CD40 on NOD CD4 T cells contributes to their activation and pathogenicity

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Our goals in this study were to investigate conditions under which T cells from NOD mice express CD40 and to determine how CD40 on autoreactive CD4 T cells contributes to their pathogenicity in T1D. Using CD40-positive diabetogenic T cell clones and CD4 T cells from NOD mice, we examined expression of CD40 upon activation through the TCR and costimulation through either CD28 or CD40. Our results indicate that CD40 expression is increased upon activation with antigen/MHC and that activation of NOD CD4 T cells through TCR/CD40 rapidly induced CD40 expression. Furthermore, CD40 costimulation promoted T cell proliferation to the same extent as costimulation through TCR/CD28. Importantly, costimulation of CD4 T cells through CD40 also interfered with T cell homeostasis by altering regulation of CTLA-4 expression. Through CD40–CD154 blocking studies, we demonstrated that signaling between T cells through CD40 and its ligand contributes to activation of pathogenic T cells and that blocking CD40 on T cells abrogates their ability to transfer diabetes. Thus, costimulation through CD40 on NOD T cells contributes to their pathogenicity by providing additional pathways for activation and by inhibiting upregulation of CTLA-4 during T cell activation.

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1. Introduction

CD40 is a member of the TNFR superfamily and is expressed on a wide variety of cells including antigen-presenting cells (APC), neurons, and endothelial cells [1]. The ligand for CD40, CD154, was originally identified on activated T cells and interaction between CD40 and CD154 results in bi-directional signals essential for the development of adaptive immunity [2,3]. CD40 uses a cytoplasmic tail to recruit TNF receptor-associated factors (TRAF), which are key to signaling through CD40, leading to the activation and nuclear translocation of transcription factors such as NF-κB or NFAT in a cell type dependent fashion [4]. Activation of the CD40 signaling pathway leads to a variety of effects such as class switching in B cells [5], cytokine secretion in macrophages [6,7], and upregulation of adhesion molecules on endothelial cells [8].

Aberrant expression of CD40 is implicated in many autoimmune diseases, including lupus, rheumatoid arthritis and multiple sclerosis (reviewed in Refs. [9,10]), and CD40 signaling is known to participate in the pathogenesis of autoimmune diseases. A specific example of CD40 expression in autoimmunity is its presence on T cells. Studies in the non-obese diabetic (NOD) mouse have shown that CD4+CD40+ T cells, but not CD4+CD40− T cells, transfer diabetes [11] and that the size of the CD4+CD40+ T cell compartment increases with the age of the mouse [12]. In addition, CD40-positive T cells have recently been described in human type 1 diabetics [13], strongly indicating a link between ectopic CD40 expression and autoaggressive phenotype. In another recent report, Munroe and colleagues demonstrated that chronic exposure to an autoantigen was necessary for T cells to signal through CD40 in the C57BL/6 model of collagen-induced arthritis (CIA) [14]. Although several studies have indicated that CD40 is functional on CD4 T cells [15–17], regulation of CD40 expression on T cells has been little documented and is poorly understood.

The two major costimulatory pathways for activation of pathogenic T cells in NOD mice involve signaling through CD28-B7 and CD40–CD154. T cell costimulation through CD28 is generally thought to be protective as CD28-deficient NOD mice develop accelerated diabetes [18] and treatment of young NOD mice with stimulating anti-CD28 monoclonal antibodies (Mab) prevents the disease [19], although differential effects of CD28 engagement by B7-1 or B7-2 on T cell function underscore the complexity of the CD28/B7 pathway [20]. On the other hand, there is a positive correlation between the engagement of the CD40/CD154 pathway and pathogenicity. The genetic disruption of CD154 (NOD CD154−/−) [21] and the treatment of NOD mice with blocking anti-CD154 Mab [12,22] abrogate disease. However, therapies aimed at targeting
pathogenic T cells via the disruption of the CD40/CD154 pathway using anti-CD154 MAb have been unsuccessful in humans due to thromboembolic complications [23]. The finding that expression of CD40 on T cells correlates with an autoimmune phenotype might open new avenues for diagnosis and/or immunotherapy.

The goals of this study were to determine the conditions under which CD4 T cells in NOD mice express CD40 and to investigate how signaling through CD40 on NOD T cells affects their diabetogenicity. Using T cell clones specific for beta cell antigens, we have asked how activation by autoantigen influences CD40 expression on autoreactive T cells. To analyze more precisely the signals involved in CD40 expression on T cells, we established a system to stimulate highly purified NOD CD4 T cells in the absence of APC, using activating monoclonal antibodies against CD3, CD28, and CD40. We demonstrate that T cell activation in the presence of TCR/CD40 signaling, but not TCR/CD28 signaling, upregulates CD40 on primary CD4 T cells and that costimulation through either CD28 or CD40 had similar effects on T cell proliferation. Interestingly, we show that expression of CTLA-4 on T cells in autoimmune mice is further induced after signaling through CD28 but not through CD40 during T cell activation. In addition, we tested the hypothesis that CD40 on the surface of CD4 T cells might interact with CD154 on activated T cells and investigated whether this interaction could provide a means for autonomous activation and costimulation of autoreactive T cells in type 1 diabetes. Lastly we examined whether blocking CD40 on diabetogenic T cell clones would alter their pathogenicity in an adoptive transfer model of T1D.

2. Materials and methods

2.1. Mice

NOD, and NOD.scid breeding mice were initially acquired from The Jackson Laboratory or the Barbara Davis Center for Childhood Diabetes (Denver, CO), and were bred and housed in specific pattern-free conditions at the University of Colorado Denver Center for Laboratory Animal Care (CLAC). NOD.scid mice were housed in sterile isolation cages. BALB/c mice were purchased from The Jackson Laboratory. NOD.scid litters (6–10 days old) were used as recipients in adoptive transfer experiments. Breeding mice and experimental animals were monitored for development of disease by urine glucose. All procedures used were in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines and approved by the UCD IACUC.

2.2. Culture and expansion of T cell clones

The BDC panel of diabetogenic T cell clones was established from spleen and lymph nodes of diabetic NOD mice as previously described [24–26], and were restimulated every 2 weeks with a beta cell granule membrane fraction obtained from beta cell tumors as a source of Ag (referred to as beta-memb) [27], irradiated NOD spleen cells as APC, and EL-4 supernatant as a source of IL-2 (40 U/ml). For primary T cell induction, sorted CD4 T cell preparations were confirmed to contain >95% CD4 positive T cells as determined by flow cytometry. Primary mouse CD4 T cells were cultured in CM supplemented with EL-4 supernatant as a source of IL-2 (40 U/ml). For primary T cell induction, sorted CD4 T cells (1 x 10^6 cells/ml) were incubated for 20 min at 37 °C with anti-CD3 biotin (2 μg/ml) ± unconjugated anti-CD28 (2 μg/ml) or anti-CD40 biotin (4 μg/ml). Cells were then cross-linked with avidin (2–4 μg/ml), plated in 6- or 24-well plates and resuspended at a final concentration of 2.5 x 10^5 cells/ml.

2.5. Bone marrow-derived dendritic cell preparation

Bone marrow-derived dendritic cells (bmDC) were prepared from 2–3 months old NOD mice after removing the bone marrow cells from tibias and femurs by flushing with CM. After red blood cell lysis (Sigma–Aldrich, St. Louis), the remaining cells were counted and plated at 1 x 10^6 cells/well in 6-well plates in RPMI supplemented with l-Glutamine at 2 mM (Gibco, NY) and GM-CSF at 4 ng/ml (BioSource International) for 2 days. Non-adherent cells were removed by gently swirling the plate; the supernatant was centrifuged at 300 x g and 2 ml of cell free supernatant was added back to each well. New medium was added every other day; bmDC were harvested on day 7 for assay.

2.6. Antibodies used for surface staining, induction and blocking

The 1C10 (anti-CD40, rat IgG2a) hybridoma was a kind donation from Frances Lund (Trudeau Institute) and the MR1 (anti-CD154, Ham IgG) hybridoma was a kind donation from Jill Sutters (PhD, Professor of Immunology, University of Louisville, KY). For surface staining, cells were first incubated with Fc Block (93; BioLegend) and then with antibodies to surface molecules: allophycocyanin-rat anti-CD4- (eBR2a; eBioscience), FITC-Rat IgG2a isotype control- (eB82a; eBioscience), FITC-Ham anti-CD3- (145-2C11; BioLegend), FITC-Rat anti-CD40- (1C10; produced in house using the FluoTag kit from SIGMA according to manufacturer's recommendation), PE-Rat IgG2a isotype control- (Pharmingen), PE-Rat anti-CD40- (3/23; Pharmingen), PE-Ham isotype control- (HTK888; BioLegend), PE-Ham anti-CD154- (MR1; BioLegend), PE-Ham anti-CD28- (37.51; BioLegend), PE-Ham anti-CD69- (H1.2F3; BioLegend) and PerCP-Cy5.5-Rat anti-CD11b- (M1/70; Pharmingen).

Antibodies for intracellular staining were: allophycocyanin-Rat anti-CD4- (GK1.5; BioLegend), PE-Ham IgG isotype control- (HTK888; BioLegend) and PE-Ham anti-CTLA-4- (UC10-489; BioLegend); antibodies were used at optimal concentrations determined empirically.

Antibodies used for T cell cross-linking included biotin-Ham anti-CD3- (145-2C11; BioLegend), purified-Ham anti-CD28 (37.51; BioLegend), Biotin-Ham anti-CD28 (37.51; Pharmingen), Biotin-Rat anti-CD40 (1C10; produced in house using the EZ-link bioconjugation kit from Pierce according to manufacturer's recommendations), Biotin-Rat anti-CD40 (3/23; Pharmingen) and Biotin-Rat IgG2a (RTK2758; BioLegend) were used at concentrations indicated. For blocking CD154, cells (5 x 10^6 cells/ml) were activated in the presence of 10 μg/ml of MR1 antibody before culture at 37 °C in a 24-well plate.
2.7. Surface and intracellular staining

For surface staining, cells were resuspended at 1 × 10^6–10^7 cells/ml in a 96-well plate in staining buffer (PBS, 0.5% BSA) containing a master mix of antibodies at appropriate dilutions and stained for 30–60 min on ice. For intracellular staining, cells were fixed in 2% formaldehyde for 10 min in the dark. Cells were washed once in staining buffer and resuspended in permeabilization buffer (staining buffer plus 0.5% saponin). Cells were then stained in permeabilization buffer containing a master mix of antibodies at appropriate dilutions and stained for 30 min on ice. Cells were washed three times in staining buffer before analysis on a FACScalibur flow cytometer (BD Biosciences).

2.8. Proliferation assay

For staining with CFSE, cells (5–10 × 10^5 cells/ml) in PBS were incubated at 37 °C for 10 min with 2.5 μM CFSE (Invitrogen). After washing, cells were activated with monoclonal antibodies as described above and cultured for 72 h. Cell divisions were analyzed on a FACScalibur flow cytometer using the FlowJo software (Tree Star, Inc., OR).

2.9. Adoptive transfer of T cell clones and recovery of diabetogenic T cells from pancreas

For disease transfer experiments, expanded cell cultures were harvested after 4 days with cell dissociation buffer enzyme-free Hanks’ based (GIBCO). For blocking experiments, T cell clones harvested after 4 days with cell dissociation buffer enzyme-free T cells from pancreas 2.9. Adoptive transfer of T cell clones and recovery of diabetogenic Star, Inc., OR).

2.10. Monitoring of disease

Mice were monitored daily for elevated urine glucose. Pancreata were harvested at the time of disease onset or 45–48 days after adoptive transfer of T cells; tissue was fixed in formalin, embedded in paraffin, and sectioned. Histology scores were generated from multiple sections throughout each pancreas to allow for more thorough examination of the entire pancreas. Scores represent examination of >100 islets from each treatment group.

2.11. Flow cytometry analysis

For each experiment, gates were set to exclude dead cells based on FSC/SSC and to include only CD4^+ cells and quadrants were set so that less than 1% of the cells were stained with the appropriate isotype control. Flow cytometry assays were performed on a FACScalibur (BD Biosciences) or FACScan and for data analysis, the geometric mean fluorescence intensity (MFI) was calculated for each sample using FlowJo software (Tree Star, Inc., OR). We chose to use the geometric mean fluorescence since this statistic is not weighted by outliers at the high end of the distribution. The ΔMFI represents the difference in geometric mean fluorescence intensity between the isotype control staining and the CD40 staining.

2.12. Statistics

Statistical significance was determined by a two-tailed Student’s t-test. A p-value ≤ 0.05 was considered significant.

3. Results

3.1. CD40 is upregulated on diabetogenic T cell clones upon antigen stimulation

We previously reported that CD40 was expressed on the NOD-derived diabetogenic CD4 T cell clones from the BDC panel and that the presence of CD40 on these T cell clones correlated with their pathogenicity [11]. It has been suggested that CD40 upregulation on T cells is caused by chronic activation by autoantigen [11,14] and to test this hypothesis in NOD, we used the T cell clone BDC-2.5 to investigate how antigen stimulation affects CD40 expression. As illustrated in Fig. 1A, we observed that T cells stimulated with islet antigen and spleen cells as a source of APC upregulate CD40 expression on their surface compared to CD40 levels on resting BDC-2.5. When we stimulated the clone with islet antigen and a purified population of bone marrow dendritic cells (bmdC) as APC, we found that CD40 expression was four times higher than when spleen cells were used; when the form of antigen was an agonist peptide mimotope, the bmdC results were more than double those obtained with islet cells as antigen. CD40 expression...
was not upregulated in control cultures of BDC-2.5 with APC in the absence of antigen (data not shown). These data suggest that expression of CD40 on diabetogenic clones increases with the strength of the signal through TCR.

Since we observed that autoreactive T cells upregulate CD40 upon antigen stimulation in vitro, we wanted to know if CD40 would be upregulated on a pathogenic T cell clone after adoptive transfer. As previously reported [24], the diabetogenic clone BDC-2.5 migrates to the pancreas where it is stimulated by its antigen, resulting in an autoimmune destruction of pancreatic β islets. After adoptive transfers into NOD.scid littermates with BDC-2.5, mice were sacrificed 2, 5 and 9 days after injection, and their pancreata were harvested for ex vivo analysis of CD40 expression on T cells. As shown in Fig. 1B, only 10% of the BDC-2.5 clones expressed CD40 before transfer. The T cell clones recovered from the pancreas after adoptive transfer showed gradually increasing surface CD40 levels with time, increasing to 24% of T cell clones being CD40 positive 9 days after transfer. These data are consistent with the in vitro observation that CD40 levels are upregulated upon antigen stimulation on this pathogenic clone and indicate that CD40 increases on T cells during the autoimmune process of T1D.

3.2. Signaling through CD40 – but not CD28 – upregulates CD40 expression on primary NOD CD4 T cells

To determine whether stimulation of primary CD4 T cells through TCR can upregulate CD40 on their surface, sorted CD4 T cells from spleen and lymph node cells of 8–12-week-old NOD mice were activated using antibodies to CD3, CD28 and CD40 and assessment of CD40 expression was measured by flow cytometry. As shown in Fig. 2A, sorted CD4 T cells cultured in IL-2 express only low levels of CD40 (there was no difference in ΔMFI between cells cultured in medium only or cells cultured in medium supplemented with IL-2). After activation with an anti-CD3 antibody, 33% of the T cells became CD40-positive, indicating that activation of primary NOD T cells through TCR upregulates surface CD40 levels.

To determine how costimulation through CD28 or CD40 affected CD40 expression, sorted NOD CD4 T cells were activated with anti-CD3 plus an anti-CD28 antibody, or anti-CD3 plus an anti-CD40 (clone 3/23) antibody. Cells were harvested after 1 day of culture and stained for CD4 and CD40 (clone 1C10) or a matching isotype control. As shown in Fig. 2A, when cells were activated through CD3/CD28, CD40 levels were comparable to cells activated with anti-CD3 alone, indicating that signaling through CD28 did not affect CD40 expression. In contrast, when the CD4 T cells were activated through CD3/CD40, 90% of the T cells became CD40-positive, suggesting that costimulation of CD4 T cells through CD40 during TCR activation sends a positive signal with respect to CD40 expression and reinforces CD40 upregulation induced by TCR activation. When the T cells were activated through CD3/CD28/CD40, the ΔMFI of CD40 staining was comparable to that of CD3/CD40 alone, and thus costimulation through CD28 in addition to CD40 costimulation does not further increase CD40 levels on NOD T cells (data not shown). The differences observed in CD40 staining levels after T cell activation indicate that costimulation through CD40 but not CD28 can upregulate CD40 on the surface of CD4 T cells.

To further demonstrate that CD40 was upregulated upon stimulation through CD3/CD40, we examined the time course of CD40 expression upon activation. CD40 expression began to increase within 1.5 h after activation through CD3/CD40 and reached a maximum after about 4 h (Fig. 2B). Due to the short time frame (<8 h) in which these experiments were conducted, there were no significant changes in cell numbers. In contrast, there is little or no increase in CD40 on NOD CD4 T cells activated through CD3 or CD3/CD28. The right-hand panel of Fig. 2B shows that expression of CD40-ligand CD154 is strongly induced upon stimulation through CD3 or CD3/CD28 but not through CD3/CD40. These data indicate that there are marked differences in the temporal expression of

![Fig. 2](image-url)
CD4 and CD154, depending on which costimulatory pathway is engaged, CD28 or CD40.

3.3. Costimulation through CD40 enhances T cell proliferation

The IL-2 receptor (IL-2R) and IL-2 play a central role in the clonal proliferation of T cells and CD25 upregulation is essential to obtain a functional IL-2R. It was shown that costimulation through CD40 on T cells upregulates CD25 expression and enhances IL-2 production when compared to activation by anti-CD3 alone [14]. This would suggest a potential role for CD40 costimulation in T cell proliferation. As we had observed enhanced expression of CD25 on NOD T cells upon activation through CD3/CD40 compared with anti-CD3 alone (data not shown), we asked whether CD40 engagement on NOD CD4 T cells would provide effective signals for T cell proliferation. Sorted CD4 T cells from lymph nodes of 2–3-month-old NOD mice were activated through CD3, CD3/CD28 or CD3/CD40. The results represented in Fig. 3A show that IL-2 alone did not induce T cell proliferation. As expected, stimulation through CD3 induced a modest T cell proliferation. In contrast, activation through either CD3/CD28 or CD3/CD40 induced substantial T cell proliferation with a doubling in T cell numbers after 3 days of culture when cells were stimulated through CD3/CD40. Using a CFSE assay, we confirmed that signaling through CD3/CD28 or CD3/CD40 induces a higher dilution of CFSE than activation through CD3 alone (Fig. 3B). Taken together, these results suggest that costimulation through CD40 is at least as effective as signaling through CD28 for T cell proliferation.

3.4. CD40 costimulation prevents CTLA-4 upregulation on NOD CD4 T cells

CTLA-4 is a major co-inhibitory molecule on T cells and plays a key role in T cell homeostasis by competitive antagonism of CD28 signals and direct negative signaling [30]. CTLA-4 expression results in down-modulation of the T cell response (cell-cycle arrest and inhibition of IL-2 production) upon antigen activation. Since CTLA-4 is induced upon activation and its upregulation is dependent on CD28 costimulation during T cell activation [20], we wanted to know how CD40 costimulation affected CTLA-4 levels. Results shown in Fig. 4 compare CTLA-4 expression on BALB/c and NOD CD4 T cells, and show that CTLA-4 is upregulated from basal levels (which were 8–12%, data not shown) on CD4 T cells after activation with anti-CD3; as expected, CTLA-4 levels are further upregulated after CD28 costimulation in both mouse strains with a slightly higher increase for BALB/c T cells compared to NOD T cells. When BALB/c CD4 T cells are stimulated through CD3/CD40, CTLA-4

Fig. 3. Costimulation through either CD40 or CD28 promotes T cell proliferation to the same level. NOD CD4 T cells were isolated from prediabetic NOD mice and resuspended in CM + IL-2 (1 × 10^6 cells/ml). (A) Sorted cells were activated with anti-CD3, anti-CD3 plus anti-CD28, or anti-CD3 plus anti-CD40. After 3 days of culture, cells were harvested and cells excluding trypan blue were counted using a hemacytometer. Results of 8 independent experiments are represented and the number of live cells counted for each cell culture condition is reported. Mean cell count values are: IL-2: 3.9 × 10^5 (±0.9 × 10^5); CD3: 1.2 × 10^6 (±0.3 × 10^5); CD3/CD28: 1.7 × 10^6 (±0.3 × 10^5); CD3/CD40: 2.0 × 10^6 (±0.7 × 10^5). *P-values < 0.05 are marked with an asterisk. (B) Sorted cells were labeled with CFSE, activated under the conditions specified above for 3 days, and analyzed by flow cytometry. Data is representative of three experiments.

Fig. 4. CD40 costimulation prevents CTLA-4 upregulation in NOD CD4 T cells. NOD CD4 T cells were isolated from (A) BALB/c females or (B) aged-matched prediabetic NOD female mice and resuspended in CM + IL-2 (2 × 10^6 cells/ml). Cells were then activated with anti-CD3, anti-CD3 plus anti-CD28, or anti-CD3 plus anti-CD40 or IL-2 alone. After 2 days of culture, CTLA-4 expression was analyzed by intracellular staining. Each dot represents the percentage of CD4^+ CTLA-4^+ cells for each experiment. *P-values < 0.05 are marked with an asterisk.
levels are not increased but are comparable to those obtained after anti-CD3 stimulation alone (Fig. 4A). We would postulate that if costimulation through CD40 on NOD T cells is similar to costimulation through CD28, the effects on CTLA-4 expression would be similar. Surprisingly, in NOD CD4 T cells, upregulation of CTLA-4 after stimulation through CD3/CD40 is actually decreased compared to CTLA-4 levels observed after stimulation through TCR alone (Fig. 4B), demonstrating that costimulation through CD40 alters normal CTLA-4 upregulation during T cell activation in NOD mice. Given that CTLA-4 levels generally correlate with down-modulation of T cell activation, our data suggest that there are differential effects of T cell costimulation through CD28 or CD40 and this may impact T cell activation in autoimmunity.

3.5. CD40 and CD154 interaction between T cells is involved in T cell activation

We again used diabetogenic T cell clones in order to investigate how CD40 and CD154 might interact during T cell activation. For these experiments, we used BDC-6.3, another clone from the BDC panel which expresses high levels of CD40 in the resting state [11] and rapidly expresses CD154 upon activation (Fig. 5A). We postulated that if CD40 and CD154 interaction between T cells participates in T cell activation, blocking this interaction during cell activation should impair T cell activation. To test this hypothesis we activated BDC-6.3 with anti-CD3 in the presence or absence of a blocking antibody to CD154 (MR1) and monitored T cell activation by assessing increases in CD69 levels. Fig. 5B shows that, as expected, CD69 levels rapidly increased upon activation of BDC-6.3, reaching the highest level 6 h after activation. When BDC-6.3 was activated in the presence of blocking antibody to CD154 (MR1), CD69 levels were significantly reduced at each time point compared to levels obtained in the absence of MR1. These data suggest that blocking CD40–CD154 interaction on BDC-6.3 during T cell activation impairs T cell activation. Similar results were observed in experiments with the clone BDC-2.5, but the differences in T cell activation were less marked, perhaps because expression of CD40 is not as high on this clone.

3.6. Blocking of CD40 on BDC-2.5 and BDC-6.3 T cell clones abrogates their diabetogenicity

To determine whether prevention of signaling through CD40 would affect diabetogenicity of T cells, we investigated the effect of blocking CD40 on T cell clones with antibody to CD40 prior to adoptive transfer. In our studies with the T cell clones, we found that anti-CD40 alone did not upregulate CD69 or induce proliferation and we, therefore, asked whether treatment of T cell clones with anti-CD40 only would inhibit their pathogenic activity. We treated BDC-2.5 or BDC-6.3 with anti-CD40, anti-CD28, or a control isotype. Following in vitro antibody treatment, T cells were adoptively transferred to NOD.scid (BDC-2.5) or to young prediabetic NOD recipients (BDC-6.3). Mice were sacrificed at onset of hyperglycemia or 45–48 days after transfer in the case of animals that did not become diabetic. As shown in Fig. 6A, in vitro treatment of the BDC-2.5 T cell clone with anti-CD40 significantly (p < 0.001) ablated diabetes transfer to NOD.scid recipients; six out of eight recipients did not become diabetic. In contrast, when BDC-2.5 was treated with an antibody to CD28, there was no significant difference relative to controls in disease incidence, although mice receiving the anti-CD28-treated T cells did develop hyperglycemia somewhat earlier than their counterparts. Similar results were obtained in transfers with the T cell clone BDC-6.3 in that T cells treated with anti-CD40 could not induce diabetes in NOD recipients. Fig. 6B is a representative photograph from the histological analysis of pancreatic tissue from mice receiving anti-CD40-treated BDC-2.5. When BDC-2.5 was treated with isotype control or CD28, more than 66% of the islets showed a complete loss of granulation. In contrast, when T cell clones were treated with anti-CD40, only 10% of islets were degranulated and over half of the islets remained completely granulated, demonstrating the protective effect of anti-CD40 treatment. These data indicate that anti-CD40 treatment of diabetogenic T cells in vitro can lead to persistent inhibition of disease in vivo.

4. Discussion

Our analysis of CD40 expression on the NOD-derived pathogenic T cell clone BDC-2.5, in vivo and in vitro, confirms the findings of others and extends them to the spontaneous NOD mouse model of autoimmunity. Munroe and Bishop [14] reported that the ability of primary T cells from normal mice (C57BL/6) to express and signal through CD40 depends on prior exposure and chronic activation by an autoantigen. Signaling through CD40 was observed only in T cells in which autoimmunity (CIA) was induced. Moreover, these authors established in the CIA model that CD40 engagement alone on primary T cells from mice immunized with type II collagen can provide necessary signals for secretion of proinflammatory cytokines (INF-γ and TNFs) and that CD40 costimulation synergizes with CD28 during T cell activation to provide necessary signals for cytokine production. We have investigated regulation of CD40 expression on both diabetogenic CD4 T cell clones and on primary
CD4 T cells from the NOD mouse. We found that our diabetogenic T cell clones, representing fully differentiated Th1 effector T cells, upregulate CD40 upon antigen activation both in vitro and in vivo, supporting our hypothesis that upregulation of CD40 on auto-antigen stimulation. On primary CD4 T cells, we found that CD40 levels are comparable to those reported on T cells from C57BL/6 immunized with type II collagen in CFA [14]. In order to further explore what signals are responsible for CD40 upregulation on NOD T cells, CD4 T cells from prediabetic NOD mice were activated in the presence or absence of activating monoclonal antibodies to CD3, CD28 and/or CD40. Our results demonstrate that signaling through CD40 in the presence of TCR activation is essential for CD40 upregulation. In previous studies on CD40 function in T cells, one difficulty encountered was obtaining sufficient numbers of CD40-positive T cells, obliging investigators to either use T cell lines transfected with CD40 or to sort on CD40-positive T cells. The spontaneous NOD model provides the advantage that CD40 expression is induced on CD4 T cells upon signaling through TCR/CD40 and, therefore, large numbers of CD40 T cells are readily generated.

Our hypothesis in this study was that CD40, through an alternative costimulatory pathway to CD28, promotes pathogenicity of CD4 Th1 effector T cells. Others have demonstrated that low levels of CD86 (B7) expression in NOD mice resulted in impaired CD28 costimulation [31]. Importantly, however, levels of expression of CTLA-4 are greatly enhanced upon CD28 engagement [32]. We previously demonstrated that CTLA-4 in NOD mice – but not in BALB/c – fails to control self-reactive T cells, suggesting that CTLA-4 expression might be regulated differently in the two mouse strains in response to self-antigen [33]. We show here that CD40...
costimulation is at least as effective as CD28 costimulation in providing necessary signals for T cell activation and proliferation, but an important difference is in the ability to regulate levels of CTLA-4 expression. Our data indicate that CD40 signaling in T cells from NOD mice prevents normal upregulation of CTLA-4, suggesting that CD40 costimulation alters inhibitory signals following T cell activation. The CD40 blocking experiments also illustrate the importance of costimulation through CD40 for T cell pathogenicity.

In conclusion, we postulate that CD40 on NOD CD4 T cells contributes to T cell pathogenicity in T1D. In our hypothetical model (Fig. 7), CD40 is expressed on CD4 T cells as a result of chronic autoantigen activation and this in turn enables autoreactive T cells to signal through CD40. Signaling through CD40 thus provides effective costimulation for activation and proliferation of autoreactive T cells and alters normal T cell homeostasis by preventing upregulation of the co-inhibitory molecule CTLA-4. During activation by autoantigens, CD154 is expressed on T cells and interacts with CD40 present on Th1 effector T cells, providing synergistic signaling and increased reactivity of these T cells. Thus blocking costimulation through CD40 on autoreactive T cells alters activation induced through CD40–CD154 interaction and also leads to inhibition of pathogenicity.

5. Conflict of interest

The authors declare no financial or commercial conflict of interest.

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