Supporting Information

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SI Text

Materials and Methods

Human Subjects. Patients with type 1 diabetes or other autoimmune diseases, ranging from lupus, multiple sclerosis, hypothyroidism, celiac disease, Crohn's, Graves, Sjogren's syndrome, and psoriasis, were recruited over a 5-year period from the Massachusetts General Hospital with full institutional approval and with informed consent.

All type 1 diabetic patients were in good health, not in renal failure, had neither received kidney transplants nor systemic immunosuppressive therapy, and had longstanding disease of at least 4-years duration. All other autoimmune patients were on standard therapy regimens for their particular disease and none were taking anti-TNF drugs like Etanercept (Embrel) or Remicade (Infliximab). Nonautoimmune controls were screened for autoimmune diseases and had no personal or family history of autoimmunity. Although the first patients and controls studied by the Ficoll method of blood separation were unblinded, all subsequent patient and control blood samples were supplied to laboratory personnel in a blinded fashion.

The design for all experiments was to simultaneously study an autoimmune blood sample paired to a non-autoimmune blood sample taken in parallel on the same day and within 1.5 h of the first sample. All blood was used fresh and was processed the same day for the cell death assays of this research project.

After informed consent, all patient and control subjects' blood was drawn into BD Vacutainer tubes (BD) containing acid citrate and dextrose or EDTA Vacutainer tubes (BD).

Blood Preparation. Peripheral blood lymphocytes (PBLs) were isolated by two major methods, using Ficoll (Fig. S1, Table S1) or nongradient methods using only magnetic beads (all other figures).

Ficoll Hypaque (Amersham Biotech) gradient centrifugation of fresh human blood followed the manufacturer's protocol. Red blood cells (RBCs) were further removed by a 5-min (on ice) incubation with NH_4Cl solution (PharMLyse (BD), Franklin Lakes, NJ). PBLs were evaluated for viability and then cultured at 5×10^5 cells/ml wells for 12 h. Viability after the Ficoll and NH_4Cl lysis was similar to that reported in the literature, with 40–60% of lymphocytes being viable and the yield representing less than 15% of CD3 T cells in fresh blood. Viability and yield were calculated using flow cytometric methods. The PBLs were cultured in RPMI-1640 media (Gibco), supplemented with 10% heat inactivated BSA and antibiotics at 37°C (100 units/ml of Penicillin; 100g/ml Streptomycin). Some PBL wells were treated with human recombinant TNF-α (R & D) at 20 ng/ml.

Subpopulations of either CD4 or CD8 human T cells were isolated from white blood cells by Dynal magnetic isolation methods (Product Nos 113–33D & 113–31D, respectively, Invitrogen). This cell separation method was perfected to the extent that daily isolated CD8 or CD4 human T cells from fresh blood were 95% viable, 95% pure, with yields of over 85% of human CD8 or CD4 T cells per ml of the starting sample. This magnetic method also allowed T cells to be free of attached beads, and therefore the membranes of the newly isolated pure cells were open for TNF binding. Cell separation methods were further standardized and automated, using the BioMek platform for uniformity of the cell preparations (Beckman Coulter).

Flow Cytometry Studies. For most flow cytometry studies, gates were set "open" for inclusion of cells of all sizesbut excluded cell debris, red blood cells, fragmented cells, and apoptotic bodies. The "open gate" was chosen because cells undergoing cell death, especially by apoptosis, can display changes in light scattering properties and for much of the data in this paper we wanted to analyze high numbers of cells per sample to ensure accuracy and capture dying cells of all shapes. A FACS machine (Becton Dickinson) was used for the analysis.

TNF receptor antibodies. TNF (Leinco Technologies) and a variety of TNFR1 and TNFR2 antibodies were purchased from the following sources for use on the magnetically separated T cells. The TNFR antibodies used were clone MR2–1 (TNFR2) and MR1–2 (TNFR1) (Cell Sciences), 80M2 (TNFR2) (Cell Sciences HM2022) (1) (2) specific to TNFR2 and Sigma T1815 (clone 22221.311; Sigma) - specific to the TNFR2 receptor. Isotype-specific control antibodies, matched to the antibody agonists, were performed at times to rule out non-specific effects of added Ig. TNF antibodies also were sometimes tested with the addition of TNF (at concentrations of 2.5 ng/ml) to observe if the agonist antibody effect was promoted, enhanced, or reversed.

Detection of cell death. For analysis of CD3 cell death within unseparated but Ficoll isolated PBLs, Propidium Iodide (PI) (Oncogene) staining was used with AnnexinV staining, a marker of apoptotic death. Although two color staining of cell death was used, no sample ever displayed cells that died with TNF undergoing necrotic death i.e., PI+AnnexinV-. CD3 lymphoid subsets were identified an anti-CD3 antibody (T cells) (Clone UEHT1; BD PharMingen). This subset-specific antibody was linked to phycoerythrin (PE).

Two 96 well plate based assays, WST-1 (Roche Applied Science) and LDH (Roche Applied Science) were used to confirm cell death versus viability. Plate-based assays carry the advantage of enabling a small volume of cells to be examined for drug dose- related responses. Plate-based assays also represent accumulated death or proliferative products so a late time point is sometimes adequate without the need to do a time course on each sample. The disadvantage of the plate-based assays is that the relative numbers generated for cell death or viability are unrelated to the absolute numbers of cells undergoing proliferation or death.

The lactate dehydrogenase (LDH) released into cell cultures is an index of cytotoxicity and evaluates the permeability of the cell membrane after cell death. Human peripheral blood CD8+ T cells were isolated using magnetic beads Dynal Beads (see above) and then plated into 96 well U-bottom plates at a concentration of 100,000 cells/well. After a 48 h incubation with different concentrations of TNF, TNFR1 and TNFR2 antibody, the culture supernatants were measured using the Roche Cytotoxicity Colorimetric Assay Kit, known as the LDH assay (Roche Applied Science) according to the manufacturer's instructions. Experiments were examined in triplicate. The absorbance was determined by Beckman Coulter DTX 880 Spectrophotometer (Beckman Coulter) at wavelength 492 nm. Test medium was used as background control. The maximum release of LDH was measured by adding 1% Triton X-100 to the cells. The absorbance value was then analyzed to determine cell proliferation or death compared to control wells using following equation: (TNF Treated – Untreated)/(High Control with Maximum LDH release – untreated).

The WST-1 assay is a cell proliferation assay that indirectly measures cell death. For WST-1 experiments, the isolated CD8 or CD4 T cells were plated into 96 well U-bottom plate with a cell concentration 100,000 cells/well. Cells were cultured overnight

at 26°C in RPMI media with 1% heat inactivated FCS. In the morning, the cells were treated with TNF or TNF receptor agonism for 1 h. After the 1 h exposure to TNF or TNF agonism, the WST-1 reagent (Roche Applied Science, Indianapolis, IN), a tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate) was added according to the manufacturer's instructions. The cleavage of WST-1 to formazan by metabolically active cells was quantified by Beckman Coulter DTX 880 Spectrophotometer (Beckman Coulter) at a wavelength 405 nm. Each experiment was performed in triplicate. Test medium was used as background control. The cells treated with various doses of ligand are presented as a percentage of proliferation compared to the untreated cells using the following equation: (TNF or TNFR2 antibody treated – untreated)/untreated).

Throughout this text we use the word viability and proliferation interchangeably and the word death and killing interchangeably. It should be acknowledged that the LDH assay can measure both cell proliferation and death but the WST-1 assay is a cell proliferation reagent that indirectly measure death by the loss of proliferation or cleavage of the WST-1 reagent. The Caspase 3/7 assay directly measures apoptosis in luminescent assay (Promega).

Detection of autoreactive or peptide-specific CD8 T cells in type 1 diabetes. Highly purified CD8 T cells from fresh human blood (within 1.5 h of venipucture) of greater than 95% purity, 95% viability and 85% yield were stained either fresh or after 12 h of culture at 24°C followed by 6 h with TNF or TNFR2 agonist and then stained with 1 μg/ml of phycoerythrin-labeled class I tetramers (Beckman Coulter, Fullerton, CA). CD8 antibodies (Becton Dickinson, NJ). For detection of autoreactive T cells to insulin, we used tetramers to HLA*0210 insulin beta 10–18 with a fragment of HLVEALYLV (Beckman Coulter #T02001). For negative control tetramers, we used two different reagents: HLA*0201 Her-2/neu with a sequence to KIFGSLAFL (Beckman Coulter #T02001), a breast cancer peptide, and HLA*0201 null without a non-specific peptide fragment (Beckman Coulter #T01044). Throughout the text we call both of these "empty tetramer reagent" or "negative tetramer reagent."

All cells were stained in the dark for 30 min and then kept on ice until flow cytometry on fresh non-expanded cells. Cells were washed twice in Hanks with 2% heat inactivated bovine serum after the tetramer staining. On average 100,000 highly pure CD8 T cells were analyzed to ensure clear data points on the Becton Dickinson FACSCalibur using the CellQuest acquisition program. If cells were to be treated with TNFR2 antibody the cells were exposed to 1 μg/ml of clone MR2–1 at a concentration of 200,000–400,000 purified CD8 T cells in 96 wells plates for 6 h at 37°C after the 12-hour overnight incubation at 28°C. All cells for tetramer staining were never frozen, cultured nor expanded before study. For calculations of insulin tetramer + cells or the elimination of these cells with TNFR2 agonism, the following equations were used, $(\text{Tetramer}^{\text{Insulin}}\_ \text{Tetramer}^{\text{Negative}})/\text{Tetramer}^{\text{Negative}}$

Statistical Analysis. Because of the well-described analysis day effect of flow cytometric data or T cell assays, every patient was simultaneously studied with one paired random non-diabetic on the same day. The differences between results in diabetic patients and controls are presented as repeated measure paired *t* test by controlling for the experiment running date. Pearson correlation was used to evaluate the relationship of percent cell death in diabetes patients with the disease duration and the age at disease onset. The differences between the effects of exposures were compared separately in patients and controls using the *t* test. We considered two-sided p value 0.05 as significant without controlling for multiple comparisons.

1. Leeuwenberg JF, Jeunhomme TM, Buurman WA (1994) Slow release of soluble TNF receptors by monocytes *in vitro*. *J Immunol* 152:4036–43.

2. Grell M, *et al.* (1995) The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* 83:793–802.

Results

Type 1 Diabetics Have a Subpopulation of T Cells That Die with TNF Exposures: Lessons from Ficoll Separated Blood. Ficoll separated PBLs from type 1 diabetic and paired controls were cultured for 12 h with or without TNF (20 ng/ml). Cells were then evaluated for cell death by flow cytometry and lymphocyte subset-specific antibodies (Table S1). An initial analysis of blood samples from 44 paired type 1 diabetes and controls exposed to TNF revealed a non-significant increase in death of CD3+ T cells in diabetic samples. Death was detected with propidium iodide (PI) and annexinV staining with an anti-CD3 antibody. We consistently observed death with AnnexinV staining, an indication of apoptotic death. Assuming that TNF triggers death in only rare autoreactive T cells, we expanded the sample size to 79 diabetics and controls to detect an effect. A greater percentage of T cells from diabetics again died by apoptosis, but still without reaching significance. To enhance the likelihood of finding a trend, the samples were powered up to 387 diabetic and controls. We found that TNF killed by apoptosis a significantly greater subpopulation of T cells ($P = 0.003$) in diabetic samples, although the frequency of TNF-sensitive cells was still small, ≈1.17% of all CD3+ cells (P-C). Since these samples represented PBLs, all samples were also evaluated for B cells and monocytes death in response to TNF expsoures. In both the diabetic and control samples, the TNF triggered death was confined to the T cells of the diabetic and not the B cell or monocyte fractions of either the patients nor controls (data not shown).

Several limitations emerged from the Ficoll study regarding standardization of blood samples and quantification of T cells sensitive to TNF-triggered death. As the most common method of separating PBLs from massive numbers of RBCs in human blood, this gradient technology produces lymphocytes of poor viability, yield and purity. By the end of isolations, Ficoll preparations typically contain 30–60% dead cells quantified by flow cytometry using forward and side scatter (Fig. S1*A*). With yields of only 20–40% of starting T cells from fresh blood, large sample sizes are necessary for consistency. Another limitation is the use of a single time point of non-synchronized T cell death triggered by TNF, which requires large cell numbers per study condition. A single monitoring time point is a blend of cell loss (dead cells already lysed) and detectable dying cells. Because TNF-triggered cell death occurs in a non-synchronized manner in blood preparations, it is difficult to optimize a single time point for flow analysis and thus standardize viability analysis between different samples (Fig. S1*B*).

To standardize T cell preparations from freshly drawn blood, we applied non-gradient separation methods. Direct positive selection of magnetically tagged CD4 or CD8 T cells yielded more viable cells (Fig. 1*A*) that were pure and more representative to the original numbers of starting cells (Fig. S1*C*). T cell separation methods were further automated using robotic platforms to allow high yields and consistently viable preparations. Fresh samples using solely magnetic separation preparations were >95% viable, >95% pure and achieved >85% yield of the starting cells in blood. This contrasted with typical preparations of Ficoll-isolated PBLs with viability of 30–60%, poor purity, and yields of only 20–40% of starting samples. A series of 256 samples from both diabetics and controls showed reproducibility of separation methods (Fig. S1*C*). The magnetic separation methods were used thereafter, eliminating the need for large sample sizes because each sample was more representative and highly reproducible of the starting blood.

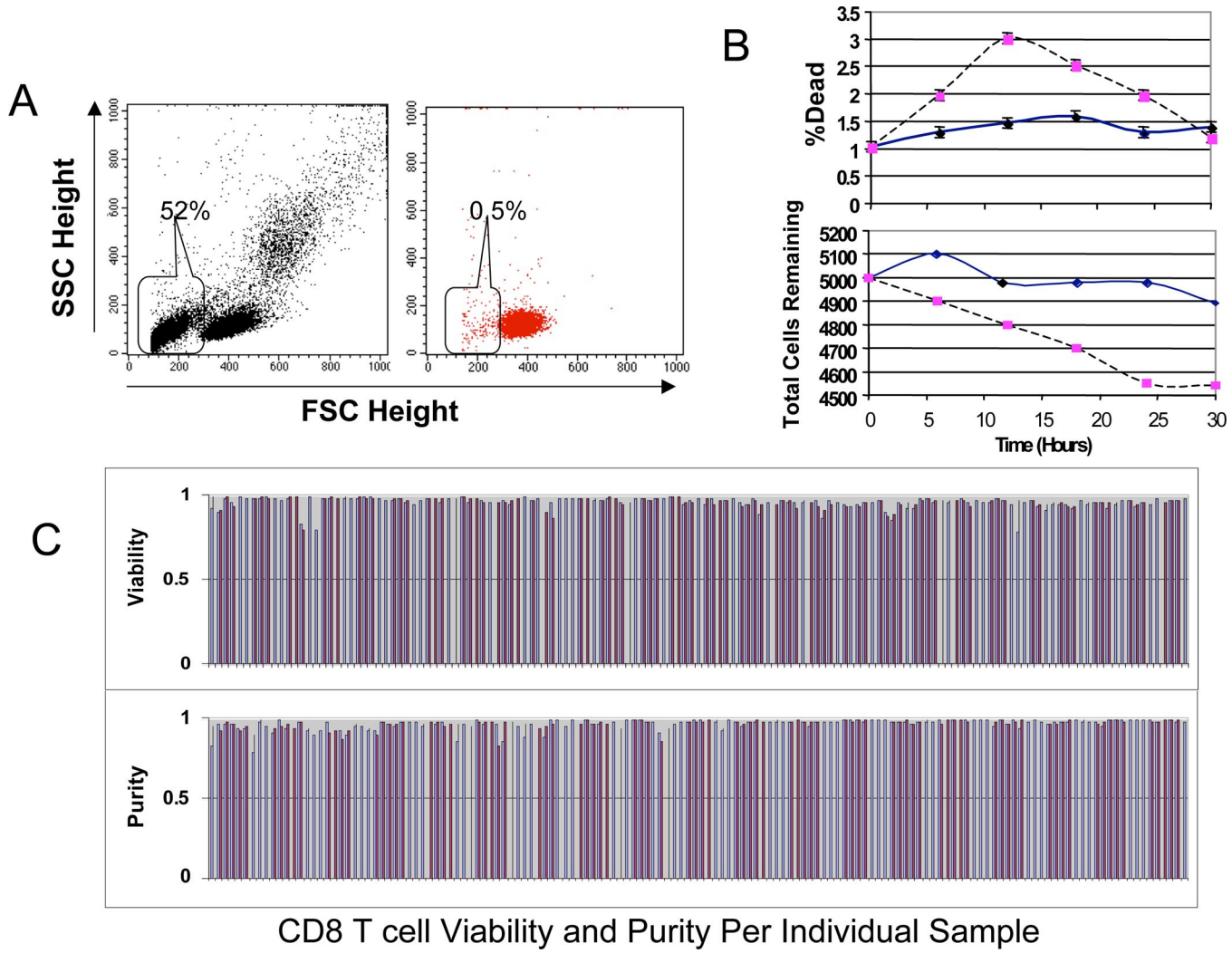

Fig. S1. Comparison of viability of lymphocytes isolated by different separation methods and time course of TNF killing with flow cytometric methods (*A*) Ficoll-separated white blood cells (WBCs) from human blood show high cell death after isolation (left histogram), with 52% rate of cell death compared to magnetic separation methods (*Right* histogram), with a rate of only 0.5% cell death. Death is measured by flow cytometry using forward scatter (FSC) versus side scatter (SSC). (*B*) Flow cytometry was used to measure death of diabetic compared to control CD8 T cells by counting of dead cells *(Top)* to total cells remaining (*Lower*) over time. (*C*) CD8 T cells separated from fresh human blood by direct magnetic separations consistently have high viability and purity ($n = 256$ blood samples from both diabetics and controls.

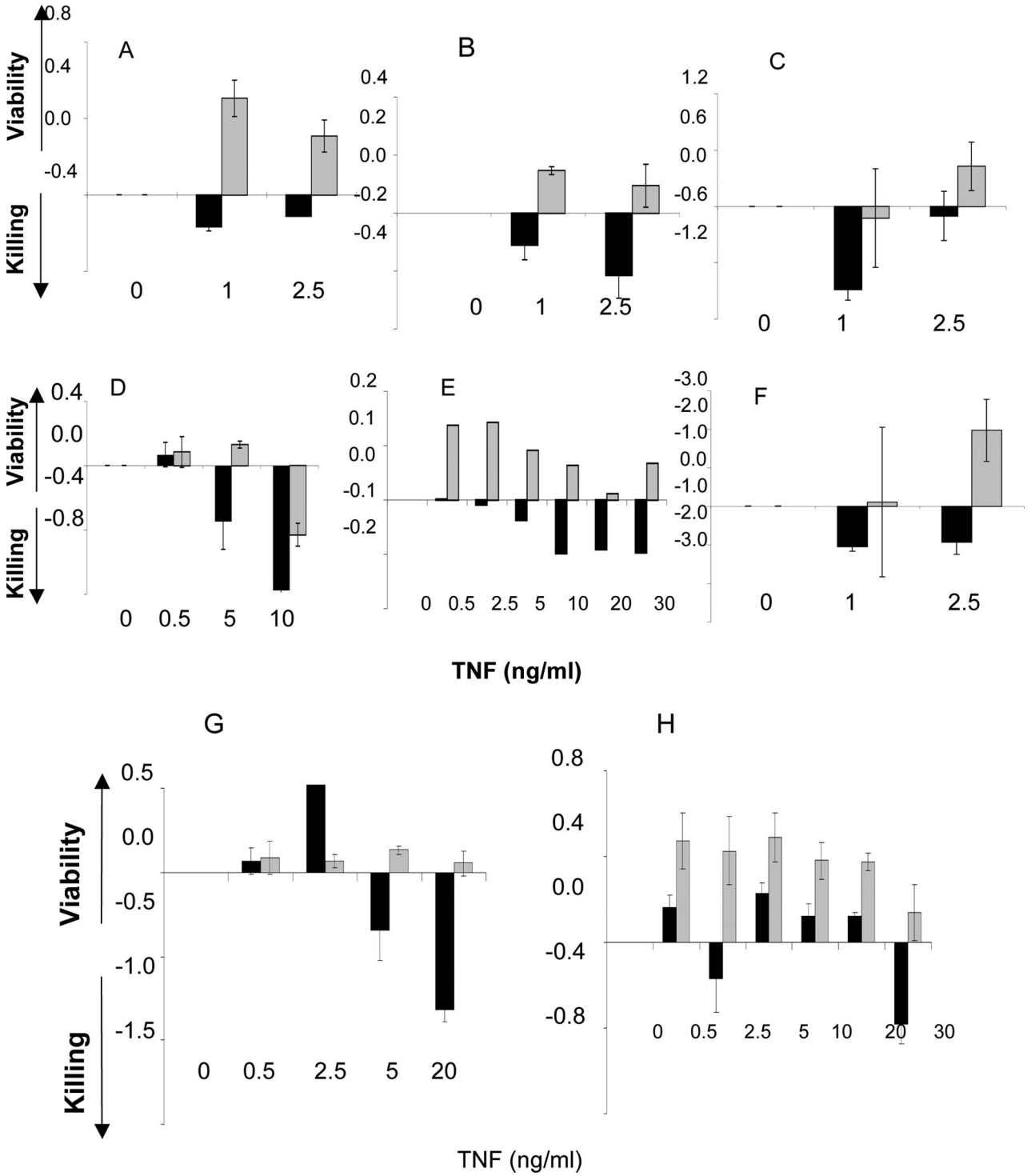

Fig. S2. TNF treatment of purified human CD8 T cells from autoimmune patients (black bar) compared to controls (shaded bar) for viability versus killing in patients with (*A*) Type 1 diabetes (*B*) Lupus (*C*) Psoriasis (*D*) Crohn's (*E*) Hypothryroidism (*F*) Multiple sclerosis (*G*) Crohn's patient who developed lupus and type 1 diabetes while taking anti-TNF therapy (*H*) Type 1 diabetic patient who at the age of 40 years developed rheumatoid arthritis. Each figure represents one patient compared to one control. The WST-1 assay was used for all patients except (*D, G*, and *H*), for which the LDH assay was used.

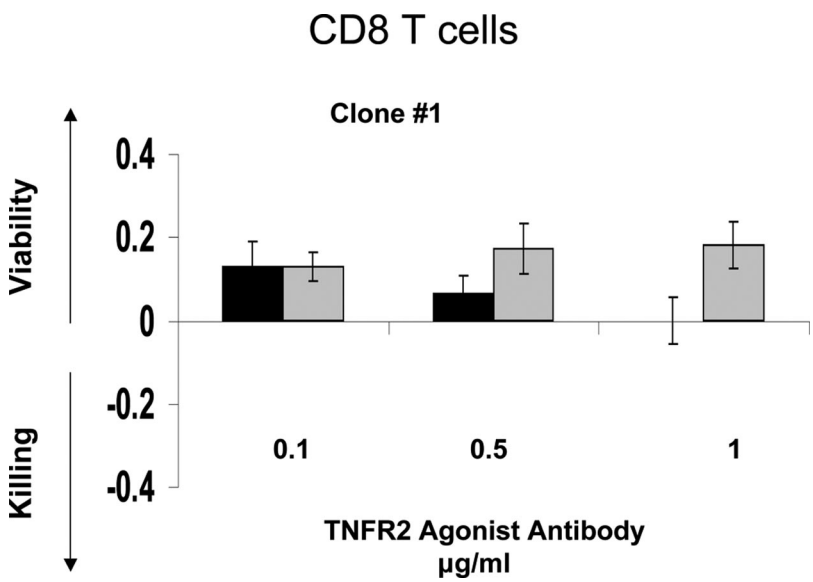

Fig. S3. Effect of TNFR2 agonist antibody clone #1 on death of Type 1 diabetic (dark bar) compared to control (shaded bar) CD8 T cells using the WST-1 assay ($n = 51$ paired samples). As in other assays, the WST-1 assay shows mild proliferation of control CD8 T cells and killing of diabetic CD8 T cells. Data from 51 paired type 1 diabetic and control samples. TNFR2 agonist clone #1 at concentrations of 0.1, 0.5, 1 μg/ml correspond to p values of 0.99, 0.17, 0.01, respectively.

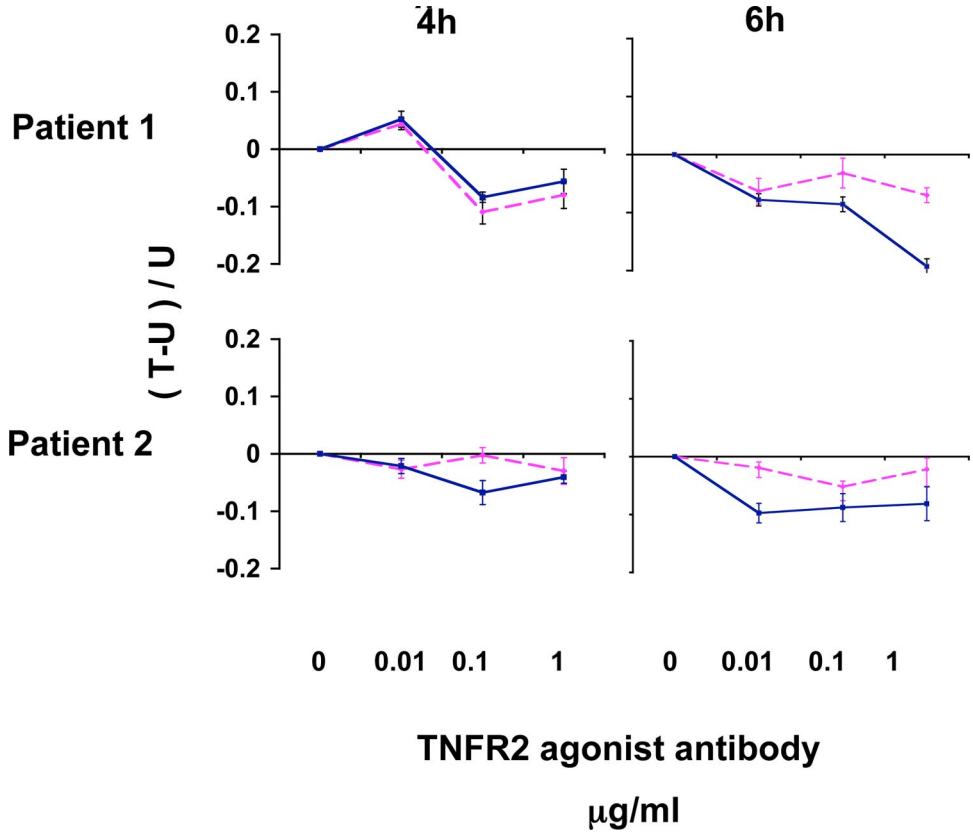

Fig. S4. TNFR2 agonist antibody clone #1 induces apoptosis of type 1 diabetic CD8 T cells (dashed line) compared to control T cells (solid line) in the Caspase 3/7 apoptotic assay. CD8 T cells from two type 1 diabetic patients are compared to those from two controls at two different assay time points of 4 h (*h*) and 6 h (*h*). Death was calculated by the caspase signal with TNFR2 treatment minus the caspase background signal without TNFR2 treatment divided by the caspase background signal.

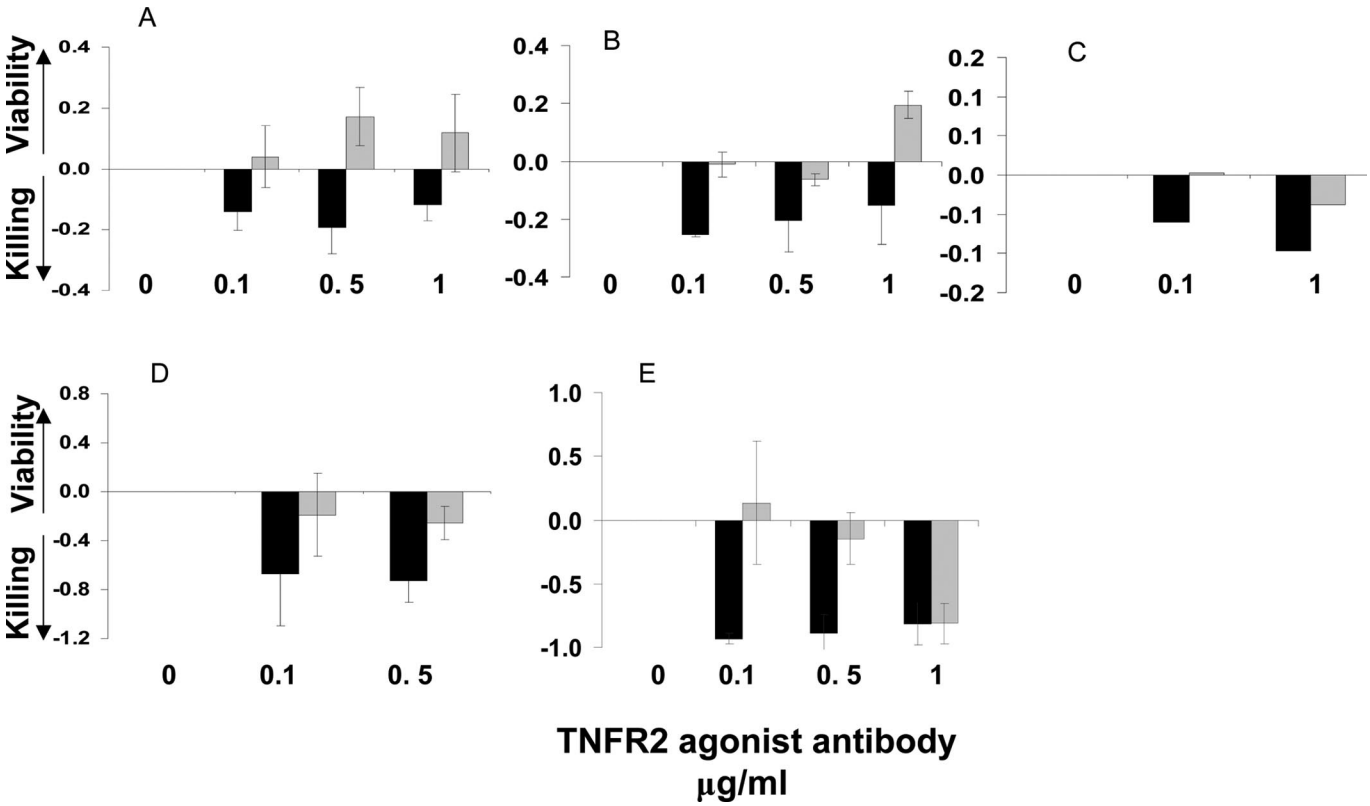

Fig. S5. TNFR2 agonist clone #1 treatment of purified human CD8 T cells from autoimmune patients (black bar) compared to controls (shaded bar) for viability versus killing. (*A*) Type 1 diabetes (*B*) Lupus (*C*) Graves (*D*) Psoriasis (*E*) Multiple Sclerosis Each figure represents one patient compared to one control.

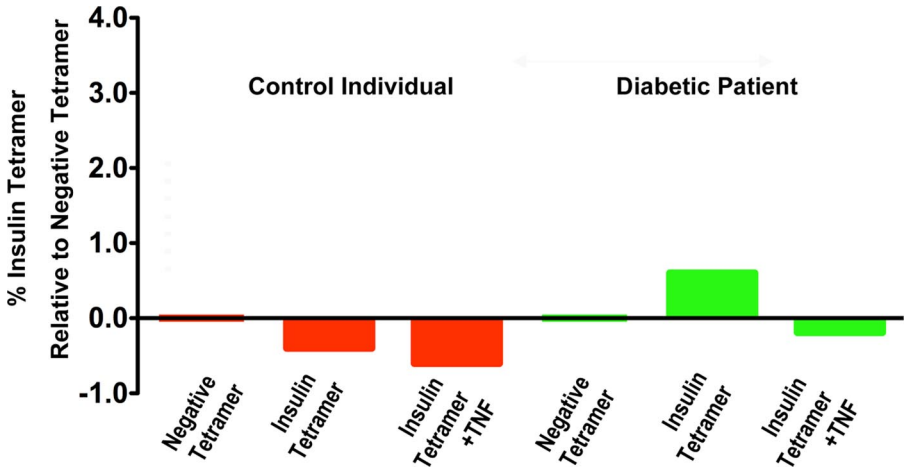

Fig. S6. Treatment of insulin autoreactive CD8 T cells with TNF kills only the pathogenic autoreactive T cells. Targeted elimination of insulin B10–18 tetramer+ T cells with a 6-hour incubation with TNF in a long-term diabetic compared to a matched control.

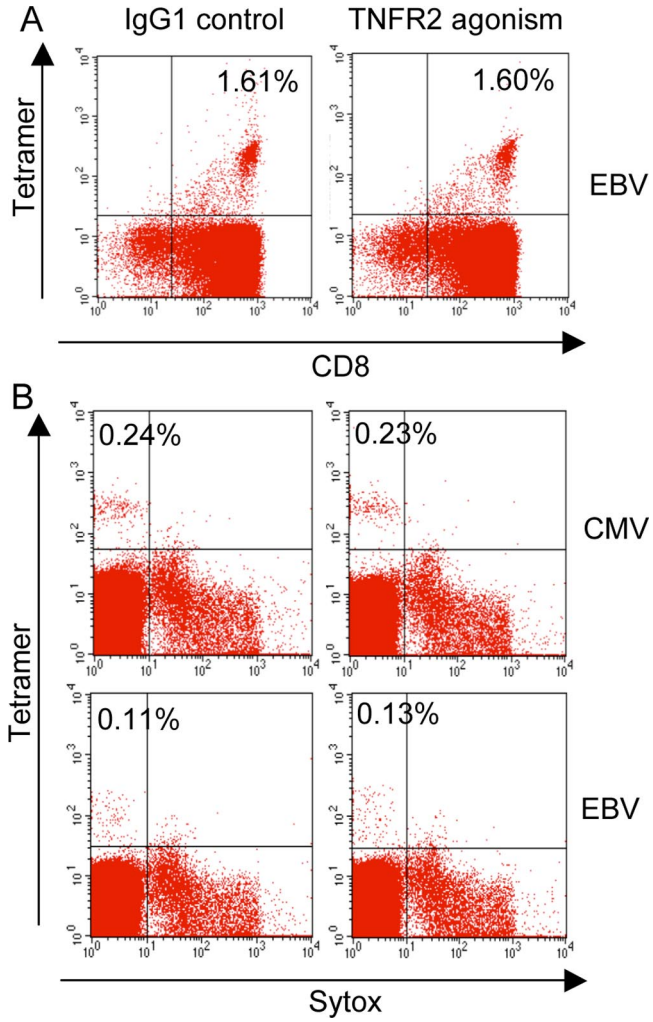

Fig. S7. Treatment of CMV or EBV CD8 T cells from two diabetic patients with TNFR2 agonist clone #1 does not cause death of activated, but not autoreactive, T cells. (*A*) CD8 T cells from a type 1 diabetic with EBV peptide reactivity do not die when exposed to TNFR2 agonist. Cell death without (IgG1 control, *Left*) or with TNFR2 agonist (TNFR2 agonist, *Right*) was monitored by looking for the disappearance of tetramer postiive cells before (1.61%) and after TNFR2 agonism (1.60%). (*B*) CD8 T cells from a type 1 diabetic with both CMV *(Top)* or EBV (*Lower*) peptide reactivity do not die when exposed to TNFR2 agonist. Cell death without (IgG1 control, *Left*) or with TNFR2 agonist (TNFR2 agonist, *Right*) was monitored with Sytox staining for direct cell death.

Table S1. A subpopulation of diabetic T cells die by apoptosis with TNF exposures; impact of sample size on death detection

	T cells exposed to TNF
	% dead +/− SEM
Paired sample size	*n* = 44
Type 1 diabetes	2.83 +/− 0.98
Controls	2.40 +/− 0.81
P-C	0.43
Paired P=	0.446
Paired sample size	*n* = 79
Type 1 diabetes	2.97 +/− 0.22
Controls	2.37 +/− 0.42
-C	0.60
Paired P=	0.223
Paired sample size	*n* = 387
Type 1 diabetes	3.52 +/− 0.08
Controls	2.35 +/− 0.02
P-C	1.17
Paired P=	0.003