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Research paper

Methods to characterize lymphoid apoptosis in a murine model of autoreactivity

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Abstract

The immune system is shaped by the random generation of lymphocytes followed by apoptosis of self-reactive cells, a process termed negative selection. The survival of these pathogenic cells in the periphery can elicit autoreactivity. We describe the development of a biomarker assay for the detection of pathogenic subpopulations of lymphoid cells in adult non-obese diabetic (NOD) mice based on disease-specific alterations in spontaneous or triggered cell death. Utilizing improved methods of cell separations, two distinct lymphoid cell subpopulations with increased susceptibility to apoptosis were identified and quantified. A subpopulation of $CD8^+$ T cells that constitutes ~3–7% of the total $CD8^+$ T cell population underwent apoptosis on exposure to low concentrations of TNF-α. Such cells were exclusively detected only in NOD mice with histologic signs of active autoreactivity. The non-T cell compartment of NOD immune system, although resistant to TNF-α-induced apoptosis, contained a subpopulation of B cells with spontaneous death by culture alone. The refined detection of small numbers of lymphoid cell subsets with quantifiable differences in apoptosis provides a possible immune biomarker for monitoring disease activity or treatment interventions.

Keywords: Apoptosis; Cytokines; Splenocytes; Biomarkers; TNF-α

1. Introduction

Apoptosis plays a central role in the development and peripheral shaping of the immune system. During lymphocyte development, autoreactive cells are eliminated by apoptosis, a process termed negative selection. Regulation of apoptosis has been found to be abnormal in a diversity of human and murine autoimmune diseases, allowing the survival of autoreactive cells in the periphery (Ohashi, 2003). A failure of antigen presenting cells to properly display a full repertoire of self-peptide in the MHC classes I or II antigen presenting structures allows self-reactive T cell formation. The cellular responses to certain death promoting cytokines that shape T cell selection in the periphery, such as free TNF-α (non-receptor bound), are deficient in certain forms of human and murine autoimmunity and may also allow self-reactive cells to survive and cause dis-

Abbreviations: NOD, non-obese diabetic; MHC, major histocompatibility complex; FBS, fetal bovine serum; PI, propidium iodide; C57, C57BL/6; FSC, forward scatter of cells; SSC, side scatter of cells.

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ease (Gabay et al., 1997; Jurewicz et al., 1999). The induction of TNF-α appears to be a highly targeted strategy to destroy autoreactive T cells in vivo, as has been shown in two different spontaneous murine models of autoimmunity (Jacob and McDevitt, 1988; Ryu et al., 2001). The therapeutic administration of anti-TNF-α in humans worsens certain forms of autoimmunity or induces new forms of autoimmunity in some clinical settings (van Oosten et al., 1996; Anonymous, 1999; Sandborn and Hanauer, 1999; Sicotte and Voskuhl, 2001; Shakoor et al., 2002; Anonymous, 2003).

The routine detection of freshly isolated autoreactive cells in humans or mice with autoimmunity has been difficult. In large part, antigen-specific T cells have been sought as indicators of active disease. These cells are extremely rare, require a prior knowledge of specific antigen specificity, and in vivo cell numbers vary depending upon the stage of disease and the type of autoreactivity. As an alternative to the detection of antigen-specific T cells, the detection of broader populations of poorly selected lymphoid cells with a phenotype of altered apoptosis offers an alternative. Indeed for many years, human and murine researchers observed altered apoptosis in unseparated blood or spleen cells populations in a diversity of autoreactive diseases (Emlen et al., 1994; Rose et al., 1997; Perniok et al., 1998). To date, these observations have not been expanded into more tightly controlled biomarker assays that allow an individual animal or human to be evaluated for the degree of the defect or the cellular subpopulation possessing the altered apoptosis defects. The ability to detect autoreactive immune cells directly would make it possible to monitor these cells during disease progression and treatment.

The development of new cell-based autoimmune detection methods based on induced or accelerated apoptosis requires freshly isolated lymphoid cells. These fresh cells need to have reproducible viability and yield prior to the assay start. Lymphoid cells in murine models of autoimmunity have traditionally been harvested from the spleen. A growing literature shows the inadequacy of traditional splenocyte isolations (Hsueh et al., 2002). This highlights the need to develop methods that eliminate harsh gradient cell separations, lengthy centrifugations, and applications of toxic red blood cell lysis methods or cell transfers that create time delays prior to analysis. The recent successful multi-center efforts to meticulously refine the most basic steps of mouse splenic B lymphocyte cell separations demonstrate the success of standardized fresh splenocyte studies (Sambrano et al., 2002). These concerted efforts on devising new lymphocyte isolations methods have yielded viable cells at the start, reproducible cellular representations and allowed multi-center efforts to define normal signal transduction networks of B-lymphocytes from the spleens of normal mice (Hsueh et al., 2002). A similar standardization of all lymphoid cell isolations is necessary for the development of reliable apoptotic markers for autoimmune subpopulations.

The present study develops a new method for the rapid isolation of splenocytes with high yield and consistent viability from diabetes-prone NOD and control mice. We then optimized culture conditions for these fresh cells in order to quantify two forms of lymphoid cell death i.e. cell death induced by TNF-α and spontaneous apoptosis in vitro. With this approach, we have identified a quantifiable subpopulation of T cells, with co-expression of CD8, that selectively undergo cell death on exposure to TNF-α. We also identify a subpopulation of non-T cells that selectively undergo cell death with culture alone. The procedures and culture conditions described are conveniently transferable and provide a suitable beginning for the format of similar quantitative studies of the numbers of autoimmune cells with altered apoptotic programs.

2. Materials and methods

2.1. Animals

Female NOD mice were obtained from Taconic Farms (Germantown, NY), and C57BL/6J (C57) and BALB/c mice were from The Jackson Laboratory (Bar Harbor, ME). All experiments were performed with NOD mice older than 12 weeks but before the onset of hyperglycemia, which typically occurs at 20 to 30 weeks of age. The NOD mice were maintained under pathogen-free conditions and screened for the onset of diabetes by monitoring of body weight and blood glucose concentration. Diabetes was diagnosed when two consecutive blood glucose concentrations exceeded 300 mg/dl.

2.2. Isolation and culture of splenocytes

Splenocyte isolation was optimized to yield the highest splenocyte viability and maximum overall cell yield. Mice were killed by cervical dislocation, and the spleen was removed through an abdominal incision, placed in a sterile petri dish containing RPMI medium supplemented with 10% FBS, and gently inflated by repeated injection of 1 to 2 ml of the medium with a 22-gauge needle. The fluid that leaked out of the spleen,

which contained dislodged splenocytes, was collected. Additional splenocytes were collected by gentle extrusion of tissue with blunt-tip forceps through a small opening created at one end of the spleen; the loose clumps of tissue were readily dissociated into single cells by repeated gentle pipetting. The collected cells were then passed through a 40-μm mesh filter to yield a crude splenocyte preparation. Purified splenocytes or lymphocyte subpopulations were cultured at 34 °C and 95% humidity in RPMI medium supplemented with 10% FBS and antibiotics.

2.3. Removal of RBCs

RBCs were removed from the crude splenocyte preparation in one of three ways: (i) The cells were resuspended in 10 ml of NH_4Cl lysis buffer [140 mM NH_4Cl, 17 mM Tris–HCl (pH 7.65)], incubated for 10 min at room temperature, and then washed once with RPMI medium containing 10% FBS. (ii) Cells suspended in RPMI medium containing 10% FBS (5 ml) were layered on top of 5 ml of Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) in a 15-ml conical centrifuge tube and centrifuged at $1300\times g$ for 10 min at 4 °C, after which those present at the interface of the two layers were collected and washed once with 10 ml of RPMI medium containing 10% FBS. (iii) Cells (5×10^7) suspended in RPMI medium containing 5% FBS and were incubated for 15 min at 4 °C with magnetic beads conjugated with rat mAbs to mouse Ter119 (Miltenyi Biotec, Auburn, CA), washed once, and applied to a magnetic LS column (Miltenyi Biotec); the cells that passed through the column were washed once with RPMI medium supplemented with 10% FBS.

2.4. Fractionation of splenocyte subpopulations

For the separation of T cells from other splenocytes, we used a Pan T Cell Isolation Kit (Miltenyi Biotec), which includes a mixture of magnetic beads linked to antibodies specific for CD45R (B cells), DX5 (NK cells), CD11b (dendritic cells, monocytes–macrophages, granulocytes), and Ter119 (erythrocytes) and therefore removes virtually all non-T cells from a mixed cell population. For a similar negative selection of non-T cells, we used a mixture of magnetic beads conjugated with antibodies to CD90 (T cells) and to Ter119 (erythrocytes). For the positive selection of B cells or monocytes–macrophages, we used magnetic beads conjugated with antibodies to CD19 or to CD11b, respectively (Miltenyi Biotec). CD4 and CD8 T cells were isolated by positive selection using magnetic beads conjugated with antibodies against CD4 or CD8, respectively (Miltenyi Biotec).

2.5. Quantitation of cell viability, cell loss and apoptosis

Cell preparations were analyzed with a FACScalibur flow cytometer (BD Biosciences, San Jose, CA) after culture for 20–24 h in RPMI medium supplemented with 10% FBS or in RPMI medium with or without 50 ng/ml mouse TNF-α added (Sigma). Apoptotic cells were detected by staining either with propidium iodide (PI) alone or with a mixture of PI and FITC-conjugated annexin V (TACS Annexin V-FITC Apoptosis Detection Kit; R&D Systems, Minneapolis, MN). In the latter approach, early apoptotic cells were defined as cells positive for staining with annexin V only, whereas late apoptotic cells were defined as cells positive for staining with both PI and annexin V. Cell viability was also assessed by staining with trypan blue; portions of cell suspension (25 μl) were diluted fivefold with 0.05% trypan blue (Sigma, St. Louis, MO) in PBS and examined with a hemacytometer. All samples were also studied for total cells remaining in case the dead apoptotic cells were no longer part of the PI positive cell population.

2.6. Statistical analysis

Comparisons between groups were performed with the one-tailed Student's *t*-test. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Increased death rate of NOD splenocytes in culture

Freshly isolated unstimulated splenocytes die in culture (Sun et al., 1992; Perandones et al., 1993; Zhang et al., 1995; Rinner et al., 1996; Sodja et al., 1998). With this fact as a starting point for the detection of subpopulations of autoreactive cells with an increased susceptibility to apoptosis, we isolated cells from the spleen of C57 and late-stage prediabetic NOD mice (12 to 17 weeks of age). RBCs were initially removed by density gradient centrifugation with Ficoll-Paque Plus. The purified splenocytes were cultured at a density of 5×10^6 cells per well (100 μl) in flat-bottom 96-well plates and their viability was evaluated at various times by staining with trypan blue. Unstimulated control (C57) and NOD splenocytes both gradually died in

culture. However, the rate of viability loss was markedly greater for NOD splenocytes than for the C57 cells (Fig. 1).

Given that the viability of primary splenocytes depends on cell density, we repeated the cell survival assays at different cell densities. In this instance, we removed RBCs from the crude splenocyte preparations with the use of magnetic beads conjugated with antibodies to Ter119, a procedure that is less harsh as compared to both Ficoll gradient centrifugation and NH_4Cl-induced RBC lysis (see below). The splenocytes were plated at densities of 10,000, 25,000, 50,000, or 100,000 cells per well (100 μl) in U-bottom 96-well plates and cultured for 24 h, after which cell viability was determined by staining of dead cells with PI and flow cytometry (see below). The viability of both C57 and NOD splenocytes increased with cell density (Fig. 2A). The viability of the NOD splenocytes, however, was again markedly lower than that of the C57 cells at all cell densities with the exception of 10,000 cells per well. Taking into account these data as well as the economic utilization of cells, we chose a cell density of 25,000 cells per well in U-bottom 96-well plates as our standard condition for characterization of cell death. The U-shaped bottom of the culture wells causes the cells to settle in the middle of the wells, resulting in a high local density even at low cell numbers (Fig. 2B); in contrast, the cells are situated farther apart in flat-bottom wells at the same seeding density.

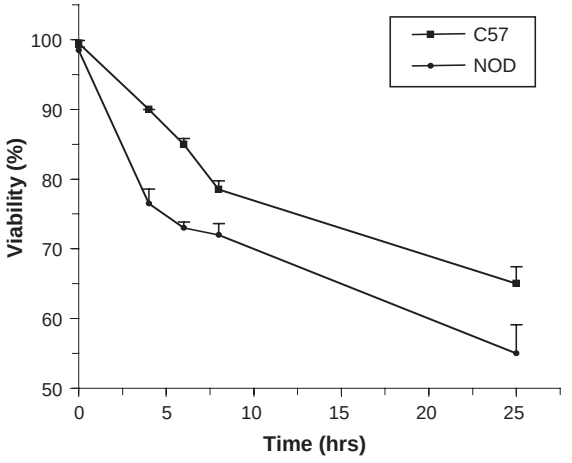

Fig. 1. Comparison of the rates of loss of viability for NOD and C57 splenocytes in culture. Crude splenocyte preparations were freed of RBCs by density gradient centrifugation on Ficoll-Paque Plus and then cultured at a density of 5×10^6 cells per well (100 μl) in flat-bottom 96-well plates. Cell viability on the basis of trypan blue exclusion was determined at the indicated times. Two representative experiments are shown performed in triplicates. A total of seven separate experiments were performed. The (●) indicate values for NOD mice; the (■) indicate values for C57 mice.

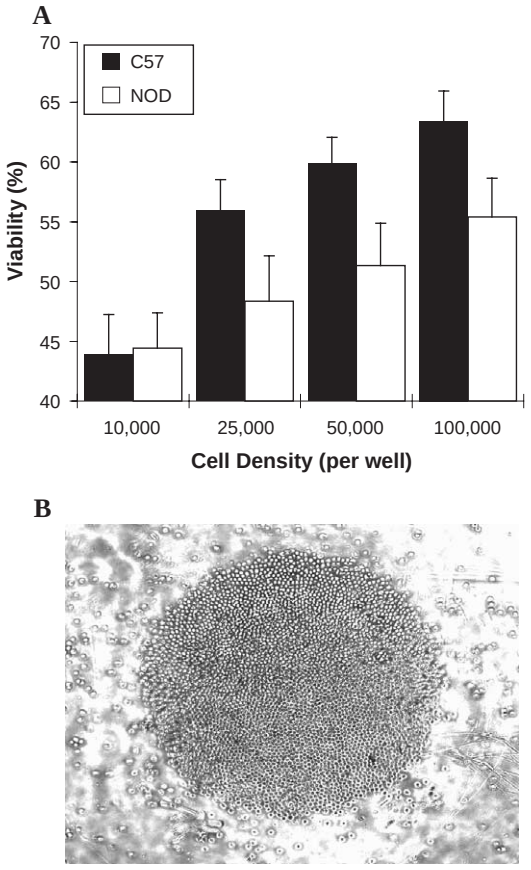

Fig. 2. Cell density dependence of NOD and C57 splenocyte viability in culture. (A) RBCs were removed from crude splenocyte preparations with magnetic beads linked to antibodies to Ter119, and the remaining cells were seeded at densities of 10,000 to 100,000 cells per well (100 μl) in U-bottom 96-well plates. Cell viability was determined after 20–24 h by staining of dead cells with PI and flow cytometry. Data are from three experiments done on separate days. (B) NOD splenocytes plated at a density of 10,000 cells per well as in (A) were examined by phase-contrast microscopy after culture for 24 h. The U-shape of the well bottom causes the cells to settle at the middle of the well, creating a high local cell density. Original magnification, 100×.

3.2. Dependence of splenocyte viability on the method of RBC removal

Given that an overall low viability of freshly isolated splenocytes might be expected to hamper the ability to detect and quantify subpopulations of cells with an increased susceptibility to apoptosis, we sought to optimize the method for cell isolation from the spleen of normal mice. For the dissociation of splenic tissue, we first tried the traditional method of processing the

spleen through a cell strainer with a 40-μm mesh. However, the viability of cells isolated by this procedure, as measured by trypan blue exclusion, was variable and frequently as low as 85%. We therefore evaluated several variations of this standard method to try to improve the initial cell viability. We eventually selected a procedure that involves inflating the spleen with culture medium to loosen the tissue, followed by harvesting of the tissue by scraping and further dissociation of cell clumps by gentle pipetting (see Materials and methods). This approach consistently yielded a high initial viability of >95% as determined by various assays.

We next evaluated various methods for removal of RBCs from the crude splenocyte preparation. Cell death was monitored on the basis of flow cytometric analysis of cells stained with annexin V and PI. We thus compared the viabilities of splenocytes that had been cultured for 20–24 h after the removal of RBCs by NH_4Cl-induced lysis, Ficoll gradient centrifugation, or magnetic separation with antibodies to Ter119 (which is expressed on mature mouse RBCs and erythroid precursor cells, but not on lymphoid or myeloid cells). In the experiment shown in Fig. 3A, the viability of the cultured splenocytes was lowest for the cells purified by NH_4Cl lysis (34.4%), intermediate for the cells purified by density gradient centrifugation (51.2%), and highest for the cells purified by magnetic cell sorting (72.5%). Statistical data from repeat experiments ($n=21$, 9 and 14 independent experiments) show the NH_4Cl separations, with respect to viability, were inferior to Ficoll ($p=0.005$) and Ficoll separation viability was inferior to magnetically separated cells ($p=0.00006$). NH_4Cl was also inferior to magnetically separated cells ($p=5\times10^{-9}$). We selected magnetic cell sorting with antibodies to Ter119 as

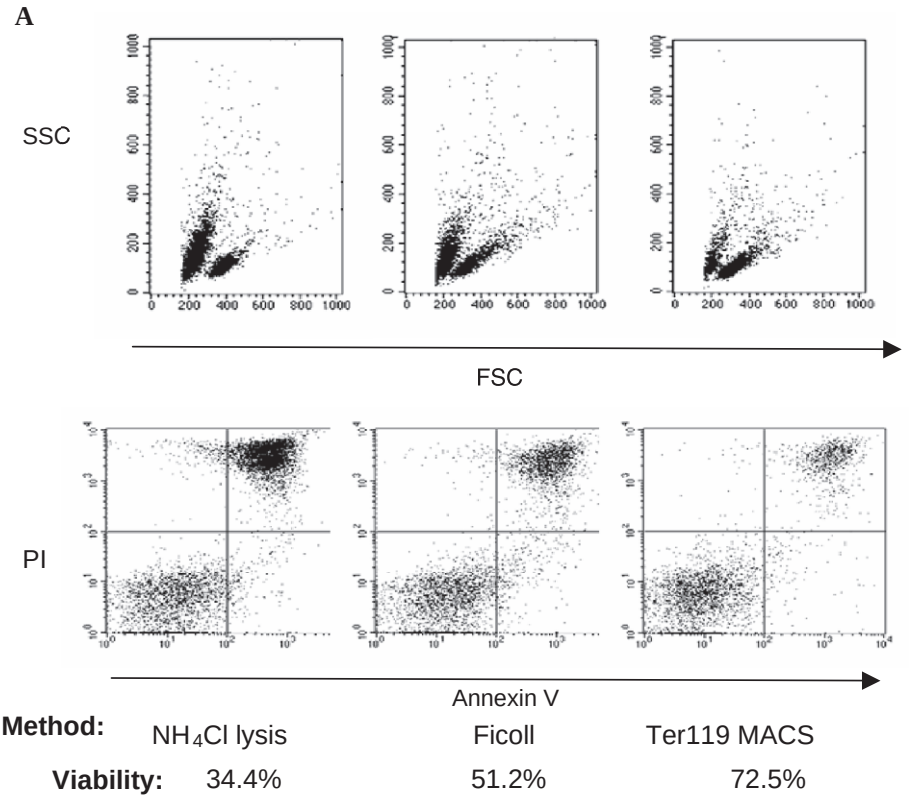

Fig. 3. Dependence of splenocyte viability on the method of RBC removal and localization of live and dead splenocyte populations on flow cytometric plots A. RBCs were removed from crude normal splenocyte preparations by either NH_4Cl-induced lysis, Ficoll gradient centrifugation, or magnetic separation (MACS) with antibodies to Ter119. The purified splenocytes were then cultured for 24 h before determination of cell viability by flow cytometric analysis of cells stained with annexin V and PI (live cells were defined as cells negative for both PI and annexin V staining). Results from a representative experiment are shown in (A). FSC, forward scatter; SSC, side scatter. (B). Localization of live and dead splenocyte populations on flow cytometric plots of SSC versus FSC. Purified C57 splenocytes were cultured for 20–24 h, stained with annexin V and PI, and analyzed by flow cytometry. Plots of SSC versus FSC (i) as well as of PI versus annexin V staining for ungated cells (ii) or for cells gated on the basis of the R1 (iii) or R3 (iv) regions identified in the scatter plot are shown.

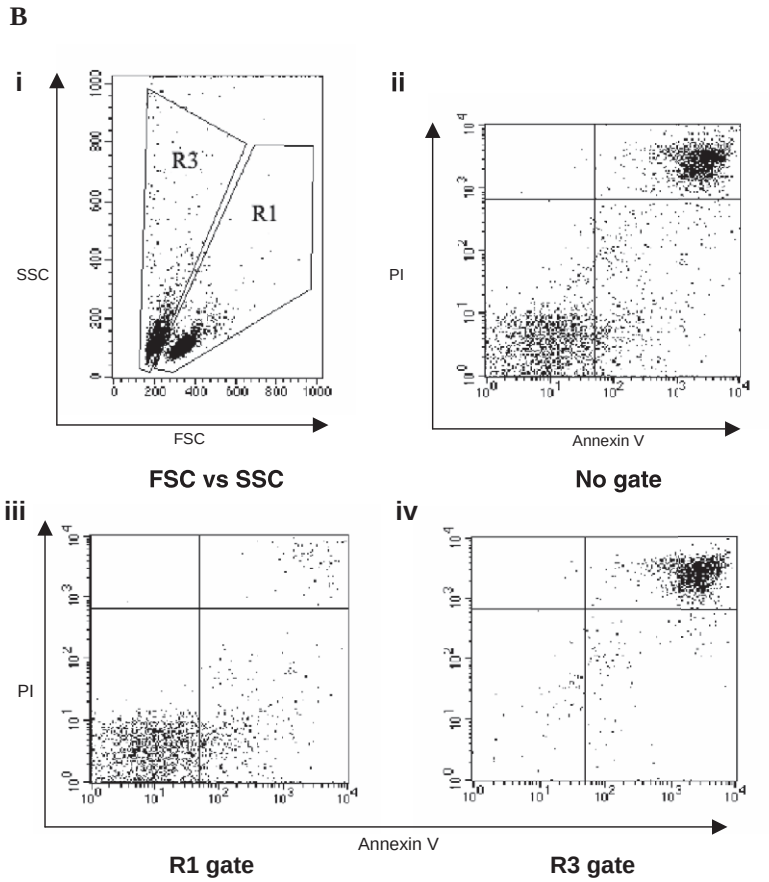

Fig. 3 (*continued*).

our approach for removal of RBCs from crude splenocyte preparations.

We determined the locations of live and dead populations of normal splenocytes (purified by magnetic cell sorting and cultured for 20–24 h) on flow cytometric plots of forward scatter (FSC) versus side scatter (SSC). The cells appeared as two major populations (R1 and R3) on such plots (Fig. 3B, i). The plot of PI versus annexin V staining for ungated cells revealed populations that remained unstained (live) or that stained with both annexin V and PI (late apoptotic or dead) (Fig. 3B, ii). We then determined the plots of PI versus annexin V staining for the cells corresponding to regions R1 (Fig. 3B, iii) and R3 (Fig. 3B, iv). These plots revealed that most live cells localized to the R1 region, whereas most late apoptotic and dead cells were restricted to the R3 region. This observation is consistent with the fact that cells shrink and become irregular in shape during apoptosis, resulting in a decrease in FSC and an increase in SSC. Thus, by gating for R1 or R3, it was possible to identify predominantly live or predominantly dead cell populations, respectively.

3.3. Differential apoptotic susceptibility of splenic T cells and non-T cells

The spleen contains a variety of immune cell types, which can be classified as T cells (mostly $CD4^+$ and $CD8^+$ cells) or non-T cells (mostly B cells and monocytes/macrophages). To determine whether the increased death rate of NOD splenocytes in culture was generalized or restricted to specific cell subsets, we used magnetic cell sorting to obtain T cell and non-T cell fractions of splenocytes from NOD and control mice. Both cell fractions were isolated by negative selection. For the isolation of T cells, we thus removed B cells, NK cells, dendritic cells, monocytes–macrophages, granulocytes, and erythroid cells. For the isolation of non-T-cells, we removed T cells and erythroid cells. The use of negative selection ensured that the cells to be studied had not been exposed to antibodies that bind to surface receptors and thereby trigger cell activation.

We first compared the viabilities of a mixture of T cells and non-T cells (using only anti-Ter119); of T cells

alone; and of non-T cells alone, that had been isolated from splenocytes of control mice and cultured for 20–24 h. In the experiment shown in Fig. 4A, the viabilities determined by flow cytometric analysis of PI- and annexin V-stained cells were 69.0% for T cells, 37.7% for non-T cells, and 51.9% for the mixture of T cells and non-T cells. The rate of spontaneous death in culture was thus greater for non-T cells than for T cells. We also determined the viability of T cell and non-T cell subpopulations of control and NOD splenocytes after culture for 20–24 h at different seeding densities (Fig. 4B). The viability of T cells was relatively independent of cell density and did not differ substantially between control mice and NOD mice although NOD T cells overall had a slightly higher viability at all densities. In contrast, the viability of the non-T cells increased with cell density and was greater for the control cells than for the NOD cells. This latter observation was found to be statistically significant on repetition of the experiment (Fig. 4C), with the viabilities of control and NOD non-T cells averaging $53.7 \pm 3.0\%$ and $47.9 \pm 3.6\%$, respectively.

To determine which non-T cell types were most sensitive to death in culture, we further fractionated the non-T cell population into cells that express either CD11b (monocytes–macrophages) or CD19 (B cells)

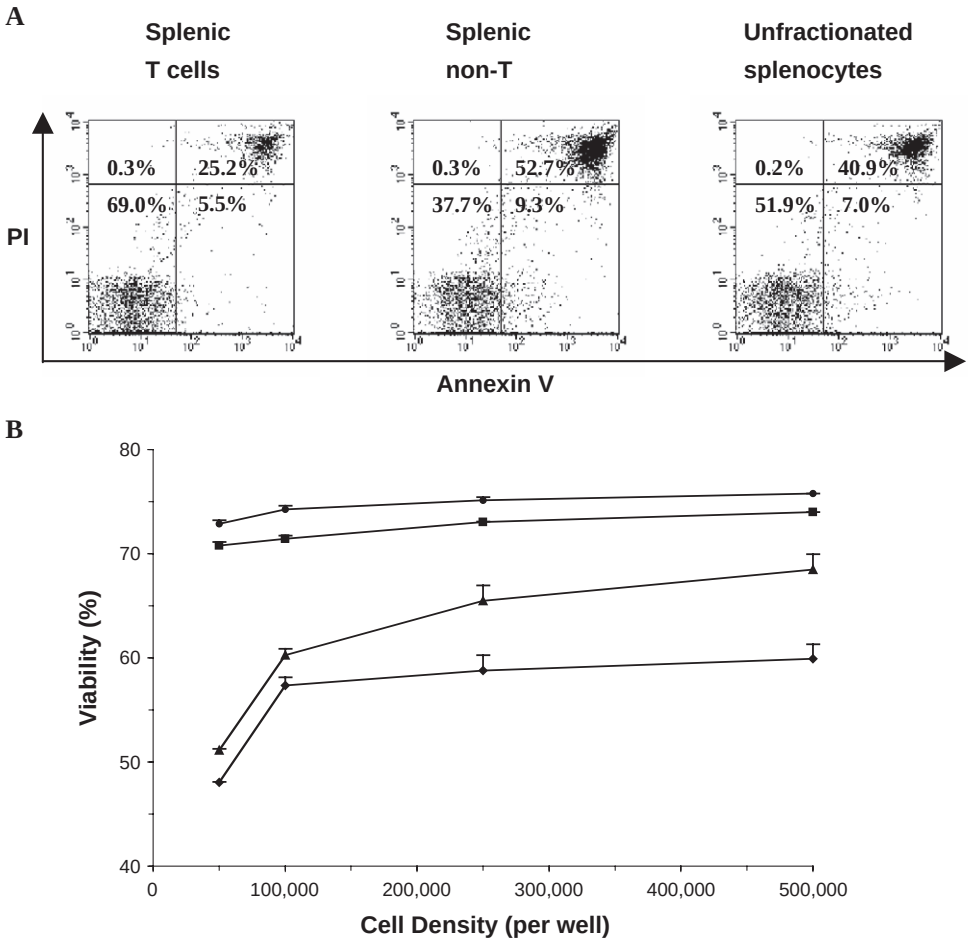

Fig. 4. Comparison of the susceptibilities of T cells and non-T cells isolated from C57 (squares, triangles) and NOD (circles, triangles) splenocytes to spontaneous apoptosis in culture. (A) Freshly isolated splenocytes of C57 mice were fractionated into T cells (circles, squares) or non-T cells (triangles, diamonds), or simply freed of RBCs (nonfractionated splenocytes), and the various cell subpopulations were then cultured for 20–24 h before staining with PI and annexin V and analysis by flow cytometry. (B) T cell and non-T cell fractions isolated from C57 or NOD splenocytes were plated at the indicated densities and cultured for 24 h, after which cell viability was determined as in (A). Data are from 3 representative experiments. (C) Viabilities of non-T cell fractions of C57 and NOD splenocytes determined after culture for 20–24 h as in (A). Data are means ± S.E.M. of values from 12 independent experiments. (D) The non-T cell subpopulations of C57 and NOD splenocytes were further fractionated into monocytes or B cells, and the resulting cell fractions were cultured and assayed for cell viability as in (A). Data are from 3 representative experiments and are expressed as the percentage of initially viable cells that died during culture.

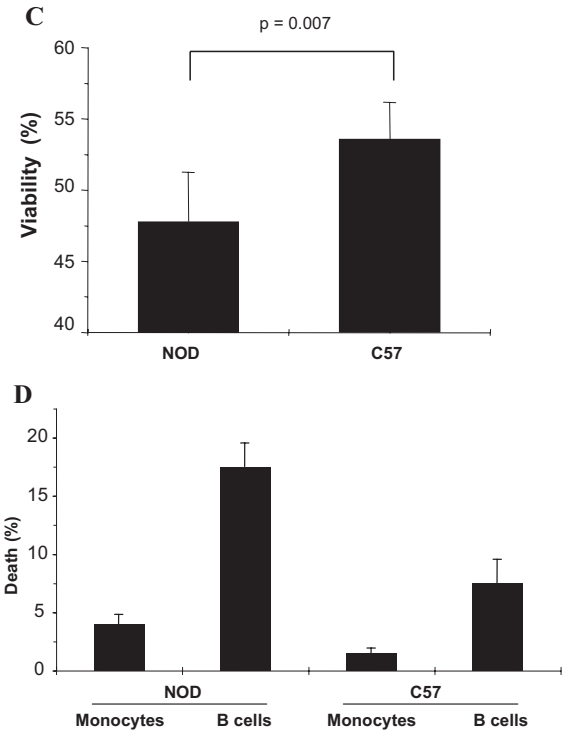

Fig. 4 (*continued*).

by positive selection. Whereas culture of each cell subpopulation for 24 h resulted in substantial cell death, the extent of such death was greater for B cells than for monocytes–macrophages for both NOD and control cells. Greatest cell death was observed for B cells from NOD (Fig. 4D).

3.4. Enhanced sensitivity of a subset of NOD splenocytes to TNF-α-induced apoptosis

Flow cytometric analysis of cells stained with a combination of PI and annexin V is able to distinguish late apoptotic or dead cells (positive for both PI and annexin V) from live cells (negative for both PI and annexin V) and early apoptotic cells (PI negative, annexin V positive). However, given that: (1) this approach cannot distinguish between late apoptotic and dead cells; (2) that annexin V binds nonspecifically to B cells (Dillon et al., 2000); (3) that we did not detect necrotic cells (PI positive, annexin V negative) in our death assays; and (4) that FITC–annexin V is relatively expensive for large-scale use, we adopted a simpler flow cytometric analysis of cell death that is based on FSC of PI-stained cells (see below).

We previously showed by both trypan blue exclusion and flow cytometric analysis of PI- and annexin V-stained cells that NOD splenocytes isolated by Ficoll gradient centrifugation after NH_4Cl treatment contain a subpopulation of cells that exhibit an increased sensitivity to TNF-α-induced apoptosis (Hayashi and Faustman, 1999; Hayashi et al., 2000). To confirm this defect with our newly developed cell isolation and death detection protocols, we removed RBCs from crude control (C57) or NOD splenocyte preparations by magnetic cell sorting with anti-Ter119 and then plated the purified splenocytes in U-bottom 96-well plates at a density of 25,000 cells per well in 100 μl of RPMI medium in the absence or presence of mouse TNF-α (50 ng/ml). After culture for 24 h, the cells were stained with PI (2 μg/ml in PBS) and analyzed by flow cytometry. Plots of PI fluorescence versus FSC revealed two spatially distinct populations of live and dead cells (regions R1 and R2, respectively; see Fig. 5). Cellular debris (low FSC values) was detected to the left of region R1 but was ignored in our analysis (Fig. 5A). We counted the number of events in regions R1 and R2 and used the results to calculate percentage viability. Whereas culture with TNF-α increased the viability of C57 splenocytes for 50.6% to 54.1% (a change of 6.9%), it reduced that of NOD splenocytes from 50.2% to 47.3% (a change of –5.8%) (Fig. 5B). A protective effect of TNF-α on normal mouse splenocytes especially CD8 cells has been described previously (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996; Kuhtreiber et al., 2003).

3.5. NOD T cells are sensitive to TNF-α induced apoptosis; a defect in the CD8 subpopulation

Using the PI-FSC viability assay, we next determined whether the T cell or non-T cell subpopulation is responsible for the increased sensitivity of unseparated NOD splenocytes to TNF-α-induced apoptosis. We thus isolated T cells and non-T cells from splenocytes of late prediabetic NOD mice and normal C57 mice by negative selection, cultured them for 24 h in the absence or presence of TNF-α (50 ng/ml), and then determined their viability. Whereas C57 splenic T cells showed a minimal or no change in viability in response to TNF-α, the NOD splenic T cells showed a statistically significant decrease in viability (Fig. 6A, B). In contrast, there was no significant difference in TNF-α sensitivity between the non-T cell subpopulations of C57 and NOD splenocytes (Fig. 6C).

We further fractionated the T cell population into $CD4^+$ or $CD8^+$ cells by positive selection with antibo-

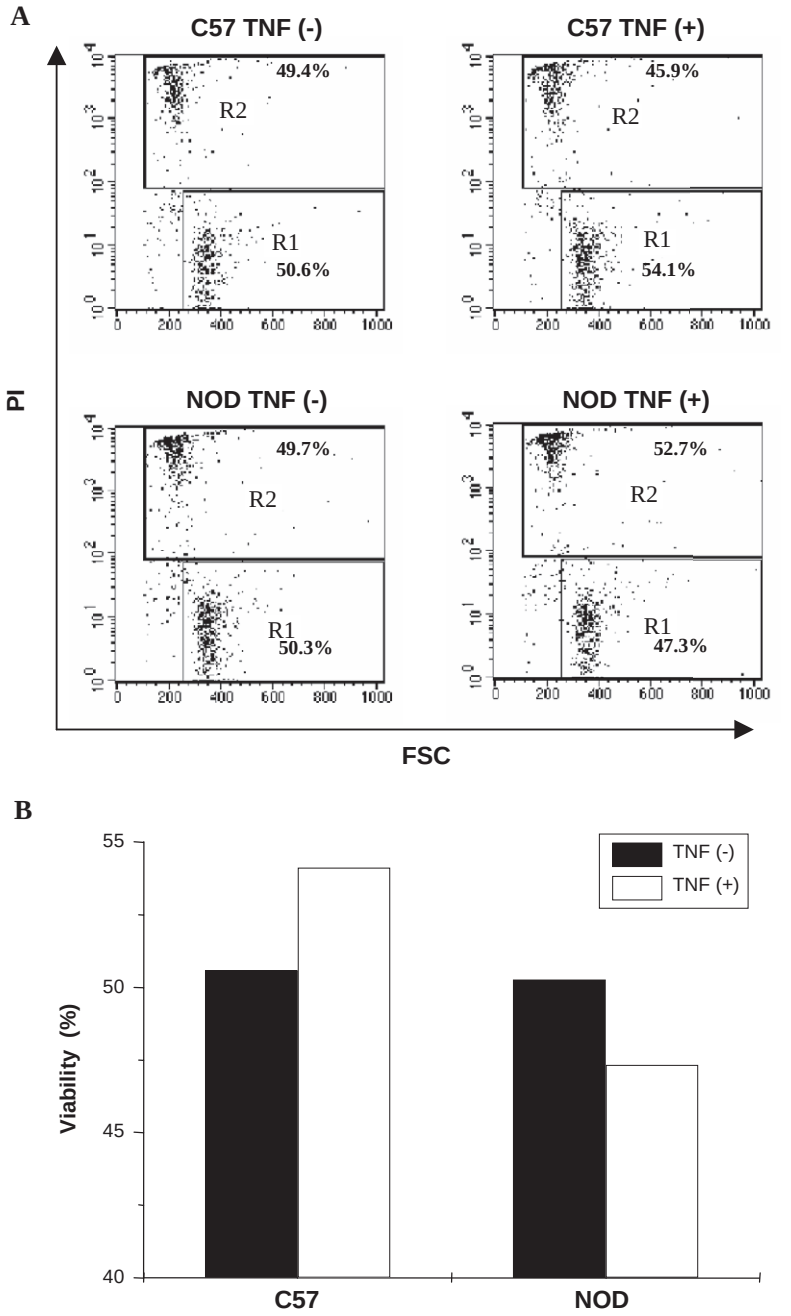

Fig. 5. Increased sensitivity of a subpopulation of NOD splenocytes to TNF-α-induced apoptosis in culture. (A) Freshly isolated splenocytes from C57 and NOD mice were cultured for 20–24 h at an initial density of 25,000 cells per well in U-bottom 96-well plates containing RPMI medium with or without TNF-α (50 ng/ml). The cells were then stained with PI and analyzed by flow cytometry. Plots of PI fluorescence versus FSC resolved the splenocytes into two spatially distinct populations of live (R1) and dead (R2) cells. (B) Percentage viability of C57 and NOD splenocytes for the experiment shown in (A). Data are from a representative experiment. Statistics are presented in Fig. 6.

dies to these antigens and magnetic cell sorting. The purified cells were cultured for 20–24 h with or without TNF-α (50 ng/ml) and then assayed for viability and total cells remaining. Isolated $CD4^+$ cells from both C57 and NOD mice showed identical trends (Fig. 7D). In contrast, $CD8^+$ cells from C57 mice all showed an increase in viability in response to TNF-α; the $CD8^+$ cells from NOD mice manifested cell subset-specific

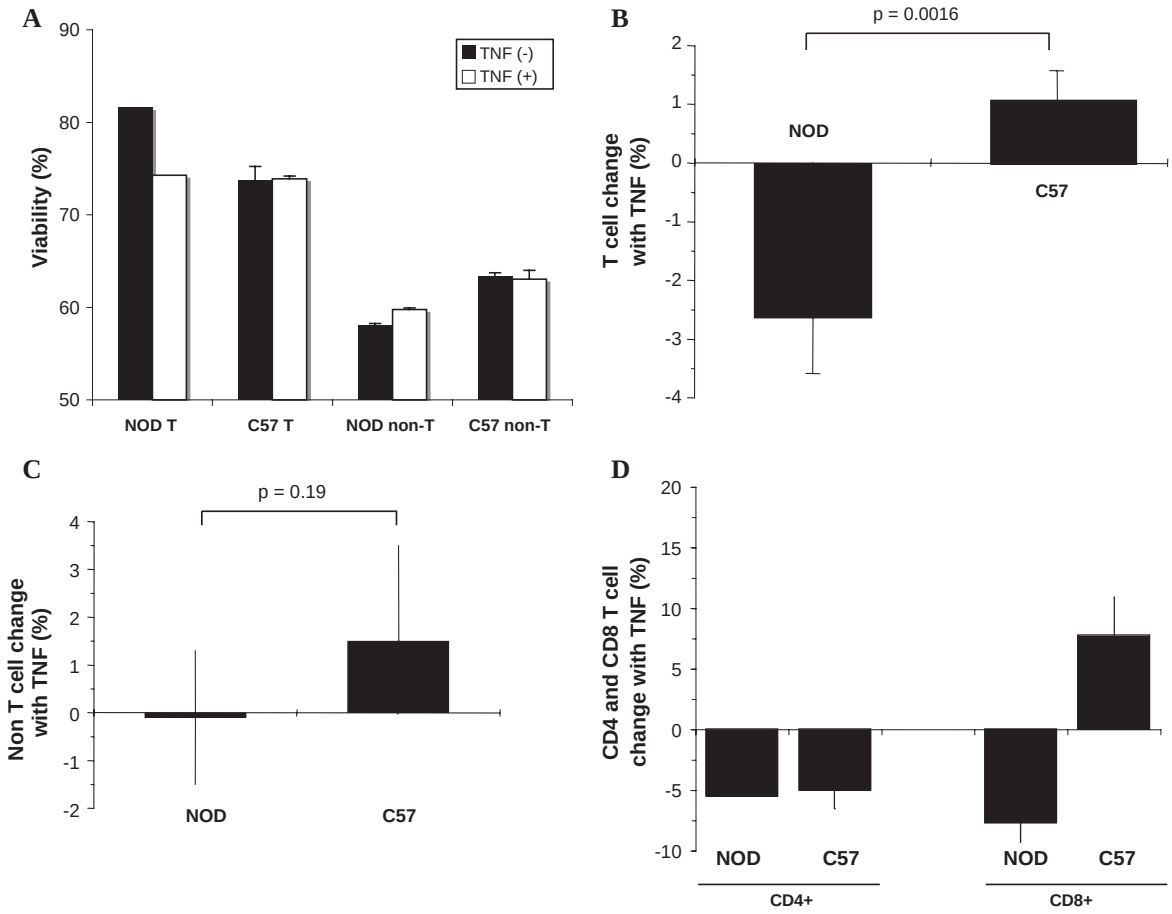

Fig. 6. Increased sensitivity of NOD splenic $CD8^+$ T cells to TNF-α-induced apoptosis. (A) T cells and non-T cells were isolated from splenocyte preparations of NOD and C57 mice, cultured for 24 h with or without TNF-α (50 ng/ml), and assayed for viability by flow cytometric analysis of PI-stained cells (plots of PI fluorescence versus FSC). Data are from 4 and 6 representative experiments. (B and C) Effects of TNF-α on T cell (B) and non-T cell (C) viability as determined in experiments similar to that shown in (A). Data are expressed as the TNF-α-induced change in viability or cell remaining and are means ± S.E.M. of values from 17 and 12 independent experiments, respectively. (D) The T cells isolated from C57 and NOD splenocytes were further fractionated into $CD4^+$ or $CD8^+$ cells and analyzed for TNF-α sensitivity as in (A). Data are from 2 representative experiments performed as triplicates and are expressed as the TNF-α-induced loss in viable cells. Similar experiments were performed a total of 7 times.

death (Fig. 6D). These results indicate that the TNF-α sensitivity of NOD splenic T cells exclusively resides in the $CD8^+$ compartment.

3.6. The sensitivity of NOD splenic T cells to TNF-α-induced apoptosis is correlated with age and extent of insulitis

The penetrance of diabetes in female NOD mice in our animal facility is ~75%. Given that we used late-stage prediabetic mice (>12 weeks of age) for our experiments, some of these mice would be expected not to progress to hyperglycemia. Although such animals (nonresponders) never become diabetic and their pancreatic islets remain free of invasive insulitis, their islets do develop peripheral insulitis, a hallmark of nonprogression. We hypothesized that the splenic T cells of nonresponders would not be sensitive to TNF-α-induced apoptosis. Of the 17 NOD mice whose splenic T cells were analyzed for sensitivity to TNF-α-induced apoptosis (Fig. 6B), 4 animals yielded T cells that showed little or no response to TNF-α. This is the expected number of nonresponders based on a penetrance of 75%. We therefore compared the extent of insulitis in pancreatic tissue of such non-responding mice with that of animals with TNF-α-sensitive T cells.

The islets of NOD mice with T cells sensitive to TNF-α-induced apoptosis typically exhibited pronounced invasive insulitis that resulted in islet destruction (Fig. 7A). In contrast, islets of NOD mice whose T cells did not manifest TNF-α-induced apo-

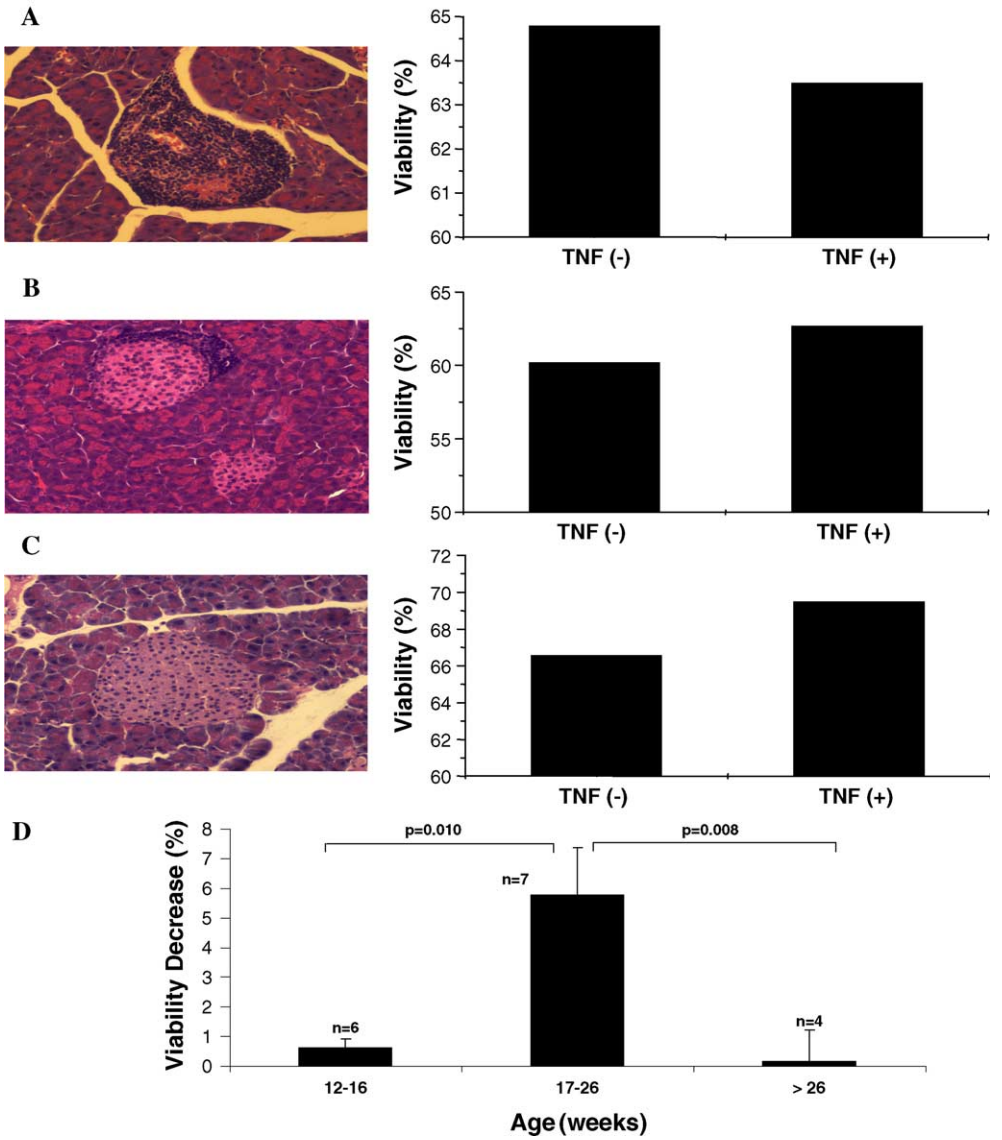

Fig. 7. Correlation of the sensitivity of splenic T cells to TNF-α-induced apoptosis in vitro with age and the extent of insulitis in NOD mice. Freshly prepared splenic T cells from NOD (A and B) and C57 (C) mice were assayed for their sensitivity to TNF-α as in Fig. 7A and the pancreases of the same animals were evaluated by histological staining with hematoxylin–eosin. NOD mice with splenic T cells that were sensitive to TNF-α-induced apoptosis showed marked invasive insulitis and islet destruction (A). In contrast, most of the islets of NOD mice whose splenic T cells did not manifest TNF-α-induced death showed peripheral or no insulitis (B). The islets of control C57 mice, whose T cells did not exhibit TNF-α-induced apoptosis, showed no insulitis (C). (D) The highest TNF-α sensitivity of the NOD T cells occurs during the time period where the NOD are expected to turn diabetic.

ptosis typically showed only peripheral insulitis or no insulitis (Fig. 7B), with only the occasional islet exhibiting invasive insulitis. The islets of C57 control mice were free of insulitis (Fig. 7C).

We also were able to correlate the sensitivity to TNF-α induced apoptosis of the NOD T cells with disease duration. We divided the same group of 17 NOD mice into three sub-groups according to their age at assay time; 12–16 weeks old (group A), 17–26 weeks old (group B), and older then 26 weeks (group C); and calculated the average TNF-α induced decrease in viability for each group (Fig. 7D). The highest TNF-α sensitivity was found for T cells from group B with a decrease in viability of $5.78 \pm 1.60\%$ ($n=7$), as compared to $0.64 \pm 0.27\%$ ($p=0.010$, $n=6$) for group A and $0.16 \pm 1.05\%$ ($p=0.008$, $n=4$) for group C. There was no statistically significant difference between group A and group C ($p=0.35$). These results correlate well with

the age at which the animals are expected to become diabetic.

4. Discussion

This report describes a standardized and reproducible procedure for the purification and culture of splenic lymphoid cell subsets from the mouse. This allows the identification and quantification of lymphocytes in the autoimmune NOD model with alterations in apoptosis sensitivity. With refined methods of harvesting and culturing fresh viable splenocytes, we have identified and quantified a subpopulation of NOD T cells, predominantly of the $CD8^+$ subtype, that undergoes apoptosis upon exposure to a low concentration of TNF-α in vitro. In mature NOD mice that are progressing to disease, these cells constitute 3–7% of the splenic T cell population. T cells from normal control mice, cultured under the same conditions, exhibit a relative increase in viability of ~7% that is likely attributable to the described normal induction of pro-survival signaling through NF-κB after TNF-α exposure (Beg and Baltimore, 1996; Van Antwerp et al., 1996).

We have also identified a subpopulation of splenic non-T cells in NOD mice that undergoes spontaneous apoptosis during culture. These cells are predominantly of B cell origin and constitute ~10% to 15% of the total non-T cell population under our culture conditions. Therefore with refined splenic isolation and culture conditions, at least two distinct subpopulations of lymphoid cells in mature NOD mice possess an altered sensitivity to apoptosis. Non-T cells isolated from individuals with lupus or Sjögren's syndrome also exhibit an increased sensitivity to spontaneous apoptosis in culture; these cells were also shown to comprise mostly B lymphocytes (Emlen et al., 1994; Gross et al., 2000). Patients with systemic sclerosis, a connective tissue disorder characterized by vascular abnormalities and excessive collagen synthesis, similarly possess PBLs with an increased susceptibility to culture-induced death (Stummvoll et al., 2000). Therefore, both spontaneous forms of murine and human autoimmunity have detectable subpopulations of lymphoid cells with altered death set points.

Diverse human and murine autoimmune diseases are characterized by the presence of lymphoid cells in the thymus and periphery with an altered sensitivity to death. This phenotype of disease appears to arise from a common denominator across several autoimmune diseases—a variety of errors in signaling by NF-κB. This transcription factor plays a tightly regulated role that is central to the immune system for the regulation of genes involved in cell fate, resistance to apoptosis and cytokine balance (Baldwin, 1996). An NF-κB signaling defect has been identified in monocytes of humans with Crohn's disease (Hugot et al., 2001; Ogura et al., 2001), an autoimmune condition of the intestine. The enhanced sensitivity of T cells in NOD or NZB mice to TNF-α has also been attributed to defective NF-κB signaling in a death pathway essential for lymphoid development and selection (Valero et al., 2002; Hayashi and Faustman, 2003). Additionally, the widespread introduction of anti-TNF-α therapies, aimed at inhibiting the cytopathogenic effects of TNF-α and NF-κB signaling, instead has uncovered a sizable fraction of patients developing new autoimmune diseases or aggravated forms of diseases such as lupus, multiple sclerosis and even diabetes (van Oosten et al., 1996; Bloom, 2000).

The subpopulations of lymphocytes with an enhanced susceptibility to apoptosis are pathogenic in NOD mice (Rabin et al., 1994; Hayashi and Faustman, 1999; Leslie et al., 1999; Ryu et al., 2001; Wakeland et al., 2001; Kuhtreiber et al., 2003). These cells are thus not merely indirect biomarkers of autoreactivity or by-products of the disease process, but rather contributors to the disease. The accumulating human and murine evidence in autoimmune models shows anti-TNF-α therapies can exacerbate or induce new autoimmune disease in humans. Additionally, low TNF-α activity may predispose to some forms of human autoimmune disease and autoreactive T cells have heightened TNF-α induced apoptosis. The mechanism of TNF-α therapeutic effect appears to be due to the direct T cell death of a highly pathogenic T cell subpopulation and in animal models this direct TNF-α killing hampers disease and prevents transfer of disease with T cells form diabetic hosts (Kodama et al., 2005). This data supports the contention that the TNF-α susceptible T cells are antigen specific and directly related to the disease process. It is possible to achieve permanent disease reversal in end-stage diabetic NOD mice by a two-component therapeutic protocol aimed at re-establishing apoptosis of diseased cell populations in adult mice (Ryu et al., 2001; Kodama et al., 2003). One component of this approach involves the elimination of pathogenic memory T cells by inducing a transient increase in TNF-α levels. Furthermore, brief exposure of splenocytes from diabetic NOD mice to TNF-α in culture markedly reduces the incidence of disease transfer to naïve male NOD cohorts, and the forced expression of TNF-α results in reversion of already diabetic NOD mice to a nondiabetic state (Ryu et al., 2001; Christen and Von Herrath, 2002). The ability to identify and quantify

pathogenic autoreactive cells in vitro would facilitate both research into pathogenesis of diabetes as well as the diagnosis and monitoring of treatment of this disease in both the NOD mouse and humans.

Only select female cohorts of NOD mice comprising about 85% of the colony progress to disease expression characterized by pronounced hyperglycemia and subsequent death. The islets of these cohorts exhibit massive invasive insulitis. The spleens of these animals, leading up to diabetes, have TNF-α sensitive T cell subpopulations. Our biomarker assay also identified splenic T cells did not exhibit enhanced sensitivity to TNF-α-induced apoptosis when disease progression did not occur i.e. aged normoglycemic NOD females. Histological examination of the pancreas of such mice revealed that the islets did not exhibit invasive insulitis, indicating that these animals were TNF-α unresponsive and would not have developed diabetes.

The development of better biomarkers based on cellular defects is important but difficult. It is becoming increasingly clear that the traditional methods of RBC lysis or Ficoll gradients to prepare splenocytes or PBLs can cause extensive damage to the cells. This makes the cells less suitable for functional studies and this is a particular problem in death assays. Similar conclusions have also been reached by the member laboratories of the Alliance for Cellular Signaling (AfCS). Indeed, in a detailed AfCS research report, Hsueh et al. (2002) have defined the essential splenic B cell isolation and culture conditions for meaningful signal transduction studies (Sambrano et al., 2002). This report also emphasizes the requirement of gentle methods to optimize cell viability.

At least two hurdles remain to the adaptation of our biomarker assay to human autoimmune diabetes. First, it will be necessary to sample peripheral blood rather than splenocytes. Given that human blood consists mostly of RBCs, the negative selection procedures that removed T cells or non-T cells from mouse splenocyte preparations are likely to be less effective with human blood as the starting material. Second, unlike NOD mice, humans with autoimmune diabetes are genetically diverse.

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