

Pathogenicity of T helper 2 T-cell clones from T-cell receptor transgenic non-obese diabetic mice is determined by tumour necrosis factor- α

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Summary

Autoimmune diabetes is predominated by a T helper 1 (Th1) response at the expense of an impaired Th2 response. Although T cells producing Th2 cytokines are generally thought to counter a Th1 response, there have been reports of Th2 T-cell clones with pathogenic activity, including one previously reported by us in which the Th2 T-cell clone was derived from a T-cell receptor transgenic (TCR-Tg) mouse bearing pathogenic TCR. In this study, our goal was to determine whether Th2 T-cell clones derived from a TCR-Tg in which the autoantigen was absent would be pathogenic and if so, to investigate possible mechanisms by which the Th2 T-cell clone could promote disease. We found that a Th2 T-cell clone derived from the 6.9 TCR-Tg/non-obese diabetic (NOD).C6 mouse in which 6.9 T cells do not encounter autoantigen, produced Th2 cytokines but not interferon- γ . This Th2 T-cell clone, like the previous one we had isolated from the 2.5 TCR-Tg/NOD mouse, also turned out to be pathogenic. Intracellular staining revealed that these Th2 T-cell clones produce low levels of tumour necrosis factor- α (TNF- α) *in vitro*, and after adoptive transfer, they migrate to the pancreas where they produce TNF- α as well as Th2 cytokines (interleukin (IL)-4, IL-10). Induction of disease was prevented by administration of soluble TNF- α receptor to recipient mice, suggesting that the diabetogenicity of these Th2 T-cell clones is caused by their low level production of TNF- α .

Keywords: autoimmunity; cytokines; diabetes; inflammation; Th1/Th2 cells

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Introduction

Type 1 diabetes (T1D) is a T-cell mediated chronic autoimmune disease characterized by lymphocytic infiltration and the selective destruction of the insulin-secreting β -cells of the islets.¹ CD4 T helper 1 (Th1) T cells promote inflammation, lead to or exacerbate autoimmune disease, and are clearly implicated in the pathology of diabetes.^{2,3} We have studied the role of Th1 T cells in pathogenesis of autoimmune diabetes through adoptive transfer studies with a panel of islet-reactive, diabetogenic CD4 Th1 T-cell clones derived from non-obese diabetic (NOD) mice.^{4,5} These T-cell clones rapidly induce disease in 1–2-week-old

NOD or NOD-severe combined immunodeficiency (NOD.*scid*) recipients and recent analysis of their effector function *in vivo* shows that they produce large amounts of interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α) in the inflammatory site.⁶ Whereas the contributions of both CD4 and CD8 effector T cells to pathogenesis have been widely studied and well established, the role of Th2 T cells in autoimmune disease has been far more controversial and is poorly understood.

Numerous reports have suggested that Th2 T cells or cytokines inhibit the onset or severity of insulin-dependent diabetes mellitus (IDDM) in NOD mice. For example, it was reported that interleukin (IL)-4 treatment

Abbreviations: CM, complete medium; IC, islet cells; ICOS, Inducible Costimulatory Molecule; i.p., intraperitoneal; NO, nitric oxide; NOD, non-obese diabetic; PEC, peritoneal exudate cells; s.c., subcutaneous; sTNFR, soluble TNF receptor; T1D, type 1 diabetes; TCR, T cell receptor; Tg, transgenic.

promotes the expansion of regulatory CD4⁺ Th2 cells *in vivo* and prevents the onset of insulinitis and IDDM mediated by autoreactive Th1 cells.⁷ Also, the systemic administration of both IL-4-expressing plasmid DNA and recombinant IL-4 inhibited the development of insulinitis in a dose-dependent manner.⁸ The protective effects of IL-10 were indicated in a report in which daily subcutaneous administration of IL-10 to NOD mice was shown to delay the onset of disease and significantly reduce the incidence of diabetes.⁹ Expression of IL-10 in T cells was observed to provide protection against type 1 diabetes¹⁰ and function of regulatory T cells induced by oral insulin therapy was associated with ability to secrete IL-10 as well as IL-4.¹¹ Effects of both cytokines together were investigated in another study in which it was found that the combined administration of mouse IL-4 and IL-10 plasmids could prevent the development of autoimmune diabetes in NOD mice.¹²

To investigate the role of Th2 T cells in regulation of autoimmune diabetes, we have produced Th2 T-cell clones from T-cell receptor transgenic (TCR-Tg) mice on the NOD background. The 2.5 TCR-Tg and the 6.9 TCR-Tg mice were developed using TCR from BDC-2.5 and BDC-6.9, two V β 4 T-cell clones in our panel of diabetogenic CD4 Th1 T-cell clones.⁵ We previously described a Th2 T-cell clone 2.5 Tg/T2-X, derived from the 2.5 TCR-Tg NOD mouse, that produces the Th2 cytokines, IL-4, IL-5, IL-6 and IL-10, but no IFN- γ .¹³ Despite its Th2 cytokine profile, 2.5 Tg/T2-X is pathogenic. It can induce disease in young NOD mice but not in immunodeficient NOD.*scid* recipients,¹³ suggesting that disease induction by the Th2 clone occurs by a different mechanism than that used by Th1 clones which can transfer disease to both young NOD and NOD.*scid* mice. The goal of the present study was to investigate how a Th2 T-cell clone, producing the typical Th2 cytokines and no IFN- γ , can cause disease. One possibility is that the Th2 T-cell clone from the 2.5 TCR-Tg mouse bears an autoaggressive TCR (originally from a pathogenic Th1 T-cell clone BDC-2.5) because T cells in the transgenic animal are activated very early because of their β -cell autoantigen specificity. To address this question, we derived another Th2 T-cell clone, 6.9 Tg.C6/T2, from a second TCR-Tg mouse, the 6.9 TCR-Tg on the NOD.C6.*scid* background, which lacks the autoantigen for the BDC-6.9 TCR. The T cells in the 6.9 TCR Tg/NOD.C6.*scid* mouse are therefore 'naïve' at any age, but are readily activated in the presence of NOD islet antigen, either in culture or *in vivo* upon transfer to NOD.*scid* recipients.¹⁴ Because the mouse from which the 6.9 Tg.C6/T2 clone was derived also bore the *scid* gene mutation, the T cells obtained from this animal were monoclonal. We report here that even though the T cells from which the 6.9 Tg.C6/T2 clone was derived had not been previously exposed to their autoantigen *in vivo*, this Th2 T-cell clone is also pathogenic.

In this study, we have compared properties of the two Th2 T-cell clones, one derived from transgenic cells that may have been 'primed' *in vivo* and the second from T cells that had never seen antigen. We provide an extensive analysis of the effector function of these two T-cell clones and how they differ from Th1 effector T cells. We also provide data to indicate that the diabetogenicity of the Th2 T-cell clones is determined by the low levels of TNF- α they produced.

Materials and methods

Mice

All mice were bred and maintained under pathogen-free conditions at the Center for Laboratory Animal Care at the University of Colorado Health Sciences Center (Denver, CO). NOD.*scid* mice were originally obtained from the breeding colony at the Barbara Davis Center for Childhood Diabetes (Denver, CO) or purchased from The Jackson Laboratory (Bar Harbor, ME). NOD or NOD.*scid* mice were used as recipients in adoptive transfer experiments. All procedures used were in accordance with Institutional Animal Care and Use Committee guidelines and approved by the UCHSC Animal Care and Use Committee.

The 6.9 TCR-Tg/NOD.C6 mouse strain¹⁴ was maintained by intercrossing. In some cases, 6.9 TCR-Tg/NOD.C6 mice were bred with NOD.C6.*scid* to obtain 6.9 TCR-Tg/NOD.C6.*scid* mice. Animals were genotyped by polymerase chain reaction (PCR) of tail DNA for the presence of the BDC-6.9 TCR, followed by PCR amplification of the DNA-dependent protein kinase catalytic subunit gene, with subsequent *AluI* digestion to identify transgene-positive mice homozygous for the *scid* mutation.¹⁵ There is no incidence of diabetes in 6.9 TCR-Tg/NOD.C6 or NOD.C6.*scid* mice.

Derivation of Th2 T-cell clones

To derive the Th2 T-cell clone, 6.9 Tg.C6/T2, we followed methods previously published¹³ with a few modifications in cytokine supplementation to the culture. Spleen cells (1×10^6) from an 8-week-old 6.9 TCR-Tg/NOD.C6.*scid* male donor were cultured with granule membrane protein (10–20 μ g) from β -cell tumours as a source of β -cell antigen and 2.5×10^7 irradiated NOD spleen cells as antigen-presenting cells (APC) in a total volume of 20 ml complete medium (CM). Cultures were also supplemented with IL-2 (0.5% supernatant from phytohaemagglutinin (PHA)-stimulated EL-4 cells), IL-4 (0.5% of X63-IL-4 supernatant), and anti-IFN- γ monoclonal antibody (mAb) (2.5% of XMGI.2 supernatant). CM was prepared by supplementing Dulbecco's modified Eagle's minimal essential medium (Life Technologies, Gaithersburg, MD) with 44 mM sodium bicarbonate, 0.55 mM

L-arginine, 0.27 mM L-asparagine, 1.5 mM L-glutamine, 1 mM sodium pyruvate, 50 mg/l gentamicin sulphate, 50 μ M 2-mercaptoethanol (2-ME), 10 mM HEPES, and 10% fetal bovine serum (FBS). T cells were incubated at 37° with 10% CO₂ for a 2-week cycle. Like the TCR of the parent clones BDC-2.5 and BDC-6.9, the Th2 T-cell clones 2.5 Tg/T2-X and 6.9 Tg.C6/T2 are both V β 4.

Subculture of T-cell clones

Four days following antigen/APC stimulation of resting T cells after the 2-week cycle, T-cell clones were expanded for 3–4 days in CM, supplemented with IL-2, and in some cases, IL-4. Optimal culture conditions for expansion varied for Th1 and Th2 T-cell clones and were determined for each clone type empirically. Expanded cell cultures were used for adoptive transfer experiments and *in vitro* intracellular staining.

Cytokine assays

At the end of each 2-week growth cycle, T-cell clones were assayed for cytokine production. In a 96-well plate, responding T cells (4×10^4) were incubated with 5×10^4 peritoneal exudate cells (PEC) as APC and 5×10^3 irradiated islet cells (IC) as antigen, or were stimulated with concanavalin A (Con A; 2 μ g/ml) at 37°, 10% CO₂. Supernatants were harvested after 72 hr (for antigen/APC) or 48 hr (Con A) and assayed by specific sandwich enzyme-linked immunosorbent assay (ELISAs) for the cytokines IL-2, IL-4, IL-5, IL-6 and IFN- γ . For transforming growth factor- β 1 (TGF- β 1) ELISA, 6×10^5 resting T cells were stimulated by plate-bound anti-CD3 (1 μ g/ml) and anti-CD28 (1 μ g/ml) overnight.

ICOS staining on the T-cell clones

After expansion for 4 days T-cell clones were harvested and suspended at 1×10^7 cells/ml; 1×10^6 cells were incubated with a mAb to inducible costimulatory molecule (ICOS) (E17G9, BD Pharmingen, San Diego, CA) and anti-V β 4 mAb (KT4, BD Pharmingen) for 30 min in 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The expression of ICOS and V β 4 was analysed by flow cytometry.

Inhibition of IFN- γ production by T cells or NO by macrophages

At the end of each 2-week growth cycle, Th1 T-cell clones (1×10^4 cells), were incubated with 2.5×10^4 PEC as APC and 5×10^3 irradiated IC as antigen at 37° and 10% CO₂ for 72 hr, with or without the 6.9 Tg.C6/T2 Th2 T-cell clone (4×10^4 cells/well). Supernatants were assayed by specific cytokine sandwich ELISA for IFN- γ .

For the nitric oxide (NO) assay, 1×10^5 macrophages (PEC) were stimulated by LPS (25 ng/ml) at 37°, with or without Th2 T-cell clones, 6.9 Tg.C6/T2 or 2.5 Tg/T2X, and 1×10^4 IC as antigen. Supernatants were harvested after 72 h and analysed by the Griess assay.¹⁶ Briefly, 50 μ l of supernatant was mixed with an equal volume of Griess reagent (Sigma, St Louis, MO) and incubated at room temperature for 10–15 min. The optical density of each sample was read at 570 nm.

Adoptive transfer of 6.9 Tg.C6/T2 T-cell clone

Recipients of Th2 T-cell clone transfers were 6–14 day-old NOD or NOD.*scid* mice which received two intraperitoneal (i.p) injections of 1×10^7 cells, one week apart. In cotransfer experiments, 1×10^7 diabetic spleen cells or pathogenic Th1 T-cell clones were cotransferred with an equal number of the 6.9 Tg.C6/T2 clone cells. Mice were monitored daily for increase in urine glucose (Diasix, Bayer, Elkhart, IN) starting at 6 days after the first injection. A positive urine glucose reading was followed by a blood glucose test. Mice were killed when the blood glucose was equal to or higher than 18 mM. Non-diabetic mice were killed 4 weeks after the first T-cell injection.

Treatment of NOD mice with soluble TNF receptor

Soluble TNF receptor reagent (PEG-sTNF-R1) was obtained from Amgen (Thousand Oaks, CA). Young (<2 weeks old) NOD recipients were treated with 200 μ g PEG-sTNF-R1, three times a week for 2 weeks, with injections administered either subcutaneously (s.c) or i.p. The Th2 T-cell clone 2.5Tg/T2-X (1×10^7 cells) was transferred to NOD recipients one day after the first injection of PEG-sTNF-R1. Mice were monitored daily for increase in urine glucose; positive readings were confirmed by blood glucose testing.

Histology

Pancreas tissue was removed for histological analysis as described previously.^{17,18} Briefly, each pancreas was fixed in 10% formalin for at least 24 hr and then embedded in paraffin. Tissue sections were stained with haematoxylin and eosin (H&E) to determine the extent of mononuclear cell infiltration, or with aldehyde-fuchsin (AF) to assess islet cell degranulation.

In vitro intracellular cytokine staining

Resting T cells were expanded for 3 days and then placed at 1×10^7 cells/well into a six-well plate coated with 1 μ g/ml anti-CD3 and 1 μ g/ml anti-CD28 for overnight incubation at 37°, 10% CO₂. Uncoated wells were used as controls. GolgiPlug (BD Pharmingen) was added (1 : 1000 v/v%) for

4–5 hr, and then the cells were harvested, washed and suspended in 0.5% BSA/PBS containing GolgiPlug (1 : 1000 v/v%) at 1×10^7 cells/ml. Cell suspensions were added to a 96-well round bottom plate (1×10^6 cells/well) and analysed for surface staining with antibodies to CD4 and V β 4 for 30 min. Cells were then fixed with 2% formaldehyde for 10 min at RT and stained in permeabilization buffer (0.5% saponin, 0.5% BSA in PBS) with anticytokine antibodies for 30 min. Anticytokine antibodies were from BD Pharmingen or Caltag (Carlsbad, CA) and included: Alexa Fluor 488-conjugated anti-IL-4 (BVD6-24G2), fluorescein isothiocyanate (FITC)-conjugated anti-IL-10 (JESS16E3), Alexa Fluor 488-conjugated anti-IFN- γ (XMG1.2), APC-conjugated anti-TNF- α (MP6-XT22), or negative isotype control antibodies. Samples were analysed by flow cytometry.

Ex-vivo intracellular cytokine staining

For *ex-vivo* staining, the pancreas was removed from each recipient mouse; for the Th1 T-cell clone, optimal recovery of immune cells from the pancreas was between 4 and 6 days following transfer, whereas for the Th2 T-cell clones, two injections were administered to recipients and pancreas was harvested 13 days after the first injection. The tissue was digested in collagenase-containing buffer for 30 min at 37°, and then homogenized with 1 : 1000 (v:v) Golgi Plug in CM to obtain a single cell suspension. Cells were incubated with 400 ng/ml phorbol 12-myristate 13-acetate (PMA) and 4 μ g/ml ionomycin as a stimulus, or in CM alone as an unstimulated control, at 37° for 5 hr. The cells were surface stained with anti-CD4 (GK1.5), anti-V β 4 (KT4), and anti-CD11b (M1/70). For intracellular cytokine staining, cells were fixed in 2% formaldehyde, permeabilized in PBS containing 0.5% saponin/0.5% BSA and stained with the same anti-mouse cytokine antibodies described above. An isotype control antibody was used at the same concentration. Non-specific antibody binding was blocked by preincubation of the cells with blocking anti Fc γ RII/III mAb 2.4G2 (0.25 μ g/ 1×10^6 cells) in 0.5% BSA/PBS. Samples were analysed by flow cytometry.

Results

Th2 T-cell clones secrete Th2 cytokines and TGF- β 1 *in vitro*

To determine the cytokine profile of the new Th2 T-cell clone 6.9 Tg.C6/T2, production of IL-2, IL-4, IL-5, IL-6, IL-10 and IFN- γ was analysed by ELISA after stimulation either with antigen (NOD islet cells) or Con A in the presence of APC. As shown in Fig. 1(a), cytokines secreted by the 6.9 Tg C6/T2 T-cell clone were detected only at low levels upon stimulation with antigen/APC

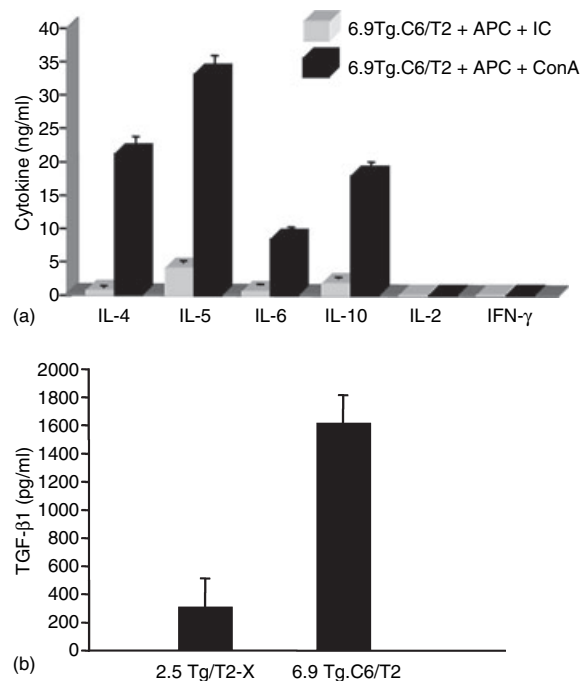


Figure 1. The Th2 T-cell clone 6.9 Tg.C6/T2 exhibits a Th2 cytokine profile and produces TGF- β 1. (a) Responding T cells (4×10^4) were incubated with 5×10^3 irradiated islet cells (IC) as antigen and 5×10^4 peritoneal exudate cells (PEC) as antigen-presenting cells (APC), or were stimulated with Con A (2 μ g/ml) at 37°, 10% CO₂. Supernatants were harvested after culture for 72 hr (with antigen) or 48 hr (with Con A) and assayed in duplicate by specific cytokine sandwich ELISAs. Results are from a representative assay; assays were repeated a minimum of three times. (b) Resting T cells (6×10^5) were stimulated with plate-bound anti-CD3 (1 μ g/ml) and anti-CD28 (1 μ g/ml) overnight. Supernatants were collected and assayed in duplicate by TGF- β 1 ELISA. The Th1 T-cell clone BDC-2.5 did not produce TGF- β 1.

compared to high levels with Con A, but in both cases conformed to a typical Th2 profile; the clone did not make IL-2 or IFN- γ . The results were similar to those with Con A if the T-cell clone was stimulated by plate-bound anti-CD3 (data not shown) and indicate that the Th2 cytokine profile of the 6.9 Tg.C6/T2 T-cell clone closely resembled that of the previously described Th2 T-cell clone 2.5 Tg/T2-X.¹³

To further define their cytokine profile, the Th2 T-cell clones were tested for secretion of TGF- β 1 upon stimulation with plate-bound anti-CD3 and anti-CD28 antibodies. In the assay illustrated in Fig. 1(b), the 6.9 Tg.C6/T2 T-cell clone produced about 5-fold more TGF- β 1 than 2.5 Tg/T2-X; no TGF- β 1 was secreted by the Th1 T-cell clone BDC-2.5.

Th2 T-cell clones also produce TNF- α

Recent research has suggested that a combination of cytokines, especially TNF- α , IFN- γ and IL-1 β , provides

the primary pro-inflammatory impetus in development of diabetes.^{19–21} TNF- α , in particular, is a potent inflammatory cytokine involved in the development of insulinitis and autoimmune diabetes.²² To investigate whether TNF- α could be detected in the Th2 T-cell clones, we tested for the presence of this cytokine by intracellular staining after activation with anti-CD3 and anti-CD28. As shown in Fig. 2, intracellular staining of 6.9 Tg.C6/T2 revealed that around 30% of the 6.9 Tg.C6/T2 cells expressed TNF- α . Over two-thirds of this TNF- α -positive population also expressed IL-4 or IL-10 (Fig. 2b),

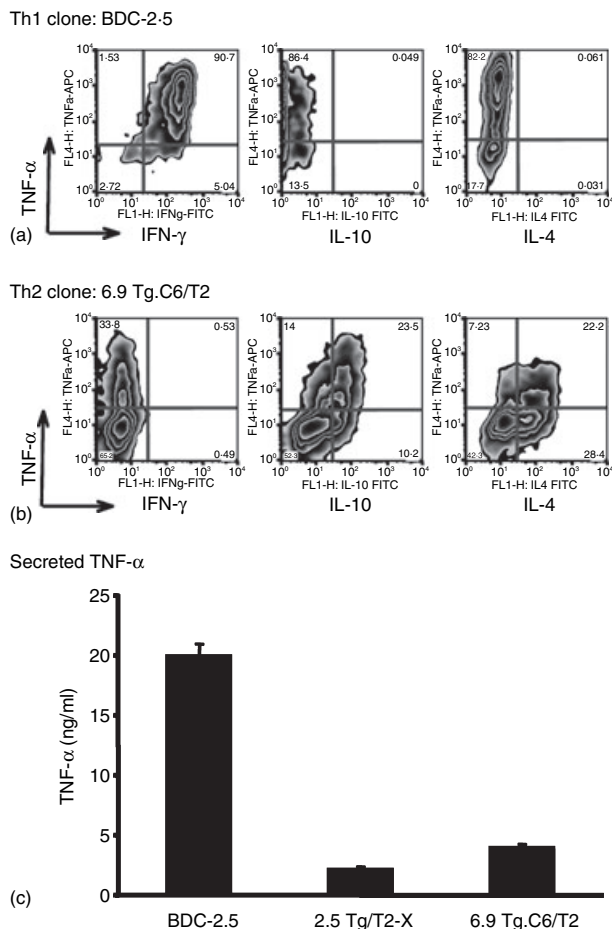


Figure 2. The 6.9 Tg.C6/T2 Th2 T-cell clone expresses TNF- α , as well as IL-4 and IL-10, *in vitro*. T-cell clones were expanded in sub-culture with IL-2, with (Th2 clone) or without (Th1 clone) IL-4 for 3 days. After being stimulated with plate-bound anti-CD3 (1 μ g/ml) and anti-CD28 (1 μ g/ml) overnight, the cells were harvested and stained for surface markers with anti-CD4 and anti-V β 4 mAb. The cells were then fixed in 2% formaldehyde and stained with anti-IFN- γ , anti-IL-4, anti-IL-10, and anti-TNF- α . Samples were analysed by flow cytometry on a FACSCalibur. (a) Intracellular cytokine staining of the Th1 T-cell clone BDC-2.5. (b) Intracellular cytokine staining of Th2 T-cell clone, 6.9 Tg.C6/T2. (c) Secreted TNF- α in culture supernatants was tested by specific ELISA.

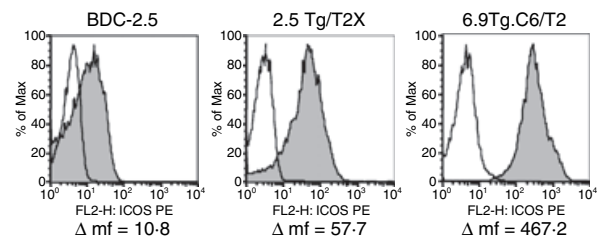


Figure 3. The 6.9 Tg C6/T2 T-cell clone expresses ICOS at high levels. T-cell clones were expanded in culture medium supplemented with IL-2 (for Th1 clones) or with IL-2 and IL-4 (for Th2 clones) for 4 days. Surface expression of ICOS was analysed by flow cytometry, gating on V β 4-positive cells.

whereas the Th1 T-cell clone BDC-2.5 expressed IFN- γ and TNF- α , but no IL-10 or IL-4 (Fig. 2a). The Th2 T-cell clone, 2.5 Tg/T2-X, showed similar levels of TNF- α by intracellular staining (data not shown). The secretion of this cytokine by both Th2 clones was confirmed by ELISA (Fig. 2c).

ICOS is expressed on Th2 T-cell clones

Because 6.9 Tg.C6/T2 expressed relatively high levels of IL-10 (Fig. 1a), we investigated ICOS expression on the surface of this T-cell clone. As predicted, ICOS expression was high on the cell surface of the 6.9 Tg.C6/T2 T-cell clone and almost 50-fold higher than ICOS on the Th1 T-cell clone BDC-2.5, which produces IFN- γ and IL-2, but no IL-10 (Fig. 3). The Th2 T-cell clone 2.5 Tg/T2-X, which secretes less IL-10 than 6.9 Tg.C6/T2, expressed lower levels of ICOS but was still 5 times higher than the BDC-2.5 Th1 T-cell clone. The higher levels of ICOS on the surface of the Th2 T cells may suggest a role for ICOS in maintaining the Th2 phenotype.

Th2 T-cell clones inhibit *in vitro* production of IFN- γ by diabetogenic Th1 T-cell clones

A number of studies have indicated that IFN- γ has a critical role in the induction of insulinitis and subsequent development of diabetes.^{23,24} As the 6.9 Tg.C6/T2 T-cell clone can produce high levels of Th2 cytokines such as IL-4 and IL-10 (Fig. 1a), and low levels of TGF- β (Fig. 1b), we asked whether this Th2 clone could affect secretion of IFN- γ by Th1 T-cell clones. Inhibition of IFN- γ secretion by 6.9 Tg.C6/T2 was tested by culturing the Th2 T-cell clone with either of two pathogenic Th1 T-cell clones BDC-2.5 and BDC-6.9. As shown in Fig. 4, the production of the pro-inflammatory cytokine IFN- γ by pathogenic Th1 T-cell clones in response to islet antigen and APC was significantly reduced in cocultures with the 6.9 Tg.C6/T2 T-cell clone. Similar results were obtained with a second Th2 T-cell clone, 2.5Tg/T2-X (data not shown).

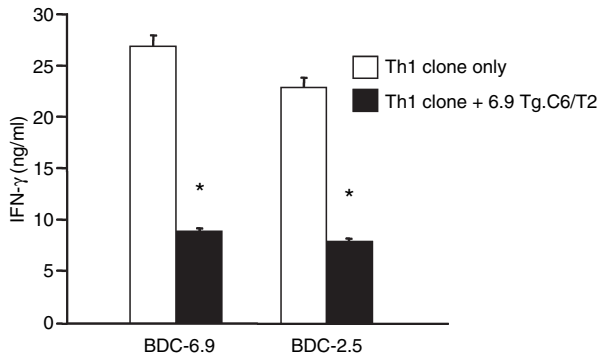


Figure 4. IFN- γ production of Th1 T-cell clones is inhibited by Th2 T-cell clones *in vitro*. Th1 T-cell clones BDC-2.5 or BDC-6.9 (1×10^4 cells) were incubated with 2.5×10^4 PEC as APC and 5×10^3 irradiated IC as antigen at 37° , 10% CO_2 for 72 hr, in the presence or absence of the 6.9 Tg.C6/T2 (4×10^4) Th2 T-cell clone. Supernatants were assayed in duplicate by IFN- γ ELISA. The asterisk (*) indicates $P < 0.001$. This experiment was performed a minimum of three times; results shown are from a representative assay.

The 6.9Tg.C6/T2 T-cell clone inhibits the production of nitric oxide by macrophages

Nitric oxide (NO) is produced mainly by macrophages and can interact synergistically with many pro-inflammatory cytokines such as IFN- γ , TNF- α , and IL-1 β , leading ultimately to destruction of pancreatic β -cells.^{25,26} We investigated whether the 6.9 Tg.C6/T2 T-cell clone could affect the ability of macrophages to produce NO. PEC, consisting of about 80% activated macrophages, were collected by peritoneal lavage of adult NOD mice three days after i.p. injection of thioglycollate. After stimulation of PEC with lipopolysaccharide (LPS), culture supernatants were assayed for NO using the Griess reagent.¹⁶ As shown in Fig. 5, in comparison to PEC stimulated with LPS

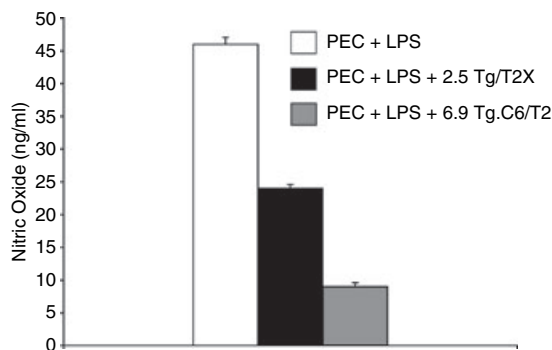


Figure 5. LPS-stimulated production of nitric oxide (NO) by macrophages is inhibited by Th2 T-cell clones. Macrophages (1×10^5 PEC) were stimulated by LPS (25 ng/ml) for 72 hr at 37° , 10% CO_2 , with or without the Th2 T-cell clones. In cultures containing T cells, 1×10^4 IC were added as antigen. NO production was tested by the Griess assay.

only, NO production was reduced by about 80% when PEC were cocultured with 6.9 Tg.C6/T2 T cells and by about 50% when cocultured with 2.5 Tg/T2-X. No inhibition was observed with Th2 T cells in the absence of islet antigen, indicating that cytokines produced by the Th2 T-cell clones upon activation with antigen/APC accounted for the suppressive effects on NO production by macrophages. This conclusion is reasonable in light of the previously reported observation that Th2 cytokines (IL-4 and IL-10) can synergize with TGF- β to inhibit macrophage cytotoxic activity.²⁷

Despite exhibiting some inhibitory properties *in vitro*, the Th2 T-cell clones are pathogenic *in vivo*

The inhibitory effects of the 6.9 Tg.C6/T2 T-cell clone on diabetogenic Th1 T cells *in vitro* are illustrated in Figs 4 and 5, and indicated that the 6.9 Tg.C6/T2 T-cell clone might be able to prevent or delay diabetes *in vivo*. To investigate the activity of this clone *in vivo*, 6.9 Tg.C6/T2 was transferred in two separate experiments into either young NOD mice or NOD.*scid* recipients. By 14 days post-transfer, hyperglycaemia was present in young NOD mice (Fig. 6). Histological analysis showed complete islet degranulation and extensive mononuclear intraislet infiltration in all young NOD recipients (data not shown). Progression of disease was very similar to that observed in a previous study with the 2.5 Tg/T2-X Th2 and BDC-2.5 Th1 T-cell clones¹³. As was also observed with the 2.5 Tg/T2-X clone, the 6.9 Tg.C6/T2 Th2 T-cell clone was diabetogenic only in NOD mice and not in young NOD.*scid* recipients, none of which developed hyperglycaemia up to 2 months after transfer. At this time point, the NOD.*scid* mice were killed and pancreatic sections

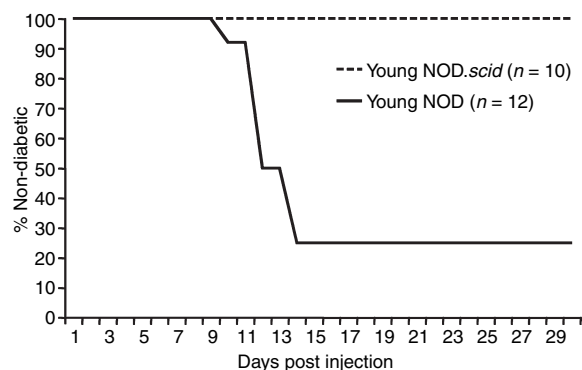


Figure 6. The 6.9 Tg.C6/T2 Th2 T-cell clone can transfer diabetes to young NOD but not NOD.*scid* mice. Recipients (6–14 days of age) received two i.p. injections (1 week apart) of 6.9 Tg.C6/T2 (1×10^7 cells). Six days after the first injection, urine glucose readings were taken daily and diabetes was confirmed by blood glucose measurement. The graph represents the combined results from several experiments in which a total of 12 NOD and 10 NOD.*scid* recipients were tested; disease developed only in NOD mice.

revealed little to no infiltration and no degranulation of the islets. These results are markedly different from transfers in very young NOD.*scid* recipients with either the BDC-2.5 Th1 T-cell clone or diabetic spleen cells, both of which lead to rapid and extensive islet damage and hyperglycemia.¹³

Diabetogenic Th2 T-cell clones produce TNF- α *in vivo* following adoptive transfer

There could be several reasons why the Th2 T-cell clones cause rather than prevent diabetes. Possibly these cells do not migrate to the pancreas of young NOD.*scid* mice after transfer, or if they do migrate to the pancreas, they either fail to secrete protective cytokines or they secrete inflammatory cytokines. To investigate the activity of the Th2 T-cell clones *in vivo*, we performed adoptive transfers into NOD.*scid* mice to determine whether the cells could be recovered from the pancreas and if so, whether they

were making the same cytokines as *in vitro*. As shown in Fig. 7, when the Th2 T-cell clone 6.9 Tg.C6/T2 was compared to the Th1 T-cell clone BDC-2.5 in the same experiment, both clones could be recovered from the pancreas. However, whereas other studies in the lab have shown that BDC-2.5 could be detected in the pancreas by 24 hr after a single injection, peaking by 4–6 days,⁶ the presence of the Th2 T-cell clone was readily detectable only after two injections and at least 10 days after the first injection of cells. Few CD4 T cells could be recovered from NOD.*scid* spleen at either 1 or 2 weeks after transfer of either Th1 or Th2 T-cell clones. Intracellular staining of the Th2 T cells that had migrated to the pancreas indicated that these cells exhibited a cytokine profile *in vivo* similar to that observed *in vitro*, with about two-thirds of the CD4 T cells producing TNF- α , IL-4, and about one-third producing IL-10 (Fig. 7a). In contrast, the Th1 T-cell clone BDC-2.5 expressed high levels of TNF- α and IFN- γ *ex vivo* but no IL-4 or IL-10 (Fig. 7b).

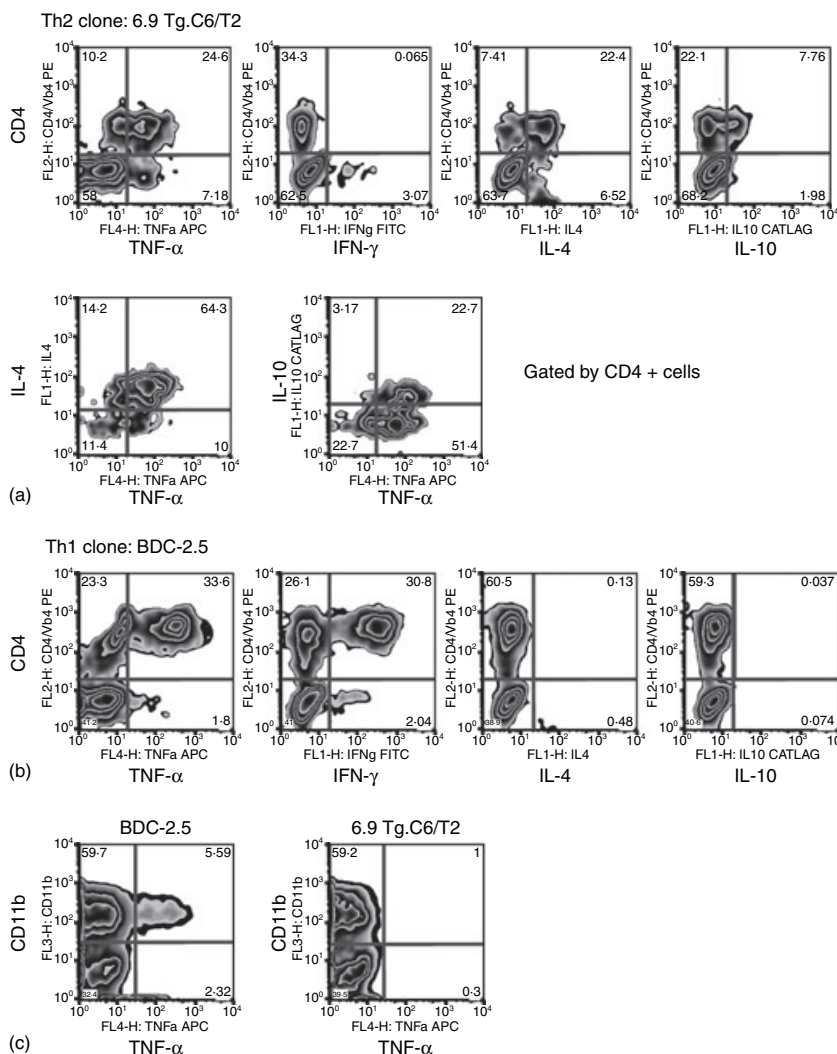


Figure 7. *In vivo* the 6.9 Tg.C6/T2 Th2 T-cell clone migrates to the pancreas and produces IL-4, IL-10, and TNF- α , but no IFN- γ . The 6.9 Tg.C6/T2 clone was administered to NOD.*scid* recipients (7–10 days old) in two injections (1×10^7 cells/injection), 1 week apart. BDC-2.5 was used as the Th1 control clone in recipient mice receiving one injection of cells. Pancreata were removed (6 days later for BDC-2.5 or 13 days after the first injection of cells for 6.9 Tg.C6/T2), digested in collagenase-containing buffer, and homogenized to make single cell suspensions. The cells were stimulated with PMA (400 ng/ml) and ionomycin (4 μ g/ml) for 4 hr at 37°, 10% CO₂. The cells were surface stained with antibodies to CD4, V β 4 and CD11b, and then fixed in 2% formaldehyde. Intracellular cytokine staining was performed by using rat anti-mouse mAb to IL-4, IL-10, TNF- α , and IFN- γ . Non-specific antibody binding was blocked by preincubation of cells with a blocking antibody (anti-Fc γ R2/III mAb, 2-4G2). Samples were analysed on a FACSCalibur. (a) *Ex vivo* analysis of Th2 T-cell clone 6.9 Tg.C6/T2. (b) *Ex vivo* analysis of Th1 T-cell clone BDC-2.5. (c) Macrophage recruitment and expression of TNF- α after adoptive transfer with BDC-2.5 or 6.9 Tg.C6/T2.

Diabetogenic Th2 T-cell clones recruit but do not activate macrophages following adoptive transfer

Previous work from our laboratory has shown that the effector function of diabetogenic CD4 Th1 T-cell clones is partially manifested through recruitment of macrophages to the pancreas, a result not obtained when a non-pathogenic T-cell clone was used.⁶ In the presence of Th1 cytokines, macrophages are then activated to make inflammatory mediators such as IL-1 β and TNF- α (J. Cantor and K. Haskins, manuscript submitted). When we investigated whether macrophages were recruited to the pancreas following transfer of the Th2 T-cell clones to young NOD.*scid* recipients, we found the number of CD11b-staining cells in the pancreas was almost equivalent to that seen in transfers with the diabetogenic CD4 Th1 T-cell clones. However, unlike macrophages recruited to the pancreas by Th1 T-cell clones, the CD11b-staining cells in the pancreas after transfer of Th2 T-cell clones produce little to no TNF- α , as indicated in Fig. 7(c), and suggesting that these macrophages are not activated to effector status.

Soluble TNF receptor can inhibit disease transfer by a Th2 T-cell clone in young NOD mice

To further investigate the contribution of TNF- α to Th2 T cell pathogenicity, we carried out adoptive transfer experiments with a Th2 T-cell clone in young NOD mice treated with a soluble TNF receptor (sTNFR) reagent. TNF- α can trigger TNFR1 expression on APC and islet beta cells, leading to enhanced presentation of beta cell antigens to autoreactive T cells and increased beta cell apoptosis.²⁸ Mice received injections of sTNFR, either i.p. or s.c., 1 day before receiving T-cell clones and five times after transfer, with sTNFR injections spaced 1 day apart. As shown in Fig. 8, control mice receiving the 2.5Tg/T2-X Th2 T-cell clone alone went diabetic in two weeks after clone transfer, whereas almost all of the recipients (12/14) treated with sTNFR maintained normal blood glucose levels for at least 2 months.

Discussion

In previous work, we found that a Th2 T-cell clone isolated from the 2.5 TCR-Tg/NOD mouse was highly pathogenic *in vivo*.¹³ In the present study, our goal was to investigate the mechanism by which Th2 T-cell clones obtained from TCR-Tg mice could induce autoimmune diabetes *in vivo* and we provide here the first report of how Th2 T-cell clones can induce pathogenesis rather than protection. We have extensively characterized the properties of two Th2 T-cell clones, derived from autoreactive TCR-Tg mice, to define their effector function *in vitro* and *in vivo*. These T-cell clones display a Th2 cytokine profile

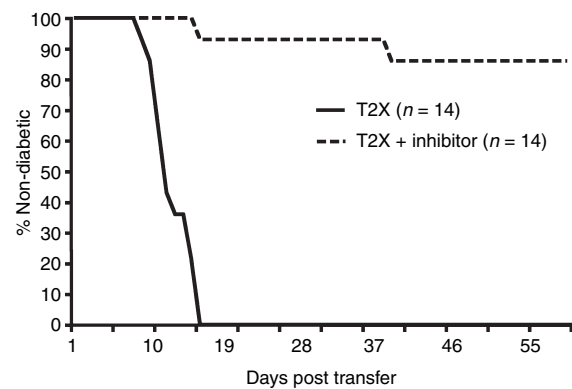


Figure 8. Disease transfer by Th2 T-cell clones is inhibited by treatment of mice with soluble TNF receptor. PEG-sTNF-R1 (200 μ g) was injected into young NOD recipients (<2 weeks old) three times per week for 2 weeks by either subcutaneous or i.p. injection. The 2.5Tg/T2-X Th2 T-cell clone (1×10^7 cells) were transferred to NOD recipients one day after the first injection of PEG-sTNF-R1.

and make TGF- β . In cocultures with Th1 T-cell clones they suppress IFN- γ secretion and if added to cultures of PEC, they inhibit LPS-stimulated production of NO. Although these Th2 T-cell clones can inhibit Th1 T cell function *in vitro*, they are nonetheless pathogenic in adoptive transfers to young NOD mice. Interestingly, they do not transfer disease to NOD.*scid* mice, unlike transfers with Th1 T-cell clones which rapidly cause disease in both NOD and NOD.*scid* recipients 7–14 days of age. *Ex vivo* analysis of T cells recovered from the pancreas of NOD.*scid* mice revealed that Th2 T-cell clones do migrate to the pancreas of recipients and produce low levels of the inflammatory cytokine TNF- α , as well as IL-4 and IL-10. However, they do not make IFN- γ *in vitro* or *in vivo* (Figs 1, 2 and 7). It has been postulated by others that there is synergy between TNF- α and IFN- γ , and that both cytokines are critical for development of diabetes.²⁹ In the absence of IFN- γ , TNF- α alone may be not enough to trigger diabetes in NOD.*scid* mice. On the other hand, cotransfer of either Th2 T-cell clone into NOD.*scid* recipients, together with a Th1 T-cell clone BDC-6.3 which produces IFN- γ but is not diabetogenic in NOD.*scid*, results in diabetes.

Another result of pathogenic T-cell activity in the pancreas is the recruitment and subsequent activation of macrophages.⁶ Although TNF- α is produced by the Th2 T-cell clones, the fact that they cannot transfer diabetes to NOD.*scid* recipients could be the result of a cytokine environment that is not sufficiently pro-inflammatory to activate infiltrating macrophages and cause destructive inflammation. This theory is based on our observation that although CD11b-staining cells are found in the pancreas after adoptive transfer with the Th2 T-cell clones to NOD.*scid* mice, the macrophages are not activated. On the other hand, in young NOD mice, host T cells are recruited to the pancreas and our previous studies indicate first that

few diabetogenic T cells are needed in the site to initiate this process,³⁰ and second that Th2 T-cell clones are, like Th1 T-cell clones, able to recruit host T cells to the pancreas in NOD neonates.¹³ In spite of the fact that Th2 cytokines are produced, the amount of TNF- α made by the Th2 T-cell clones in NOD mice may be sufficient to attract and activate other host inflammatory immune cells.

This study of adoptive transfer of disease with Th2 T cells complements our results from other diabetes induction models, suggesting that TNF- α is a key cytokine in initiating recruitment and activation of inflammatory cells in the pancreas.⁶ We hypothesize that the Th2 T-cell clones that we have isolated from TCR-Tg mice are diabetogenic due to the production of low levels of TNF- α : the TNF- α produced is sufficient to recruit host T cells in the NOD mouse and result in diabetes, but in the NOD.scid in the absence of other effector T cells, the Th2 T-cell clones are not enough to cause disease. To further test this hypothesis, we carried out experiments to block TNF- α function in recipient mice. By treating NOD mice with a soluble TNF receptor reagent in conjunction with adoptively transferring a Th2 T-cell clone, we could abrogate the diabetogenicity of the Th2 T-cell clone. This result confirms earlier findings by others on the importance of TNF- α in autoimmune diabetes^{31–33} and provides further evidence that this cytokine is a potentially important target in immunotherapy of T1D, as it is in other autoimmune diseases.

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