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ARTICLE

# Transgenic expression of murine chemokine decoy receptor *D6* by islets reveals the role of inflammatory CC chemokines in the development of autoimmune diabetes in NOD mice

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**Abstract**
*Aims/hypothesis* Autoimmune diabetes results from a progressive destruction of insulin-producing beta cells in the pancreatic islets by chemokine-attracted lymphocytes. Because islet cells in NOD mice produce chemokines during the development of autoimmune diabetes, we investigated the role of inflammatory CC chemokines in disease progression in these mice.

*Methods* We generated a transgenic NOD mouse model that overproduces the inflammatory CC chemokine decoy receptor D6 in pancreatic islets.

*Results* The frequency of diabetes and insulitis scores of transgenic mice were decreased significantly, compared with non-transgenic control littermates. Transgenic expression of *D6* (also known as *Ccbp2*) did not affect systemic lymphocyte development or alter: (1) the T cell subsets

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such as T helper (Th)1, Th2 and T regulatory cells; or (2) antigen-presenting cells such as dendritic cells or macrophages. The percentages and numbers of T and B lymphocytes were decreased significantly in the pancreas. Activation status, autoantigen-specific proliferation and diabetogenicity of lymphocytes were also markedly reduced.

*Conclusions/interpretation* Inflammatory CC chemokines play a critical role in the development of autoimmune diabetes. Transgenic expression of *D6* in pancreatic islets of NOD mice reduced this pathogenic process by suppressing activation of autoreactive lymphocytes and by reducing migration of lymphocytes to the pancreas.

**Keywords** Chemokine · D6 · NOD mice · Type 1 diabetes

**Abbreviations**

| | |
|---|---|
| CCL | CC chemokine ligand |
| CCR | CC chemokine receptor |
| FOXP3 | Forkhead box P3 |
| GFP | Green fluorescent protein |
| hThy1 | Human Thy-1 cell surface antigen |
| m*D6* | Murine *D6* |
| mThy1.1 | Mouse thymus cell antigen 1, theta |
| PIL | Pancreas-infiltrating lymphocytes |
| PLN | Pancreatic lymph nodes |
| p*INS*-m*D6* | Insulin promoter-driven murine *D6* construct |
| SCID | Severe combined immunodeficient |
| Th | T helper |
| Treg | T regulatory |

## Introduction

Type 1 diabetes mellitus results from the destruction of insulin-producing beta cells in the islets of the pancreas and has been identified as a T cell-mediated autoimmune disease [1]. The development of type 1 diabetes is usually diagnosed in young patients, so this disease is also referred to as juvenile- or childhood-onset diabetes. The NOD mouse spontaneously develops T cell-dependent beta cell destruction that resembles human type 1 diabetes and serves as an animal model of this autoimmune disease [2].

Chemokines are small heparin-binding proteins that induce the migration of circulating leucocytes to sites of inflammation or injury. They include the CC, CXC, $CX_3C$ and XC chemokine families [3]. The CC chemokine ligand (CCL) family is the largest group, members of which can be defined as homeostatic chemokines or inflammatory chemokines, depending on their biological functions. Inflammatory CCLs include CCL1 to CCL5; their main function is to attract mononuclear cells to sites of chronic inflammation [4].

The pathogenesis of type 1 diabetes involves the infiltration of immune cells into the pancreas, a process dependent on the attraction of these cells by chemokines. Islet cells in NOD mice have been reported to produce high amounts of CCL2 and CCL5 compared with BALB/c mice [5, 6]. Moreover, CC chemokine receptor (CCR)4-related chemokines such as CCL17 and CCL22 are involved in the pathogenesis of autoimmune diabetes in NOD mice [7]. CCL5 is produced on the islets of NOD mice and neutralisation of CCL5 by an anti-CCR5 antibody decreases the severity of insulitis and prevents islet destruction [8]. On the other hand, Grewal et al. demonstrated that overproduction of CCL2 on the beta cells of C57BL/6×C3H mice leads to insulitis [9]. Transgenic expression of *CCL2* by beta cells led to insulitis and islet destruction, and promoted the development of diabetes in C57BL/6×DBA mice [10]. Furthermore, beta cell-specific production of a pan-chemokine blocking protein, M3, which was originally encoded in the genome of murine γ–herpesvirus 68, inhibited the migration of lymphocytes to islets and completely prevented the onset of diabetes in NOD mice [11]. Collectively, these results indicate that chemokines participate in the pathogenesis of autoimmune diabetes and that modulation of these chemokines affects disease progression and severity. However, the physiological roles of individual chemokine families in autoimmune diabetes have not been fully elucidated.

D6 is an inflammatory CC chemokine decoy receptor that exhibits a 7-transmembrane-domain similar to that of conventional chemokine receptors [12, 13], yet lacks the ability for intracellular signal transduction. It is mainly produced in the placenta and on the endothelial cells of lymphatic afferent vessels in the skin, gut and lung [14]. D6 binds to a broad range of inflammatory CC chemokines, including most ligands for CCR1 to CCR5 [15], and targets them for intracellular degradation [16–20]. Strikingly, D6-deficient mice displayed delayed clearance of cutaneous inflammatory CC chemokines and developed a pathological condition in skin that resembled human psoriasis, indicating that D6 is involved in resolution of the cutaneous inflammatory response [21]. Another study has also demonstrated an increased inflammatory response in the skin of D6-deficient mice [22]. That study also demonstrated that the amount of CCL2 and the numbers of lymphocytes in skin-draining lymph nodes were increased in D6-deficient mice, supporting a critical role of D6 in the control of lymphocyte migration in lymph nodes [22].

To investigate the potential role of inflammatory CC chemokines in the pathogenesis of autoimmune diabetes, we generated a transgenic NOD mouse that expresses *D6* (also known as *Ccbp2*) specifically on pancreatic beta cells. By analysing the process of diabetes and the status of immune responses, we demonstrated that inflammatory CC chemokines played a critical role in the development of autoimmune diabetes in this animal model, Moreover, transgenic expression of *D6* in pancreatic islets was able

to effectively reduce this pathogenic process by suppressing the activation of autoreactive lymphocytes and reducing migration of lymphocytes to the pancreas.

## Methods

*Animals* Inbred NOD/Sytwu mice ($K^d$, $D^b$, $L^d$, I-$A^{g7}$) and NOD/severe combined immunodeficient (SCID) mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and subsequently bred at the animal centre of the National Defense Medical Center in Taipei, Taiwan, under specific pathogen-free conditions. T1/T2 double transgenic NOD mice were generated by back-crossing T1/T2 double transgenic BALB/c mice to NOD mice for more than 12 generations, as described [23]. Experiments were conducted in accordance with institutional guidelines and were approved by National Defense Medical Center's Institutional Animal Care and Use Committee.

*Real-time RT–PCR* Total RNA was prepared from splenocytes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then reverse-transcribed by SuperScript III Reverse Transcriptase (Invitrogen). Real-time RT-PCR was performed by iQ SYBR Green PCR supermix (Bio-Rad, Hercules, CA, USA) in an iCycler (Bio-Rad). Fold changes were measured using the $\Delta\Delta C_t$ method. The sequences of primers used in this experiment are shown in electronic supplementary material (ESM) Table 1.

*Generation of murine D6 transgenic NOD mice* The *D6* gene of the NOD mouse was cloned from genomic DNA of mouse tail prepared by phenol–chloroform extraction and amplified by PCR. The PCR products were cloned into p*INS* plasmid for the generation of transgenic mice [24]. Murine *D6* (m*D6*) DNA was inserted into the p*INS* plasmid to generate an insulin promoter-driven murine *D6* construct (p*INS*-m*D6*). The linearised DNA fragment carrying p*INS* and *D6* DNA was purified and microinjected into the pronuclei of NOD embryos at the one-cell stage, then implanted into pseudopregnant (BALB/c×FVB) F1 females. Southern blotting was performed to confirm the presence of the p*INS*-m*D6* transgene. All transgenic mice used in our study were heterozygous for the *D6* transgene.

*Western blot* Protein samples were separated on 10% (wt/vol.) SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The presence of D6 and β-actin on the membrane was detected with anti-D6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-β-actin antibody (Sigma-Aldrich, St Louis, MO, USA), respectively. The signals were detected by an imaging system (LAS-3000; Fujifilm, Tokyo, Japan).

*Assessment of insulitis and diabetes* Pancreatic tissues were obtained from 14-week-old transgenic or non-transgenic mice and the severity of insulitis was scored on haematoxylin–eosin-stained sections and classified as described [25]. Urine glucose concentrations (glycosuria) were measured weekly or every other day using Chemstrips (Boehringer Mannheim, Indianapolis, IN, USA). Mice with urine glucose concentration >27.75 mmol/l on two consecutive tests were defined as diabetic.

*T cell proliferation* Splenocytes were isolated from the spleen of 8-week-old non-transgenic or m*D6* transgenic mice. T cell proliferation was performed as described [26, 27]. Splenocytes were treated with TRIS-buffered ammonium chloride to lyse the erythrocytes. After washing, splenocytes were resuspended at $5\times10^6$ cells/ml in RPMI 1640 medium. Cells ($5\times10^5$ cells/well) were stimulated with an immobilised anti-CD3 antibody (BD Biosciences, Pharmingen, San Diego, CA, USA), concanavalin A or islet antigens. The islet antigens were prepared by two cycles of freeze and thaw on islet cells isolated from young male NOD mice (less than 7 weeks old) and dissociated by cell dissociation buffer (Invitrogen). Lysed islet cells ($5\times10^4$) were added to each well. The incorporation of methyl-[$^3$H] thymidine was detected at 72 h with a liquid scintillation counter (TopCount; Packard Instruments, Meriden, CT, USA).

*Flow cytometry* Lymphocytes were taken from the spleen, pancreatic lymph nodes (PLN) or pancreas of m*D6* transgenic or non-transgenic mice. Isolated cells were stained with antibodies to mouse CD4, CD8, CD19, CD25, CD11b, CD11c, CD80, CD86 and forkhead box P3 (FOXP3) (BD Biosciences), and to human CD90 (hThy1, clone 5E10) and mouse CD90.1 (mThy1.1, clone OX-7) (BD Biosciences). Flow cytometry was performed using a FACSCalibur (BD Biosciences).

*Adoptive transfer* Splenocytes of female m*D6* transgenic or non-transgenic NOD donors were treated with TRIS-buffered ammonium chloride for erythrocyte depletion and $2\times10^7$ cells were injected into female NOD/SCID mice via the retro-orbital plexus. In lymphocyte transfer-induced diabetes experiments, 6-week-old female m*D6* transgenic mice or their non-transgenic control littermates were selected as recipients and injected with $2\times10^7$ splenocytes taken from 14-week-old female NOD donors using the procedure described above. Diabetes was assessed as described earlier.

*Isolation and analysis of pancreas-infiltrating lymphocytes* Pancreas-infiltrating lymphocytes (PIL) were isolated as follows. Collagenase XI (Sigma-Aldrich) was injected into the common bile duct of 14-week-old m*D6* transgenic or non-transgenic mice and the pancreases were isolated. After

washing with RPMI-1640 medium, islets and lymphocytes in the pancreas were separated by a density gradient using Histopaque 1077–1 (Sigma-Aldrich). The cells separating into the density gradient interface were collected and incubated for 1 to 2 min with 1 ml cell dissociation buffer (GIBCO Invitrogen) at room temperature. After passing them through a cell strainer, suspended single cells were counted and stained with antibodies for flow cytometry.

*Statistics* Data are presented as the mean±SD or mean± SEM. The significance of any difference in the frequency of diabetes was determined by Kaplan–Meier survival analysis. For the remaining experiments, $p$ values were calculated using two-tailed Student's $t$ tests. Differences were considered significant at $p<0.05$.

## Results

*Chemokine expression profiles in the islets of NOD mice and the generation of mD6 transgenic NOD mice* To assess the expression kinetics of chemokines in the islets of NOD mice, we used real-time RT-PCR to measure the expression of chemokines in isolated islets from NOD mice at different ages and compare it with that in islets from 12-week-old Balb/c mice. Our results indicate that NOD islets express higher levels, but diverse kinetics, of chemokines, including several members of inflammatory CC chemokines, compared with Balb/c islets (ESM Fig. 1). To investigate the potential of an organ-specific murine *D6* transgene in the prevention of autoimmune diabetes, we used a p*INS*-m*D6* construct to create a transgenic NOD mouse model expressing the transgene in a beta cell-specific manner. The p*INS*-m*D6* construct contains the insulin promoter, the first and part of the second non-coding exons of insulin gene, an intron from rabbit β-globin, mouse *D6* DNA and a poly-A signal from rabbit β-globin (Fig. 1a). Transgenic positive m*D6* NOD mice can be distinguished easily from their non-transgenic littermates by PCR genotyping with P1 and P2 primers (Fig. 1b). Southern blot analysis of tail genomic DNA indicated that the founder mouse carried five to ten copies of the transgene (Fig. 1c). To determine the level of transcription of the *D6* transgene specifically, we performed RT-PCR using the P1 and P2 primers. Except for thymus and pancreas, none of the organs tested (lung, heart, liver, spleen and intestine) from transgenic mice transcribed RNA of transgenic m*D6* (Fig. 1d). A trace of transgenic *D6* expression in the thymus could be caused by the effect of the autoimmune regulator (Aire) [28]. These results

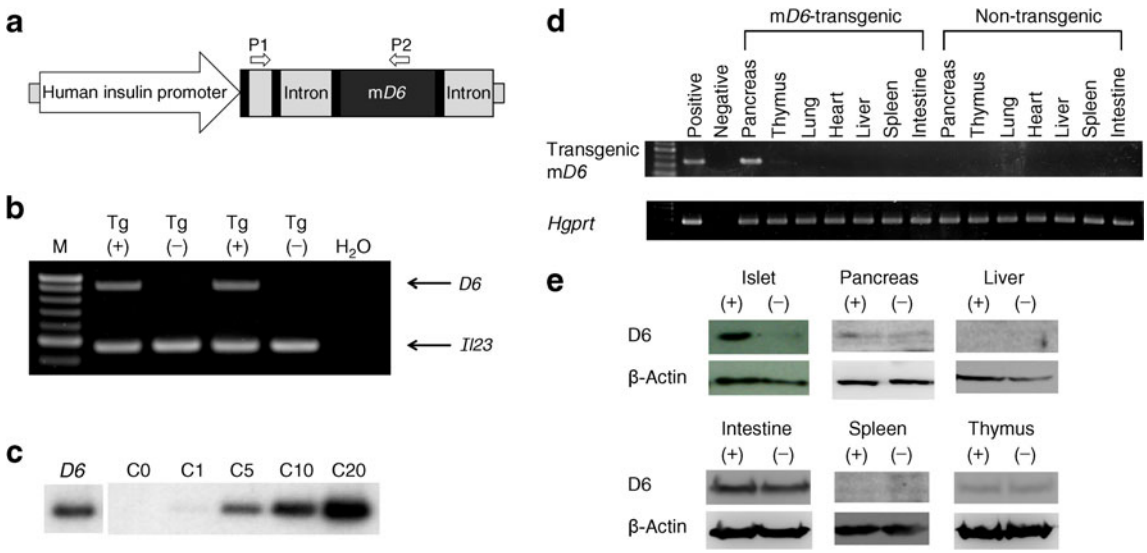


**Fig. 1** Generation of m*D6* transgenic NOD mice. **a** Scheme of the transgene construct. The black areas represent exons and the grey areas represent introns. A forward primer (P1) targeting the second exon of the insulin gene and a backward primer (P2) targeting the coding region of m*D6* were designed to specifically evaluate p*INS*-m*D6* transcription. **b** Genomic DNA was extracted from the tails of m*D6* transgenic (Tg) or non-transgenic mice. The extracted genomic DNA was then amplified by PCR using primers P1 and P2. *D6* indicates the product of the transgene of m*D6*. The *Il23* (also known as *Il23a*) gene was amplified as an internal control for genomic DNA PCR. **c** Southern blot analysis of the transgenic D6 signal in m*D6* transgenic founders is denoted as *D6*. The standard C0 to C20 was prepared by different copy numbers of the linearised p*INS*-m*D6* plasmid, which was used in the microinjection for generation of m*D6* transgenic NOD mice. Southern blot analysis revealed that there were five to ten copies of the transgene in the genome of the p*INS*-m*D6* transgenic founder mouse. **d** Tissue-specific expression by m*D6* transgenic NOD mice. Total RNA purified from various organs of m*D6* transgenic and non-transgenic mice was reverse -transcribed to cDNA using oligo-dT primers and further amplified by PCR using primers P1 and P2 for transgenic m*D6* expression. *Hgprt* (also known as *Hprt*) was used as an internal control. **e** Test organs were prepared from m*D6* transgenic and non-transgenic mice and the abundance of D6 protein was measured by western blotting. β-Actin was used as a protein loading control

demonstrated that m*D6* transgenic NOD mice specifically expressed transgenic *D6* in the pancreas, indicating the high stringency of the insulin promoter. Western blot analysis also confirmed overproduction of D6 at the protein level in islets from m*D6* transgenic NOD mice (Fig. 1e).

To evaluate the neutralisation effects of transgenic *D6* on islets, we detected the amount of two D6-binding chemokines (CCL2 and CCL5) and one D6-non-binding chemokine (CXCL10) as control. The amount of CCL2 and CCL5 was decreased in the islets of m*D6* transgenic mice, compared with non-transgenic littermates. In contrast, the level of CXCL10 was similar in islets between m*D6* transgenic and non-transgenic mice (ESM Fig. 2). These data suggest that overproduction of D6 by islets specifically decreased the amount of inflammatory CC chemokines such as CCL2 and CCL5 in the islets of NOD mice.

*Expression of D6 in NOD islets reduced the severity of insulitis and development of diabetes* To investigate the protective effects of organ-specific and membrane-bound expression of transgenic *D6* on autoimmune diabetes, we analysed the incidence of spontaneous diabetes and the severity of insulitis in m*D6* transgenic mice. The m*D6* transgenic NOD mice were significantly prevented from developing autoimmune diabetes compared with non-transgenic littermates ($p<0.01$; Fig. 2a), demonstrating islet-specific protection of transgenic *D6* in NOD mice. Furthermore, the severity of insulitis in m*D6* transgenic mice was significantly reduced (Fig. 2b). To determine whether the *D6* transgene prevents islet inflammation, we compared the insulitis scores of transgenic and control mice at 8, 14 and 20 weeks. The severity of insulitis was reduced significantly at all ages examined (Fig. 2c). These results indicate reduced infiltration of lymphocytes to pancreatic islets in m*D6* transgenic mice.

*Immune cell development in mD6 transgenic NOD mice* To investigate whether overexpression of D6 on pancreatic beta cells would alter the development of lymphocytes, we analysed the populations of lymphocytes taken from spleen, PLN or pancreas using flow cytometry. There were no significant differences between m*D6* transgenic and non-transgenic mice in the populations of $CD4^+$, $CD8^+$ T or $CD19^+$ B cells in spleen, PLN or pancreas (Fig. 3a–c).

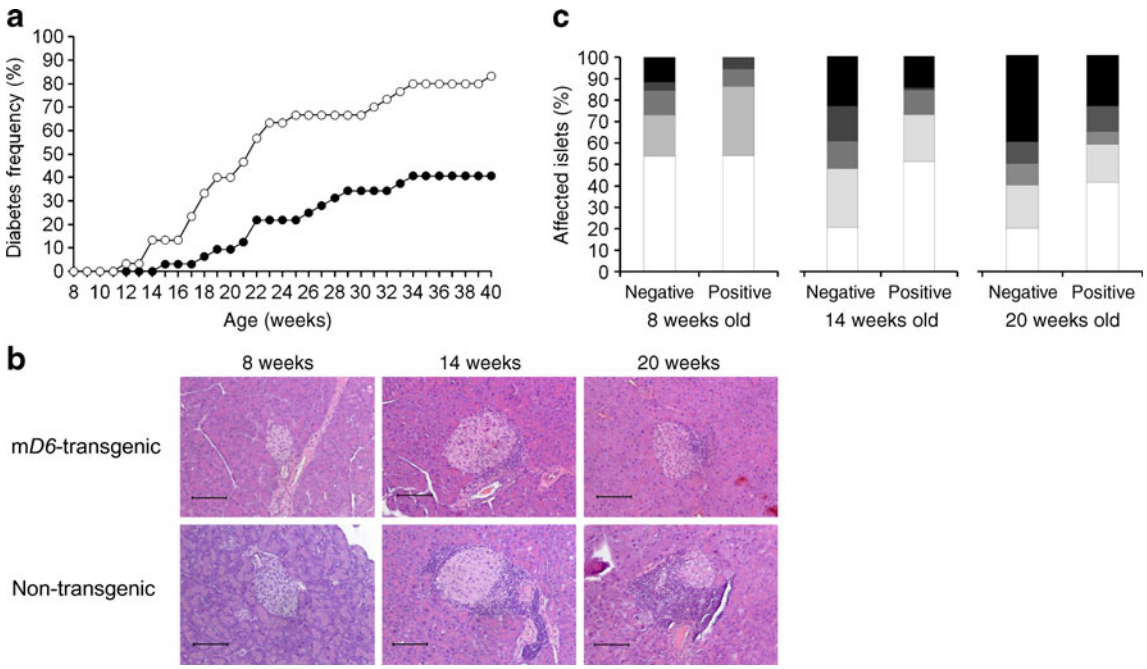


**Fig. 2** Characterisation of the diabetogenic process in m*D6* transgenic NOD mice. **a** Spontaneous diabetic frequency of female m*D6* transgenic NOD mice. Spontaneous diabetes in female m*D6* transgenic NOD mice (black circles, $n=32$) or their control littermates (white circles, $n=30$) was monitored by weekly measurement of glycosuria. Compared with non-transgenic control littermates, which started to develop diabetes at 12 weeks of age, m*D6* transgenic mice first developed diabetes after 15 weeks, indicating a delay in disease onset. At 20 weeks of age, the incidence of diabetes in control mice increased to 40%, but the incidence in m*D6* transgenic mice was only 9%. After 25 weeks, more than 66% of non-transgenic mice had developed diabetes, but the incidence in m*D6* transgenic mice was still only around 22%. **b** Histopathology of the severity of insulitis in m*D6* transgenic NOD mice. Pancreases prepared from m*D6* transgenic or non-transgenic mice of different ages as labelled were fixed and embedded for histopathology using haematoxylin and eosin staining. Scale bars 100 μm. **c** The severity of insulitis was examined on haematoxylin and eosin-stained sections of pancreas from m*D6* transgenic or non-transgenic NOD mice at the indicated ages. Black, destructive insulitis; dark grey, severe insulitis; medium grey, intra-insulitis; light grey, peri-insulitis; white grey, intact islets

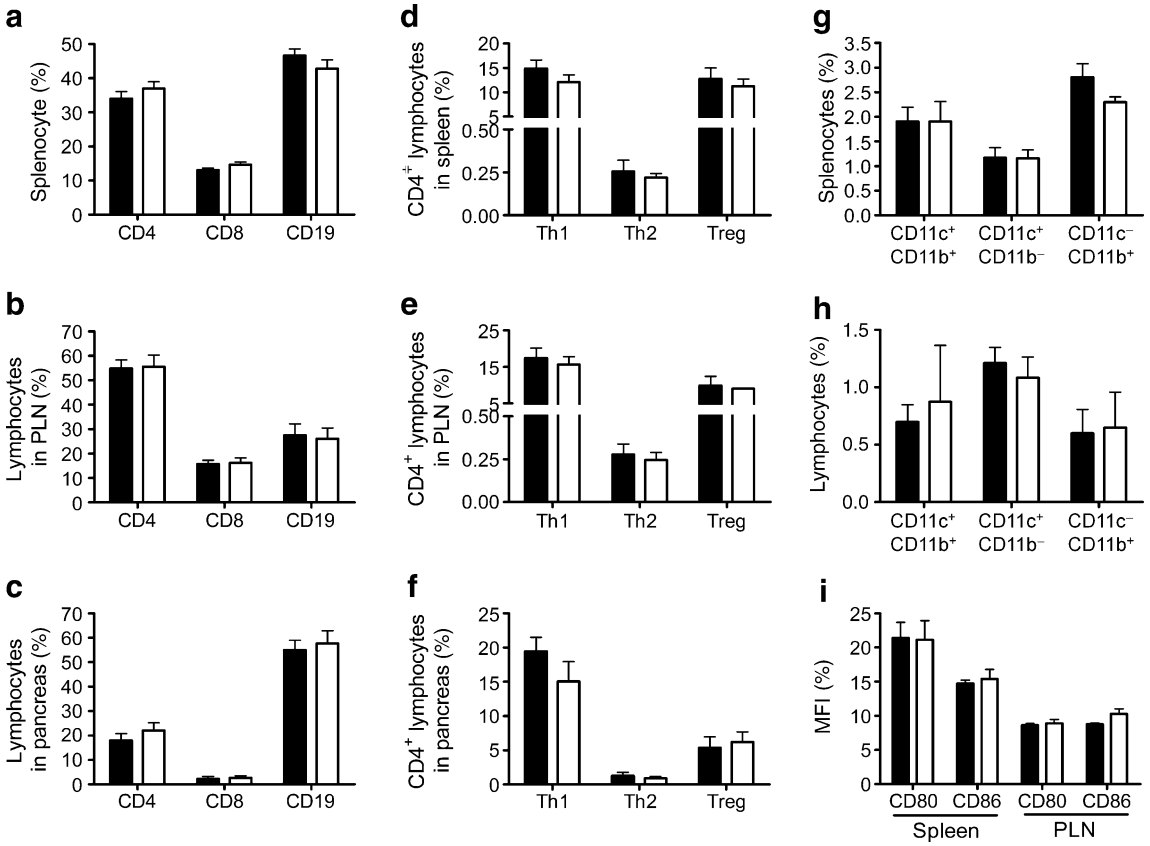


**Fig. 3** Immune cell development in m*D6* transgenic NOD mice. **a** Composition of lymphocytes in the spleen ($n$=5), (**b**) PLN ($n$=4) and (**c**) PIL ($n$=5) from 8-week-old m*D6* transgenic mice (black bars) and non-transgenic controls (white bars) was analysed by flow cytometry. **d** The percentages of Th1 ($CD4^+hThy1^+$), Th2 ($CD4^+mThy1.1^+$) and Treg cells in the spleen ($n$=4) and (**e**) PLN ($n$=4) from 8-week-old m*D6*/T1/T2 triple transgenic (black bars) or T1/T2 double transgenic (white bars) NOD mice were analysed by flow cytometry. **f** The percentages of Th1 ($CD4^+IFN\text{-}\gamma^+$), Th2 ($CD4^+IL\text{-}4^+$) and Treg lymphocytes in pancreatic tissues of m*D6* transgenic (black bars) or non-transgenic (white bars) mice were subjected to intracellular cytokine staining and analysed by flow cytometry ($n$=4). **g** Composition of CD11b- or CD11c-positive cells in the spleen or (**h**) PLN of m*D6* transgenic (black bars, $n$=3) or non-transgenic (white bars, $n$=3) mice was analysed by flow cytometry. **i** The abundance of CD80 or CD86 on $CD11c^+$ dendritic cells in the spleen or PLN of m*D6* transgenic (black bars, $n$=3) or non-transgenic (white bars, $n$=3) mice was determined by mean fluorescence intensity (MFI) in flow cytometry. Data are expressed as the mean±SEM. There were no significant differences between m*D6* transgenic and non-transgenic mice

It is known that an imbalance between T helper (Th)1 and Th2 responses [29], and a relative deficiency in $CD4^+CD25^+FOXP3^+$ regulatory T (Treg) cells [30, 31] and pathological dendritic cells [32–37] predispose NOD mice to developing autoimmune diabetes. To investigate whether the protective effect of transgenic *D6* might work through regulation of lymphocyte development, we crossed m*D6* transgenic mice with T1/T2 double transgenic mice to generate m*D6*/T1/T2 triple transgenic mice. T1/T2 double transgenic mice bear two transgenes: (1) human *THY1* under control of the murine *Ifn-γ* (also known as *Ifng*) promoter; and (2) murine *Thy1.1* (also known as *Thy1*) under control of the murine *Il4* promoter. Using these mice, the kinetic development of Th1 and Th2 cells could be measured directly by detecting the presence of human Thy-1 cell surface antigen (hThy1) (a T1 marker) and mouse thymus cell antigen 1, theta (mThy1.1) (a T2 marker), respectively. The percentages of IFN-γ-producing ($hThy1^+$), IL-4-producing ($mThy1.1^+$) or Treg cells in spleen, PLN and pancreas (Fig. 3d–f) were similar between m*D6*/T1/T2 triple (black bar) and T1/T2 double (white bar) transgenic mice, suggesting that overexpression of transgenic m*D6* by islets did not alter systemic or local lymphocyte development in NOD mice.

To investigate whether overexpression of D6 on beta cells would affect the development of antigen-presenting cells such as macrophages and dendritic cells, we analysed the distribution of $CD11c^+$ and/or $CD11b^+$ cells in spleen and PLN by flow cytometry. There were no significant differences between m*D6* transgenic and non-transgenic mice in the populations of $CD11c^+CD11b^+$, $CD11c^+CD11b^-$ or $CD11c^-CD11b^+$ cells (Fig. 3g, h), indicating that the development of macrophages and dendritic cells was not affected by overexpression of m*D6*. We also assessed the activation status of dendritic cells by determining the levels of the co-stimulatory molecules CD80 or CD86 on

$CD11c^+$ cells. The mean fluorescence intensity values of CD80 or CD86 were similar between m*D6* transgenic mice and non-transgenic mice in spleen and PLN (Fig. 3i), indicating that overproduction of D6 by islets did not influence activation of dendritic cells in these tissues.

*Production of D6 by islets reduced infiltration and activation of lymphocytes in islets* To investigate whether overproduction of D6 by islets would impede migration of lymphocytes to the pancreas, we used flow cytometry to quantify lymphocytes among the cells isolated from the pancreas of m*D6* transgenic mice. The percentages and numbers of $CD3^+$ T cells and $CD19^+$ B cells were decreased significantly compared with the cells from non-transgenic mice (Fig. 4a, b). Thus, overproduction of the CC chemokine decoy receptor D6 by islets effectively impeded the migration of T and B cells to the pancreas.

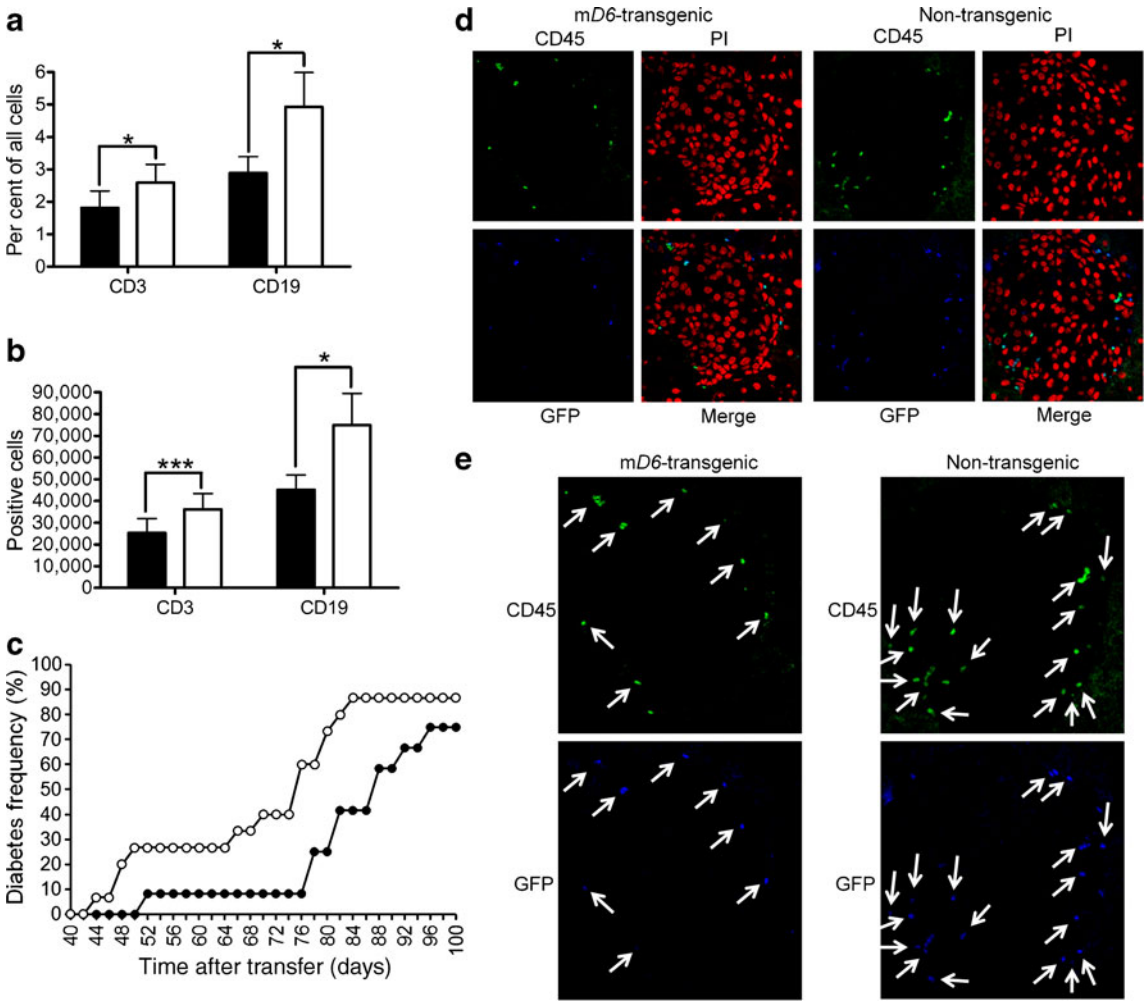


**Fig. 4** The infiltration of lymphocytes in the pancreas of m*D6* transgenic NOD mice was reduced. **a** The percentages and (**b**) cell numbers of $CD3^+$ and $CD19^+$ cells in cells isolated from pancreatic tissues of 12-week-old m*D6* transgenic (black bars) or non-transgenic (white bars) mice were analysed by flow cytometry. The proportions and cell numbers of $CD3^+$ cells ($n$=4) and $CD19^+$ cells ($n$=5) in m*D6* transgenic mice were significantly less than in non-transgenic controls. Data are expressed as the mean±SEM; *$p$<0.05 and ***$p$<0.001. **c** Characterisation of lymphocyte transfer-induced diabetes progression in m*D6* transgenic NOD mice. Female 6-week-old m*D6* transgenic and non-transgenic mice were injected intravenously with $2\times10^7$ splenocytes isolated from 14-week-old female NOD mice. The incidence of diabetes in m*D6* transgenic (black circles, $n$=12) and non-transgenic (white circles, $n$=15) recipients was monitored by testing glycosuria every other day. The incidence was significantly lower in m*D6* transgenic mice ($p$<0.05). **d** GFP-positive splenocytes were transferred to m*D6* transgenic NOD mice and their non-transgenic control littermates. The infiltration of transferred splenocytes in the islets at day 65 was assessed by immunofluorescence assay. Pancreas tissues paraffin-embedded sections were rehydrated and heated in 0.1 mol/l citrate buffer. Sections were probed with with Cy5-conjugated anti-GFP antibody (blue spots) and FITC-conjugated anti-CD45 antibody (green spots). Propidium iodide (PI) was used for nuclear counterstain (red spots). Images were captured on a Zeiss confocal LSM510 microscope (Zeiss, Thornwood, NY, USA). **e** Images showing the signals of CD45 or GFP were magnified. Double-positive cells are indicated by white arrows

To further investigate whether D6 overproduction in situ would protect mice from diabetes by reducing the infiltration of pathogenic lymphocytes to islets, we performed experiments on lymphocyte transfer-induced diabetes. About 40% of the non-transgenic mice developed diabetes 70 days after transfer; however, more than 90% the m*D6* transgenic recipients remained diabetes-free (Fig. 4c). About 90% of non-transgenic mice became diabetic by 84 days after transfer, but only 41% of transgenic recipients were diabetic, suggesting that islet-specific production of D6 not only delayed, but also reduced the infiltration of pathogenic lymphocytes to islets. To examine whether the inhibition of transferred diabetes in m*D6* transgenic recipients resulted mainly from delayed migration of transferred splenocytes, we performed the lymphocyte transfer-induced diabetes experiment by transferring splenocytes positive for green fluorescent protein (GFP) into m*D6* transgenic or non-transgenic NOD mice. The GFP-positive splenocytes were isolated from GFP-transgenic NOD mice in which transgenic GFP was driven by the ubiquitin promoter. We assessed the infiltration of transferred splenocytes in islets at day 65 after transfer, using immunofluorescence assay. We found that most CD45-positive cells in islets are GFP-positive, indicating that the development of diabetes in recipients was mainly due to the transferred splenocytes (Fig. 4d, e). We also observed that infiltrated immune cells in m*D6* transgenic recipients were much less numerous than in non-transgenic recipients, suggesting that overexpression of D6 on islets inhibited the migration of immune cells into islets.

The protective effect of the *D6* transgene in m*D6* transgenic mice is likely to result from a reduction of lymphocyte infiltration to the pancreas. To further evaluate whether this reduction of lymphocyte infiltration was caused by alteration of T cell activation status in m*D6* transgenic mice, we used flow cytometry to analyse early activation of surface marker CD69 on $CD4^+$ or $CD8^+$ T cells in the spleen, PLN and pancreas. The percentage of CD69-positive cells in $CD4^+$ lymphocytes was significantly reduced in the PLN and that of CD69-positive cells in $CD8^+$ lymphocytes was significantly reduced in the PLN and PIL of m*D6* transgenic mice (Fig. 5a, b), indicating that reduction of T cell activation in transgenic NOD mice is associated with m*D6* overexpression.

*The diabetogenicity of splenocytes was reduced in mD6 transgenic NOD mice* It is possible that the protection observed in transgenic mice was caused by modulation of the development of autoreactive T cells by transgenic expression of *D6*. We therefore performed adoptive transfer experiments and characterised the pathogenicity of T lymphocytes in m*D6* transgenic NOD mice. NOD/SCID

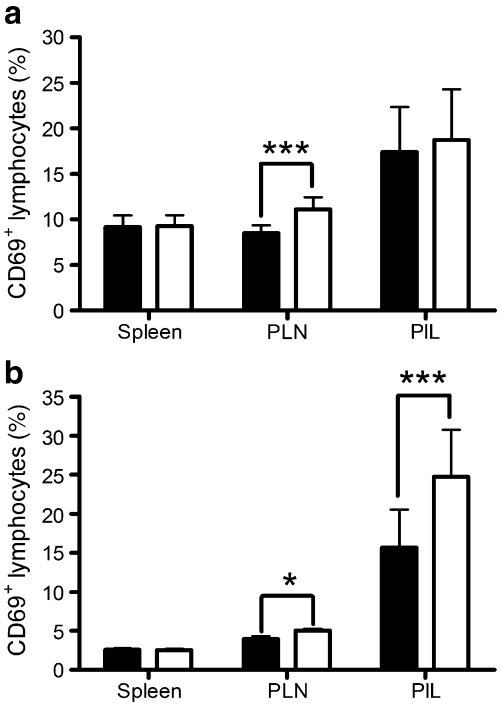


**Fig. 5** Activation of CD4 and CD8 T cells in m*D6* transgenic NOD mice. Abundance of the early activation marker, CD69, on lymphocytes from spleen, PLN and PIL of m*D6* transgenic (black bars, $n=6$) or non-transgenic (white bars, $n=6$) mice was analysed by flow cytometry. **a** The percentage of $CD4^+CD69^+$ T cells in PLN was significantly lower in the m*D6* transgenic mice. **b** The percentage of $CD8^+CD69^+$ T cells in the PLN and PIL of m*D6* transgenic mice was significantly lower than in non-transgenic mice. Data are expressed as the mean±SEM; $^*p<0.05$ and $^{***}p<0.001$

recipients were injected intravenously with splenocytes from 12-week-old transgenic or control mice and progression of diabetes in these two groups was compared. The diabetes incidence was significantly lower in recipients transferred with splenocytes from m*D6* transgenic mice (Fig. 6a). These results indicate a significant inability of splenocytes in m*D6* transgenic mice to induce diabetes and suggest a critical role of transgenic *D6* in regulating autoimmunity in NOD mice. To further characterise the proliferation potential of splenocytes in m*D6* transgenic mice, we stimulated those cells with an anti-CD3 antibody, with concanavalin A or with islet antigens in vitro. Splenocytes isolated from m*D6* transgenic and non-transgenic mice exhibited similar proliferative capacity under stimulation with anti-CD3 antibody or concanavalin A (Fig. 6b, c), indicating that the responses of lymphocytes to non-specific mitogenic stimulators were not altered in m*D6* transgenic mice. However, when stimulated with islet antigens, the proliferation of splenocytes from m*D6* transgenic mice was significantly lower than in non-transgenic splenocytes (Fig. 6d), suggesting that the reduction of splenocyte proliferation in m*D6* transgenic mice was islet antigen-specific.

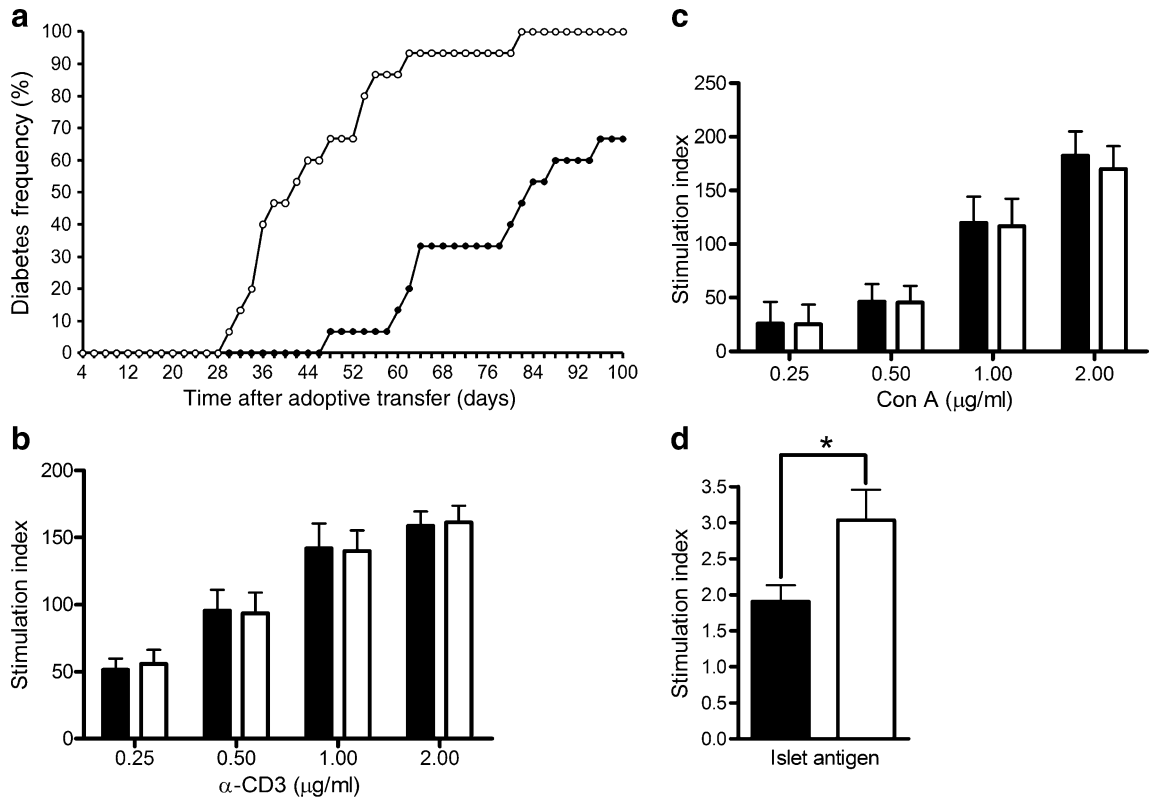


**Fig. 6** Characterisation of the pathogenicity of splenocytes in m*D6* transgenic NOD mice. **a** The incidence of diabetes in NOD/SCID recipients with splenocytes adoptively transferred from 12-week-old m*D6* transgenic (black circles, $n=15$) or non-transgenic (white circles, $n=15$) mice was assessed by testing glycosuria every other day. In recipients with splenocytes transferred from non-transgenic controls, diabetes first developed on day 30 after transfer. By contrast, in mice receiving splenocytes from m*D6* transgenic mice, the first instance of disease was on day 48. On day 82, all recipients transferred with control splenocytes became diabetic, while disease incidence in recipients with *D6* splenocytes was still only 46.6%. **b** Proliferative capacity of T cells in m*D6* transgenic NOD mice. Splenocytes isolated from m*D6* transgenic (black bars) or non-transgenic (white bars) mice were stimulated with plate-bound anti-CD3 antibody ($n=4$) or (**c**) concanavalin A (Con A, $n=5$) at the indicated dosages. **d** Proliferative response of splenocytes to islet antigens in m*D6* transgenic NOD (black bar) or non-transgenic (white bar) mice. The stimulation index of splenocytes taken from m*D6* transgenic mice was significantly reduced by stimulation with islet antigens compared with non-transgenic mice; $n=3$; $^*p<0.05$. Data are expressed as the mean± SEM

## Discussion

Chemokines have been reported to be involved in the development of autoimmune diabetes. Martin et al. demonstrated that blockade of chemokines from their ligands by transgenic overproduction of the chemokine-blocking protein M3 in beta cells protected NOD mice from developing diabetes [11]. However, the M3 protein, originally encoded in the genome of murine γ-herpesvirus 68, blocks interactions between almost all chemokines and their corresponding receptors; therefore, the physiological role of individual chemokine family members in the pathogenesis of autoimmune diabetes in NOD mice remains to be elucidated. To investigate whether inflammatory CC chemokines might play a role in the development of autoimmune diabetes, we generated a transgenic NOD mouse model that expresses the inflammatory CC chemokine decoy receptor D6 on beta cells. This significantly reduced the infiltration of lymphocytes into the pancreas and decreased the incidence of diabetes in NOD mice, indicating that inflammatory CC chemokines play a critical role in the pathogenesis of autoimmune diabetes in NOD mice. Moreover, based on the biological function and natural existence of D6 in human beings, our results further imply a therapeutic potential for D6 manipulation in the prevention or treatment of human type 1 diabetes.

To further investigate the protective mechanism(s) mediated by transgenic overexpression of *D6* by islets, we analysed the systemic development of lymphocytes by flow cytometry. Our results indicated that the populations of $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells were not altered in the spleen, PLN or pancreas of transgenic mice compared with control littermates. The protective mechanism in *M3* transgenic mice is caused, at least in part, by a reduction of $CD11b^+CD11c^+$ dendritic cells in the PLN and islets. However, the percentages of dendritic cell subsets in

spleen or PLN between m*D6* transgenic and non-transgenic mice were similar, suggesting that transgenic *D6*-mediated protection is not likely to act through direct modulation of dendritic cell development. Our results also show that levels of the maturation markers CD80 and CD86 on dendritic cells were not altered in m*D6* transgenic mice. Instead, the infiltration of $CD3^+$ lymphocytes and $CD19^+$ B cells in the pancreas were significantly reduced in m*D6* transgenic mice, suggesting that CC chemokines and CXC chemokines might modulate the pathogenesis of autoimmune diabetes differentially in NOD mice.

It has been reported that D6 reduces the local gradient of inflammatory CC chemokines at sites of inflammation [15, 16] and thus plays an important role in the resolution of pathological inflammatory conditions [38]. To further investigate whether overexpression of inflammatory CC chemokine decoy receptor D6 on beta cells would impede the migration of pathogenic lymphocytes to islets, we evaluated the lymphocyte transfer-mediated induction of diabetes. Our results demonstrated that the protective mechanism in m*D6* transgenic mice is through blockage of the migration of pathogenic lymphocytes to islets.

In the *M3* transgenic mouse model, in situ production of M3 by islets not only delays the migration of diabetogenic cells into the islets, but also decreases the frequency and/or impairs the effector function of pathogenic lymphocytes in peripheral lymphoid organs. Thus, NOD/SCID recipients with splenocytes transferred from *M3* transgenic NOD mice were more resistant to the development of disease than mice receiving splenocytes from non-transgenic littermates [11]. In this study, we also observed a significant decrease in the incidence of diabetes among recipients with splenocytes transferred from m*D6* transgenic mice. Interestingly, around 65% of recipients eventually developed diabetes, suggesting that the biological effect mediated by M3, a wide spectrum immune cell migration inhibitor, is stronger than that of the murine endogenous chemokine decoy receptor D6. However, this high selectivity of D6 in immune cell migration blockage might provide a safety advantage in future clinical applications.

By evaluating the activation status of lymphocytes, we found that the percentage of activated $CD4^+$ T cells was reduced in PLN and that of activated $CD8^+$ T cells was reduced in PLN and pancreas. This suggested that reduction of lymphocytes mediated by D6 overproduction in the pancreas might subsequently influence the activation status of lymphocytes in PLN. Furthermore, in adoptive transfer experiments, we observed a significant decrease in disease development, in terms of onset and incidence, in recipients with splenocytes transferred from m*D6* transgenic mice, suggesting that *D6* overexpression in situ somehow affects the generation and/or effector function of autoreactive lymphocytes in peripheral lymphoid organs. To further address whether the reduction in pathogenicity of splenocytes from m*D6* transgenic donors was beta cell-specific, we performed in vitro proliferation assays by stimulating splenocytes with anti-CD3 antibody, concanavalin A or islet-specific antigens. The proliferation capacity of splenocytes was reduced under stimulation with islet antigens, suggesting that the D6-mediated protective effect in transgenic mice was islet antigen-specific. These results further imply a possible critical role of inflammatory CC chemokines in the generation and/or activation of diabetogenic lymphocytes in NOD mice.

In conclusion, our study demonstrated that the transgenic expression of inflammatory CC chemokines decoy receptor *D6* by pancreatic islets prevented autoimmune diabetes by delaying the migration and decreasing the activation of lymphocytes in the islets of NOD mice. These results suggest that inflammatory CC chemokines might promote the migration of lymphocytes to the pancreas and influence the activation of autoreactive lymphocytes in NOD mice. This study also provides a theoretical basis for *D6*-based genetic manipulation in islets as a potential therapeutic strategy for the prevention and/or treatment of autoimmune diabetes.

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**Duality of interest** The authors declare that there is no duality of interest associated with this manuscript.

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