Reactivated CD4+ Tm cells of T1D patients and siblings display an exaggerated effector phenotype with heightened sensitivity to activation-induced cell death

Michael Lei Bian¹, Oscar Haigh³, David Munster¹, Mark Harris², Andrew Cotterill², John J. Miles³⁴⁵ and Slavica Vuckovic¹³⁵

¹Mater Research, Translational Research Institute, Brisbane, QLD, 4102 Australia

²Mater Children’s Hospital, Brisbane, QLD, 4102, Australia

³QIMR Berghofer Medical Research Institute, Brisbane, QLD, 4006, Australia

⁴Institute of Infection and Immunity, Cardiff University School of Medicine, Heath Park, Cardiff, Wales, United Kingdom

⁵School of Medicine, The University of Queensland, Brisbane, QLD, 4102, Australia

**Title page:** CD4+ Tem and AICD in T1D patients and siblings

**Corresponding author:** Dr Slavica Vuckovic, Bone Marrow Transplantation Laboratory, QIMR Berghofer Medical Research Institute, QLD 4006.

Phone: +61-7-3845-3991; Fax: +61-7-3845-3509; Email: slavica.vuckovic@qimrberghofer.edu.au.

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Abstract

It has been proposed that dysfunction in effector memory contribute to autoimmunity in T1D. Using a unique cohort of age- and sex-matched T1D patients, non-affected siblings and unrelated control children we undertook a detailed analysis of proliferation, activation, effector responses and apoptosis in reactivated CD4^+Tm cells during TCR stimulation. Across cohorts, there was no difference in the proliferation of reactivated CD4^+Tm cells. In T1D patients and siblings, CD4^+Tm cells easily acquired activated CD25^+ phenotype and effectively transitioned from a central (CD62L^+Tcm) to an effector memory (CD62L^-Tem) phenotype with an elevated cytokine ‘signature’ comprising IFN-γ and IL-10 in T1D patients and IFN-γ in siblings. This exaggerated Tem phenotype also exhibited an exaggerated immune shutdown with heightened sensitivity to AICD and Fas-independent apoptosis. Apoptosis resulted in the elimination of half of the effector memory in T1D patients and siblings compared to third of the effector memory in controls. This data suggests genetic/environmental driven immune alteration in T1D patients and siblings that manifests in exaggerated CD4^+Tem response and its shutdown by apoptosis. Further immunological studies are required to understand how this exaggerated CD4^+Tem response fits within the pathomechanisms of T1D and how the effector memory can be modulated for disease treatment and/or prevention.
**Introduction**

Type 1 diabetes (T1D) is an autoimmune disease that results from progressive T cell-mediated destruction of insulin-producing β-cells in the pancreatic islets (1). Although multiple immune cell lineages participate in the pathogenesis of T1D, memory CD4⁺T cells (T(m)) play a key inflammatory role in both inducing and maintaining the autoimmune response (2). The central role for CD4⁺Tm cells in driving disease was recently highlighted in a clinical trial that showed that drug-mediated blocking of T cell activation slowed β-cell destruction (3). Disease progression could also be prevented in the nonobese diabetic (NOD) mouse model by artificially guiding CD4⁺Tm cells away from an 'effector' memory phenotype towards a 'regulatory' memory phenotype (4). These studies clearly point towards CD4⁺Tm cells as a promising immune checkpoint target for T1D treatment.

CD4⁺Tm cells comprise functionally distinct central memory (Tcm) and effector memory (Tem) subsets and their relative numbers in the peripheral circulation and tissues vary depending on the level of antigen exposure (5). CD4⁺Tcm cells express CD62L and CCR7, home to lymph nodes and self-renew. In contrast, CD4⁺Tem cells lack CD62L, migrate to tissues and differentiate into Th1, Th2 or Th17 lineages (6). Also contained within CD4⁺Tm cells are autoreactive cells recognizing several β-cell antigens including GAD65, proinsulin, insulin, chromogranin A and islet amyloid polypeptide (2). Autoreactive CD4⁺Tm cells can be found in both healthy individuals and in T1D patients (7) and their activation (8, 9) possibly through accidental cross-reactivity (10) with pathogen-derived antigen(s) (11) leads to destruction of β-cells in a MHC-restricted manner.

CD4⁺Tm cells re-exposed to pathogen or self-antigen differentiate into effector cells producing multiple cytokines that can modulate CD8⁺T cell function, innate immunity and antibody production by plasma cells (6). Alternatively, reactivated CD4⁺Tm cells can differentiate into induced regulatory T cells (iTr) which are important in maintaining self-tolerance via cell surface regulatory receptors and/or inhibitory cytokines (11). After the clearance of a pathogen, a large fraction of CD4⁺Tem cells undergo contraction. This contraction phase involves activation-induced
cell death (AICD). Two distinct but converging pathways mediate AICD in CD4⁺Tm cells including: (i) the extrinsic death receptor pathway (comprising Fas [CD95] or tumour necrosis factor receptor 1 [TNF-R1]) and (ii) the intrinsic mitochondrial pathway (12). In healthy individuals, it has been suggested that Fas mediated apoptosis has a major role in the elimination of CD4⁺Tem cells reactivated by the T cell receptor (TCR) activation pathway (13).

Enhanced effector responses, manifested by elevated levels of the Th1 (IFN-γ and TNF-α) and the Th17 (IL-17) cytokine ‘signatures’ drive CD4⁺Tm induced pathology in both T1D patients and the NOD mouse (2, 11, 14). The intrinsic mechanisms that favour effector versus iTr lineage commitment in CD4⁺Tm cells of T1D patients (as well as in healthy subjects) remain unclear and controversial. Loss-of-function mutations in Fas and FasL genes in mice leads to T cell-mediated autoimmunity/lymphoproliferative pathology (15-17). Interestingly, mutations in the Fas system have been detected in patients with T1D (18) and collectively, these observations suggest that problems with effector immune response shutdown perhaps caused by a defect in the death receptor apoptotic pathway may be a contributing factor for pathological autoimmunity in T1D.

In line with this presumption, in this study we asked whether intrinsic differences in CD4⁺Tem responses and/or their shutdown by apoptosis may discriminate T1D patients from healthy subjects. Using a unique cohort of age- and sex-matched T1D patients, non-affected siblings and unrelated control children we detailed analysis of proliferation, activation, effector responses and apoptosis in CD4⁺Tm cells reactivated through CD3/CD28 stimulation, which mimics in vivo stimulation with cognate pathogen and/or self-derived antigen. We found that reactivated CD4⁺Tm cells from both T1D patients and siblings activated easily and transitioned from central memory to effector memory cells very efficiently leading to ‘exaggerated’ Tem phenotype with an elevated cytokine ‘signature’ comprising IFN-γ and IL-10 in T1D patients and IFN-γ in siblings. Likewise, in both T1D patients and their siblings we observed an ‘exaggerated’ Tem shutdown effect with heightened sensitivity to AICD which results in their elimination by apoptosis.
Research Design and Methods

A total of 31 children with T1D, 45 non-affected siblings and 29 age- and sex-matched unrelated control children (Queensland Diabetic Center, Mater Children’s Hospital, Brisbane) were enrolled into the study (Table 1). This study was approved by the human research ethics committee of the Mater Children’s Hospital. Informed consent was obtained from parents or guardians.

Immunophenotype analysis: The following anti-human mAb were used: purified CD8α, HLA-DR, CD45RA, CD11c, CD14, CD16, CD19, CD20, CD56 (Coulter Immunotech, NSW), CD34, CD66c, CD235a (BD Bioscience, NSW), CD3-Pacific Blue, CD4-FITC, Fas-FITC, CD45RO-PE, CD8α-PE-Cy7, CD25-APC, CD62L-APC, CD45-APC-H7, Caspase-3-PE, Annexin-V-PE (BD Bioscience), FasL-PE (BioLegend, WA). Expression of surface or intracellular molecules was evaluated by flow cytometry (BD FACS-LSRII) and analyzed by FlowJo v7.6.5 software (TreeStar Inc.). Absolute counts of CD3⁺T, CD4⁺T and CD8⁺T cells in the lysed whole blood of T1D patients, siblings and unrelated controls were calculated using multiparametric flow cytometry and TruCOUNT beads (19).

CD3/CD28 stimulation assay: CD4⁺Tm cells were purified from peripheral blood mononuclear cells (PBMC) by negative separation excluding CD8⁺/CD45RA⁺/HLA-DR⁺/CD11c⁺/CD14⁺/CD16⁺/CD19⁺/CD20⁺/CD34⁺/CD56⁺/CD66c⁺/CD235A⁺ cells (AutoMACS, Miltenyi Biotec, NSW). The pure negative fraction comprised 90-95% CD4⁺CD45RO⁺Tm cells. Unlabelled or carboxyfluorescein succinimidyl ester (CFSE)-labelled CD4⁺Tm cells (1 x 10⁵ cell/well) were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 U/ml), 10 mM HEPES (Life Technologies, NSW) and 10%v/v of autologous serum in the presence (reactivated cells) or absence (resting cells) of plate-coated anti-CD3 mAb (2 µg/ml, eBioscience, SA) and soluble anti-CD28 mAb (1 µg/ml, BD Bioscience, NSW) for five days. To address strength of CD3 signalling, in some experiments plate bound anti-CD3 mAb was used across a range of concentrations (1, 2, 5, 10 µg/ml, Supporting data Fig 2). In cross-culture assays, autologous serum was replaced with allogeneic serum derived from
siblings or unrelated controls or with fetal calf serum (FCS, Life Technologies, VIC). Proliferation dynamics of the reactivated and resting CD4\(^+\)Tm cells was assessed by \(^3\)H-thymidine uptake (1 µCi/well, Perkin Elmer, VIC) and kinetics of proliferation was assessed by CFSE dilution assay at day 1, 3 and 5 of culture.

**Apoptotic assay:** Apoptosis was evaluated in reactivated and resting CD4\(^+\)Tm cells at days 1, 3 and 5 of culture using Annexin-V and 7AAD (Sigma Aldrich, NSW) staining to discriminate early and late stage of apoptosis (Annexin-V\(^+\)7AAD\(^-\) and Annexin-V\(^-\)7AAD\(^+\) cells). To block apoptosis, the antagonistic anti-Fas ZB4 mAb (2.5 µg/ml, Merck Millipore, VIC) or the isotype control mAb (2.5µg/ml, IgG1 NALE, BD Bioscience, NSW) was added at day one of culture. Efficiency of the antagonistic anti-Fas ZB4 mAb to block Fas-induced apoptosis was validated in Jurkat cells stimulated with anti-Fas CH-11 mAb (20 µg/ml, Merck Millipore, Supporting data Fig 3).

**Cytokine screening:** IFN-γ, IL-10, IL-4, IL-2, IL-6, TNF-α and IL-17 were measured in the supernatants of CD3/CD28 stimulated CD4\(^+\)Tm cells collected at days 1, 3 and 5 of cultures using a cytometric bead array (CBA) assay as per the manufactur’s instructions (BD Bioscience). Cytokine levels were determined from calculated standard curves (FCAP Software).

**Quantification of apoptotic gene expression:** CD3/CD28 stimulated CD4\(^+\)Tm cells collected at days 1 and 5 of culture were lysed in Qiagen RLT buffer and total RNA was extracted with Qiagen RNeasy Mini kit (Qiagen, VIC). cDNA was synthesized using an iScript cDNA synthesis kit and PCR thermal cycler (Bio-Rad, NSW). The qPCR assay was performed on the Viia7 Real Time PCR System (Applied Biosystems, Life Technologies, VIC) to determine the pro-apoptotic BIM-L, BAD, BAX and BAK and the anti-apoptotic BCL2, BCLXL (BCL2L1) and BCLW (BCL2L2) gene expressions. Expression of β-actin (ACTB) was used as a house-keeping gene (HKG) for normalisation. The following primers were used: BIM-L: F:5'-AYC CYC AAG ACA ACO GGA GCC C-3'; R:5'-ATG GAA GCC ATT GCA CTG AGA-3'; BAD: F:5'-GCA GCC ATC ATG GAG GCG CT-3'; R:5'-CTG GGC TCC TCC CCC ATC CC-3'; BAX: F:5'-GCC CTG TTG CTT CAG GGT TTC TCC TCC CCC ATC CC-3'; BAK: F:5'-GCC TGA TCC CGT CCT CCA CTG AG-3'; R:5'-CTG GGA CGT CCT GCT CCT CAG AAG C-
3'; BCL2: F:5'-TGG GAT GCC TTT GTG GAA CTA T-3'; R:5'-AGA GAC AGC CAG GAG AAA TCA AAC-3';
BCLXL: F:5'-GGT CGC ATT GTG GCC TTT-3'; R:5'-TCC TTG TCT ACG CTT TCC ACG-3'; BCLW: F:5'-CTG
ACC CGG CTC CAC GCT GG-3'; R:5'-TCT GCC ACC AGA GCC GT-3'; ACTB: F:5'-CC GTA CGC CAA
CAC AGT GC-3'; R:5'-AT CTC CTG CTT GCT GAT CC-3' (GeneWorks Hindmarsh, SA).

**Statistical Analysis:** Analysis of variance and Chi-squared test was used to compare age and
gender respectively between cohorts. Data within subject groups was compared by Mann-Whitney
test and between groups by Kruskal-Wallis test and post-hoc multiple comparisons. Spearman’s
correlation test was used to analyse the association between clinical variables (age, age of diagnosis,
disease duration, C-peptide, islet autoantibodies) and continuous variables (proportion of total,
activated or apoptotic CD4^+Tm cells). Data were analysed using GraphPad Prism 6.02.
Results

**T1D is associated with decreased absolute counts of CD4\(^+\) Tm cells in peripheral blood**

Across the three cohorts, there was no statistical difference in the percentage or absolute counts of CD4\(^-\) T cells defined within the total CD3\(^+\) cell compartment (Fig 1B). There was also no statistical difference in the percentage or absolute counts of CD4\(^-\) Tn cells or percentage of CD4\(^+\) Tm cells defined within total CD4\(^+\) cells. In T1D patients, percentage or absolute counts of CD4\(^+\) Tm cells did not correlate with disease duration or age of diagnosis (data not shown). However, there was a statistically significant reduction in the absolute counts of CD4\(^+\) Tm cells in T1D patients when compared with unrelated controls (Fig 1B, \(P = 0.024\)). The absolute CD4\(^+\) Tm cell count was also reduced in siblings but this did not reach statistical significance when compared with controls (Fig 1B, \(P=0.07\)). The lower counts of CD4\(^+\) Tm cells in T1D patients and siblings resulted from a reduction in CD4\(^+\) Tcm cells (Fig 1B). The alteration in CD4\(^+\) Tm cell counts did not occur in isolation, being associated with lymphopenia and reduced numbers of CD3\(^-\) T, CD8\(^+\) T and CD8\(^+\) Tm cells in T1D patients and their siblings (Table 1, Supporting data Fig 1). Neutropenia and reduced monocytes counts was only evident in T1D patients (Table 1).

**Increased sensitivity of CD4\(^+\) Tm cells to TCR activation in T1D patients and siblings**

Using pure ex vivo CD4\(^+\) Tm cells and anti-CD3/CD28 stimulation we compared the proliferation dynamics of T cells from T1D-patients, siblings and unrelated controls in vitro (Fig 2). There was no inter-group difference in the proliferation dynamics of CD4\(^+\) Tm cells by TCR stimulation (Fig 2A). We also examined the kinetics of proliferation in the reactivated CD4\(^+\) Tm cells post CD3/CD28 stimulation through CFSE labelling and multiparametric flow cytometry. Based on the level of CFSE expression at day 1, reactivated CD4\(^+\) Tm cells underwent more than four divisions between day three and five of culture, with the proliferation index of 2.32, 2.27 and 2.59 and frequency of divided parent cells of 47.9\%, 42.0\% and 54.3\%, respectively in T1D patients, siblings and control (Fig 2B). While there was no statistical difference in the proliferation potential of CD4\(^+\) Tm cells, an
increased percentage of CD4\(^+\) Tm cells from T1D and siblings were activated by CD3/CD28 stimulation (Fig 2C). The CD4\(^+\) Tm cells from T1D patients and siblings underwent heightened activation regardless of the concentration of the plate bound anti-CD3 mAb in the range of 1–10 µg/ml (Supporting data Fig 2). This increase in activated CD4\(^+\)CD25\(^+\) Tm cells was not due to an increase in iTr cells (CD4\(^+\)CD25\(^+\)Foxp3\(^+\)) since iTr cells accounted for a similar minority (<15% of CD4\(^+\) Tm cells) across all cohorts (data not shown). These results showed that while CD4\(^+\) Tm cells of T1D patients-and siblings exhibited similar proliferation dynamics and kinetics when compared to controls, they were acutely more 'sensitive' to TCR-induced activation.

**Altered functional phenotype of CD4\(^+\) Tm cells from T1D patients and siblings after TCR-induced activation**

Given the differences in CD4\(^+\) Tm cell post-activation status between cohorts, we next performed an in depth analysis of cell phenotype and function (Fig 3). After CD3/CD28 stimulation, CD4\(^+\) Tm cells from healthy controls maintained CD62L\(^+\) Tcm and CD62L\(^-\) Tem cell compartment in size similar to that at day 1 of culture (Fig 3A). This was in contrast to CD4\(^+\) Tm cells of T1D patients and siblings, where the percentage of CD62L\(^+\) Tcm cells was markedly lower after five days of culture (Fig 3A). Thus, in T1D patients and siblings, CD4\(^+\) Tm cells effectively transitioned to CD62L\(^-\) Tem cells after TCR-induced activation.

Another functional difference related to cytokines secreted by CD4\(^+\) Tm cells after activation (Fig 3B). Unlike healthy controls, CD4\(^+\) Tm cells from T1D patients elevated IFN-\(\gamma\) and IL-10 whereas CD4\(^+\) Tm cells from siblings elevated IFN-\(\gamma\) production post CD3/CD28 stimulation. Although CD3/CD28 stimulation failed to increase amount of secreted IL-4 across cohorts, IL-4 production appeared to be the best sustained in reactivated CD4\(^+\) Tm cells from T1D patients (Fig 3B). Kinetic studies determined that the rise in IFN-\(\gamma\) and IL-10 cytokine secretion occurred between day 3-5 of culture (data not shown). Across cohorts, there was no difference in the production of IL-6, TNF-\(\alpha\) and IL-17 (data not shown). Of note, CD3/CD28 stimulation reduced the amount of secreted IL-2 by CD4\(^+\) Tm cell across all cohorts, tentatively suggesting that reactivated CD4\(^+\) Tm cells are taking
up the IL-2 that they produce via an autocrine process. Overall, the data suggests that TCR-induced stimulation promotes a Tem cell phenotype in both T1D patients and siblings, characterised by up-regulation of IFN-γ and IL-10 in T1D patients and up-regulation of IFN-γ in siblings.

**Reactivated CD4⁺Tm cells of T1D patients and siblings exhibit increased rates of Fas-independent apoptosis**

Given the previous observations that CD4⁺Tem cells are prone to Fas mediated AICD which results in their elimination by apoptosis (13, 20), we next examined CD4⁺Tm apoptosis kinetics in T1D patients, siblings and controls (Fig 4). Across all cohorts, we observed that resting CD4⁺Tm cells underwent comparable rates of apoptosis (Fig 4A). In contrast, after CD3/CD28 stimulation, apoptosis rates of CD4⁺Tm cells in T1D patients and siblings were markedly elevated when compared with controls (Fig 4A). Also elevated apoptosis rates of CD4⁺Tm cells in T1D patients and siblings was evident at the concentration of anti-CD3 mAb in the range of 1–10 µg/ml (Supporting data Fig 2). Kinetic studies determined that most apoptosis occurred between day 3-5 of culture (data not shown). Apoptotic cells were predominantly in the early stage of apoptosis with a minority (<10% of apoptotic cells) in late apoptosis (data not shown). Apoptosis in reactivated CD4⁺Tm cells of T1D children and siblings was also associated with increased frequencies of cells expressing the active form of Caspase-3 (Fig 4B). Interactions with serum proteins was excluded as a possible cause of differential CD4⁺Tm apoptosis rates since comparable rates were observed regardless of the serum source (Fig 4C).

The death receptor (Fas) was detected on the surface of the majority of reactivated CD4⁺Tm cells in all subject groups, but with a higher frequency in T1D patients and siblings than in controls (Fig 5A). In contrast to universally high Fas expression, FasL was expressed on a minority of reactivated CD4⁺Tm cells in all subject groups but again with a higher frequency in T1D patients and siblings (Fig 5A). Treatment with the antagonistic anti-Fas ZB4 mAb, which blocks the cognate interaction between Fas and FasL, reduced Fas expression on reactivated CD4⁺Tm cells in all subject groups and blocked Fas-induced apoptosis in Jurkat cells but did not alter the levels of apoptosis of
reactivated CD4+ Tm cells in any subject group (Fig 5B, C, Supplementary Fig 3). Increased Fas and FasL expression in reactivated CD4+Tm cells of T1D patients and siblings was coupled with an increase of pro-apoptotic BIM-L, BAD, BAX and BAK transcripts compared to healthy controls (Fig 5D). Reactivated CD4+Tm cells of T1D patients showed a statistically significant spike in BAD expression that separated them from healthy controls (Fig 5D). Interestingly, the anti-apoptotic transcripts BCL-2, BCL-XL and BCL-W remained unchanged in reactivated CD4+Tm cells across all cohorts (Fig 5D). These data suggested that reactivated CD4+Tm cells of T1D patients and siblings are acutely susceptible to AICD through a Fas-independent mechanism.

**Apoptosis in reactivated CD4+Tm cells is biased towards effector cells**

Given that CD4+Tm cells in T1D patients and siblings were highly susceptible to AICD, we next sought to determine which ‘type’ of memory cell was prone to AICD. In healthy controls, we observed that the majority of apoptosis occurred in non dividing cells (Fig 6A). In contrast, CD4+Tm cells of T1D patients and siblings showed consistently high levels of apoptosis across every cell division (Fig 6A). Given that CD4+Tm cells encompass functionally distinct lineages (5), we next subdivided the analysis of apoptosis into Tcm (CD62L+) and Tem (CD62L-) lineages. Majority of CD4+Tm cells undergoing apoptosis were within the Tem lineage, which encompassed >80% of apoptotic cells across each cohort (Fig 3B). Importantly, we observed that the apoptotic bias towards Tem cells was more pronounced in T1D patients and siblings compared with controls eliminating approximately half of the cells from the Tem lineage in T1D patients and siblings but only one third of CD4+Tem cells in controls (Fig 6C). In contrast to the Tem lineage, the Tcm lineage remained largely resistant to AICD, however, the Tcm lineage of T1D patients and siblings still displayed greater rates of AICD when compared with controls (Fig 6C). These data suggest the cells from the Tem lineage are highly susceptible to AICD in T1D patients and siblings.
Discussion

T1D is one of the most heritable common diseases, with high sibling relative risk and the largest concordance rate in monozygotic twins amongst the autoimmune diseases (21, 22). Thus, if we are to understand the mechanisms of T1D pathophysiology and intervene with preventative or curative therapies, we must look carefully at the causes of immune dysfunction in patients and their at risk siblings. To address this gap in knowledge, we undertook a high definition analysis of CD4⁺Tm cell composition and function in an extremely well characterised age- and sex-matched cohort of children comprising T1D patients, their non-affected siblings and unrelated healthy controls.

Interestingly, we identified a distortion in the global CD4⁺Tm cell repertoire with T1D patients having lower absolute counts of CD4⁺Tm cells compared with healthy controls. In both T1D patients and their siblings lower absolute counts of CD4⁺Tm cells did occur at the expense of CD4⁺Tcm cells. This reduction in CD4⁺Tm cells was associated with reduced CD3⁺T, CD8⁺T and CD8⁺Tm cells counts and lymphopenia in T1D patients and their siblings. Given this distortion in global CD4⁺T cell memory, we next asked whether there was a problem with T cell proliferation, activation and/or effector immune response shutdown in T1D patients and their siblings. No inter-group differences in cell proliferation was observed indicating that CD4⁺Tm cells of T1D patients and their siblings were not senescent, exhausted or defective in cell replication. However, we observed a difference in cell phenotype post stimulation with a spike in the activated CD4⁺CD25⁺Tm cells in T1D patients and siblings consistent with reported presence of activated CD25⁺CD3⁺T cells in T1D patients and siblings (8).

The increased activation of CD4⁺Tm cells was further reinforced by the observation that reactivated CD4⁺Tm cells of T1D patients and siblings were capable of ‘exaggerated’ effector responses through efficient ‘switching’ from central memory (CD62L⁺) to effector memory (CD62L⁻). This phenotype may provide some evolutionary benefit. For instance, compared with healthy controls, reactivated CD4⁺Tm cells of T1D patients and siblings can better upregulate IFN-γ. IFN-γ is critical for clearing bacteria, viruses and cancers (23, 24) and higher levels may result in more ‘efficient’ T
cell surveillance. Indeed, there is some evidence of decreased risk of particular cancers in T1D patients (25, 26). In line with this idea, we have recently identified a reduced rate of infection with common childhood viruses in T1D patients and siblings when compared to age-, sex- and geographically-matched children (Miles, personal communication), however, a larger cohort is needed to categorically establish this link. Elevated levels of IL-10 production by reactivated CD4+ Tm cells in T1D patients may also be significant given that this cytokine is known to mediate the destruction of β-cells in the NOD mouse (27). Sustained levels of the multifunctional pleiotropic cytokine IL-4 in reactivated CD4+ Tm cells of T1D patients is also of interest given the central role of this cytokine in guiding the alternative fates of T cell differentiation (28). Additionally, it appeared that reactivated CD4+ Tm cells of T1D patients and their siblings rapidly taking up secreted IL-2 via an autocrine process. Determining whether any differences in cytokine consumption between T1D patients siblings and controls, however, will require additional experimental approaches (e.g. qPCR and intracellular cytokine staining).

A striking observation in CD4+ Tem cells of both T1D patients and siblings was a heightened sensitivity to AICD. Apoptosis in CD4+ Tem cells occurred via TCR stimulation and might be expected to result from Fas-mediated death receptor pathways (13). Although we observed upregulation of Fas and FasL in reactivated CD4+ Tm cells there was upregulation of pro-apoptotic BIM-L, BAD, BAX and BAK transcripts which are associated with the mitochondrial apoptotic pathway. Additionally, we observed upregulation of the active form of Caspase-3 shared by both apoptotic pathways. Of note, only in reactivated CD4+ Tm cells of T1D patients was a significant upregulation of pro-apoptotic gene BAD observed which discriminated this disease cohort from the other subject groups. Elevated AICD rates in CD4+ Tem cells from both T1D patients and siblings could not be prevented by blocking the Fas receptor. This suggests that there may be a defect in the Fas death receptor pathway via altered lipid raft microdomains (13) or defects in assembly of the Fas-associated dead-inducing signalling complex (29). Alternatively, upregulation of BIM-L, BAD, BAX and BAK transcripts suggests that mitochondrial apoptotic pathways may be mainly
responsible for the increased rate of apoptosis in CD4\(^+\)Tem cells. Finally, although CD4\(^+\)Tem cells of T1D patients and siblings displayed similar susceptibility to apoptosis, we cannot rule out that apoptosis could differ at the clonotype level. It is possible that in T1D patients or siblings who go on to develop disease, autoreactive effector cells may be particularly resistant to apoptosis. Therefore, if would be of key interest to further subdivide the CD4\(^+\)Tem cell lineage with additional phenotypic/functional markers and full TCR repertoire analysis.

Previous investigations of CD4\(^+\)T cell biology in T1D have been restricted to the comparison of patients with unrelated healthy controls (30, 31). These comparisons will likely miss critical genetic and environmental cues that underpin CD4\(^+\)Tm cell pathomechanisms in T1D patients and siblings (32). In our study, we identified several novel aspect of CD4\(^+\)Tm cells biology common to T1D patients and siblings including reduced absolute CD4\(^+\)Tm and CD4\(^+\)Tcm counts, ‘exaggerated’ CD4\(^+\)Tem phenotype with an elevated IFN-\(\gamma\) ‘signature’, heightened sensitivity to activation and AICD. These features of the CD4\(^+\)Tm cells common to T1D patients and siblings associated with lymphopenia (32) but not with clinical variables such as age, age of disease, C-peptide or autoantibody status and are evident before the onset of autoimmunity and islet antibody detection.

In conclusion, this study has revealed a novel ‘hyperactive’ phenotype of reactivated CD4\(^+\)Tm cells of T1D patients and their siblings. In these cells, we found an exaggerated ‘activation switch’ (activated CD25 receptor and Tem phenotype) was counterbalanced by an exaggerated ‘shutdown switch’ (AICD). Whether an exaggerated ‘activation switch’ herein induced in reactivated CD4\(^+\)Tm cells by polyclonal TCR stimulation also operates across other T cell lineages (e.g. CD8\(^+\)T cell lineages) as well as in response to antigen specific stimulation is warranted in further studies. Precisely how this exaggerated ‘activation switch’ in CD4\(^+\)Tm cell phenotype relates to \(\beta\)-cell destruction will require a thorough immunological dissection. Nonetheless, this data helps further define the mechanisms underlying T1D pathophysiology and illuminates new biologically relevant surface receptors, cytokines, biochemical pathways and specific immune cells lineages for therapeutic targeting.
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Author Contributions: M.L.B. performed experiments, analysed data and contributed to the manuscript preparation. O.H. performed experiments and contributed to data interpretation. D.M. contributed to the experimental design and data interpretation. M.H. and A.C. oversaw clinical sampling, provided clinical data and reviewed/edited the manuscript. J.J.M. contributed to research data interpretation and manuscript writing. S.V. designed the study, contribute to data interpretation and wrote the manuscript.
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<td>NR</td>
<td></td>
</tr>
<tr>
<td>IA-2A only</td>
<td>3 (10%)</td>
<td>0 (0%)</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>GADA&amp;IA-2A</td>
<td>20 (65%)</td>
<td>1 (2%)</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Mean HbA1c (%,[mmol/mol], mean ± SD)</td>
<td>8.3 ± 1.4 [68 ± 15.7]</td>
<td>NR</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>C-peptide (nmol/L, median (range))</td>
<td>0.17 (0.1-1.2)</td>
<td>NR</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Subjects with high risk HLA-DQ haplotype ‡</td>
<td>16 (94%)</td>
<td>NR</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Age-corrected BMI (Z-score, mean ± SD)</td>
<td>0.68 ± 0.89</td>
<td>NR</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>WBC (1 x 10^7/mL, mean ± SD)</td>
<td>6382 ±1551***</td>
<td>6896 ±1641***</td>
<td>8780 ±1848</td>
<td>H0.001</td>
</tr>
<tr>
<td>Lymphocyte (1 x 10^9/mL, mean ± SD)</td>
<td>2494 ±639***</td>
<td>2510 ±779**</td>
<td>3403 ±922</td>
<td>0.0008; 0.0021</td>
</tr>
<tr>
<td>Monocytes (1 x 10^9/mL, mean ± SD)</td>
<td>503 ±172**</td>
<td>588 ±169</td>
<td>629 ±204</td>
<td>0.041</td>
</tr>
<tr>
<td>Neutrophil (1 x 10^9/mL, mean ± SD)</td>
<td>2981 ±1018**</td>
<td>3399 ±1121</td>
<td>4178 ±1303</td>
<td>0.004</td>
</tr>
<tr>
<td>Eosinophil (1 x 10^9/mL, median (range))</td>
<td>220 (75-1110)</td>
<td>259 (38-1190)</td>
<td>365 (78-1274)</td>
<td>0.211</td>
</tr>
<tr>
<td>Basophil (1 x 10^9/mL, median (range))</td>
<td>39.0 (5-104)</td>
<td>43 (16-95)</td>
<td>49 (14-155)</td>
<td>0.176</td>
</tr>
<tr>
<td>Platelets (1 x 10^6/mL, mean ± SD)</td>
<td>296 ±67</td>
<td>294 ±56</td>
<td>313 ±89</td>
<td>0.930</td>
</tr>
</tbody>
</table>

Categorical variables (gender and islet autoantibody) are presented using counts and percentages. Parametrically distributed data are presented as mean ± SD and non-parametrically distributed data as median with minimum and maximum ranges. ‡One sibling was positive for GAD&IA-2&ZnT8 autoantibody, but did not develop T1D during the course of this study. †High risk HLA-DQ haplotypes included DQA1*05:01/DQB1*02:01 (DQ2) and/or DQA1*03:01/DQB1*03:02 (DQ8). Age-corrected body mass index (BMI) Z-score (standard deviation score) was calculated based on population data published by WHO. There was no difference in BMI Z-scores between boys and girls. Denote: a, difference between T1D patients and controls; b, difference between siblings and controls. WBC, white blood cells. NR, no record.
Figure legend

**Figure 1. CD4$^+$ T cell counts in the peripheral blood of T1D patients, siblings and unrelated control children.** (A) Representative dot plots show gating steps used to define (B) frequency and absolute counts of the CD4$^+$T cells and their lineages in the lysed whole blood samples of T1D patients, siblings (Sib) and unrelated control children (Control). Data are presented as median. Tn, naïve T cells; Tm, memory T cells; Tcm, central memory T cells; Tem, effector memory T cells.

**Figure 2. Proliferation and activation of the CD4$^+$Tm cells post CD3/CD28 stimulation.** Pure ex vivo CD4$^+$ Tm cells of T1D patients, siblings and control were cultured for 5 days in the presence (reactivated) or absence (resting) of anti-CD3/CD28 mAb (anti-CD3 mAb 2 µg/ml; anti-CD28 mAb 1 µg/ml). (A) Proliferation assessed by $^3$H-thymidine uptake. (B) CFSE intensity of reactivated CD4$^+$Tm cells cultured for 1 day (serve as the control to locate the undivided [division 0] cell position), 3 days and 5 days. Progressive divisions (1, 2, 3, 4, >4) are apparent by even two-fold dilutions of the CFSE. Computer fitting used to determine proliferation index and proportion of divided parent cells (median, 25-75% quartiles and the minimum and maximum ranges). (C) Activation assessed by the expression of CD25 (representative dot plots; bar graph, median, 25-75% quartiles and the minimum and maximum ranges).

**Figure 3. Functional phenotype of CD4$^+$Tm cells after TCR-induced activation.** (A) Representative dot plots show gating steps used to define the CD62L$^+$Tcm and CD62L$^-$Tem cells within CD4$^+$ Tm cells at day 1 and 5 of culture (median, gates are set up for live CD4$^+$ Tm cells inclusion based on FSC&SSC). (B) Secreted cytokines were measured in culture supernatants collected at day 1 and 5 of culture (median, 25-75% quartiles and the minimum and maximum ranges). Solid lines indicate cytokine detection limit evaluated from standard curves.

**Figure 4. Apoptosis in reactivated CD4$^+$Tm cells.** (A) For Annexin V$^+$ 7AAD$^-$ apoptotic cell quantification, gates are set up open for inclusion of CD4$^+$ Tm cells of all sizes but exclusion of the cell debris (representative dot plots). The percentage of apoptotic cells within the reactivated and
resting CD4+ Tm cells at day 1 and 5 of culture (median, scatter plot). (B) Caspase-3 expression within the reactivated (open histograms, scatter plots with median) and resting CD4+ Tm cells (close histograms) measured at day 1 and 5 of culture. (C) The percentage of apoptotic cells within the reactivated CD4+ Tm cells at day 5 of culture with either autologous serum, allogeneic serum (from siblings or unrelated control) or FCS (mean ± SEM).

Figure 5. Dead receptor and mitochondrial apoptotic pathways in reactivated CD4+Tm cells. (A) The percentage of Fas+ and FasL+ cells within the reactivated CD4+ Tm cells at day 1 and 5 of culture (representative dot plots; bar graphs with median, 25-75% quartiles and the minimum and maximum ranges). (B) The percentage of Fas+ cells and (C) apoptotic cells within the reactivated CD4+ Tm cells at day 5 of culture with anti-Fas ZB4 or isotype control mAb (bar graphs with median, 25-75% quartiles and the minimum and maximum ranges). (D) Expression of pro-apoptotic BIM-L, BAD, BAX, BAK and anti-apoptotic BCL-2, BCL-XL and BCL-W transcripts relative to HKG (AGTB) in reactivated CD4+ Tm cells at day 1 and 5 of culture (bar graphs with median, 25-75% quartiles and the minimum and maximum ranges).

Figure 6. Occurrence of apoptosis across cell division and within CD4+Tcm and CD4+Tem lineages. (A) Computer fitting used to define the proportion of apoptotic cells (mean ± SEM) within undivided (division 0) and divided (divisions 1, 2, 3, 4, >4) reactivated CD4+Tm cells at day 5 of culture. (B-C) For apoptotic cell quantification in reactivated CD4+Tm cells at day 5 of culture gates are set up open for inclusion of all apoptotic cells but exclusion of the cell debris (representative dot plots). (B) The percentage (median) of CD4+ Tcm and CD4+ Tem cells within apoptotic gate. (C) The percentage of apoptotic cells (median) within CD4+Tcm and CD4+Tem cells.
### Diabetes

**A**

Gated on CD3^+^ T

- CD4
- CD8

**B**

Gated on CD3^+^ T

- % CD4^+^ T
- % CD4^+^ Tn
- % CD4^+^ Tm
- CD4^+^ T (x 10^3/ml)

Gated on CD4^+^ T

- % CD4^+^ Tn
- % CD4^+^ Tm
- % CD4^+^ Tcm
- % CD4^+^ Tem
- CD4^+^ Tcm (x 10^3/ml)
- CD4^+^ Tem (x 10^3/ml)

**Notes:**

- T1D N=22
- Sib N=37
- Control N=22

* P = 0.024

^P = 0.07

^P = 0.34

^P = 0.91

^P = 0.031

Bian et al. Revised Fig 1.
Diabetes

A

$^{3}H$-thymidine uptake (counts per minute)

Reactivated Resting

B

Events

Day 1

Day 3

Day 5

CFSE

T1D

Sib

Control

$>4$ $4$ $3$ $2$ $1$ $0$ Divisions

Bian et al. Revised Fig 2.
Bian et al. Revised Fig 4.
Supporting Figure 1. CD3+T, CD4+T and CD8+T cell counts in the lyed whole blood of T1D patients, siblings and unrelated control children calculated by multiparametric flow cytometry and TruCOUNT beads. (A) Representative dot plots show gating steps used to define frequency and absolute counts of CD3+T, (B-C) CD4+T (shown in Fig 1) and CD8+T cell lineages. Data are presented as median; Tn, naive T cells; Tm, memory T cells; Tcm, central memory T cells; Tem, effector memory T cells; Temra, terminally-differentiated effector T cells.

<table>
<thead>
<tr>
<th>Anti-CD3 mAb (µg/ml)</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD28 mAb (µg/ml)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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</table>

Supporting Figure 2. Reactivated CD4+Tm cells from T1D patients and siblings maintain the phenotype of heightened activation and sensitivity to AICD in response to increasing concentrations of the plate bound anti-CD3 mAb. Pure ex vivo CD4+ Tm cells of T1D patients (T1D, n=3), siblings (Sib, n=3) and control (n=3) were cultured for 5 days in the absence of CD3/CD28 stimulation or in the presence of increasing concentrations of the plate coated anti-CD3 mAb (1, 2, 5 and 10 µg/ml) combined with anti-CD28 mAb (1 µg/ml). Activation assessed by the expression of CD25 (top panel) and apoptotic cells by AnnexinV/7AAD staining (bottom panel) at day 5 of culture (left: representative dot plots; right: bar graph, mean ± SEM).
Supporting Figure 3. Treatment with the antagonistic anti-Fas ZB4 mAb reduced Fas induced apoptosis in Jurkat cells. Jurkat cells co-cultured with and without CH-11 mAb (20μg/ml) were treated with 0, 1, 2.5 or 5 μg/ml of ZB4 mAb or 5μg/ml of isotype control mAb. (A) Representative dot plots show apoptosis (Annexin+/7AAD+ cells) induced in Jurkat cells by CH-11 mAb in the presence of isotype control mAb or increasing concentration of ZB4 mAb. (B) Antagonist anti-Fas ZB4 mAb masked the Fas expression on Jurkat cells (left panel) and reduced the proportion of apoptotic Jurkat cells induced by CH-11 mAb (right panel). Data are of two independent experiments, and are presented as mean ± SEM.