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Patients with Type 1 Diabetes Display Selective Defect in Antigen Receptor Mediated T Cell Apoptosis

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ABSTRACT

Type 1 diabetes is an autoimmune disease in which insulin-producing beta cells are destroyed by auto-reactive T lymphocytes. Studies in mice indicate that incomplete deletion of self-reactive T-cells and compromised peripheral tolerance mechanisms can contribute to the manifestation of autoimmune diabetes. In patients with type 1 diabetes, defects in T regulatory cell numbers and function have been previously reported. In this study, we have ascertained the integrity of activation-induced cell death, a mechanism of peripheral T cell tolerance, in long-standing type 1 diabetes patients. Activation of peripheral blood derived T cells from non-diabetic individuals with a T cell mitogen and interleukin-2 rendered them susceptible to subsequent T-cell receptor/CD3-mediated apoptosis, as indicated by the dissipation of the mitochondrial membrane potential and activation of intracellular caspases. In contrast, similarly activated T lymphocytes from type 1 diabetes patients failed to undergo apoptosis when challenged with a bacterial superantigen or anti-CD3 antibody. Supplementation of T cell cultures with interleukin-4 or interleukin-18 failed to restore self-tolerance. However, both the expression of the Fas receptor and its ability to transduce apoptotic signal were comparable in T cells of type 1 diabetes patients and controls. Additionally, no marked difference in the T cell subsets was observed between controls and diabetes patients under all activation conditions analyzed. These data suggest that the abnormality in T-cell receptor-mediated apoptosis is cell autonomous in long-standing type 1 diabetes patients, which in addition to other defective peripheral tolerance mechanisms, likely to contribute to the manifestation of autoimmune diabetes.

INTRODUCTION

Most auto-reactive T cells are deleted by apoptosis in the thymus during development. However, some auto-reactive T cells escape to the periphery where they become pathogenic if not

regulated by tolerance mechanisms. One such peripheral tolerance mechanism is activation-induced cell death (AICD). Activation of naïve T-cells renders them susceptible to apoptosis when the TCR/CD3 complex is subsequently triggered following ligation with immobilized anti-CD3 antibody or the bacterial superantigen Staphylococcal enterotoxin B (SEB) [1-5], which binds to specific TCR V_β segments outside of the antigen binding site [6]. AICD is thought to be due to the interaction between Fas and membrane associated Fas ligand expressed as a consequence of TCR-mediated activation in previously activated T-cells [3]. Impaired AICD due to the dysfunctional Fas receptor in mice with *lpr* mutation and defective Fas ligand expression in mice carrying the *gld* mutation leads to severe lymphoproliferation, characterized by the accumulation of $CD3^+CD4^-CD8^-B220^+$ T cells and autoimmune lupus disease [7]. Inherited dominant negative mutation of the Fas receptor has been implicated in autoimmune lymphoproliferative syndrome (ALPS) in pediatric patients [8-9]. Although controversy exists as to the role of Fas: Fas ligand system in negative selection of potentially auto-reactive T-cells in the thymus, it is generally accepted that Fas-mediated AICD is crucial for peripheral T cell homeostasis [10].

Type 1 diabetes (T1D) is a chronic, autoimmune disorder characterized by T cell-mediated loss of insulin producing β-cells [11-12]. Autoantigen-specific T-cells have not only been detected in T1D patients but also in healthy individuals who share the same susceptible HLA-DR allele [13], indicating that mechanisms exist in non-diabetic individuals to control manifestation of autoimmune diabetes despite the presence of putative auto-reactive T-cells. It was also noted that the T lymphocytes from most patients with long-standing T1D and non-diabetic siblings displayed anergy, lack of activation, to a variety of autoantigens *in vitro* although this was not related to the disease course [14]. It is possible that this anergic state may compromise antigen receptor-mediated activation and acquisition of sensitivity to AICD in auto-reactive T lymphocytes. Consistent with this notion, T lymphocytes of NOD mice, which develop spontaneous T1D similar to humans, displayed resistance to anti-CD3 antibody-mediated AICD *in vitro* [15]. A previous

report indicated that *in vitro* activated T lymphocytes from patients with T1D underwent Fas-mediated apoptosis comparable to controls whereas patients with T1D and ALPS displayed resistance to Fas mediated apoptosis [16], indicating the association of genetically inherited Fas resistance with T1D patients displaying ALPS selectively. However, it remains to be determined whether the T-cells from T1D patients without ALPS can transmit apoptotic signal independent of the Fas receptor and preferably through the antigen receptor. We addressed this important issue in T lymphocytes derived from long standing T1D patients who do not display ALPS.

MATERIALS AND METHODOLOGY

Study subjects

Patients (3 females and 9 males) with long-standing T1D (n = 12) ranging from 16 to 41 yrs of age were recruited for this study with informed consent at the Diabetes Research Institute of the University of Miami Miller School of Medicine. One female and three male patients also had concomitant hypothyroidism and no other autoimmune diseases were detected. The HbA1c ranged from 6 to 9.4 in these patients. Twelve unrelated healthy volunteers with no family history of T1D were also recruited for this study. The protocol was approved by the Institutional Review Board of the University of Miami Miller School of Medicine.

T cell activation

Venous blood was withdrawn from patients and controls after overnight fasting. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO) and their viability was determined using trypan blue dye as described previously [17]. Viable cells were re-suspended at 5 x 10^6/ml of RPMI 1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, antibiotics, non-essential amino acids, sodium pyruvate, and 2-ME and activated with 10 μg of phytohemagglutinin A (PHA-P)/ml (Sigma-Aldrich) as described [17]. Cultures were supplemented with recombinant derived human IL-2 (50 U/ml, eBioscience, San Diego, CA), IL-4 (100 U /ml, eBioscience) or IL-18 (300 ng/ml, Medical and Biological Laboratories CO. Ltd., Woburn, MA) on days 1 and 3. Cells were harvested on day 5 or 6

and used for phenotyping and apoptosis assays.

Phenotypic analysis

Freshly isolated PBMC were analyzed on a flow cytometer and lymphocytes were gated based on forward- and side scatter light properties. Staining of cells treated with CD3-PE, CD4-APC, CD8-PE, CD25-PE, CD95-FITC (BDIS, Mountain View, CA) and CD152-PE (Coulter, Miami, FL) was determined after subtracting the background fluorescence emitted by cells stained with isotype-matched mouse Ig coupled with FITC, PE or APC. The CD4$^+$ T cells were further analyzed for the co-expression of CD25 or CD152.

Apoptosis induction and analysis

After 5-7 days of culture, 6 X 10^5 T cells/ml were incubated with 1 µg of anti-Fas antibody (CH-11, Upstate Biotech, Billerica, MA), 50-µM etoposide, 10 µg of Staphylococcal enterotoxin B (SEB, Sigma-Aldrich) or 10 µg of PHA-P. T cells were also incubated in a 24-well tissue culture plate previously coated with 10 µg of purified goat anti-mouse antibody followed by 10 µg of anti-human CD3 antibody, clone CRIS-7 (eBioscience). After overnight culture, viability was determined by flow cytometry after incubation with 50 nM tetramethylrhodamineethylester (TMRE, Invitrogen) for 20 min at 37° C [18]. Intracellular detection of activated caspases (IDAC) was performed by incubating cells with FITC-VAD-FMK (Promega, Madison, WI) for 45 min at 37° C and analyzed after the addition of propidium iodide (PI, Boehringer-Mannheim, Indianapolis, IN) as described previously [19].

Statistics

Statistical analysis was performed using an unpaired two-tailed Student's t test (GraphPad Prism, San Diego, CA).

RESULTS AND OBSERVATIONS

Correlation between activated caspases and mitochondrial dysfunction in T lymphocytes

It is well established that previously activated peripheral blood human T lymphocytes and un-manipulated Jurkat T cell leukemia undergo apoptosis in response to stimulation through the TCR/CD3 complex and the Fas receptor, characterized by DNA fragmentation, compromised plasma membrane integrity,

externalization of phosphatidylserine and intracellular acidification [17-18]. In addition, apoptosis induced by the stimulation of Jurkat cells with an anti-Fas antibody resulted in the dissipation of the inner mitochondrial transmembrane potential and activation of intracellular caspsases, respectively detected by using the potentiometric dye TMRE and FITC conjugated pancaspase inhibitor [17-18]. However, such a correlation has not been demonstrated in untransformed human T lymphocytes, by flow cytometry. Therefore, we first determined whether a similar correlation exists between the activation of caspases and mitochondrial dysfunction in normal T lymphocytes undergoing apoptosis through the antigen receptor. To this end, PBMC isolated from non-diabetic controls were stimulated with the T cell mitogen PHA and recombinant IL-2 for 5-6 days and subjected to apoptosis induction through the antigen and Fas receptors [17]. Representative data shown in Fig. 1a indicate that treatment of activated T-cells with the anti-Fas antibody induced the activation of caspases, as determined by the binding of the cell-permeable fluorochrome conjugated pancaspase inhibitor, FITC-VAD-FMK, consistent with the data we showed earlier in Jurkat cells [19]. A majority of these cells also had compromised plasma membrane permeability, as evidenced by PI uptake, indicating that they were at the late stage of apoptosis (Fig. 1a, upper right quadrant). Similarly, ligation of the TCR with SEB also induced the activation of caspases. Since SEB binds to many TCRV$_\beta$ segments such as 1, 3.2, 6.4, 12, 14, 15, 17 and 20 [6], the data indicate that activation with PHA rendered T-cells susceptible to apoptosis through the antigen receptor in multiple T-cell subsets. Similar levels of caspase-dependent apoptosis was also induced by re-stimulation of T-cells with PHA, that binds to all TCR regardless of antigen specificity [20]. Etoposide, a topoisomerase II inhibitor, which induces apoptosis in activated but not resting T-cells [21], triggered robust caspase-dependent apoptosis in activated T lymphocytes (Fig. 1a). Activation of caspases following treatment with anti-Fas antibody, SEB, PHA or etoposide was accompanied by mitochondrial dysfunction, as indicated by substantial reduction in the retention of TMRE in the mitochondrial matrix (Fig. 1b, upper right quadrants). These data demonstrate a good correlation between the intracellular

activation of caspases and mitochondrial dysfunction in control T lymphocytes induced to undergo apoptosis through the TCR/CD3 complex or the Fas receptor. Therefore, in subsequent

experiments we determined the induction of apoptosis through the antigen receptor by assessing the mitochondrial transmembrane potential by flow cytometry.
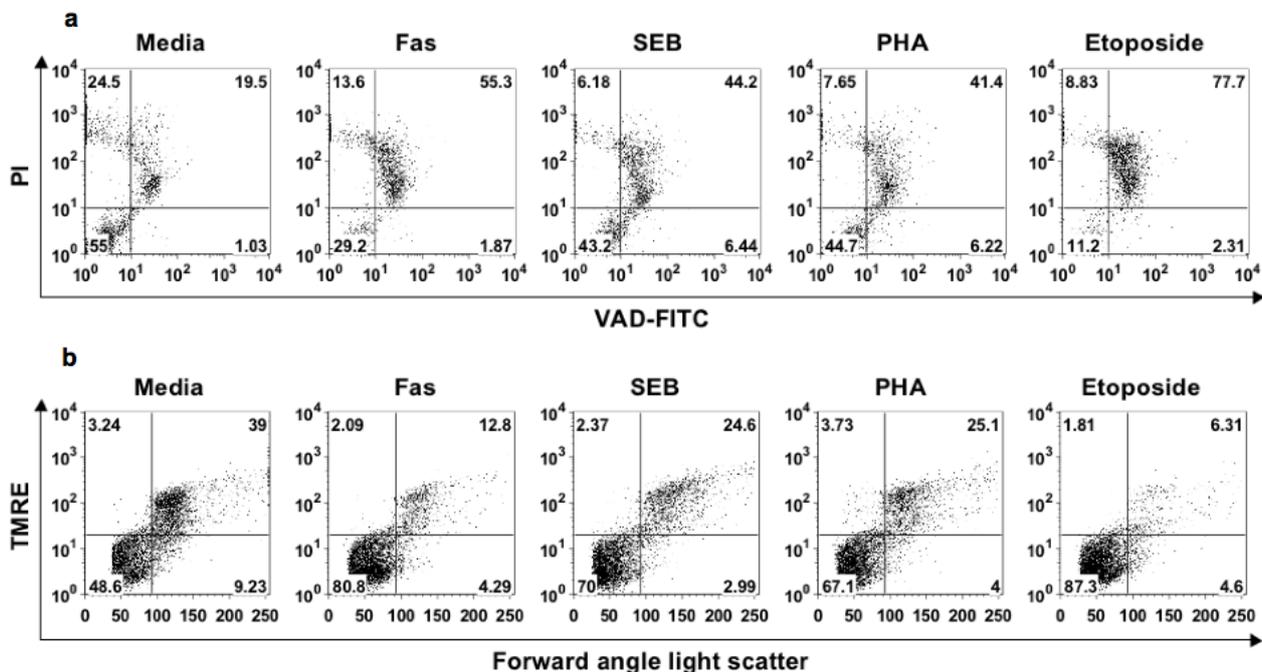
Figure 1.

Fig. 1. Correlation between intracellular activated caspases and mitochondrial dysfunction in apoptotic T cells.

Normal human PBMC were activated with PHA + IL-2 for 5 days and then challenged with anti-Fas antibody, SEB, PHA, or etoposide. After overnight culture, intracellular activated caspases were determined by incubating treated cells with VAD-FITC and PI was added just before analysis on a flow cytometer (**a**). Mitochondrial function was analyzed on a flow cytometer following incubation of cells with TMRE (**b**). Data shown are representative of 6-10 different normal individuals.

Selective impairment of TCR/CD3 mediated AICD in patients with T1D

It was next determined whether *in vitro* activated T lymphocytes from patients with long-standing T1D differ from controls in their ability to undergo AICD through the TCR/CD3 complex. This was tested in short-term T cell lines generated by activating PBMC with PHA and recombinant IL-2 for 5 to 6 days since this method of activation rendered control T-cells susceptible to apoptosis through the antigen and Fas receptors [1-3, 17, 22]. These T cell lines were then challenged with TCR ligands and apoptosis was ascertained by the exclusion of TMRE. As expected, ligation of the TCR either with immobilized anti-CD3 antibody or the superantigen SEB triggered apoptosis in T lymphocytes of non-diabetic controls (Fig. 2a). In contrast, activated T cells from patients with long-standing T1D displayed significant resistance to

apoptosis induction (<10% apoptosis) by anti-CD3 antibody (in 6/8 of patients, 75%) or SEB (5/8 of patients, 63%). However, stimulation with the anti-Fas antibody CH-11 resulted in the induction of robust apoptosis uniformly in T lymphocytes of all control and patients with T1D. These results indicate that T lymphocytes of long-standing T1D patients display selective resistance to antigen receptor mediated apoptosis. Substitution of cultures with recombinant IL-4 (Fig. 2b) did not influence the resistance of T-cells to undergo apoptosis in response to stimulation with anti-CD3 antibody (6/7 of patients, 86%) or SEB (7/7 of patients, 100%). Although supplementation of T-cell cultures with recombinant derived human IL-18 also did not reduce the frequency of control T-cells undergoing apoptosis when stimulated with anti-CD3 or SEB, it did, however, reduce the levels of apoptosis (Fig. 2c). Incubation of T-cells from T1D patients with IL-18 did not alter the frequency

of apoptosis resistance when stimulated with anti-CD3 antibody (5/8 patients, 63%) or SEB (5/8 patients, 63%). Taken together, these data indicate that T-cells from patients with long-standing T1D are selectively resistant to TCR/CD3-**Figure 2.**

mediated apoptosis and supplementation of T-cell cultures with different lymphokines during *in vitro* activation did not restore sensitivity to AICD in these cells.
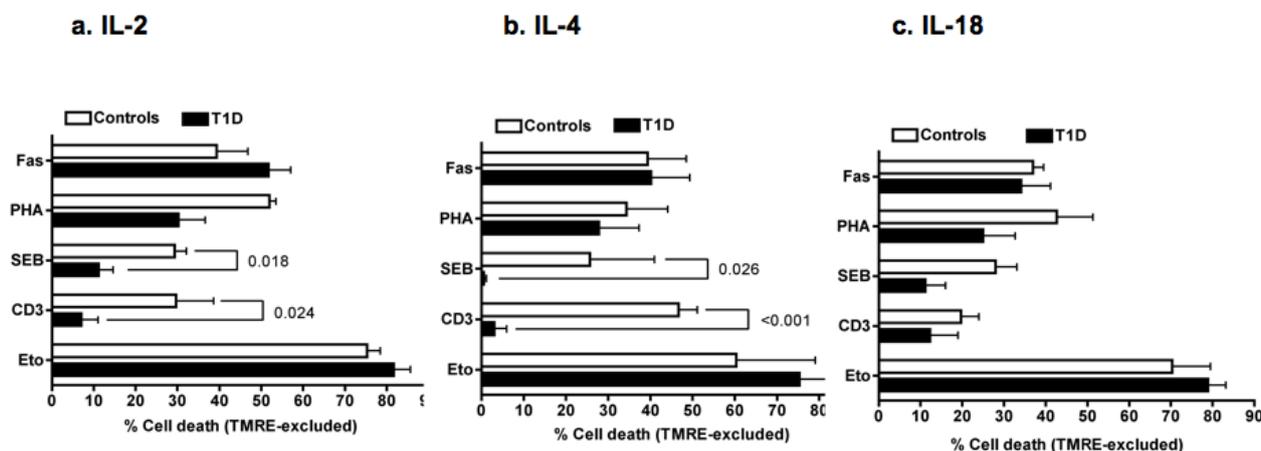

Fig. 2. Resistance to antigen receptor-mediated apoptosis in T1D patients

The PBMC derived from control (n=4) and patients with T1D (n=7-10) were cultured with PHA + IL-2 (**a**), PHA + IL-4 (**b**) or PHA + IL-18 (**c**) for 5 to 6 days and the T lymphocytes were treated with anti-Fas antibody, PHA, SEB, immobilized anti-CD3 antibody or etoposide. After overnight culture, the frequency of apoptotic cells was determined by the exclusion of TMRE by flow cytometry. The statistical significance between control and subjects with T1D is indicated.

In contrast to the marked reduction in TCR/CD3-mediated apoptosis of T-cells derived from patients with T1D, reactivation with the T cell mitogen PHA induced modest but not significant reduction in the level of apoptosis, compared to control T cells regardless of the cytokines used for stimulation *in vitro* (Fig. 2a, b and c). Similarly, activation with etoposide induced similar levels of apoptosis in both diabetic and control T cells regardless of whether the cultures were supplemented with IL-2, IL-4 or IL-18 (Fig. 2a, b and c). Since etoposide induces apoptosis in activated but not resting T lymphocytes [21], similar levels of apoptosis observed in T lymphocytes of patients with T1D indicated that these cells were comparably activated as control T-cells but were unable to transduce apoptotic signal(s) selectively through the TCR/CD3 complex.

Apoptosis resistance is not due to altered T cell activation

We next examined whether altered activation of T lymphocytes could account for the abnormal resistance to TCR/CD3-mediated apoptosis observed in T1D patients. Data shown in

Fig. 3a indicate that the total numbers of $CD3^+$ T cells were slightly lower in freshly isolated PBMC of patients with T1D in comparison to controls. However, culture of PBMC derived from T1D patients and control subjects with the T cell mitogen PHA and IL-2, IL-4 or IL-18 for 5 to 6 days yielded similar (>85%) numbers of $CD3^+$ T cells (data not shown). The frequency of $CD4^+$ cells was slightly lower in PBMC of patients with T1D and in T cell lines activated with PHA and IL-2, IL-4 or IL-18 (Fig. 3a). On the other hand, the numbers of $CD8^+$ T cells were higher in PBMC of patients with T1D than in controls (Fig. 3b). While $CD4^+$ cells proliferated modestly, the numbers of $CD8^+$ cells from controls and subjects with T1D increased markedly (2 to 3-fold) after stimulation with the T cell mitogen plus IL-2, IL-4 or IL-18. Both the PBMC and the T-cell lines cultured with PHA and IL-2, IL-4 or IL-18 from controls and patients with long-standing T1D displayed comparable numbers of Fas-expressing cells (Fig. 3c). Thus, the T cells from patients with long-standing T1D responded to stimulation with a T cell mitogen comparably to controls and the addition of exogenous lymphokines did not markedly alter the T cell activation profiles.

To further analyze whether the T-cells of patients with long-standing T1D could respond to mitogenic stimulation differently from control T-cells, we analyzed the expression of other activation-associated determinants. As seen in Fig. 4a, the frequency of the $CD4^+CD25^+$ cells in freshly isolated PBMC of patients with T1D and controls was similar. *In vitro* activation of PBMC derived **Figure 3.**

from controls and T1D patients with PHA similarly increased the numbers of $CD4^+$ T cells co-expressing the activation-associated determinant, CD25. Supplementation with IL-2, IL-4 or IL-18 induced comparable numbers of $CD4^+$ T cells co-expressing CD25 in both controls and patients with T1D.
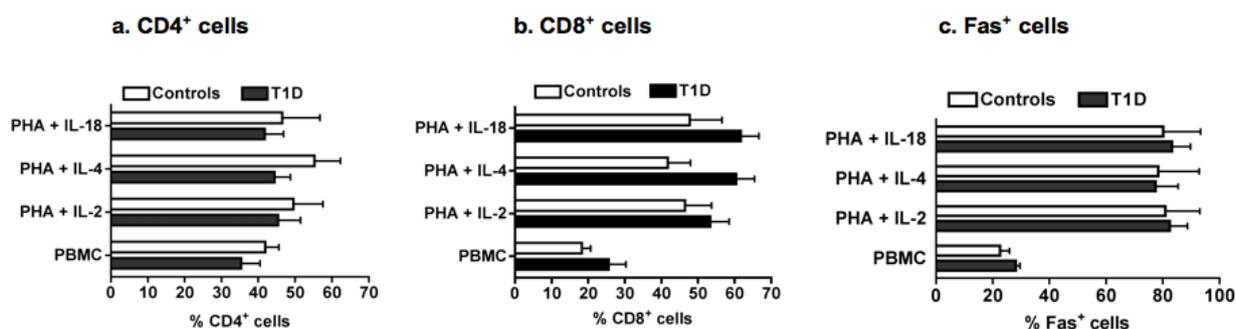

Fig. 3. Equal activation of T cell subsets in controls and subjects with T1D

Total numbers of $CD4^+$ (**a**), $CD8^+$ (**b**) and Fas^+ cells (**c**) cells were determined in freshly isolated PBMC and after culture with PHA + IL-2, PHA + IL-4 or PHA + 1L-18 for 5 to 6 days from controls (n=4-10) and patients with T1D (n=6-8) for 5 to 6 days. The differences between controls and T1D patients are not statistically different.
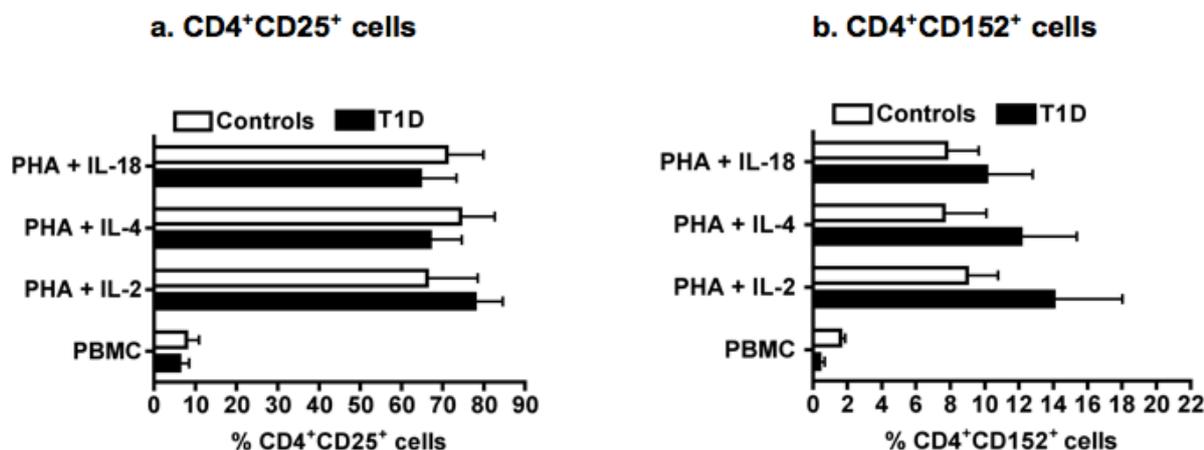
Figure 4.

Fig. 4. Comparable levels of activation of T regulatory cells in T1D patients

The numbers of $CD4^+CD25^+$ (**a**) and $CD4^+CD152^+$ cells (**b**) were determined in freshly isolated PBMC from controls (n=10) and patients with T1D (n=10). Similar determination was performed in T cells cultured with PHA + IL-2, PHA + IL-4 and PHA + IL-18 for 5 to 6 days. The differences between controls and patients with T1D are not statistically significant.

The PBMC of controls and patients with T1D did not contain significant numbers of $CD4^+$ cells co-expressing CD152 or CTLA-4 (Fig. 4b), implicated in the regulation of T1D in NOD mice [23]. Activation of PBMC from T1D patients with PHA and IL-2, IL-4 or IL-18 slightly but not

significantly increased the frequency of these cells. The numbers of $CD4^+CD152^+$ cells were somewhat higher in activated T lymphocytes of T1D patients than in similarly activated control T-cells. Collectively, these data indicate that the inability of activated T-cells from patients with

long-standing T1D to undergo antigen receptor mediated apoptosis could not simply be due to abortive T cell activation programs since the expression of activation associated determinants such as Fas, CD25 and CD152 is comparable to controls.

DISCUSSION

The data presented herein demonstrate for the first time that the T lymphocytes of long-standing T1D patients display a lesion in the apoptotic pathway coupled to the TCR/CD3 complex whereas the Fas pathway remains fully functional in these patients. It was shown that the ligation of the TCR/CD3 complex resulted in Fas ligand expression and its subsequent interaction with the Fas receptor activated the apoptotic pathway in pre-activated normal human T lymphocytes [1, 3, 22, 24]. Apoptosis induced in this fashion was partially blocked by the addition of Fas. Fc fusion protein, indicating that AICD via the TCR/CD3 complex engages the Fas pathway [1, 22, 24]. Stimulation of the Fas receptor recruits the adaptor molecule FADD via a death domain interaction with the Fas receptor [25]. This leads to the binding of the procaspase-8 through the death effector domain interaction and the formation of the death-inducing signalling complex followed by proteolytic auto-activation of caspase-8. Activated caspase-8 cleaves the proapoptotic member of the Bcl-2 family, Bid and the resulting t-Bid targets mitochondria to induce the release of cytochrome c and subsequent sequential activation of caspase-9 and caspase-3 resulting in the externalization of phosphotidylserine and eventual apoptosis [3, 7, 24-25]. In this extrinsic death pathway, mitochondrial dysfunction is a central and irreversible event [26-28], which correlates well with the activation of caspases, as determined by western blotting [25-28] and flow cytometry [19]. Mitochondrial dysfunction as indicated by the loss of the potentiometric dye TMRE is a manifestation of caspase-dependent apoptosis [18-19, 27-28]. Several possibilities may explain the split tolerance of T lymphocytes to negative signalling from patients with long-standing T1D. Inasmuch as membrane-bound Fas ligand is essential for Fas-mediated apoptosis [29], it is possible that the lack or defective expression of membrane-bound Fas ligand following TCR/CD3 ligation may account

for the apoptosis resistance observed in the T lymphocytes of patients with T1D. Another possibility is the lack of expression of Daxx, which is recruited to the Fas receptor upon Fas ligand engagement and promotes T-cell homeostasis [30]. Although the mouse with *gld* mutation exhibits defective Fas ligand expression, which accounts for the lymphoproliferative disorder [7], similar mutation of the Fas ligand gene has not been reported in patients with autoimmune conditions. Whereas several negative transmembrane adapter proteins have been implicated in the control of lymphocyte development and activation [31], it is not clear whether they are involved in the regulation of TCR-mediated AICD. Further work is necessary to elucidate the mechanisms underlying the selective resistance of T lymphocytes derived from long-standing T1D patients to TCR-mediated apoptosis.

Our data demonstrate that the expression and function of the Fas receptor in activated T lymphocytes of T1D patients without other associated autoimmune conditions are comparable to controls. However, resistance to Fas mediated apoptosis was previously reported in T1D patients, which was characterized by lower caspase-8 activation [32], reduced caspase-3 expression [33] and up-regulation of c-FLIPshort, an antagonist of Fas signalling [32]. It should be noted that Fas resistance was restricted only to a proportion of T1D patients who also had additional autoimmune diseases such as ALPS and was characterized by lower levels of the apical enzymes involved in the Fas pathway, caspase-8 and -9 [16, 34]. Previous studies established that Fas resistance in rare pediatric patients with the X-linked ALPS was due to the inherited dominant negative mutation of the Fas gene, resulting in defective apoptotic signalling through the Fas receptor [8-9]. Somatic fusion of the Fas-resistant T cells with Fas-sensitive HUT78 cells generated Fas resistant hybrid cells, suggesting the dominant negative nature of the Fas receptor in T1D patients with ALPS [16]. Taken together, these data suggest that the Fas resistance phenotype observed in patients with T1D and ALPS [16, 34] is due to a genetically inherited defect of the Fas receptor associated with ALPS [8-9] and not a trait of autoimmune diabetes in general. This explains why the T lymphocytes from our small cohort of T1D patients (n=12) without associated ALPS

failed to display Fas resistance and further work is necessary to validate this important finding in a large number of T1D patients.

It was demonstrated that activation of mouse naïve T lymphocytes required exogenous IL-2 or IL-4 for the acquisition of sensitivity to AICD through the TCR [4, 35]. Consistently, we observed that the addition of recombinant derived IL-2 or IL-4 rendered T lymphocytes from normal individuals sensitive to both TCR/CD3- and Fas-mediated apoptosis. In contrast, activation with IL-2, IL-4 or IL-18 failed to restore sensitivity of T-cells from T1D patients to undergo TCR-mediated apoptosis, indicating that this resistant phenotype is an invariant trait associated with T1D and not amenable to manipulation with lymphokines. Interesting to note is that the treatment with IL-18 diminished the level of TCR/CD3-mediated apoptosis in normal human T lymphocytes. This could be due to the induction of blocking factor(s) by IL-18 that interfere with the manifestation of apoptosis through the antigen receptor. Although the underlying mechanisms have not been deciphered, it was observed that serum levels of IL-18 were higher in newly diagnosed T1D patients [36]. Treatment of NOD mice with recombinant IL-18 accelerated autoimmune diabetes, indicating a pathogenic role for this cytokine in diabetes manifestation [37]. It is important to discern whether the up regulation of IL-18 in subjects with T1D is linked to the down regulation of TCR/CD3-mediated apoptosis demonstrated herein.

Re-stimulation of activated T lymphocytes from T1D patients with the T cell mitogen PHA induced comparable levels of apoptosis in spite of defective antigen receptor mediated apoptosis. Although the superantigen SEB can bind to multiple TCR V_β elements such as 1, 3.2, 6.4, 12, 14, 15, 17 and 20 [6], and anti-CD3 antibody can stimulate all T cells, they are not as potent as PHA in inducing apoptosis. Since PHA can bind to the TCR as well as CD2 [20], it can deliver stronger signals than ligation of the TCR or CD3 alone. This could compensate for the inefficient delivery of apoptotic signals through the TCR/CD3 complex and override compromised apoptosis in T lymphocytes of T1D patients. This is similar to our earlier demonstration of the circumvention of impaired Fas-receptor mediated apoptosis by reactivation of T lymphocytes with PHA in asthma patients [17]. Taken together, these results are consistent with the notion that apoptosis induction through the extrinsic pathway, such as by the engagement of the TCR is a function of the strength of the apoptotic stimulus and defective or insufficient signalling via the extrinsic pathway can be compensated by polyclonal activation.

Several T cell abnormalities including secretion of IL-2 [38-40] and IFN-γ [41] and impaired signalling through the TCR/CD3 complex with normal co-stimulatory pathway were reported in patients with T1D [42]. Although decreased frequency of the naturally occurring T regulatory cells of the phenotype, CD4$^+$CD25$^+$ was initially reported in the peripheral blood of T1D patients [41], subsequent studies failed to confirm this observation [43-45]. Our data also showed no substantial decrease in the frequency of this type of T regulatory cells in long-standing T1D patients in comparison to controls. In addition, polyclonal activation with PHA and IL-2/IL-4/IL-18 resulted in the generation of equal numbers of CD4$^+$ T cells expressing the IL-2Rα, CD25 in both controls and T1D patients. However, increased resistance to CD4$^+$CD25$^+$ naturally occurring T regulatory cell-mediated suppression was noted in long-standing as well as newly diagnosed type 1 diabetes patients [46]. Thus, it remains possible that the function of CD4$^+$CD25$^+$ naturally occurring T regulatory cells may be compromised without altering their numbers in long-standing and newly diagnosed T1D patients.

Our data suggest that CD4$^+$CD152$^+$ cells, implicated in the regulation of autoimmune diabetes in NOD mice [23], are numerically similar in the peripheral blood of long-standing T1D patients and controls. In addition, activation with PHA resulted in comparable numbers of these cells in these two groups, regardless of the lymphokines used for activation. In data not shown, we observed that the total numbers of CD3$^+$ T cells either in the peripheral blood or after *in vitro* activation with PHA plus IL-2, IL-4 or IL-18 did not differ between controls and T1D patients. Moreover, the numbers of Fas$^+$ cells after activation with PHA and various lymphokines were not different in these two groups. Previous studies indicated lowered frequency of CD4$^+$ cells than CD8$^+$ cells in the peripheral blood of T1D patients [47-48] and we observed a modest increase in the numbers of CD8$^+$ cells in our cohort of T1D patients. Mitogenic stimulation *in vitro* did not

restore the 2:1 ratio of CD4 and CD8 cells, normally found in controls, irrespective of activation with various lymphokines, indicating that this is an invariant characteristic of T1D patients. Inasmuch as both CD4^{+} and CD8^{+} T cells are susceptible to anti-CD3 mediated apoptosis in pre-activated T lymphocytes [4] and both CD4^{+} and CD8^{+} T cells have been shown to be refractory to AICD through the TCR/CD3 complex in autoimmune diabetes prone NOD mice [49-50], the increased proportion of CD8^{+} cells in T1D patients could not be primarily attributed to the observed resistance to TCR/CD3 mediated apoptosis in T1D patients. Taken together, these data indicate that neither numeric alteration nor abortive activation of T cells as defined by the expression of CD25, Fas/CD95 and CD152 could be attributed to the selective perturbation of antigen receptor mediated T cell apoptosis in patients with T1D.

CONCLUSION

Our results revealed that defective TCR/CD3 mediated apoptosis is a cell autonomous inherited trait in long-standing T1D patients. We anticipate that this inherited defect may also be apparent in newly diagnosed T1D patients like other genetically inherited defects, which were found in both long-standing and newly diagnosed T1D patients [46]. Taken together, these data suggest that defective peripheral tolerance mechanisms including compromised TCR/CD3-mediated apoptosis may contribute significantly to the retention and function of potentially auto-reactive T lymphocytes involved in the manifestation of autoimmune diabetes. Further work is necessary to understand the biochemical mechanisms of this interesting and important phenomenon.

LIST OF ABBREVIATIONS

AICD – Activation induced cell death

ALPS – Autoimmune lymphoproliferative syndrome

IDAC – Intracellular detection of activated caspases

IL – Interleukin

FITC-VAD-FMK – FITC conjugated to Valine Alanine Aspartic acid fluoromethyl ketone

PHA – Phytohemagglutinin A

PBMC – Peripheral blood mononuclear cells

SEB – Staphylococcal enterotoxin B

T1D – Type 1 diabetes

TCR – T cell receptor

TMRE – Tetramethylrhodamineethylester

CONFLICTS OF INTEREST

The authors declare no conflict of interests.

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