Diabetologia (1998) 41: 1502–1510

**Diabetologia**


# Lymphoid Hyperplasia, CD45RB$^{high}$ to CD45RB$^{low}$ T-cell imbalance, and suppression of Type I diabetes mellitus result from TNF blockade in NOD→NOD-*scid* adoptive T cell transfer

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**Summary** Sustained antibody-mediated inhibition of tumor necrosis factor (TNF) activity offers protection against Type I (insulin-dependent) diabetes mellitus in non-obese diabetic (NOD) mice. The mechanism of this effect, however, has remained obscure: TNFα might be required for the development of specific immune responses to islet antigens or it could directly participate in destruction of beta cells. In this study, autoimmune destruction of beta cells was initiated in NOD-severe combined immunodeficient (*scid*) mice by transfer of NOD splenic T-cells to induce diabetes. The blockade of TNFα activity was achieved during a narrow window of time after transfer. Transient inhibition of TNFα greatly reduced the number of islet lymphocytes and the incidence of diabetes in recipients of prediabetic NOD spleen cells. Protection extended beyond the interval of effective TNF blockade. Furthermore, the protective effect was only observed if cells were obtained from 6-week-old donors. The suppression of autoimmunity was reversible in the context of adoptive transfer as indicated by the transfer of splenocytes from the primary recipient to a second NOD-*scid* host led to a diabetic outcome. The blockade of TNFα was accompanied by a considerable increase in spleen size and doubling of the total splenocyte count, suggesting that TNFα might normally eliminate a transplanted T-cell subset within the recipients. Further analysis showed an increase in the absolute count of CD4 + T cells and pronounced distortion of the CD45RB$^{high}$ to CD45RB$^{low}$ ratio, with a relative augmentation in the CD45RB$^{low}$ count in the spleen. TNFα appears to regulate the number and subtype distribution of a transplanted T cell population. [Diabetologia (1998) 41: 1502–1510]

**Keywords** TNF, Type I diabetes mellitus, cytokines, autoimmunity, immunoregulation.

Abnormal changes in the islets of non-obese diabetic (NOD) mice can be observed as early as the fourth postnatal week [1]. Islet infiltration by mononuclear cells (predominantly lymphocytes) is followed by glycosuria, ketosis and overt diabetes [1]. This process closely mimics that seen in humans with Type I (insulin-dependent) diabetes mellitus. Disease transmission can be accomplished by adoptive transfer of T-cells from NOD mice to histocompatible, T-cell deficient recipients [NOD-severe combined immunodeficient (*scid*) mice] [2]. Within 150–200 days [2] 80 % of NOD-*scid* mice infused with spleen cells from prediabetic NOD females develop diabetes.

The presence of tumor necrosis factor (TNFα) mRNA expressing cells at very early stages of islet infiltration in spontaneous insulitis as well as in the transfer of diabetic spleen cells from diabetic NOD mice into young syngeneic recipients suggests that TNFα adds to the early development of Type I diabetes [3, 4]. This has also been suggested by studies in which the blockade of TNFα activity was imposed in

Received: 9 March 1998 and in revised form: 2 June 1998

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*Abbreviations*: FACS, fluorescence-activated cell sorting; HBSS, Hanks' balanced salt solution; NOD, non-obese diabetic; PFU, plaque forming unit; *scid*, severe combined immunodeficiency; TNF, tumor necrosis factor; IL-1, interleukin-1; IFN, interferon.

NOD mice through repeated treatment with a monoclonal antibody against the cytokine [5–7]. It has been shown that neutralization of TNFα in early postnatal life can effectively prevent diabetes [7], whereas treating neonatal mice with TNFα decreased the latency and increased the incidence of disease [5, 6]. Daily treatment with TNFα beginning at 4 weeks of age was found to delay the onset of diabetes [8] and release of cytokines interleukin (IL-1) and interferon (IFN) gamma. Investigators have recently reported that NOD mice are protected from spontaneous diabetes after permanent neutralization of TNFα by high blood concentrations of soluble TNF receptor p55 human FcIgG3 fusion molecule resulting from the expression of a transgene [9].

The time-dependent effect of TNFα exposure on development of Type I diabetes has not been explained. Furthermore, there is no detailed understanding of the relation between TNFα and *any* autoimmune disease. Two general hypotheses could explain the actions of this cytokine. Firstly, TNFα is a pro-inflammatory cytokine that could participate in the destruction of tissues [10, 11] targeted by cellular components of the specific immune system perhaps causing direct cytolysis of beta cells or acting indirectly by recruiting cells that accomplish the same effect. Secondly, it is possible that TNFα regulates the specific immune response to the tissue at risk. Such regulation could consist in the stimulation of cell proliferation or in the removal of cells by apoptosis. Precedent for the latter mechanism is the closely related Fas ligand/Fas receptor system, which mediates the apoptosis required to prevent the development of autoimmunity [12, 13]. A specific group of cells that is normally removed by TNFα has, however, still to be identified. It is not established that TNFα is responsible for the apoptotic removal of any population of somatic cells, though it has been reported that the combination of TNFα, interleukin1$\beta$ and interferon-$\gamma$ induces deoxyribonucleic acid strand breaks and apoptosis in human islet cells [14].

In Type I diabetes, discrimination between these two possible mechanisms might be accomplished using a system in which transient blockade of TNFα activity is imposed prior to the development of insulitis. Furthermore, the effect of blockade might best be monitored using cells that have already been primed to cause insulitis, but in a host that has not been subjected to immune assault. This set of conditions applies in NOD-*scid* mice that are recipients of spleen cells transplanted from NOD animals. Accordingly, we studied TNFα blockade in an adoptive transfer model of Type I diabetes [15]. To do so, we made use of an adenoviral transduction system yielding endogenous expression of a chimeric TNFα inhibitor protein [16]. This molecule consisted of the extracellular domain of the human TNFα receptor connected to the Fc or heavy chain of mouse immunoglobulin [17]. After infusion of adenovirus encoding the inhibitor, plasma inhibitor concentration is reliably maintained at a level of 0.1 to 1 mg/ml for a period of 42 days. Thereafter, inhibitor expression is effectively silenced [17]. The efficacy of transient TNFα blockade is indicated because it renders animals highly susceptible to infection by *Listeria monocytogenes* but resistant to the lethal effect of lipopolysaccharide [17]. Thus, this TNF inhibitor encoding adenovirus has been shown to partially attenuate endotoxic shock, the condition under which the highest circulating concentrations of TNF have been shown to be present. A control adenovirus, encoding $\beta$-galactosidase, served as a marker of transduction efficiency and persistence but did not affect TNFα production or activity in vivo [17]. As endpoints in this study, we determined the effect of transient TNF blockade upon the incidence of insulitis and overt diabetes and upon the number and subtype distribution of T-cells in the spleen and pancreas of reconstituted NOD-*scid* mice.

## Materials and methods

*Mice.* Female, 6-week-old NOD/LTJ ( NOD) and NOD/LTSZ-SCID-J (NOD-*scid*) mice were obtained from Jackson Laboratories, Bar Harbor Maine, USA. The mice were maintained in a sterile environment and were checked either once or twice a week for glycosuria, and those which developed glycosuria were scored as diabetic. Normally, diabetic mice died within a few days to weeks after development of glycosuria. In some instances, however, animals were kept alive by daily intraperitoneal injection of NPH insulin, 0.02 U per mouse, in order to enable harvesting and secondary transfer of spleen cells. To measure TNF inhibitor in the plasma, heparinized blood was sampled from mice on several occasions after infusion of the adenoviral vector. A western blot was used for inhibitor detection.

*Cell preparation and transplantation.* Female NOD mice were killed at 6 weeks old by $CO_2$ narcosis. Their spleens were removed and minced in HBSS (Hanks Basic Salt Solution). Spleen cells ($50 \times 10^6$) were injected into the lateral tail vein of 6-week-old NOD-*scid* female mice as described previously [4].

*Adenovirus vector encoding βgalactosidase or TNF inhibitor.* The recombinant adenovirus carrying the TNF inhibitor gene and that carrying the $\beta$-galactosidase gene were constructed as described previously [16]. Virus stocks were purified over a cesium chloride gradient and maintained in small aliquots at –80 °C before use. Plaque forming units (PFU) ($5 \times 10^9$) of each virus were injected into the lateral tail vein of recipients 1 h after splenocyte transplantation.

*Western blot detection of the TNF inhibitor.* Mice were bled at 18, 42 and 70 days after adenoviral transduction to determine plasma concentrations of the TNF inhibitor by western blot analysis. Samples (0.1 μl) of plasma treated with heparin (10 U/ml blood) were resolved by SDS-PAGE (10 % polyacrylamide gel) under reducing conditions and transferred to a nitrocellulose membrane. Recombinant human soluble TNF receptor Type I (R& D Systems, Minneapolis, Minnesota, USA) (50 ng) was applied to the gel as a standard. After blocking the

membrane with 5% milk in tris-buffered saline (TBS) containing 0.05% sodium azide, the blot was exposed to mouse anti-human soluble TNF receptor Type I polyclonal detection antibody (R&D Systems). Bound antibody was allowed to react with alkaline phosphatase conjugated goat anti-mouse IgG (Sigma, St. Louis Mo., USA). Measurement was by densitometric comparison of the bands in each lane to the standard.

*Histology and assessment of insulitis.* Pancreases from the NOD-*scid* transplant recipients, subsequently injected with either the TNF inhibitor- or $\beta$-galactosidase-encoding adenovirus, were fixed in formalin and embedded in paraffin. Each pancreas was cut and stained with hematoxlin and eosin. Islets ($n = 50$) of the 10 TNF inhibitor and 6 $\beta$-galactosidase recipient mice were analysed by light microscopy. The severity of insulitis was assessed as peri-insulitis (islet surrounded by few lymphocytes and insulitis (lymphocytic infiltration into the interior of islets). In addition, the inflammatory infiltrate was evaluated and classified for each islet according to the following grading system: (0) intact islet, (1) area of mononuclear cell infiltration within an islet less than 25%, (2) 25–50%, (3) greater than 50%, (4) final stage of insulitis characterized by small retracted islets with or without residual infiltrate [18].

*Cell count and preparation of splenic and pancreatic CD4 + enriched cells.* Spleens were weighed and cells were counted in a Coulter Counter (Coulter Electronics, Inc. Hialeah, Fla., USA). Enriched CD4 + splenic T cells were prepared by initially lysing erythrocytes with 0.92% $NH_4Cl$ solution. Splenic T cells enriched with CD4 + were prepared by depleting CD8 + T cells with anti-CD8 antibody (Gibco Life Technology, Gaithersburg, Md., USA), and adsorbed rabbit complement. Briefly, cells were incubated with antibody at a dilution of 1:10 for 30 min; 1:8 dilution of adsorbed rabbit complement was added and incubation was carried out for an additional 30 min at 37 °C. The cell suspension was centrifuged at 1500 rpm for 10 min after CD8 + T-cell lysis. The pellet was then resuspended in 200 µl of staining solution (made by combining 0.5 l HBSS, 0.5 l phosphate-buffered saline (PBS), 1 g bovine serum albumin (BSA), and 1 g sodium azide) as described previously [19]. On some occasions (noted in the text), CD4 + T cells were exposed to 0.92% $NH_4Cl$ solution to remove erythrocytes and then purified using an anti-CD4 column (Gibco Life Technology).

Purified splenic and pancreatic CD4 + T cells were obtained initially by subjecting the whole, minced organs to vigorous pipetting. After coarse fragments had settled for 5–10 minutes at unit gravity, the overlying cell suspension was removed and centrifuged at 1 500 rpm to collect a pellet. The pellet was resuspended in PBS containing 2–5 mmol/l EDTA and 1.0% BSA. The cells were then incubated with anti-CD4 magnetic beads and passed through a MACS magnetic cell sorter (Miltenyi Biotec, Auburn, Calif., USA). The adherent cells were then eluted from column [20].

*Fluorescence-activated cell sorting (FACS) analysis.* Spleens were harvested from NOD-*scid* recipients of NOD spleen cells 90–180 days after the injection of adenovirus encoding either the TNF inhibitor or $\beta$-galactosidase. An aliquot of lymphocytes was counted in a Coulter counter to estimate the total number of cells harvested. The remaining cells were incubated with fluoroisothiocyanate (FITC) labelled anti-CD4, anti-CD-8, or anti-CD3 antibodies (Pharmingen, San Diego, Calif., USA) [19, 21]. In addition, purified CD4 + splenic and pancreatic T cells were stained for surface expression of CD45RB with fluoroisothyiocyanate labelled anti-CD45RB antibody (Pharmingen). The cells were analysed by fluorescence-activated flow cytometry (FACStar; Becton Dickinson, San Diego, Calif., USA) [19].

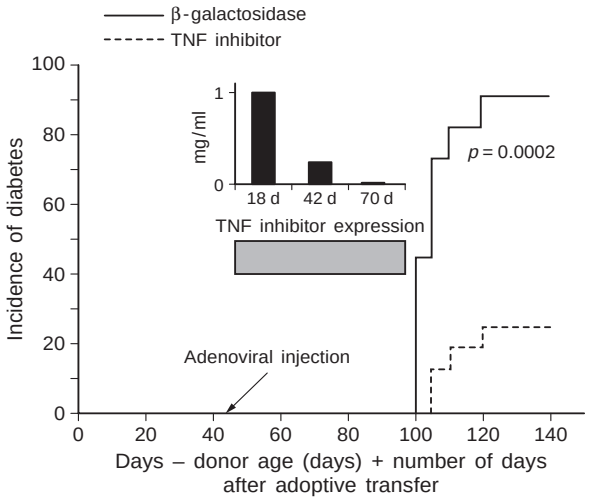



**Fig. 1.** Effect of transient inhibition of TNF on incidence of diabetes in NOD-*scid* recipients of prediabetic NOD mice splenic cells. Splenocytes from 6-week-old prediabetic NOD mice were transferred into 4- to 6-week-old NOD-*scid* recipients. Within 1 h thereafter, mice received either $5 \times 10^9$ PFU of $\beta$-galactosidase encoding adenovirus ($n = 14$) (——) or $5 \times 10^9$ PFU of TNF inhibitor encoding adenovirus ($n = 11$) (-----). The mice were checked once or twice a week for glycosuria. Inset: at 18, 42, or 70 days after adenoviral injections 0.1 µl of plasma from three mice that had received the TNF inhibitor was separated by SDS-PAGE in a 10% gel. TNF inhibitor was made visible with a polyclonal antibody against extracellular domain of 55 kD TNF receptor. Bars indicate the mean concentration of inhibitor assayed at each time point. Standard deviation among assay values was less than 10% of each mean with no detectable level at day 70

## Results

*Transient inhibition of TNF reduces the incidence of diabetes in NOD-scid recipients of NOD spleen cells.* Splenocytes were obtained from 6-week-old NOD females and infused into NOD-*scid* animals that were treated with adenovirus encoding the TNF inhibitor or adenovirus encoding $\beta$-galactosidase (controls). Of 11 NOD-*scid* controls 10 developed diabetes by day 120. By contrast only 4 of the 14 NOD-scid animals that had received the TNF inhibitor encoding adenovirus developed diabetes ($p = 0.0002$; $\chi^2$ generalized Wilcoxen analysis of survival curves, Fig. 1). For 60 additional days, 10 TNF inhibitor-treated and 6 control mice were observed. None developed diabetes. There was no difference in the incidence of diabetes between 7 control NOD mice that had received no adenovirus and 11 that had received the control $\beta$-galactosidase encoding adenovirus ($p = 0.846$). The TNF inhibitor was detected in the plasma of mice that were inoculated with the inhibitor-encoding adenovirus 18 days, 6 weeks and 10 weeks after infusion of the viral vector (Fig. 1). Maximum concentrations of the inhibitor were observed at 18 days; lower concentrations at 6 weeks, and no inhibitor was detectable 10 weeks after transduction nor was inhibitor activity

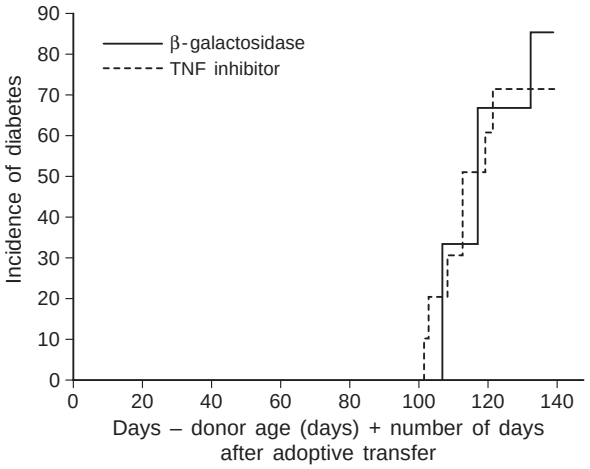



**Fig. 2.** Effect of transient inhibition of TNF in NOD-*scid* recipients of prediabetic 9-week-old NOD mice splenic cells on incidence of diabetes. Splenocytes from 9-week-old prediabetic NOD mice were transferred into 9-week-old NOD-*scid* recipients. Within 1 h thereafter, mice received either $5 \times 10^9$ PFU of $\beta$-galactosidase encoding adenovirus ($n = 6$) (—) or $5 \times 10^9$ PFU of TNF inhibitor encoding adenovirus ($n = 10$) (——). The mice were checked biweekly for glycosuria

detectable in plasma. Thus, TNFα neutralization effectively ceases between 42 and 70 days after infusion of the adenoviral vector. Yet, diabetes did not develop after inhibitor concentrations waned. It can therefore be concluded that TNFα fulfills an essential permissive effect within the first weeks following adoptive transfer, allowing primed T-cells to mount a lethal autoimmune attack in the NOD-*scid* recipient.

This strong protective effect was maintained if the inhibitor-encoding virus was given to recipients of splenocytes from 6-week-old donors. If, however 9-week-old NOD mice were used for transfer, 70 % of 10 TNF inhibitor recipients developed diabetes by 133 days as compared with 84 % of the 6 $\beta$-galactosidase recipients (Fig. 2). Hence, TNFα seems to fulfill an essential diabetogenic function given that T cells are derived from a donor 42 days old at the time of transplantation. For a successful autoimmune assault however, TNFα is no longer required if the donor T-cell population is derived from a mouse that is 63 days of age.

*Larger spleen size and higher splenocyte count in adoptive transfer recipients that are blocked in their responses to TNFα.* To determine whether the protective effect wrought by TNFα inhibition was associated with alteration of the number or helper subtype profile of immune cells, we first examined the spleen size and total lymphocyte count of NOD-*scid* mice that were reconstituted using cells from 42-day-old NOD donors, before treatment with the TNF inhibitor adenovirus or the control adenovirus. The spleen was larger in 200-day-old reconstituted NOD-*scid* mice that were blocked in their response to TNF as compared with controls that received the $\beta$-galactosidase encoding adenovirus [$0.0743 \pm 0.0035$ ($n = 6$) vs $0.060 \pm 0.005$ g ($n = 4$) ($p = 0.045$)]. Concomitantly, a higher splenocyte count was observed in the TNF inhibitor group ($24.3 \pm 2.8 \times 10^6$ vs $11.6 \pm 2.4 \times 10^6$; $p = 0.006$; Welch's T test).

Spleens from NOD-*scid* mice that had received spleen cells from 63-day-old NOD donors were weighed at 133 days after transplantation and showed no difference in splenic weights between the $\beta$-galactosidase and the TNF inhibitor recipients' nor any difference from the control NOD→NOD-*scid* mice that had received no adenovirus after transplanation ($0.0733 \pm 0.0133$ vs $0.0773 \pm 0.005$ vs $0.0713 \pm 0.0028$).

*The relative ratio of CD4 + /CD8 + T cells and the percentage of CD4 + T cells are higher in TNF inhibitor recipient mice than in β-galactosidase recipient mice.* Type I diabetes is a T-cell mediated disease. Therefore, efforts to understand the large increase in total splenic lymphocyte count and the protection from diabetes that were conferred by TNF blockade were directed towards quantitative analysis of T-cell subsets. Initially, the percentage of CD4 + and CD8 + cells in the spleens of recipient mice and the CD4 + :CD8 + ratio were examined. The CD4 + :CD8 + ratio (Fig. 3) was higher in mice exposed to the TNF inhibitor than in the controls ($4.5 \pm 0.21$ vs $3.2 \pm 0.57$; $p = 0.06$) or in prediabetic NOD mice ($4.5 \pm 0.21$ vs $2.2 \pm 0.13$; $p = 0.001$). Of note, the CD4 + T cell population comprised 22 % ± 2.7 % of the spleen cells in the diabetic $\beta$-galactosidase recipient group, 37 % ± 5 % in the non-diabetic recipients of the TNF inhibitor, and 27 % ± 2 % in the prediabetic NOD mice (Fig. 3). Thus, the overall expansion of the lymphocyte population occurring in mice subjected to TNF blockade reflected a disproportionate increase in the fraction of CD4 + cells.

*An excess of CD45RB$^{low}$ cells accounts for expansion of the CD4 + compartment in mice exposed to the TNF inhibitor.* The expression of the CD45RB surface marker on T cells is related to prior T-cell activation (T-cell memory) [22–26] and is high on T cells that have not been exposed to antigens (naïve T cells) but low in those T cells that have already been exposed to antigens (activated or memory cells). Mice exposed to the TNF inhibitor (Fig. 4) exhibited a notable distortion of the CD45RB surface expression, with a diminished percentage of splenic CD45RB$^{high}$ cells in the CD4 + compartment as compared with controls ($36.5 \pm 0.5$ vs $21.5 \pm 0.5$; $p = 0.002$) and an increase in the percentage of splenic CD45RB$^{low}$ cells ($30.5 \pm 0.5$ vs $37 \pm 0.5$; $p = 0.01$). If, however, 9-week-old NOD mice were used for transfer, the CD45RB surface expression was similar to that observed in the $\beta$-galactosidase recipients (Fig. 5).

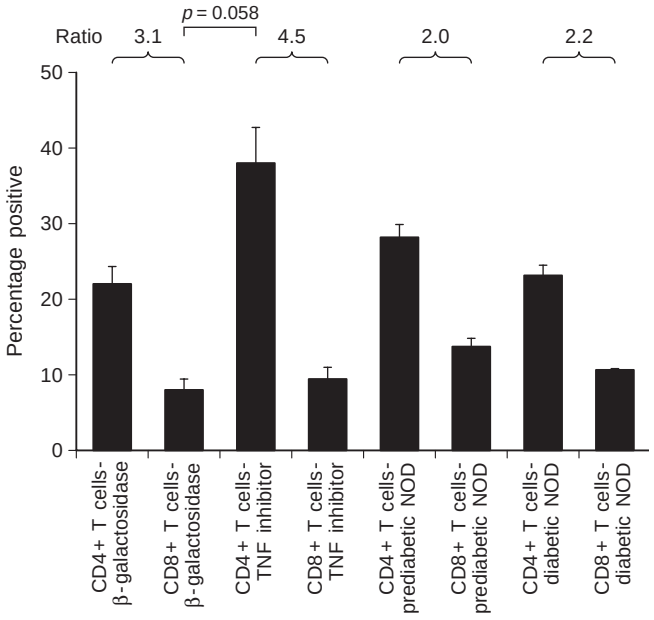



**Fig. 3.** The percentage of CD4 + splenic lymphocytes and the splenic CD4 + :CD8 + ratio in nondiabetic TNF inhibitor recipients and $\beta$-galactosidase recipients. Splenocytes were harvested between 132 and 202 days after transplantation from nine TNF inhibitor recipient mice and six $\beta$-galactosidase recipient mice, as well as from 5 prediabetic NOD and 5 diabetic NOD mice. The cells were stained with anti-CD4, anti-CD8 and anti-CD3 antibodies. The fraction of CD3 + cells expressing each of the other cell surface markers was analysed by FACS. The mean percentage of lymphocytes expressing CD4 or CD8 or both antigens, and the CD4 + :CD8 + ratio were measured for each of the four groups of mice. Error bars indicate the standard deviation of percentage of CD4 + and CD8 + determinations in each group of mice. "Ratio" refers to the mean of the CD4 + :CD8 + ratios calculated for each mouse in each of the four groups. Significance of differences between CD4:CD8 ratios were tested using analysis of variance (ANOVA), applied to ratios measured in individual mice

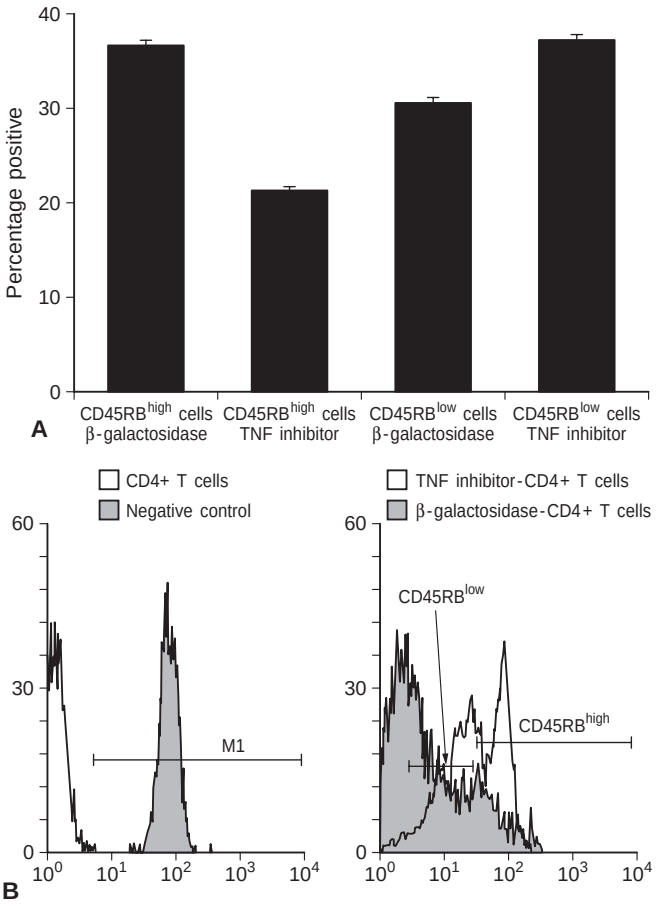



**Fig. 4 A,B.** The percentage of splenic CD45RB$^{high}$ cells in TNF inhibitor recipients and $\beta$-galactosidase recipients. **A** Purified CD4 + splenocytes from three TNF inhibitor recipient mice and three $\beta$-galactosidase recipient mice were assessed by FACS analysis for CD45RB cell surface marker. The percentage of cells expressing high and low levels of this surface marker was averaged for each group. The significance of the difference between CD45RB expression in the two groups ($p = 0.01$) was determined by Student's $t$-test. **B** Left panel: a FACS scan from a representative CD4 + splenic T cell purification experiment. Right panel: a FACS scan showing the CD45RB expression profile of purified CD4 + splenic T cells from a pool of inhibitor recipients and from $\beta$-galactosidase recipients. M1: the window of cells used in assessment of CD4 or CD8 positivity or both

*Peri-insulitis occurs both in mice exposed to TNF inhibitor and in controls, however, the severity of the lesion is diminished in the former group.* Sections of pancreatic tissue were harvested from mice during an interval between 130 days and 225 days after adoptive transfer and adenoviral injection. Mice blocked in their responses to TNF$\alpha$, like control animals, showed peri-insular mononuclear infiltrates. In control animals transduced with the $\beta$-galactosidase-encoding virus, many of the islets were fully infiltrated by lymphoid cells and few, if any, intact beta cells could be observed. The inflammation score in 100 % of the 25 islets examined in the control adenoviral recipients was between 2 and 4 (25–50 % inflammation, > 50 % inflammation or completely retracted islet). Only 14 % of the 50 islets analysed from the TNF inhibitor recipients scored 2,3 or 4 ($p < 0.0001$) (Table 1) and 62 % scored either 0 or 1 (peri-insulitis).

Measurement of pancreatic lymphocytes (Fig. 6 a) showed a fivefold diminution in the number of T cells within the islets of prediabetic mice that had been treated with the inhibitor compared with control animals. The relative proportion of CD4 + and CD8 + cells within inflammatory lesions was examined by FACS analysis. Although the number of CD4 + and CD8 + cells was greatly diminished in mice that had been treated with the inhibitor, a 1:1 ratio of CD4 + :CD8 + cells was observed in each group (Fig. 6 b). Both CD45RB$^{high}$ and CD45RB$^{low}$ CD4 + cells were seen in the islets. As distinct from the profile of splenic T cells, the percentage of CD45RB$^{high}$ cells within the pancreas of mice treated with inhibitor was increased by TNF$\alpha$ blockade

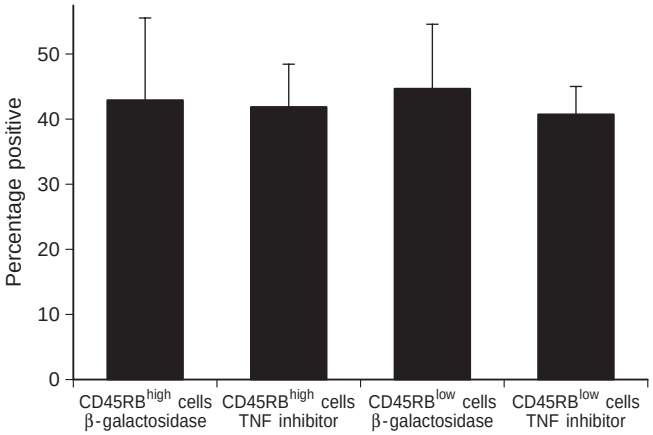



**Fig. 5.** CD45RB cell surface marker after TNF blockade in recipients of 9 week old NOD spleen cells. Splenocytes from eight NOD-*scid* mice that had received 9 week-old NOD spleen cells and either the TNF inhibitor encoding adenovirus or the control $\beta$-galactosidase encoding adenovirus were assessed for levels of the CD45RB cell surface marker by FACS analysis. The percentage of cells expressed high and low levels of this surface marker was averaged for each group. The significance was assessed by a Student's *t*-test

(Fig. 6c). The numbers of both $CD45RB^{high}$ and $CD45RB^{low}$ CD4 + T cells, however, were far lower in the pancreatic tissue of inhibitor-treated mice than in controls.

*Secondary transfer of T cells from NOD-scid mice yields a uniformly diabetic outcome.* Splenocytes ($6–8 \times 10^6$) obtained from each of eight primary recipients of NOD splenocytes (132–202 days after transplantation) that had been protected by TNFα blockade were injected into each of eight näive NOD-*scid* hosts. All recipients developed diabetes within 80 days. Similarly, five out of five NOD-*scid* mice that were transplanted with $6–8 \times 10^6$ cells obtained from five age-matched diabetic control mice (that had received the $\beta$-galactosidase encoding adenovirus at the time of primary transfer) developed diabetes within 80 days (Fig. 7). Hence, the T cells "tolerized" by exposure to the TNF inhibitor retain a latent ability to produce autoimmune diabetes. A second cycle of adoptive transfer was sufficient to permit resumption of auto-aggressive activity, provided a TNFα blockade was not instituted in the recipient.

## Discussion

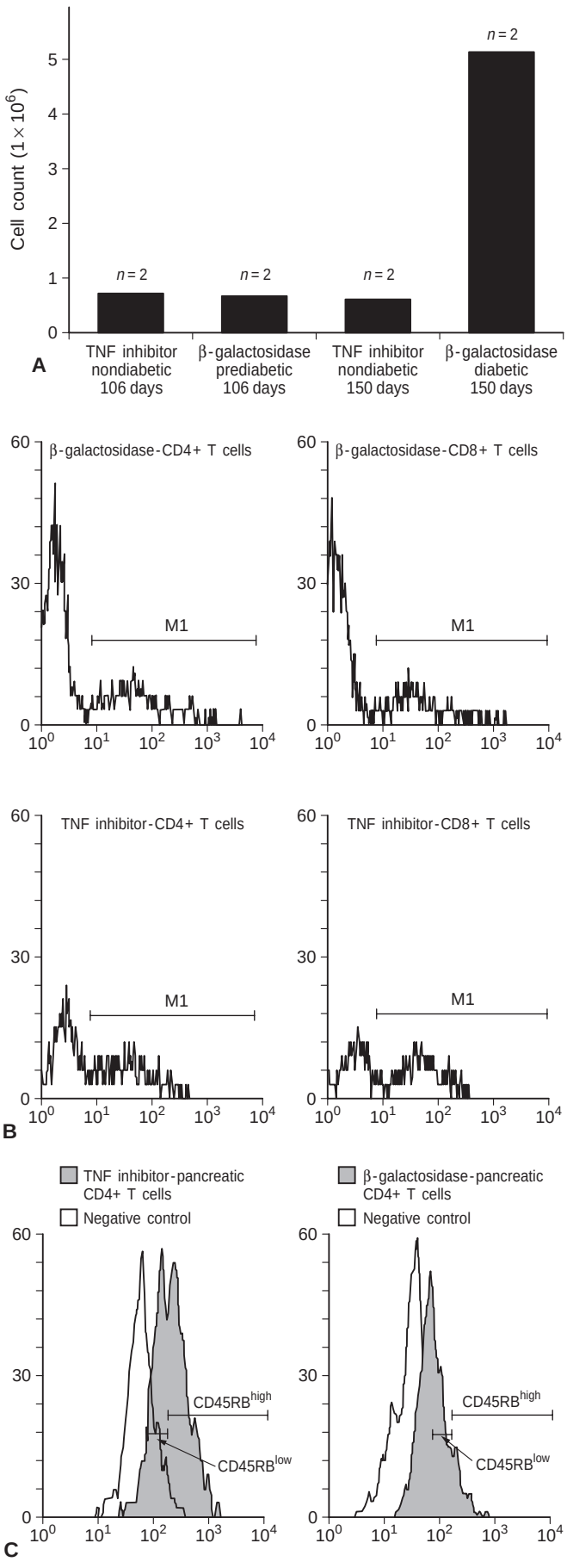
An ameliorative effect of TNFα blockade has been established in several authentic and experimental autoimmune diseases, including rheumatoid arthritis [27], Crohn's disease [28], experimental allergic encephalomyelitis [29], and Type I diabetes [1, 2]. The protective mechanism responsible for the effects has remained obscure. Although TNF might participate in the inflammatory reaction that leads to destruction of target tissues in each disease, it could also govern the development of the specific immune response.

**Table 1.** Severity and inflammatory infiltrate in islets of mice after TNF blockade

| Inflammatory Infiltrate[a] | $\beta$-galactosidase | TNF inhibitor |
|---|---|---|
| 0 | 0 % | 44 % |
| 1 | 0 % | 42 % |
| 2 | 8 % | 12 % |
| 3 | 52 % | 2 % |
| 4 | 40 % | 0 % |
| Severity | $\beta$-galactosidase | TNF inhibitor |
| 0 | 0 % | 46 % |
| 1 | 0 % | 16 % |
| 2 | 100 % | 38 % |

[a] Insulitis is shown as the percentage of infiltrated islets per total islets scored. For each experimental group (TNF inhibitor $n = 9$) and ($\beta$-galactosidase $n = 6$) at least 3 hematoylin and eosin stained sections were analysed by light microscopy. An average of 6 islets per animal were analysed

We have shown that in NOD mice with Type I diabetes, the net effect of TNFα blockade is ultimately reflected in the number of T cells that reside within islets in the pancreas. The diminished infiltrative response is unexpected given the large increase in the number of T cells in these mice. A nearly even ratio of CD4 + and CD8 + cells was observed both within islets of mice blocked in their responses to TNFα and in controls. A distortion of the $CD45RB^{high}$ to $CD45RB^{low}$ ratio was observed, however, both within the islets and within the spleen of animals subjected to TNF blockade. Within the islets, a relative increase in the fraction of $CD45RB^{high}$ to $CD45RB^{low}$ ratio resulted from transient TNF inhibition. Within the spleen, an opposite effect prevailed. These findings hint that a fundamental modification of specific immune function is elicited as a consequence of TNFα blockade. Though TNFα's ability to induce expression of adhesion molecules or molecules involved in lymphocyte homing could explain part of the decreased inflammation associated with blockade, the change in the CD4 + activation markers is unrelated to the induction of such molecules. Therefore, the effect on diabetes is specific and mediated by T cells. Transient blockade of TNFα activity prevents the development of diabetes if enforced during a window of time that precedes islet destruction in untreated mice. The protective effect is essentially permanent, long outlasting the presence of the inhibitor. These observations are consistent with the view that TNFα is somehow required for the development of a specific immune response directed against the beta cells [4], whatever direct role it has in early beta-cell dysfunction or death. Since the T cells that were transferred to näive NOD-*scid* recipients had been presensitized to islet antigens in the NOD environment (many of the donors had developed insulitis, but as yet none




had overt diabetes), it can be inferred that the post-priming phase of the response is critical. The striking effect of donor T-cell age on the efficacy of blockade bespeaks a narrow interval of time during which TNFα is of determinative importance in the development of Type I diabetes. Blockade of TNFα in a recipient of 9-week-old donor cells cannot avert insulitis and diabetes. Hence, TNFα has presumably already exerted its effect within the donor, before the harvest of its cells.

These results differ from previous reports [4, 5, 9] in a number of important ways. A single dose of an adenoviral vector encoding a TNF inhibitor protein halted the development of diabetes in the NOD→NOD-*scid* transfer model of diabetes, whereas previous studies [5] relied upon repeated treatment with anti-TNF antibodies in neonatal NOD mice. Furthermore, the model we used has allowed assessment of the effect of TNF on NOD spleen cells obtained from donors of varying age. Transient inhibition of TNF stopped the progression of diabetes. This finding adds to the work that showed that lifelong neutralization of TNF could prevent diabetes [9]. Furthermore, our model uses spleen cells primed in an environment that facilitates TNFα-TNFR interactions. Harvested from mice that were 6 weeks of age, donor cells subjected to TNF blockade within the recipient environment failed to elicit diabetes. When harvested from mice 9 weeks of age, NOD splenic T cells were not affected by TNF blockade and caused diabetes whether or not such blockade was instituted.

That blockade of TNF activity doubles the number of splenic T cells in a stably engrafted NOD-*scid* recipient suggests TNF normally eliminates many of the cells that are transplanted. Such an explanation seems plausible as TNFα is well-known to induce apoptosis. Accompanying the increase in the number of T cells there was a pronounced distortion in the ratio

**Fig. 6A–C.** Phenotypic differences in the islet infiltrates of mice blocked in their responses to TNF. **A** Pancreatic lymphocytes from both control and experimental mice were assessed for levels of CD4 and CD8 cell surface expression. M1: the window of cells used in assessment of CD4 or CD8 positivity or both. **B** Measurement of lymphocytes within islets of animals blocked in their responses to TNF and in controls, done at 106 and 150 days after adoptive transfer. Each bar represents the mean count of pancreatic lymphocytes obtained from each of two individuals. The standard error of the mean was less than 10% for each determination. **C** Distortion of the CD45RB expression profile of pancreatic lymphocytes harvested from animals exposed to the TNF inhibitor (left) as compared with controls (right). Gating of $CD45RB^{high}$ and $CD45RB^{low}$ populations was done identically on the two samples, and shows a higher ratio of $CD45RB^{high}$ to $CD45RB^{low}$ population in mice that were transiently blocked in their response to TNF

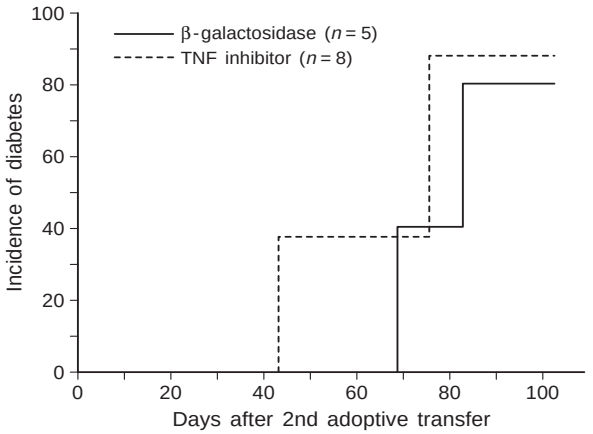



**Fig. 7.** Effect of transfer of spleen cells from NOD-scid mice that had been exposed to TNF inhibitor to a second NOD-*scid* animal. Three of eight recipients of cells taken from the spleens of mice that had been "tolerized" in their response to islets by transient exposure to the TNF inhibitor developed diabetes within 6 weeks. 100% develop diabetes within 80 days. Animals that received splenocytes from recipients of the $\beta$-galactosidase-encoding adenovirus also developed diabetes; the difference between incidence curves is not significant

of CD4 + :CD8 + cells, favouring cells of the CD4 + phenotype. Among CD4 + cells, a disproportionate number of cells of the $CD45RB^{low}$ subtype prevailed after TNFα blockade. Hence, it can be assumed that TNF normally eliminates a part of the $CD45RB^{low}$ subset following adoptive transfer, by a direct or indirect process.

Although TNFα is capable of eliciting the apoptotic destruction of tumour cells, the rationale of this activity is not clear. Deletion of the 55 kD TNF receptor, which contains a death domain and presumably triggers apoptosis in a manner mechanistically similar to the Fas antigen, does not lead to maximum accumulation of lymphoid cells as do mutations of the Fas antigen [13]. More subtle changes in lymphocyte subtype distribution are perhaps apparent only under special conditions, including adoptive transfer.

It can also be inferred that TNFα-based selection only operates within a defined interval of time during immune ontogeny, such that cells obtained from 9-week-old donors are not susceptible to the effect of blockade. It can be assumed that naïve/memory T cell activation and differentiation has, in the authentic NOD environment, progressed to completion by 9 weeks of age, yielding a fixed ratio of the two cell types.

Though animals transiently blocked in their responses to TNFα have, to date, remained non-diabetic for nearly one year after adoptive transfer of diabetogenic T cells, a second adoptive transfer, using splenocytes obtained from the initial transfer recipients, has produced a uniformly diabetic outcome. The $CD45RB^{high}$ to $CD45RB^{low}$ ratio in the second set of recipients (not shown) was not different from that observed in control animals inoculated with the $\beta$-galactosidase encoding adenovirus, or from that in untreated NOD mice. Hence, it can be surmised that the $CD45RB^{high}$ to $CD45RB^{low}$ balance is reversible in the context of adoptive transfer, though apparently stable within a given recipient at some point after reconstitution has occurred. It is not clear, however, whether this finding indicates the $CD45RB^{high}$ $CD45RB^{low}$ differentiation is reversible in the intact host or rather, that a phase of $CD45RB^{low}$ elimination occurs normally as cells populate lymphoid tissues of the recipient following transfer.

No clear evidence exists that TNFα overproduction is a primary genetic defect in Type I diabetes. The MHC linkage of TNFα notwithstanding, direct measurements of lipopolysaccharide-driven transcription from the NOD TNF allele shows it to be equivalent to the BALB/c TNF allele when both genes reside within a common environment, that is the peritoneal macrophage [30]. The cellular source of TNFα which is a decisive factor in the development of diabetes is not known. Though it is certain that lymphocytes are the relevant targets of detrimental TNFα action, they are the only cells lacking in the NOD-*scid* recipient [2] and the only cells replenished by adoptive transfer. Either the host or the graft could produce the TNFα that is required for diabetogenic effect. These two alternatives can be investigated through adoptive transfer studies in which the recipient lacks a functional TNFα gene.

In conclusion, the number and the subtype distribution of the transplanted T-cell population that reside in the pancreatic islets and the spleen of the NOD mouse is regulated by TNFα. Importantly, the effect of TNFα blockade on the T cells can be observed only within a specific window of time. Finally, these results imply that the development of diabetes can be halted by TNFα blockade even after pathological T cells have been primed in a diabetogenic environment.

*Acknowledgements.* Grant Support: National Institutes of Health K11-DK02304; the Juvenile Diabetes Foundation DK50823/JDF, and Becton Dickinson & Co. (DIRP grant) is gratefully acknowledged.

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