 7 Expression of *DNMT* and *INS* genes in human beta cells. Human islets were cultured in medium without (white bars) or with TNF-α plus IFN-γ plus IL-1β (black bars) for 48 h and then gene transcription was analysed, as for mouse islets. Data are the means ± SEM from 4 experiments, each with a single islet donor. ANOVA: **$p<0.01$, ****$p<0.0001$

Discussion

We studied epigenetic and functional changes in the insulin genes during progression of diabetes in NOD mice. There is strong clinical evidence that both functional changes and loss of beta cell mass due to killing occur during the development of type 1 diabetes [23, 24]. Our studies showed a quantitative increase in infiltrating lymphocytes during diabetes progression and a corresponding decline in both the number and function of beta cells. Earlier studies from our and other groups in beta cells from BALB/c mice showed that CpG sites within the insulin genes were unmethylated, but these initial studies did not examine how the autoimmune response might change the epigenetic signature of the genes [5, 25]. We analysed the methylation patterns of CpG sites within the *Ins1* and *Ins*2 genes by high-throughput sequencing and found changes in the methylation patterns of *Ins1* exon 2 and *Ins2* exon 1 that were inversely associated with gene transcription. We then addressed the basis of these changes, focusing initially on the soluble mediators found in islets during disease progression because these factors seemed most likely to account for the epigenetic changes. We found that IL-1β combined

with IFN-γ or IL-6 reduced insulin gene transcription and induced methylation of CpG sites within a representative exon. This treatment also increased expression of the DNA methyltransferase genes, *Dnmt3a* and *Dnmt3b*. The expression of cytokines may be related to the decrease in insulin gene transcription and increase in insulin DNA methylation that we identified at age 6 weeks. Our studies have therefore identified a novel mechanism that may operate during progression of autoimmune diabetes and account for the functional changes that occur in beta cells.

A number of environmental factors may affect gene methylation, particularly in the promoter region. Studies from other experimental settings have suggested that insulin promoter DNA methylation correlates negatively with insulin gene expression in human pancreatic islets [5, 26]. Interestingly, we did not find a relationship between the methylation of CpG sites within the *Ins2* promoter and *Ins2* gene transcription in beta cells during diabetes progression in NOD mice. It is important to note that our findings were from primary beta cells from normoglycaemic NOD mice, indicating that changes in the glucose level were not responsible for the methylation changes. Brenet et al reported that methylation of DNA downstream of the transcription start site was more tightly linked to transcriptional silencing compared with methylation in the upstream promoter region [27]. In addition to methylation, other epigenetic mechanisms such as histone modifications may be involved in the control of insulin gene transcription [28].

A significant finding of our studies is that inflammatory cytokines found in the islets during diabetes progression could induce methylation of CpG sites in the insulin genes as a result of increased DNMT expression. Obesity-induced, proinflammatory cytokines were reported to promote *Dnmt1* expression [29]. IL-6 was reported to induce DNMT1 activity and mediates *SOCS3* promoter hypermethylation in ulcerative colitis-related colorectal cancer [30, 31]. Likewise, TGF-β was reported to induce both the expression and activity of DNMT1, DNMT3A and DNMT3B; DNMT3A was shown to be required for acquisition of beta cell function in human and rodent cells [6, 32]. Our ex vivo studies of beta cells from NOD mice showed increased expression of *Dnmt3a* and *Dnmt3b* during disease progression, but our in vitro studies showed a significant increase in only *Dnmt3a* after cytokine addition. Most likely, the difference between these findings is due to the limited number of cytokines added to the cultured cells. There are likely to be many more mediators in the islets during disease progression that may affect the expression of DNA methyltransferases.

The similar changes seen in *DNMT* genes in human islets exposed to cytokines suggest that epigenetic changes may also occur in beta cells during progression of type 1 diabetes. We do not know whether these changes occur in vivo or whether they account for the transient changes in beta cell function seen in individuals at risk of developing type 1 diabetes. However, our studies have identified a potential limitation of analysing beta cell killing via measuring the levels of unmethylated insulin DNA in the circulation. Assays from our laboratory and those of others have relied on detecting unmethylated CpG sites in insulin DNA released from dying beta cells [5, 25, 33, 34]. However, methylation of CpG sites could alter detection of beta cell derived DNA in the serum.

In summary, we have shown methylation marks in the *Ins1* and *Ins2* genes that change during diabetes progression in NOD mice, and that these methylation marks appear to be caused by DNMTs that can be induced by cytokines. Our studies suggest that anti-inflammatory cytokine therapies may alter beta cell function even if they cannot curtail the ongoing cytolytic response that leads to the ultimate loss of beta cell mass.

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Duality of interest statement KCH has a patent application for an assay to detect beta cell killing in vivo. All other authors declare that there is no duality of interest associated with their contribution to this manuscript.

Contribution statement JL, JR, KCH, PLC, SD and SU-B conceived and designed the study; JL, JR, PLC and SD acquired the data; JR and KCH analysed the data; JL, JR, KCH, PLC, SD interpreted the data; JR and KCH drafted the manuscript; JL, PLC, SD and SU-B reviewed the manuscript; and SU-B revised it critically for important intellectual content. All authors revised the article and approved the final vision to be published. KCH is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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