A unique T cell subset described as CD4<sup>lo</sup>CD40<sup>+</sup> T cells (T<sub>CD40</sub>) in human type 1 diabetes

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Abstract Human T1D pancreatic lymph nodes contain diabetes-autoantigen responsive T cells but identification of such T cells in the periphery has proven difficult. Here we describe a unique T cell subset defined by CD4<sup>lo</sup> and CD40 expression (T<sub>CD40</sub>) that is significantly expanded in peripheral blood of T1D but not control or T2D subjects. The HLA-DR3 and DR4 alleles are considered high risk factors for T1D and T<sub>CD40</sub> expansion occurs in T1D subjects carrying HLA DR3 or DR4 haplotypes but, T1D subjects who do not carry either DR3 or DR4 haplotypes still have an expanded percentage of T<sub>CD40</sub> cells. Non-autoimmune subjects, even DR3+ and DR4+, do not have elevated percentages of T<sub>CD40</sub> cells. The majority of T<sub>CD40</sub> cells in T1D carry a memory phenotype and a portion of those proliferates when exposed to diabetes-associated self-antigens. A greater number of memory T<sub>CD40</sub> cells express CXCR3 when compared to CD40<sup>-</sup> memory cells and that number is significantly expanded in T1D compared to control subjects. If only total CD4<sup>+</sup> T cells are compared no difference in CXCR3 is seen. Furthermore, T<sub>CD40</sub> cells produce a Th1, pro-inflammatory cytokine profile. In healthy controls, T<sub>CD40</sub> cells have equally Th1 and Th2 profiles.

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KEYWORDS
Type 1 diabetes; Auto-reactive T cells; Autoimmunity; CD40

Introduction
Autoimmune diseases are characterized by aseptic, chronic inflammation leading to debilitating tissue destruction. Mouse T1D models show that different cell types participate in the inflammatory process. In particular, T cells infiltrate...
the pancreatic islets, leading to loss of insulin production and hyperglycemia [1–4]. It was recently demonstrated that self-antigen reactive, including pre-pro-insulin, human T cells occur in pancreatic lymph nodes of diabetic subjects [5]. The majority of these cells expressed TRAV8-3 or TRAV39 that were HLA-DR4 restricted [5]. Detecting self-antigen reactive T cells in the periphery of T1D subjects has proven to be very difficult. Using HLA tetramer technology some autoantigen responsive T cells have been detected [6,7]. The frequency of such T cells is quite low; perhaps due to the nature of the HLA tetramers, i.e. tetramers utilize one specific peptide. In this manner, only a limited number of self-antigens can be examined.

In the nonobese diabetic (NOD) mouse T1D model a unique subset of helper T cells characterized as CD4loCD40+ (TCD40) cells proved to be highly autoaggressive [1–3]. While found in naive, non-autoimmune animals, TCD40 cells consistently remain contained at low levels [1,3]. Our previous work [1–3,8] raises the question of whether this unique T cell subset occurs in human subjects, contains self-antigen reactive T cells and is likewise expanded in T1D.

In this report, through a blinded study, comparing diagnosed T1D, T2D and control subjects, we found that a subset of T cells characterized as CD4lo that express CD40 are detected in human subjects and are significantly expanded in peripheral blood of T1D but not T2D or control subjects HLA DR3 and DR4 are tightly associated with T1D [9]. TCD40 cell expansion occurs in subjects carrying HLA DR3 and DR4, but expansion of TCD40 cells is seen in T1D subjects that do not carry either the DR3 or DR4 haplotypes. Also of interest, healthy non-autoimmune subjects that carry HLA-DR3 or DR4 do not expand TCD40 cells. A significant portion of TCD40 cells have a memory phenotype with greater numbers of TCD40 cells in T1D subjects carrying a memory phenotype. A portion of TCD40 cells from T1D subjects but not from control subjects proliferate when exposed to human islets and the T1D identified self-antigens, pre-pro-insulin one of the GAD peptides and human insulin B9 peptide. A significant number of memory TCD40 cells express CXCR3, a chemokine receptor potentially associated with T cell pathogenicity and diabetes, compared to CD40+ memory T cells, especially in T1D. Mechanistically, TCD40 cells in T1D produce a Th1, pro-inflammatory cytokine panel. These data demonstrate the reliable identification of an autoantigen responsive T cell subset in human T1D that can be identified in peripheral blood.

Methods

Human T cells

Patients were recruited from the Barbara Davis Childhood Diabetes Center and the University of Colorado Denver and Health Sciences Center. T1D patients included recent onset, within 6 weeks of examination and long term, up to 40 years after initial diagnosis. The age range of patients was from 17 to 64 years of age, with clustering in 20s and 30s (shown in Table 1). T1D and T2D were diagnosed by the American Diabetes Association criteria including failed glucose tolerance test, confirmed by anti-islet or diabetes-associated self-antigen autoantibody positive tests in T1D and no autoantibody detection in T2D patients. Subjects that exhibited acute inflammation or infection were excluded from the study. Peripheral blood was collected, then diluted 1:1 with PBS and passed through Ficoll–Hypaque and washed three times with PBS. Studies were conducted under a Colorado-Municipal Institutional Review Board (IRB # 01-384) approved protocol.

Staining

In all cases, human lymphocytes, 1×10⁶, were stained with directly conjugated antibodies including: anti-CD40 (FITC, clone 5C3); anti-CD4 (PE-Cy5, clone RPA-T4); anti-CD8 (PE-Cy5 or APC, clone RPA-T8); and isotype controls all from e-Bioscience, San Diego, CA. Anti-CCR5 (APC) and anti-CXCR3 (CyChrome) are from BD-Pharmingen, Thousand Oaks, CA. Percentages reported in all cases were determined from 1×10⁶ cells to reflect actual numbers. Cells were stained after overnight incubation of cells in culture in 10% FCS-RPMI medium.

Antigen stimulation of T cells

Peripheral blood lymphocytes were isolated and passed through nylon wool columns [10] or T cells were purified using a magnetic microbead anti-CD4 antibody followed by AutoMacs (Miltenyi Corp, Auburn, CA). Cells eluted from columns were >95% CD3+. Antigen presenting cells (APC) and T cells were segregated as described [11]. Purified APCs, 1×10⁵, were incubated for 12 h with 10 μM pre-pro-insulin,

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* p=0.757.
** p=0.949.
amino acid sequence 73–90 (AGSLQPLALEGSLQKRGG); GAD 271-285 peptide (PRLIAFTSEHSHFSL); or human B9 insulin peptide (SHLVEALYLVCGERG) or with 10 picked human islets provided by the Barbara Davis Childhood Diabetes Center. Tetanus toxoid at 400 ng/ml and PHA-M at 1 μg/ml were included as positive controls and OVA as negative control. APCs were irradiated using a strata-linker ultraviolet light source, for 2 min or were treated with mitomycin C at 50 μg/ml.

**Proliferation assays**

T cells were purified by depleting CD8+, CD19+ and HLA+ and CD25+ cells from peripheral blood leukocytes that were isolated through Ficoll–Hypaque of collected blood samples. Depletions were done using a Miltenyi AutoMACS cell sorter. Samples were passed twice through the machine set to DEPL05, the most sensitive depletion setting. Purified T cells 1 × 10^6 cells, typically greater than 95% CD4+, were incubated with CFSE, 5 μM, in PBS for 45 min in the dark. Cells were washed with PBS–5% BSA two times then added to APC–antigens as described above. Cells were incubated for 4 days then stained with anti-CD45RO (PE) anti-CD40 (Cy5, fluoresces in Fl-3) and CD4 (APC that fluoresces in Fl-4) and assayed using a Becton-Dickinson FACScalibur flow cytometer and the CellQuest analysis software. Proliferation was measured as decrease in mean fluorescent intensity of CFSE compared to background, T cells labeled with CFSE and not exposed to APC.

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**Figure 1** T<sub>CD40</sub> cells in peripheral blood lymphocytes are expanded in human T1D. Percentages of CD40<sup>+</sup> T cells reported from T1D, T2D and control subjects were determined from 1 × 10<sup>6</sup> cells to reflect actual numbers. Direct percentage of CD4<sup>+</sup>CD40<sup>+</sup> and CD4<sup>+</sup>CD40<sup>+</sup> T cells are demonstrated in (A) non-autoimmune control subjects; (B) T1D subjects; (C) T2D subjects. CD4<sup>+</sup>CD40<sup>+</sup> T cells were confirmed by counter staining with CD3, then gating on CD3<sup>+</sup> T cells in (D) normal subjects and (E) T1D subjects. Dot plots showing CD8<sup>+</sup>CD40<sup>+</sup> T cells from control (F) and T1D (G) subjects. In all cases, gates were set from appropriate isotype controls. (H) Scatter plots representing the ratio of CD4<sup>+</sup>CD40<sup>+</sup> to total CD4<sup>+</sup> T cells and the mean with standard deviations for T1D, control and T2D subjects are shown. Statistical analysis was performed using single-variable and multi-variable Kruskal–Wallis one-way analysis of variance on ranks by the SigmaStat<sup>™</sup> analysis program.
Cytokine induction assays

APC–antigen complexes were washed then purified CD4+ T cells, 1 x 10^5, were exposed for 18 h in the presence of 10 μM Brefeldin A. In each experimental system, T cells were exposed to antigen-loaded APC from the same donor to avoid allogenic responses. T cells were stained with CyChrome-conjugated anti-CD4 and FITC-conjugated anti-CD40 as described above. Cells were washed with PBS/5% BSA then with PBS and incubated with 2% paraformaldehyde in PBS for 10 min at room temperature then treated with a mild detergent cell lysis buffer (BD-Pharmingen) for 10 min. Cells were incubated with PE-conjugated anti-TNFα, anti-IFNγ, anti-IL-6 or anti-IL-4 or isotype control antibody at 5 μg/ml for 45 min. T cells were washed 3 times with PBS/5% BSA then analyzed by flow cytometry. For analysis, samples were gated on CD4hiCD40+ or CD4loCD40- and levels of cytokine are reported from each subset. Levels reported are above isotype controls within each subset.

Statistical analysis

Statistical analysis was performed when appropriate, single-variable and multi-variable one-way ANOVA and Student's t test difference of the means using the SigmaStat™ analysis program.

Results

CD4loCD40+ cells are expanded in the periphery of T1D subjects

We examined T cells from peripheral blood of healthy control subjects comparing to clinically diagnosed T1D subjects in a blinded study. Representative dot plots of stained peripheral blood cells from control subjects reveal a subpopulation of cells that are CD4lo and CD40+. In the case represented this constitutes 15% of the total lymphocytes (Fig. 1A). T1D patients exhibit a substantial increase in the percentage of TCD40 cells (Fig. 1B) compared to controls (Fig. 1A). This percentage expansion was seen in all T1D subjects examined. The concern arose that expanded percentages of TCD40 cells occur due to hyperglycemia; therefore, we recruited type 2 diabetic (T2D) subjects and examined levels of TCD40 cells. The TCD40 cell percentage in T2D is statistically the same as healthy controls (Fig. 1C) demonstrating that the expanded percentages of TCD40 cells in T1D occur independently of hyperglycemia. While TCD40 cells are expanded in T1D subjects, it is noteworthy that CD4loCD40- T cell percentages are lower in T1D (as represented in Fig. 1; 29.5% in T1D compared to 41% in T2D and control subjects).

It has been suggested that human macrophages and B cells can express CD4; therefore, we gated on CD3+, which is associated with T cells. Gated CD3+ cells included the CD4loCD40+ T cell subset and importantly the TCD40 cell subset, thus restricting them to a T cell phenotype (Figs. 1D and E). We did note that CD3 expression is lower on TCD40 cells (data not shown). CD3-gating removes a population of peripheral CD40+ cells, and there were no discernable differences in levels of these cells between T1D and control patients; however, T2D subjects show an expanded CD40+, non-T cell subset.

There are reports of CD8+ autoaggressive T cells contributing to diabetes in the NOD mouse T1D model and CD8+CD40+ T cells have been reported [12–15]. However, no difference in the percentage of CD8+CD40+ T cells between T1D and control patients was detected (Figs. 1F and G). This suggests that CD8+CD40+ T cells may be less prominent in the human T1D process. Typically, there were more CD8+CD40+ T cells in non-diabetic controls than in T1D as represented in Fig. 1. In fact, a recent study suggests that some human CD8+ T cells have regulatory T cell properties [16].

In mouse studies, we determined that the ratio of CD4loCD40+ to CD4hiCD40- T cells proved predictive for T1D [3]. When the ratio of CD4loCD40+ to total CD4+ T cells is compared from T1D, T2D and control subjects a clear distinction between T1D versus T2D and controls emerges. The mean ratio of TCD40 cells to total CD4+ T cells in T1D is 45.681 ± 8.03% which is significantly (p < 0.0001) greater than the ratio for T2D, 21.42 ± 5.16%, or controls, 21.06 ± 5.15% (Fig. 1H). These data suggest that significantly elevated levels of TCD40 cells may be a discernable risk factor for T1D.

TCD40 cells and HLA expression

Current risk factors for T1D include carrying either the HLA-DR3 or HLA-DR4 haplotypes [6,9,17–20]. We therefore addressed the correlation between levels of TCD40 cells and HLA haplotypes in T1D and control subjects. Analysis was done examining means of TCD40 cells as a percentage of the total CD4 T cell compartment from subjects carrying DR4 (Fig. 2A), DR3 (Fig. 2B) and if neither DR3 nor DR4 haplotypes occurred (Fig. 2C). As expected, an expanded percentage of TCD40 cells was detected in T1D subjects carrying DR4 (Fig. 2A) or DR3 haplotypes (Fig. 2B). Even though HLA-DR3 and DR4 are strongly associated with T1D, there are T1D subjects who do not carry either of those haplotypes, suggesting that diabetes autoreactive T cells can develop in the absence of HLA-DR3 or DR4. Interestingly in T1D subjects who do not carry either DR3 or DR4, an expanded percentage of TCD40 cells occurred nonetheless (Fig. 2C). When the percentage of TCD40 cells from each HLA population were compared within T1D using a multi-variable analysis of variance test there were no significantly different groups. In all haplotypes, TCD40 cell percentages in T1D were significantly expanded above controls (Fig. 2). In control subjects with no identified autoimmune disease carrying the diabetes high risk haplotypes, DR4 or DR3, the percentage of TCD40 cells was not increased.

We determined the correlation between age of subjects and TCD40 cell percentages. The age range of subjects was from 17 to 65 years, with the majority of subjects falling between 20 and 40 years old (see Table 1). The percentage of TCD40 cells in younger T1D subjects was higher but not significantly different from percentages at other ages (p > 0.25) (Fig. 2D). In three subjects diabetes was diagnosed more than 40 years prior, yet percentages of TCD40 cells remain elevated. This suggests persistent response to different self-antigens or a distinct failure to appropriately undergo apoptosis. Regardless of age, T1D subjects always have a greater percentage of TCD40 cells (Fig. 2D). In all healthy control subjects, including those > 60 years old, TCD40
cell percentages remain contained thus demonstrating that expansion of these T cells is not age related.

A portion of $T_{CD40}$ cells from T1D subjects responds to identified diabetes self-antigens

The expanded percentage of $T_{CD40}$ cells in T1D could reflect that this T cell subset harbors autoreactive T cells. A number of GAD65 and pre-pro-insulin T cell clones were identified in pancreatic lymph nodes of T1D subjects [5]. In another study, it was shown that peripheral T cells from T1D and some control subjects respond to the GAD65 peptide [21]. In that study, T cells from T1D subjects did not require CD28 costimulation for activation; thus, the authors surmised that autoreactive T cells in T1D primarily are of memory phenotype.

We directly determined levels of memory T cells within the CD4$^{+}$ T cell subset (Fig. 3A) by measuring CD45RO expression, which identifies human memory T cells [22]. Approximately one third of the CD40$^{-}$ T cell population in both T1D (Fig. 3B) and control (Fig. 3C) subjects exhibit a memory phenotype. Within the $T_{CD40}$ cell population from T1D subjects the majority of CD40$^{+}$ cells (65%) were CD45RO$^{+}$ (Fig 3D). In the $T_{CD40}$ cell population from control subjects 45% express the memory marker (Fig. 3E). Previous reports have associated CD40 function with generation of memory T cells [15].

Because of the clear distinction in levels of memory phenotype CD40$^{+}$ T cells between control and T1D subjects, we focused on memory $T_{CD40}$ cells as potentially harboring autoreactive T cells. We compared the total proliferative response of memory CD40$^{+}$ and memory CD40$^{-}$ T cells from T1D and control subjects when exposed to diabetes-associated antigens. T cells from each donor were exposed to APC from that donor loaded with each antigen (Fig. 4). A subset of CD45RO$^{+}$CD40$^{-}$ T cells from T1D subjects were responsive to pre-pro-insulin, another
subset were responsive to GAD556 (GAD65), or to islets or to the B9 human insulin peptide (Fig. 4A). This suggests that TCD40 cells from T1D subjects do harbor autoreactive TCR bearing T cells and that different T cells within that population carry uniquely pathogenic TCR molecules. Therefore, rather than a very limited clonal expansion, there may be some expansions of several individual TCD40 cells each with a unique specificity to a self-antigen that can contribute collectively to the disease process. Additionally, a portion of CD45RO−CD40+ T cells from T1D were responsive to positive control, tetanus toxoid and PHA (Fig. 4A). Interestingly, only a very small percentage of CD45RO−CD40− T cells from T1D were responsive to the GAD271 peptide, in fact in two T1D subjects TCD40 cells were not responsive to GAD271; suggesting that T cells carrying TCR molecules that recognize this antigen are not present in these individuals. Each subject demonstrated individual response differences relative to each of the antigens tested (not shown), with only the averaged data presented here. Memory TCD40 cells from control subjects were not responsive or only slightly responsive to diabetes-associated antigens (Fig. 4A). But a portion of memory TCD40 cells from control subjects were responsive to tetanus toxoid and PHA stimulation, demonstrating that these T cells are not anergic (Fig. 4A).

These data suggest identification of a unique T cell subset wherein autoreactive T cells can be found. If it is considered that 29% of PBL are CD4+CD40+ (Fig. 1) and 65% of those cells are memory T cells (Fig. 2) then back calculations incorporating the data from Fig. 3 would mean that 0.90% of total PBL are pre-pro-insulin responsive; 1.51% are GAD556 responsive; 1.13% are islet-antigen responsive; and 0.94% are B9 human insulin responsive. A portion of CD45RO−CD40− T cells from T1D subjects were responsive to GAD556 (GAD65), islets and B9 human insulin (Fig. 4B). These data represent that if the TCD40 population is removed from consideration, then only a very small population of antigen reactive T cells would be detected in the periphery. For instance, back calculating from the CD40+ memory data presented here, on average only 0.31% of peripheral T cells could be detected as responsive to GAD556 and even fewer T cells are responsive to other antigens. These new data demonstrate, however, that by appropriately including the TCD40 population, a better...
representation of the autoreactive containing T cell subset is achieved.

**T\textsubscript{CD40} cells and chemokine receptor expression**

While direct evidence that any T cell subset attacks pancreatic islets in human studies is not available, markers for directed trafficking exist. For example, expression of certain chemokine receptors has been associated with islet infiltrates in murine studies [23,24]. Directed migration of lymphocytes is mediated by a chemokine concentration gradient. In the murine T1D model, it has been shown that islets produce the IFN\(\gamma\)-inducible chemokines, CXCL9/Mig; CXCL10/IP10 and CXCL11/TAC, and the chemokine receptor for these IFN\(\gamma\)-inducible chemokines is CXCR3, which is a Th1-associated marker [25]. In that mouse model it was shown that chemotaxis of activated T cells to \(\beta\) cells was mainly mediated by CXCR3 [25]. This suggests that expression of CXCR3 is involved in directed targeting of pathogenic T cells to islets. In human studies the association of CXCR3 with type 1 diabetes has been tenuous. One report states that CXCR3 expression on total CD4 lymphocytes was significantly lower in subjects described as having fulminant disease but was significantly higher in subjects described as having typical diabetes, i.e. ketoacidosis prone and within 6 months from diagnosis to insulin dependency [26]. Another study reported somewhat reduced expression of CXCR3 on total peripheral blood lymphocytes in children newly diagnosed with T1D [27]. Thus, some confusion exists relative to CXCR3 expression and T1D.

When we examined CXCR3 expression on total CD4\(^+\) lymphocytes from T1D and control subjects no significant differences were detected (data not shown). However, when CD4\(^+\) cells were further gated into CD40\(^+\)CD45RO\(^+\) populations, i.e. memory TCD40 cells and CD40\(^-\)CD45RO\(^-\), CD40 memory cells (Fig. 5A), discreet differences in CXCR3 expression emerge. As shown earlier, T1D have a greater number of memory phenotype T cells than control subjects (Fig. 5A). Within individuals, CXCR3 levels on gated CD4\(^+\)CD40\(^+\)CD45RO\(^+\) cells from T1D subjects were significantly \((p<0.001)\) higher than on CD40\(^-\)CD45RO\(^-\) cells (Fig. 5B). CXCR3 levels on gated, CD4\(^+\)CD40\(^+\)CD45RO\(^-\) memory cells from control subjects were higher than on CD40-negative memory cells from control subjects. When memory TCD40 cells are considered, direct comparison of memory TCD40
cells between T1D and control subjects demonstrate a significantly higher percentage (p<0.001) of CCR5+ cells in T1D (Fig. 5B).

CCR5 expression like CXCR3 is associated with Th1 cells and is activation inducible [28,29]. In memory TCD40 cells from T1D subjects, CCR5+ cells were decreased compared to CD40+ memory T cells from T1D and interestingly there was no difference in CCR5+ TCD40 cell percentages between T1D and controls (Fig. 5B; p=0.634). Memory CD40+ cells from control subjects had a markedly greater percentage of CCR5+ cells (Fig. 5B). Because fewer memory TCD40 cells from T1D and control subjects express CCR5, this suggests that TCD40 cells are not activated, relative to other memory T cells. However, the increased number of CXCR3+ memory TCD40 cells suggests these T cells are responsive to CXCR3 related chemokines, especially in T1D.

Figure 5  TCD40 cells and CXCR3 and CCR5. T cells were isolated from 6 T1D and 6 control subjects and 1 x 10⁶ cells were stained with anti-CD40 (FITC), anti-CD4 (APC), anti-CD45RO (PE) and anti-CXCR3 (Cy-Chrome). In a different staining strategy cells were stained with anti-CD40 (FITC), anti-CD4 (CyChrome), anti-CD45RO (PE) and anti-CCR5 (APC). Cells were gated on CD4 expression. (A) A representative dot plot of CD40 versus CD45RO expression. Gates were set from isotype controls for T1D and for controls. (B) CXCR3 and CCR5 levels in CD40+CD45RO+ cells (solid histograms) and CD40−CD45RO− (solid line, open histograms) and isotype controls (dashed, open histograms) were determined. Percentages reported are means and standard deviations from 5 each T1D and control subjects. Top numbers are percent within CD4+CD40+CD45RO+ cells and bottom numbers are percent within CD4+CD40−CD45RO− cells. Statistical analyses were done using Kruskal–Wallis one-way analysis of variance on ranks using SigmaStat Analysis™ or Tukey (ANOVA) test.

Effector cells function includes synthesis and secretion of cytokines including Th1 (IL-2, IFNγ and TNFα) or Th2 (IL-4, IL-5, IL-10 and TGFβ) cytokines. T1D has been linked to Th1, pro-inflammatory cytokine production [30,31]. We assessed the ability of TCD40 cells to respond to diabetes-associated self-antigens and achieve effector status. Background levels (exposure to APC alone) of Th1, TNFα and IFNγ TCD40 cells from normal subjects are very low (Figs. 6A and B). Importantly, no increase in TNFα or IFNγ producing TCD40 cells is seen in control T cells when exposed to known diabetes-associated antigens (Figs. 6A and B). TCD40 cells from T1D subjects, however, appear primed for inflammatory cytokine production. TCD40 cells exposed to APC alone exhibit increased percentages of TNFα+ (Fig. 6A) and INFγ+ (Fig. 6B) cells as compared to TCD40 cells from healthy control subjects (Fig. 6A). Importantly, when antigen challenged with the diabetes-associated antigens including pre-pro-insulin, GAD peptide and human islets, levels of the Th1 producing T cells, TNFα+ (Fig. 6A) and IFNγ+ (Fig. 6B) were significantly (p<0.01) increased in T1D demonstrating that these T cells carry autoantigen responsive TCR molecules and when exposed to these antigens produce pro-inflammatory cytokines.

IL-6 is a pro-inflammatory cytokine and increased expression is linked with autoimmunity [32–34]. We found that relatively high percentages of TCD40 cells produce IL-6 in T1D compared to T cells from control subjects (Fig. 6C). As expected, no change in IL-6+ T cells from control subjects occurred when exposed to diabetes-associated antigens. Interestingly though, no alteration in the levels of TCD40 cells from T1D subjects producing IL-6 occurred when challenged with diabetes self-antigen (Fig. 6C). This suggests that IL-6 producing TCD40 cells do not express TCR molecules that recognize the antigens tested. Nonetheless, it is striking that a pro-inflammatory cytokine emerging as definitive in autoimmune diseases is consistently increased in TCD40 cells from T1D.

Levels of IL-4 producing TCD40 cells were very low in T1D subjects and those levels did not alter when exposed to the diabetes-associated antigens (Fig. 6D). Levels of IL-4 producing T cells were equally low in control subjects and as expected, did not respond to diabetes-associated antigen challenge.

The overall T helper cell phenotype, Th1 versus Th2, was compared by stimulating TCD40 cells versus CD4+CD40+ T cells with PHA, an agglutin that activates T cells in a TCR independent manner. Upon challenge with PHA, TCD40 cells from T1D subjects exhibited a striking Th1 phenotype relative to CD40+ T cells from the same subjects (Fig. 6E). PHA-exposed TCD40 cells from control subjects favor a slightly higher Th1 phenotype compared to CD40+ T cells, but that difference is not significant (p>0.25) (Fig. 6E). A portion of TCD40 cells from T1D subjects (22%) are capable of a Th2 response under these antigen-independent activation conditions (Fig. 6E). Unlike T cells from T1D, TCD40 cells from healthy controls produced a substantial Th2 response when PHA challenged (Fig. 6E). Additionally, CD40+ T cells from healthy controls produced a more robust Th2 response than the same T cell subset from T1D subjects when PHA stimulated (Fig. 6E). While Th2 T cell clones generated
from a TCR transgenic mouse have been reported to be diabetogenic [35], typically Th2 cytokines are more strongly linked to autoimmune protection. Our result suggests that the T cell environment in control subjects is biased to favor protection while in T1D subjects the environment is biased to favor inflammation.

**Discussion**

This report describes a unique peripheral T cell subset, T_{CD40} cells, which is present in control subjects but is significantly expanded in T1D subjects. Furthermore, as we demonstrate here, T_{CD40} cells from T1D subjects but not from control subjects harbor diabetes-associated self-antigen reactive cells. Previously, we identified T_{CD40} cells as a pathogenic T cell subset in the NOD T1D model [1–3]. We showed that concurrent with progressive insulitis, T_{CD40} cells expand throughout diabetes progression [1,3]. While T_{CD40} cells are present in non-autoimmune mouse strains, there is no percentage expansion. Pathogenicity of T_{CD40} cells was defined by successful adoptive transfer of insulitis and hyperglycemia to NOD.scid recipients. Importantly T_{CD40} cells from diabetic and pre-diabetic NOD mice were equally capable of disease transfer while the conventional helper T cells, CD4^{hi}CD40^{-}, do not transfer insulin or diabetes [1,3].

A recent report described pre-pro-insulin reactive, HLA-DR4-restricted T cell clones derived from pancreatic lymph nodes of T1D patients [5]. However, identification of self-reactive T cells in the periphery has proven very difficult; likely because T cell studies typically focus on only CD4^{hi} T cells. Our studies, including the current report, demonstrate...
that autoaggressive T cells have lower CD4 expression and express CD40 [1–3,36]. This may be a consequence of autoantigen T cell activation, but non-autoimmune subjects carry CD40+ T cells albeit at a significantly lower percentage. Autoantigen reactive T cells have been identified using tetramer technology [6,7], but that approach has limitations. For example, tetramers detect only partial peptide, i.e. 12–17mer reactive T cells [7,37]. If diabetogenic T cells are represented by several different peptides, then such a strategy would not detect the gamut of ‘autoaggressive’ T cells, and thus the actual number of self-antigen reactive T cells would be underestimated. Here we show that portions of peripheral TCD40 cells isolated from T1D subjects respond to PPI, GAD, human insulin B9 peptides and whole islets. An important intrinsic difference between T1D and control subjects is that TCD40+ Cells from healthy controls are better able to produce regulatory Th2 cytokines and therefore may be better able to control autoimmunity. Furthermore, CD40− T cells from control subjects much more readily produced Th2, regulatory cytokines. These observations provide an important mechanistic difference between autoimmune and non-autoimmune conditions that now can be better defined relative to the TCD40 cell subset.

Another interesting facet of these studies addresses chemokine receptors in T1D. CCR5 and CXCR3 are Th1-associated and have been studied in mouse and in human T1D [23,25,27,38–40]. However, discrepancies in levels of CXCR3 relative to T1D are reported. In one study CXCR3 levels are reported to be increased on total CD4 lymphocytes in subjects described as having typical diabetes, but decreased in subjects with fulminant disease [26]. In another study, CXCR3 was somewhat decreased in new T1D onset children [27]. We discovered that examining total lymphocytes was not helpful in discerning CXCR3 levels but by focusing on memory TCD40 cells, a greater percentage of CXCR3 cells within a given individual are detected. CCR5 is detected on memory TCD40 cells from T1D and control subjects and is higher in control subjects. CXCR3 and CCR5 are considered activation markers [28]. However the current data suggest that TCD40 cells are not merely an activation state but that CXCR3 expression may play a more prominent role for these T cells in T1D pathogenesis.

The overarching mechanism we propose is that sustained high levels of TCD40 cells promote autoimmunity [36], in this case type 1 diabetes. In NOD mice, CD40 engagement induces RAG1/RAG2, the recombinase proteins required for altering TCR expression in peripheral T cells [2], via a process called TCR revision [41–43]. We have shown that revised T cells can be highly diabetogenic [3]. TCR revision could generate numerous potentially pathogenic T cells, including autoaggressive T cells in other autoimmune diseases, which we are investigating. While CD40 induced TCR revision can be pathogenic, coincidentally, TCR revision could prove tolerogenic depending upon appropriate conditions [36].

An important control mechanism for autoimmunity is regulatory T cells, Tregs, which suppress the action of autoaggressive T cells. Many studies have suggested that a dominant feature of autoimmunity is insufficient Tregs, thus allowing autoaggressive T cells to go uncontrolled, resulting in disease. It is essential to delineate and understand cellular differences that will account for autoimmunity. The elucidation of an autoantigen responsive T cell subset is paramount. We propose that TCD40 cells include that T cell subset.

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