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Review

The therapeutic potential of tumor necrosis factor for autoimmune disease: a mechanistically based hypothesis

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Abstract. Excess levels of tumor necrosis factor-α (TNF-α) have been associated with certain autoimmune diseases. Under the rationale that elevated TNF-α levels are deleterious, several anti-TNF-α therapies are now available to block the action of TNF-α in patients with autoimmune diseases with a chronic inflammatory component to the destructive process. TNF-α antagonists have provided clinical benefit to many patients, but their use also is accompanied by new or aggravated forms of autoimmunity. Here we propose a mechanistically based hypothesis for the adverse events observed with TNF-α antagonists, and argue for the opposite therapeutic strategy: to boost or restore TNF-α activity as a treatment for some forms of autoimmunity. Activation defects in the transcription factor nuclear factor κB leave autoreactive T cells sensitive to TNF-α-induced apoptosis. Treatment with TNF-α, by destroying autoreactive T cells, appears to be a highly targeted strategy to interrupt the pathogenesis of type 1 diabetes, lupus and certain forms of autoimmunity.

Key words. NF-κB; TNF-α; apoptosis; autoimmunity.

Introduction and overview

Anti-tumor necrosis factor-α (anti-TNF-α) therapies have been introduced for treating moderate to severe rheumatoid arthritis, Crohn's disease and other chronic inflammatory disorders. The approved therapies (infliximab, adalimumab and entanercept) are monoclonal antibodies or inhibitory molecules that block TNF-α activity [1]. The therapeutic rationale behind their development is reduction of pro-inflammatory actions of the cytokine TNF-α, which is found elevated with other cytokines in autoimmune lesions [2–4]. But TNF-α is not just a pro-inflammatory cytokine. It has also been proposed to be an immunoregulatory molecule that can alter the balance of T regulatory cells [5]**.** What if normal or even elevated TNF-α activity plays an essential role as an immune regulator that diminishes or prevents autoimmunity? If that were the case, blocking the effects of TNF-α might turn out to be counterproductive in certain forms of autoimmunity or at select stages of the disease.

TNF-α's dual physiological roles, as pro-inflammatory and immunoregulatory, might explain why anti-TNF-α therapies present a complex picture: the therapies are effective for the majority of autoimmune patients with rheumatoid arthritis with end organ destruction due to inflammation, but they worsen or induce autoimmunity for a significant minority of these patients. That kind of adverse event is consistent with numerous animal and human studies showing that reducing TNF-α activity aggravates or initiates certain forms of autoimmunity.

Intrigued by the paradoxical findings, our laboratory and others sought to investigate TNF-α's underlying role in autoimmune pathogenesis. The mechanistic evidence

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suggests, contrary to prevailing wisdom, that boosting or restoring TNF-α activity – rather than blocking it – might be therapeutic for some forms of autoimmunity. In this review article, we present a mechanistically based hypothesis that TNF-α holds potential therapeutic value because of its demonstrated capacity in animal models of autoimmunity to selectively kill, by apoptosis, autoreactive (pathogenic) T cells but not normal cells. The selective vulnerability of autoreactive T cells to TNF-α-induced apoptosis appears to stem from a variety of errors in activating nuclear factor κB (NF-κB), which is a crucial transcription factor with anti-apoptotic effects (among many other immune functions). Because our findings and others show that autoreactive T cells continue to remain sensitive to TNF-α-induced apoptosis, treatment with TNF-α appears to be a highly targeted strategy to destroy autoreactive T cells and interrupt the pathogenesis of autoimmunity.

Anti-TNF therapies can exacerbate or induce new autoimmune disease

Evidence has accumulated from clinical trials that anti-TNF-α therapies can, under certain circumstances, promote rather than quell certain forms of autoimmunity. The evidence is strongest for multiple sclerosis (MS), with studies showing that anti-TNF-α therapies exacerbate its course. An early phase I safety trial of two patients revealed that one anti-TNF-α therapy transiently increased demyelinating lesions in the central nervous system (CNS) and immune activation in the cerebrospinal fluid (CSF) [6]. A double-blind, placebo-controlled phase II safety trial of 168 patients with MS found no benefit with another anti-TNF-α drug candidate and also found more frequent and earlier exacerbations. The annualized exacerbation rate was up to 50% greater in treated versus placebo patients [7]. A case of new-onset MS has been reported in a patient with juvenile rheumatoid arthritis treated with yet a third anti-TNF-α therapy [8]. MS and/or new onset demyelination disease are also adverse events with infliximab therapy in colitis and Crohn's disease [9, 10]. Infliximab therapy in 125 Crohn's patients results, after 24 months, in a high cumulative incidence (57%) of patients developing antinuclear antibodies, two patients developing drug-induced lupus and one patient developing autoimmune hemolytic anemia [11].

In rheumatoid arthritis, therapy with diverse therapeutic forms of TNF-α antagonists in all therapeutic forms is associated with relatively common and detectable autoimmune adverse events, including demyelinating disease, confirmed forms of MS, autoimmune hemolytic anemia, type 1 diabetes, a lupus-like syndrome, and cutaneous lupus rashes. Further, 11–57% of patients develop new or elevated antinuclear antibodies, usually shortly after therapy initiation or within 1 year [1, 2, 11–15]. Approximately 7–15% of patients develop new antibodies against double-stranded DNA [13, 15]. Case reports indicate onset of systemic lupus erythematosis or a similar syndrome at 6 weeks to 14 months after treatment initiation [14, 16–19]. Rheumatoid arthritis patients treated with anti-TNF-α therapies can also develop new onset autoimmune vasculitis [20, 21]. About 9–34% of patients with Crohn's disease in clinical trials of TNF-α antagonists develop antinuclear or double-stranded DNA antibodies [4, 22, 23]. Another trial of a TNF-α antagonist reported that 50% of treated Crohn's disease patients develop positive antinuclear antibodies [24]. Progression of lupus has been reported in several patients when TNF-α antagonists were not withdrawn [23]. Recently, a case of type I diabetes was reported in a 7-year old girl undergoing treatment of juvenile rheumatoid arthritis with a TNF-α antagonist [25]. The induction of new onset autoimmunity or the occasional worsening of autoimmunity is an apparent class effect of anti-TNF-α therapy and is not unique to any given TNF-α antagonist.

Low TNF activity may predispose to some forms of autoimmune disease

Several lines of investigation in humans and animals suggest that low TNF-α activity is associated with pathogenesis of some forms of autoimmunity. Low TNF-α activity might result from gene polymorphisms reducing TNF-α expression or disrupting its production. Low TNF-α activity also might result from excess production of soluble TNF receptors. Soluble TNF receptors bind to and inactivate TNF-α [26], effectively lowering TNF levels available to bind to membrane-bound receptors, which is a necessary step for activating intracellular signaling pathways.

Autoimmune researchers provided early insights about low TNF-α production being associated with disease activity in certain groups of patients and in spontaneous animal models of autoimmunity. Jacob and colleagues [27] searched for correlations between human major histocompatibility complex (MHC) haplotypes and predisposition to lupus. TNF-α genes are found within the MHC. Gene polymorphisms in the TNF region, with other genes in the region, appear to confer risk. Patients with certain MHC haplotypes show low levels of TNF production and an increased incidence of lupus nephritis. Patients with another MHC haplotype show high levels of TNF production and decreased incidence of lupus nephritis. The investigators did not know which genes in the MHC are responsible for the association.

These genetic findings in lupus patients are consistent with experimental findings in NZB mice, an animal model for lupus. A deficiency in TNF production, created

by backcrossing NZB mice with TNF-deficient mice, is associated with acceleration of disease [28]. Similar to human lupus, the TNF-α region of the MHC contains polymorphisms with risk for lupus expression [29]. Furthermore, the spontaneous rat model of diabetic and thyroid autoimmunity (BB rat) exhibits lowered expression of TNF [30]. The latter study also linked low TNF to defective T cell maturation; TNF-α is a critical cytokine in the normal T cell negative selection in the periphery but not the thymus [30, 31].

Higher levels of circulating TNF receptors are found in association with lupus activity [32, 33]. Serum levels of both types of TNF receptors (TNF-sR55 and TNF-sR75) are elevated and are better markers of disease activity than other laboratory or clinical parameters [32]. High serum levels of soluble TNF receptors effectively lower bioavailable TNF.

In MS patients, excess TNF receptor shedding has been found in the blood [34], suggesting lower bioavailability of TNF. Higher levels of soluble TNF receptors have also been found in sera and synovial fluids of patients with rheumatoid arthritis [35]. One study reported that because soluble TNF receptors were much higher in lupus than RA patients, the relative TNF-α deficiency might be predicted to be more severe with lupus [33].

Autoreactive T cells have heightened TNF-α-induced apoptosis linked to dysfuntional NF-κB regulation

The onset of autoimmunity may be driven in part by deficient presentation of self-peptides followed by escape from apoptosis of poorly educated, naïve T cells. The released cells eventually become activated and autoreactive upon encountering the peptides of the target organ. Evidence discussed below suggests that several autoimmune diseases have a common vulnerability to TNF-α exposure: their activated, autoreactive T cells are sensitive to TNF-α-induced apoptosis. The common vulnerability appears to be linked to various errors in NF-κB signaling. The errors, while having distinct origins depending on the underlying disease, render activated, autoreactive T cells sensitive to TNF-α-triggered apoptosis.

TNF-α – and interleukin-1 (IL-1) and lipopolysaccharide (LPS) can activate transcription factor NF-κB. Activated NF-κB, in turn, has been implicated in the regulation of genes contributing to cytokine generation, expression of cell surface adhesion epitopes, lymphocyte maturation, MHC class I antigen processing and presentation, and protection from apoptosis after exposure to TNF-α [36–40]. Recognizing that autoimmune diseases have cell-specific interruptions in NF-κB processing, depending on cell type and its state of activation (as discussed below), we propose a mechanistically based hypothesis about the etiology of many common features of autoimmunity, e.g., altered cytokine generation, delayed lymphocyte maturation and the overabundance of naïve T cells [36, 41–44], and interrupted MHC class I antigen processing and presentation defects [45].

In late-stage NOD mice with progression towards clinical disease, T cells have defects in activating NF-κB upon exposure to TNF-α. In normal T cells, activation of NF-κB occurs in the cytoplasm and requires intact proteasomes to cleave the active form of NF-κB from the inhibitory protein IkB-α (after phosphorylation and ubiquination). Once released, the active form of NF-κB is free to enter the nucleus and to express target genes that prevent cell death in response to TNF-α exposure. But NOD mice have defective proteasomes that are unable to liberate NF-κB from IkB-α [46–48]. Having defective proteasomes makes a subpopulation of T cells, after low-dose exposure to TNF-α, vulnerable to rapid death in culture or in vivo [48]. The possible therapeutic benefit of TNF-α therapy is well demonstrated in the NOD mouse model. With either direct TNF-α administration or TNF-α induction, murine autoimmune disease is reversed [49]. Conversely, in the same mouse model, blockade of TNF signaling accelerates autoimmune disease [50].

In type 1 diabetes patients, a new mutation has been found in a protein (SUMO4) that regulates NF-κB activity [51]. Guo and colleagues have shown that this newly identified protein variant alters IkB-α function in antigen-presenting cells and results in altered NF-κB regulation. Since proper proteasomal processing of IkB-α is similarly important for T cells with different forms of cell surface triggering, one can reasonably anticipate that the SUMO4 protein mutation will also result in altered NF-κB activity in the T cells of type 1 diabetic patients. In short, both murine and human diabetes appear to have defects in the processing of the same protein (IkB-α) and the same lymphoid cell-signaling pathway (NF-κB).

In human lupus, NF-κB is also altered. Subsets of T cells have decreased activation of NF-κB with stimulation [52]. In this disease, it is still unknown which steps along the NF-κB signaling pathway are altered.

Several other autoimmune diseases display gene polymorphisms or defects that might alter NF-κB activity. In Crohn's disease, mutations have been found in the NOD2 gene in antigen-presenting cells, and the mutations appear to confer susceptibility to disease onset [53, 54]. Intracellular NOD2 proteins are critical for NF-κB activation. Although it is not known at this time whether the NOD2 protein plays a role in normal or pathogenic T cells, errors in NOD2 function could lower NF-κB activity in T cells exposed to TNF-α, rendering them more vulnerable to TNF-α-induced apoptosis. Scleroderma, an autoimmune disease of the skin, has now been reported to have T cell subpopulations with altered NF-κB activity with associated accelerated apoptosis [55].

TNF protects against autoimmunity

A plethora of experimental studies show that TNF-α, directly or indirectly, protects against the onset of early autoimmunity in animal models of three autoimmune diseases. We define early autoimmune disease as immunologically active disease but prior to clinical onset. Systemic administration of TNF-α suppresses or prevents onset of spontaneous autoimmune disease in a murine model of lupus [56] and in the NOD model of type 1 diabetes [57–59]. TNF-α's therapeutic action in the NOD mouse also can be delivered locally [60]. TNF-α administered to type 1 diabetic-prone BB rats prior to disease appearance also protects from autoimmune progression [58]. TNF-α administration protects against the development of experimental autoimmune encephalomyelitis (EAE), a murine model of MS [61].

In further support of these findings, numerous experimental studies in the NOD mouse find that agents that induce TNF-α, such as bacillus Calmette-Guérin (BCG) or complete Freund's adjuvant (CFA), have the same or similar effect as direct administration of TNF-α: they protect against diabetes onset or against recurrent disease in the transplanted islet tissue [62–70]. The effect appears dose-related, as repeat dosing with BCG is more effective in preventing diabetes than a single dose [71]. Indeed, chronic TNF-α administration can quell or subdue active NOD autoimmunity, especially in late-stage disease or in settings of late disease where the pathogenic upstream pathogenic naïve cells have not been eliminated. TNF-α eliminates potent effector cells, but not all potentially pathogenic T cells in various stages of activation, including quiescent cells (naïve, autoreactive T cells). TNF-α induction with the immunomodulator AS101 also delays onset of lupus in an animal model [72].

Rationale for TNF as therapy in established autoimmune disease

The previous sections have presented evidence of anti-TNF-α strategies exacerbating some forms of autoimmunity and of pro-TNF-α strategies protecting against onset or exacerbation. The foremost questions are, What is TNF-α's mechanism of action in autoimmunity? Does the mechanism justify use of TNF-α as therapy and, if so, under what circumstances?

Our hypothesis is that TNF holds potential therapeutic value because of its capacity in animal models of autoimmunity to selectively kill autoreactive (pathogenic) T cells but not normal cells. Our laboratory was the first to show that NOD mice have a defect in regulation of transcription factor NF-κB. In those mice, NF-κB dysregulation makes the pathogenic T cells selectively vulnerable to TNF-α-induced apoptosis [46]. In that study we reported that cultured immune cells from NOD mice have a proteasome defect that traces to reduced or absent expression of LMP2, a protein that forms one of the proteasome's two catalytic subunits. A defective proteasome disrupts the NF-κB pathway in the cytoplasm. The pathway normally activates NF-κB, which protects cells from TNF-α-induced apoptosis (fig. 1). The proteasome defect, in contrast, leaves T cells incapable of activating NF-κB, which makes them vulnerable to TNF-α-induced apoptosis. Unlike B cells and other immune cells, T cells do not constitutively express the active form of NF-κB. Instead,

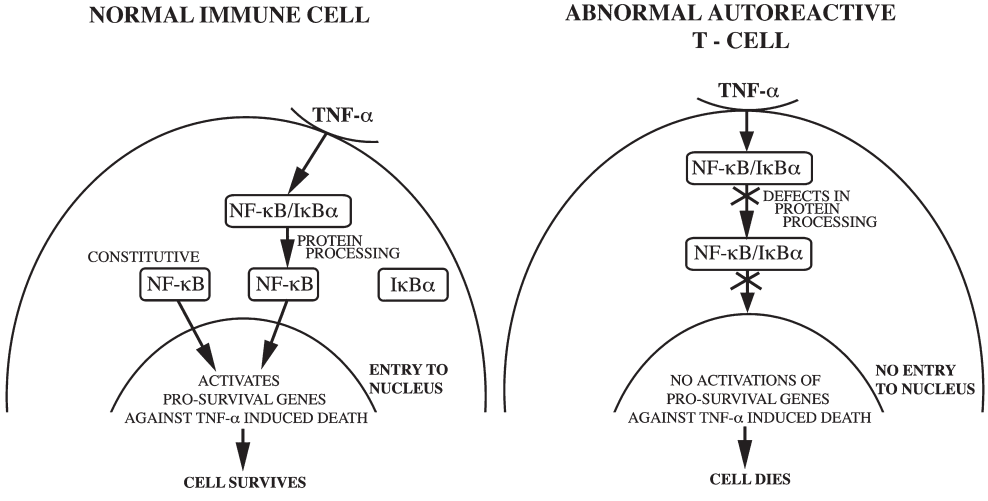

Figure 1. Response to TNF-α exposure by normal immune cells versus autoreactive (pathogenic) T cells. Normal cells either constitutively express the active form of NF-kB or have intact proteasomes that activate NF-kB by cleaving it from its bond with IkB-α. The active form of NF-κB then translocates to the nucleus to initiate expression of pro-survival genes, which counteract the apoptotic effects of TNF-α. In autoreactive (pathogenic) T cells, proteasomal defects prevent activation of NF-κB after TNF-α exposure. Without translocation of the active form of NF-κB into the nucleus, the cell dies because it is unable to express pro-survival genes.

they activate NF-κB upon exposure to TNF-α. Activation requires intact proteasomes to cleave the bond between IkB-α and NF-κB in order to produce the active NF-κB dimer (p65p50). Only the active form of NF-κB can translocate to the nucleus, bind to DNA and initiate expression of an array of anti-apoptotic proteins that counteract the pro-apoptotic effects of TNF-α [73]. If the active form of NF-κB fails to reach the nucleus, the T cell is unprotected from apoptosis when exposed to TNF-α. B cells and other immune cells are usually protected from TNF-α-induced apoptosis because they constitutively express the active form of NF-κB (fig. 1).

To confirm the importance of NF-κB in an animal model of diabetes, we found that freshly isolated NOD mouse T cells exhibit impaired degradation of IkB-α (via immunoblot analysis) and less binding of NF-κB to DNA (via a DNA binding assay) [46]. We also showed, via cell death assay, that a subset of splenocytes is highly sensitive to TNF-α-induced apoptosis in a dose-dependent manner. The greater the dose of TNF-α, the greater the degree of apoptosis (and hence less survival) in a select T cell subpopulation. Our finding of a dose-dependent, but cell-specific, increase in apoptosis with TNF-α, or a TNF-α-inducer, has been confirmed in vivo with NOD mice [74] and in vitro with cells from NZB mice [75]. Normal, freshly isolated splenocytes, on the other hand, are unaffected by TNF-α. Similarly, all populations of resting T cells, both in the NOD mouse and in normal mice, are resistant to TNF-α induction of death. Normal T cells are also resistant to TNF-α because they have intact proteasomes and normal production of NF-κB [46]. Also, nonactivated cells do not possess the intracellular signaling pathways leading to apoptosis after TNF-α exposure. B cells and other cells of the NOD mouse also survive after TNF-α exposure due to constitutive activation of NF-κB by pathways other than the T cell-specific proteasomal activation pathway [76, 77].

Abnormal NF-κB activity has been found in humans with lupus and Crohn's disease [52–54]. Only NOD mice appear to have the proteasome defect as the origin of abnormal NF-κB activity, but the role of the altered the NF-κB pathway and specific IkB-α regulation appears similar to that found in humans with type 1 diabetes [46, 51, 78, 79]. For instance, the proteosomal defect in the NOD animal model is due to a promoter mutation in the MHC-linked proteasome subunit LMP2 [79]. This proteasome defect, conferring altered NF-κB induction in mature T cells, is developmentally specific because it appears gradually and after only 5–6 weeks of age [47]. The appearance of the defect is consistent with the time course of autoimmunity. NOD mice do not manifest autoimmunity until at least 5–6 weeks of age, when they develop insulitis, and by 8 weeks of age, when they produce autoantibodies. The LMP2 subunit proteasome defect in NOD mice is apparently found in multiple cell types of lymphoid origin [80], but its impact in the proteasome (namely, dysregulation of NF-κB and TNF-α-induced cell death) is required for T cells, as contrasted with macrophages and B cells [76, 81, 82]. The lineage specificity of NF-κB activation via IκBα degradation is what makes TNF-α selectively lethal to one type of cell and, more specifically, to one subpopulation of activated T cells.

Selective TNF-induced apoptosis of pathogenic cells in the NOD mouse

The pathogenesis of type 1 diabetes stems in part from impaired activation of transcription factor NF-κB in a subpopulation of activated T cells. This defect makes these cells susceptible to rapid TNF-α-induced death. Repeat TNF-α dosing is necessary for disease suppression when the autoimmune disease is in late stages and the upstream naïve pathogeneic cells can still form the endstage highly activated autoreactive CD8+ T cells. These cells are generated from a subset of naïve T cells that are resistant to TNF-α until they become activated. Working under the hypothesis that autoreactive but naïve T cells in the NOD mouse would survive after TNF administration or induction, we tested a two-pronged strategy to determine whether permanent disease elimination was possible by eliminating both naïve and activated autoreactive T cells.

We tested this two-pronged treatment strategy in endstage diabetic NOD mice: (i) a single administration of TNF-α, or CFA (an inducer of TNF-α) to eliminate activated autoreactive T cells, and (ii) treatment with matched complexes of MHC class I molecules and self-peptides to kill newly emerging naïve, autoreactive T cells [49, 83]. The strategy permanently reversed (i.e., cured) diabetes by returning the animals to normoglycemia [49]. Remarkably, stable and robust pancreatic islet cells reappeared in the pancreas as a result of de novo regeneration from endogenous or exogenously administered adult stem cells [84]. The effectiveness of a similar, two-pronged treatment strategy in a diabetes animal model also has been found by von Herrath and colleagues [85].

Mechanisms of Treatment Efficacy

We report here further in vivo and in vitro analyses in the NOD mouse to confirm the mechanisms by which treatment was successful, especially in regard to killing pathogenic memory (i.e., previously activated) T cells. We examined the immunological pathways underlying successful treatment, characterized treatment variables, and evaluated the role of distinct peripheral lymphoid T cell subpopulations in disease and confirmed their selective death with therapy.

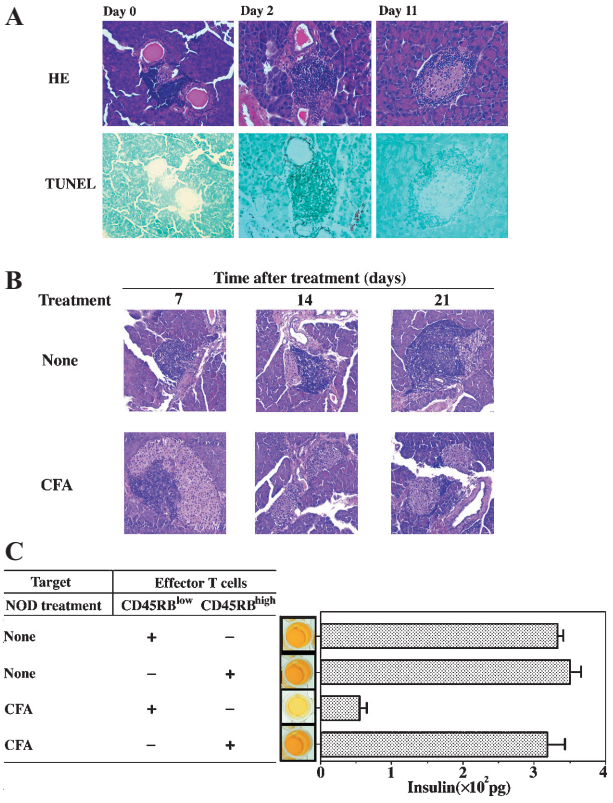

Figure 2. Islet-directed autoreactivity in vitro of T cell subpopulations from diabetic NOD mice subjected to various treatments and effect of CFA treatment on invasive insulitis in vivo. (*A*) Hematoxylin-eosin (HE) and TUNEL staining of paired serial sections derived from the pancreases of pre-diabetic NOD female mice treated (or not, day 0) with a single injection of CFA and sacrificed 2 or 11 days later. (*B*) HE staining of pancreatic sections derived from the pancreases of late pre-diabetic NOD female mice treated (or not, day 0) with a single injection of CFA and sacrificed 7, 14 or 21 days later. (*C*) Cytotoxicity assays were performed with dispersed islet cells from young NOD females. CD45RB^{low} and CD45RB^{high} subpopulations of effector T cells were isolated from the splenocytes of untreated diabetic NOD female given a single injection of CFA. Islet-directed autoreactivity was assessed by ELISA measurement of insulin release and by an associated colorimetric change of the culture medium. Data are means ± SD of triplicates from an experiment that was repeated a total of five times using different donor animals. The bottom row represents islets without any added T cells

We first found, as expected, that treatment with the TNF-α-inducer CFA rapidly produced massive apoptosis of the T cells responsible for destructive insulitis of diabetic mice (fig. 2A). The treatment also diminished insulitis by day 11. The animals were NOD females at 15–20 weeks of age, when pancreatic autoreactivity (but not hyperglycemia) is evident. We examined tissues of the pancreas 2 or 11 days after treatment with CFA alone. Paired serial tissue sections were stained by hematoxylin-eosin (HE) to detect lymphoid infiltrates and by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) technique to detect apoptotic cells. Untreated NOD animals (day 0) show destruction of islets by extensive and invasive lymphoid infiltrates. TUNEL staining (day 0) showed that the invasive infiltrates consists of viable lymphocytes. However, 2 days after CFA injection, the invasive lymphoid infiltrates are still evident, but they are now composed of apoptotic cells. By day 11, the invasive insulitis is eliminated from the central region of islets, and islet structures become evident. TUNEL staining again revealed that the islet core is viable and that the invading lymphocytes are dead. Thus, CFA treatment induces striking apoptosis of lymphoid cell infiltrates–without harming the underlying pancreatic islet structure. But CFA alone is incapable of curing established diabetes. Follow-up times in excess of 20 days after CFA injection revealed the slow and gradual reappearance of invasive insulitis (data not shown), presumably due to proliferation and invasion of naïve autoreactive T cells exposed to islet antigen.

We also used direct HE staining of pancreatic tissue to determine the extent of lymphoid infiltrates over a longer period of time (fig. 2B). Untreated NOD mice develop progressively greater insulitis over the course of 21 days. But CFA treatment eliminates invasive insulitis as early as 7 days after therapy initiation. The elimination of pathogenic insulitis persists, even with a single dose therapy, through day 21.

Two populations of pathogenic T cells, in different stages of activation, are killed by different means

In the next set of studies, we examined the impact of treatment on two subpopulations of autoreactive T cells in the mouse: CD45RB^{low} (memory or activated T cells) and CD45RB^{high} (naïve T cells). Specifically, we wanted to prove that TNF-α or TNF-α inducers only kill memory T cells (CD45RB^{low}), but have no effect on naïve autoreactive T cells.

High expression of the surface marker CD45RB identifies cells unexposed to antigen, whereas low expression signifies past or ongoing exposure to antigen [86, 87]. The maintenance of CD45RB is tightly controlled by proper and continuous MHC class I and self-peptide expression in the periphery [88]. These later memory T cells with additional surface marker identification are sometimes referred to as T regulatory cells and are recognized as deficient in autoimmunity. Human type 1 diabetes is characterized by an abundance of naïve T cells or deficiency of T regulatory T cells [41–44]. Again, this abnormality, as with the NOD mouse, is due to defective MHC class I and self-peptide presentation for central and continuous peripheral T cell selection [45, 49, 89].

While our treatment selectively eliminates distinct subpopulations, the evidence described here focuses on destruction of only one subpopulation of autoreactive T cells, namely, memory T cells, when they are isolated from treated NOD mice and examined by in vitro as-

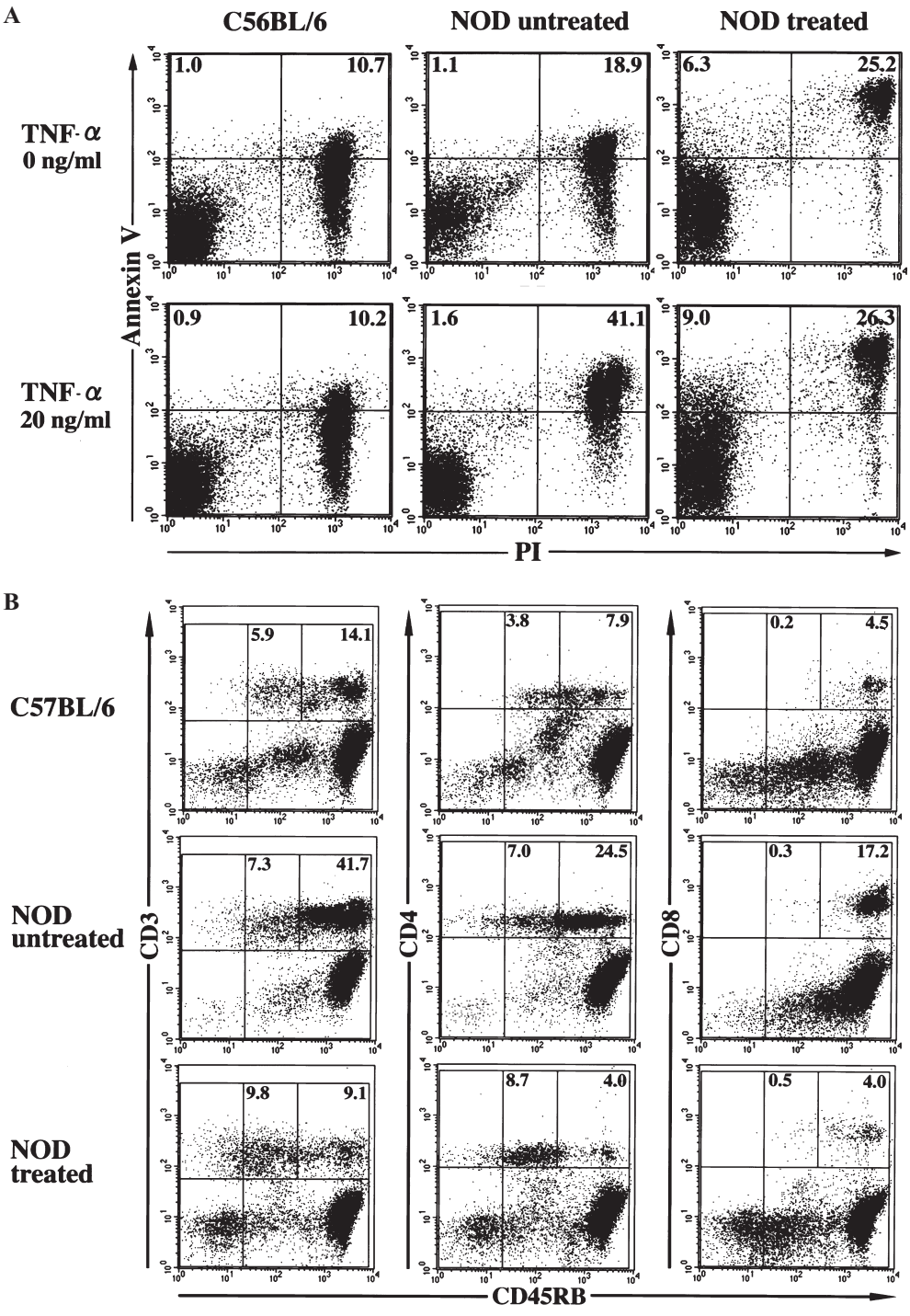

Figure 3. Elimination of autoreactive T cells and normalization of naïve and memory T cell subpopulations in NOD mice with long-term disease reversal with CFA and MHC-peptide complexes. (*A*) Effect of TNF-α on the survival of spleen cells derived from a control C57BL/6 mouse, an untreated 20-week-old NOD female or a NOD female with long-term disease reversal. Cells were incubated in the absence or presence of TNF-α (20 ng/ml) for 24 h before staining with annexin V and propidium iodide (PI) followed by flow cytometric analysis. Similar results were obtained with a total of eight mice with long-term disease reversal. (*B*) Flow cytometric analysis of $CD3^+CD45RB^+$, $CD4^+CD45RB^+$ or $CD8^+CD45RB^+$ splenocytes isolated from a C57BL/6J mouse, an untreated NOD female with diabetes or a treated NOD female with long-term restoration of normoglycemia. Similar results were obtained with a total of four mice with long-term disease reversal.

says (fig. 2C). We isolated each T cell subpopulation (CD45RB^{low} and CD45RB^{high} cells) from animals after two treatment conditions: no treatment or CFA-only treatment. Using an islet cytotoxicity assay, we assessed the ability of each subpopulation to lyse dispersed islets from a young NOD pre-diabetic mouse. A T cell subpopulation that is eliminated by our treatment would have less capacity to kill dispersed islets, which, in turn, would release less insulin. Islet death is indicated by the degree of released insulin in the media, as measured by enzyme-linked immunosorbant assay (ELISA).

With no treatment, CD45RB^{low} (memory T cells) and CD45RB^{high} (naïve autoreactive T cells) are equally capable of lysing islets. With CFA-only treatment, CD45RB^{low} (memory T cells) lyse fewer islets. This implies that CD45RB^{low} cells must have been previously killed by CFA treatment–presumably from the defect in the activation of endogenous NF-κB. In contrast, the CFA-only treatment does not kill the CD45RB^{high} cells, because their capacity to lyse islets is as vigorous as that in untreated animals. We conclude that CFA selectively kills only memory autoreactive T cells – a finding consistent with other reports of selective vulnerability of memory T cells to TNF-α-induced apoptosis [90, 91]. Our findings are also supported by those of Qin and colleagues [74], who found that BCG, another TNF-α inducer, selectively kills CD4+ and CD8+CD45RB^{low} cells in NOD mice. Naïve pathogenic cells are killed by MHC class I and self-peptide re-exposures (data not shown).

We performed another assay of apoptosis – by flow cytometry after staining with propidium iodide and fluoroscein isothiocyanate (FITC)-conjugated annexin V – to confirm that (i) treatment successfully eliminates cells that are selectively sensitive to TNF-α and (ii) no treatment still leaves cells from diabetic mice sensitive to TNF-α-induced apoptosis. We isolated splenocytes from a treated NOD mouse, an untreated NOD mouse and a normal C56BL/6 mouse to compare their in vitro sensitivity to TNF-α incubation. As shown previously [46, 48], incubation of normal (C57BL/6) spleen cells with TNF-α has no effect on cell viability. The proportion of apoptotic cells is 10.2% in the presence of TNF-α and 10.7% in its absence (fig. 3A). In contrast, there is a dramatic rise in apoptosis with TNF-α incubation, from 18.9 to 41.1%, in splenoctyes from an untreated non-diabetic NOD female. There is no increase in apoptosis in splenocytes from a previously CFA-treated female NOD mouse who had become normoglycemic for >180 days. The apoptosis levels remain nearly the same in the absence or presence of TNF-α. This implies that earlier CFA treatment had effectively killed one subpopulation of TNF-α-sensitive cells. The fact that the magnitude of apoptosis is similar, yet similarly high (25.2% and 26.3% with or without TNF-α, respectively), requires further explanation. We reported a similar finding in our earlier study ([49], Fig. 7a). We believe there is a separate subpopulation of splenocytes, apart from autoreactive T cells, which undergoes spontaneous death when cultured. Several studies of peripheral blood in lupus or systemic sclerosis patients also uncovered apoptotic lymphocytes or monocytes upon placement in culture [92–98]. The findings suggest that other immune cell populations besides autoreactive T cells are altered in autoimmunity and, consequently, that other strategies may been needed – above and beyond TNF-α therapy.

Both diabetic NOD mice and humans with diabetes previously have been shown to possess an overabundance of unstimulated or naïve T cells [41, 43, 44], defined in mice by the surface phenotype CD3+CD45RB^{high}. We therefore evaluated the impact of treatment with CFA and MHC-peptide complexes on the cell surface phenotype of peripheral CD45RB-expressing T cells. Our expectation was that treatment would reduce the overabundance of naïve cells. Using flow cytometric analysis of splenocytes, we first showed that a normal mouse (age- and sex-matched) displays about a 1:2 ratio of the two phenotypes: 5.9% are CD45RB^{low} (memory T cells) and 14.1% are CD45RB^{high} (naïve T cells). An untreated diabetic mouse, however, displays a 1:6 ratio, with 7.2% CD45RB^{low} and 41.7% of CD3+ cells CD45RB.^{high} This confirms that untreated diabetes is associated with a striking overabundance of naïve T cells (fig. 3B). The overabundance of naïve T cells is also apparent on analysis of the CD4+ and CD8+ subpopulations from the same mouse. But 180 days after treatment with CFA and MHC class I-peptide complexes, the normal ratio of low to high for CD3+ CD4+ or CD8+ T cells is resumed almost to normal in an NOD female restored to normoglycemia (fig. 3B). In other words, brief treatment, assists in bringing down the percentage of naïve cells, even after long-term follow-up.

TNF eliminates a highly pathogenic T cell subpopulation

Finally, we performed several adoptive transfer experiments to test whether treatment, in vitro or in vivo, successfully eliminates pathogenic lymphocytes. First, we established the validity of our model of adoptive transfer. We transferred splenocytes (2×10^7), which had been isolated and pooled from diabetic NOD females, to each of 21 irradiated young (4- to 8-weeks old) male pre-diabetic NOD recipients. All 21 recipients developed severe hyperglycemia within 10–25 days, with a mean ± SD of 18.4 ± 3.6 days (fig. 4, panel A), a finding commonly reported with this experimental model. Likewise, the male recipients also demonstrated pancreatic islet pathology featuring extensive and pronounced insulitis (fig. 4, panel C). The pathology is indistinguishable from that in NOD females after spontaneous onset of disease at 20–30-weeks of age.

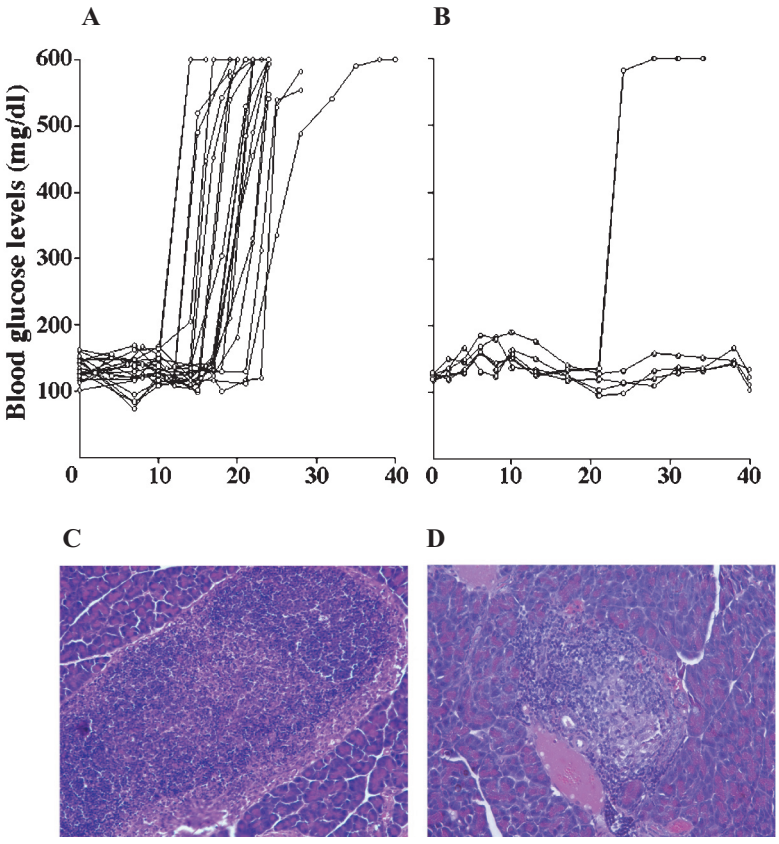

Figure 4. Elimination of autoreactive T cells by TNF-α treatment in vitro as demonstrated by adoptive transfer. Splenocytes isolated from diabetic NOD females were left untreated (panels *A* and *B*) or incubated for 24 h with TNF-α (20 ng/ml) in vitro (panels *B* and *D*) before adoptive transfer into young NOD males. The isolated cells were then transferred to young NOD males. The effects of adoptive transfer on blood glucose concentration (panels *A–B*) and on pancreatic islet histology (panels *C–D*) were determined.

Then we tested the impact of treating cells in vitro with TNF-α prior to adoptive transfer. We found that treatment nearly eliminated pathogenic cells. Splenocytes from diabetic NOD females, which had been treated with TNF-α for 24 h before adoptive transfer, failed to produce hyperglycemia in four (80%) of the five male recipients for at least 40 days (fig. 4, panel B). This finding is consistent with TNF-α-induced apoptosis of disease-causing cells. Examination of pancreatic histology 40 days after cell transfer revealed mild or moderate invasive insulitis (fig. 4, panel D). Thus, while culture with TNF-α before adoptive transfer appears to have eliminated most of the highly pathogenic cells, they were not completely eliminated since the infusion also contained naïve pathogenic T cells.

In summary, the findings presented here support the existence of autoreactive T cells, in particular memory autoreactive T cells ($CD45RB^{low}$), which are susceptible in vitro or in vivo to TNF-α-induced apoptosis. Disease reversal by combination treatment (CFA plus MHC class I-peptide complexes) assists in the normalization of peripheral ratios of naïve versus memory T cells in all populations of T cells (CD4 or CD8). Several distinct assays were used to confirm the death of autoreactive T cells with therapy, the preservation of normal pancreatic tissue and the restoration of normoglycemia. The findings support the therapeutic use of TNF or TNF inducers, such as BCG and CFA, to kill autoreactive T cells in diabetes and possibly other autoimmune diseases.

Model of TNF in autoimmune disease and therapy

In normal people, autoreactive T cells are destroyed in the bone or thymus during the process of negative selection. The process of apoptosis occurs through the T cell receptor, fas ligand or other death pathways [74, 99]. While TNF is constitutively express in the thymus [100], it does not appear to play an essential role in negative selection of early precursor T cells [31].

In people with autoimmune disease, autoreactive T cells survive after having evaded negative selection. In the NOD mouse model, their escape from negative selection occurs because of a proteasomal defect that alters protein processing, including processing of self-antigens for display on the cell surface of autoreactive T cells. Without appropriate display of self-antigens, autoreactive T cells

avoid being seen as 'self', and thus avoid targeted death. Their survival leads to onset or worsening of autoimmunity. But the underlying proteasomal defect in these cells also renders them exquisitely sensitive to TNF-α-induced apoptosis. Because of the dysfunctional proteasome, autoreactive T cells, of the activated type, do not form NF-κB, the transcription factor that would ordinarily protect them from TNF-α-induced apoptosis. Lacking functional NF-κB, they remain sensitive to TNF-α-induced apoptosis. It is that continued sensitivity to TNF-α-induced apoptosis that can be exploited for therapeutic purposes.

We propose therapy with TNF-α, or TNF-α inducers, to selectively kill autoreactive T cells in type 1 diabetes, lupus and MS. Treatment with TNF-α appears to offer a highly targeted strategy to destroy autoreactive T cells and interrupt the pathogenesis of autoimmunity. TNF does not appear to harm normal T cells or other tissues, presumably due to their active form of NF-κB. The level and timing of TNF-α therapy, as well as disease status, appear to be key variables in therapy. Pathogenic memory T cells are especially sensitive to TNF-α-induced apoptosis. Their destruction required only a single dose of therapy with a TNF-α-inducer with a short half-life. That should allay concerns that TNF-α levels become so high that they might induce inflammation.

Our findings show the benefits of TNF-α for autoimmune diabetes, but they also show that TNF-α alone is insufficient to cure the condition with one single dose. TNF-α does not eliminate the naïve autoreactive T cells, which will become pathogenic on exposure to self-antigens, because they are yet sensitive to TNF-α. To prevent eventual self-tissue recognition and activation, naïve T cells must be killed by an additional therapy with a separate death pathway.

The evidence from lupus and certain other autoimmune diseases suggests that low TNF-α levels worsen or bring on disease. The reason may be that the deficiency of TNF-α allows continued survival of autoreactive T cells. The cells previously might have escaped negative selection because of improper processing of self-antigen. In these diseases, introducing or inducing TNF-α may succeed as a therapeutic strategy.

Anti-TNF-α strategies are successful for a large percentage of rheumatoid arthritis patients. Often these patients are in advanced forms of disease, and their painful symptomatology may be driven by a strong inflammatory component to the disease process. While the mechanism of action is not fully understood, it could be that prior to treatment, TNF-α levels are so high in patients' lesions that reducing pro-inflammatory effects of TNF-α provides clinical benefit. Inflammation may be the major source of the discomfort and deformity at this stage of disease.

Our hypothesis holds that despite the apparent benefits of reducing inflammation, anti-TNF-α therapies would have no effect on the underlying autoimmune disease or might actually worsen it. Indeed, the adverse event reporting discussed previously documents the common occurrence of autoantibodies to self-antigens and the occurrence of new autoimmune disease with therapy, usually in the form of lupus, MS or even diabetes. Autoreactive T cells, according to our model, are expected to survive and proliferate in a low TNF-α environment. An alternative explanation for the success of anti-TNF-α therapies in certain autoimmune diseases such as rheumatoid arthritis is that this disease may have underlying defects that are distinct from and unrelated to the NF-κB signaling defects found in type 1 diabetes, lupus and MS.

Another alternative explanation comes from recent data suggesting that some forms of anti-TNF-α drugs bind a form of TNF-α that sits on the exterior surface of the T cell, a transmembrane form of TNF-α itself. This newly identified form of TNF-α on the external cell surface membrane allows direct anti-TNF-α binding, and this binding directly kills the T cell [101–104]. This form of T cell killing is not believed to be specific only to autoreactive T cells and most likely represents an effect similar to an immunsuppressive drug. At this early time the T cell death pathway for this killing is undefined.

These mechanistic findings about anti-TNF-α therapies appear to underscore our hypothesis that a new generation of therapies should strive for selective apoptosis of activated, autoreactive T cells.

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