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An Alternative Role for Foxp3 As an Effector T Cell Regulator Controlled through CD40

Gisela M. Vaitaitis,* Jessica R. Carter,* Dan M. Waid,* Michael H. Olmstead,* and David H. Wagner, Jr.*†

The BDC2.5 T cell clone is highly diabetogenic, but the transgenic mouse generated from that clone is surprisingly slow in diabetes development. Although defining pathogenic effector T cells in autoimmunity has been inconsistent, CD4+ cells expressing the CD40 receptor (Th40 cells) are highly diabetogenic in NOD mice, and NOD.BDC2.5.TCR.Tg mice possess large numbers of these cells. Given the importance of CD40 for pathogenic T cell development, BDC2.5.CD40−/− mice were created. Regulatory T cells, CD4+CD25highFoxp3+, develop normally, but pathogenic effector cells are severely reduced in number. Th40 cells from diabetic BDC2.5 mice rapidly induce diabetes in NOD.scid recipients, but Th40 cells from prediabetic mice transfer diabetes very slowly. Demonstrating an important paradigm shift, effector Th40 cells from prediabetic mice are Foxp3+. As mice age, moving to type 1 diabetes development, Th40 cells lose Foxp3. When Th40 cells that are Foxp3+ are transferred to NOD.scid recipients, disease is delayed. Th40 cells that are Foxp3− rapidly transfer disease. Th40 cells from BDC2.5.CD40−/− mice do not transfer disease nor do they lose Foxp3 expression. Mechanistically, Foxp3+ cells produce IL-17 but do not produce IFN-γ, whereas Foxp3− Th40 cells produce IFN-γ and IL-2. This poses a new consideration for the function of Foxp3, as directly impacting effector T cell function. The Journal of Immunology, 2013, 191: 717–725.

Autoimmune diseases are characterized by the emergence of autoaggressive cells leading to auto-Ab production and T cell–mediated aseptic inflammation. In the case of type 1 diabetes (TID), pathogenic effector T cells and other cells infiltrate pancreatic islet β cells (insulitis), leading to the loss of insulin production. An interesting aspect of this is the high degree of attack specificity confounded by the multiplicity of autoantigen responses. Immune cells must exist in homeostatic balance to prevent such outcomes, with tolerance ultimately keeping the system in check. T cell–tolerance mechanisms include energy due to inappropriate costimulation (1), TCR revision (2), activation-induced cell death (3), and regulatory T cell (Treg) control of pathogenic effector T cells (4). In autoimmunity, autoaggressive T cells (TAAAs) escape control, thus breaching tolerance mechanisms.

Clear definition of TAAAs has proven difficult and inconsistent; attempts to identify biomarkers have not always been successful. TAAAs typically are associated with a memory phenotype characterized by the rapid expression and subsequent downregulation of activation markers (e.g., CD69, CD25, and CD154). Activated human T cells express HLA-DR, with one study suggesting that HLA-DR+ and CD30 are diabetogenic T cell biomarkers (5). We defined a subset of effector T cells based on CD40 expression that proved to be highly diabetogenic in the NOD mouse model of T1D (6–10). Th40 cells produce proinflammatory cytokines and, interestingly, can produce IL-17A (Th17-defining cytokine) and IFN-γ (a Th1-defining cytokine) concurrently (11).

Th40 cell levels are predictive of diabetes onset and are highly pathogenic, as determined by passive disease-transfer experiments (6, 8–10). Given that Th40 cell numbers in spleen and pancreatic lymph nodes (PLNs) of NOD mice are significantly expanded and capable of disease transfer when isolated prior to diabetes onset, we performed a translational study to examine Th40 cells in diagnosed diabetic human subjects. In a blinded study, we correctly identified TID patients versus controls and, importantly, type 2 diabetes subjects using the Th40 identifier (12). New-onset (diagnosis < 2 wk) and long-term (diagnosis > 40 y) diabetic subjects maintain a significantly (p < 10−7) elevated percentage of Th40 T cells compared with controls (12). Additionally, CD40 expression occurs on naive and memory T cells, making CD40 expression irrelevant of activation status.

CD4+CD25high cells that express the forkhead box transcription factor Foxp3 are defined as Tregs (13). Tregs control TAAAs through cell contact and secretion of cytokines, including TGF-β and IL-10. Transfer of polyclonal NOD CD4+ T cells transduced with a Foxp3 retrovirus did not protect from diabetes, but transfer of BDC2.5 cells transduced with Foxp3 ameliorated disease for >100 d (14). Another Ag-specific (GAD protein) Foxp3-transduced T cell clone (15) did not protect from diabetes, suggesting an Ag-specificity requirement for Treg function. Foxp3 expression is required for Treg development (16) and functions as a transcriptional repressor and transcriptional activator (17). Major suppressor targets are cytokine genes, including IL-2 and IFN-γ (18), which are effector cell cytokines. Plasticity to Foxp3 expression was demonstrated; for example, it was shown that a Foxp3low and a Foxp3high population of cells exist (15). Interestingly, a subset of cells is Foxp3high RORγt+, with RORγt being the crucial transcription factor for IL-17 expression (15). Splenic Foxp3high RORγt+ cells express membrane TGF-β and CD62L, the latter targeting them to the pancreas (15). Importantly, these cells could function as Tregs but also could polarize to a Th17 effector cell phenotype (15).
The BDC2.5 T cell clone is highly diabetogenic, inducing rapid insulinitis and then hyperglycemia in NOD.scid-recipient mice (19), and it accelerates diabetes in young NOD recipients (20). Given the highly autoaggressive nature of this T cell clone, it was assumed that the TCR transgenic (TCR.Tg) mouse would be highly diabetogenic, when, in fact, it proved to be much less diabetes susceptible than the NOD mouse (21). Although typically 80% of NOD mice are diabetic by 20 wk of age, only 15 and 50% of BDC2.5.TCR.Tg mice are diabetic by 25 and 40 wk, respectively (21). BDC2.5.TCR.Tg mice generated on a RAG-knockout background experience rapid diabetes, with 100% incidence by 8 wk (21). Although BDC2.5 mice have T cells carrying a highly autoimmune TCR, Tregs are abundant in these mice (22). It was shown that a population of CD4+Foxp3+ cells occurs at a markedly increased frequency (22). Such expansions did not occur in NOD, BALB/c, or C57BL/6 mice. Furthermore, the suppressive function by classic Tregs remained intact through 20 wk of age (22).

Given all of the above considerations, we hypothesized in cells destined for effector function, acting as a temporal autoregulator. In this study, we confirm that BDC2.5 TCR.Tg mice have excessively delayed diabetes kinetics, but 100% of mice become diabetic given sufficient time. BDC2.5 mice on a CD40-knockout background do not become diabetic at all, and T cells isolated from those mice are incapable of transferring diabetes, even after extended time periods. T cells develop in BDC2.5.CD40−/− mice with fewer CD4+ cells and slightly more CD8+ cells. Classic Tregs in these mice are functionally intact, whereas pathogenic effector cells are drastically ablated. In BDC2.5 mice, Th40 cells express intracellular Foxp3 initially but lose it over time. Effector T cells from BDC2.5.CD40−/− mice do not lose Foxp3 intracellular expression and do not develop diabetes. Th40 cells isolated from younger, 6-week-old donor, or older, 22-week-old nondiabetic, BDC2.5 mice require longer time periods to transfer diabetes. Th40 cells isolated from diabetic BDC2.5 mice transfer diabetes very rapidly. In cases in which diabetes transfer is slower, a substantial portion of Th40 cells has Foxp3+, whereas rapidly disease-transferring Th40 cells are Foxp3−. When Th40 cells are recovered from diabetic recipient mice, the cells are all Foxp3−. Mechanistically, Foxp3− Th40 cells produce Th1 cytokines IFN-γ and IL-2, in addition to producing the Th17-definitive cytokine IL-17. Interestingly, Foxp3+ Th40 cells produce IL-17 but do not produce IFN-γ or IL-2. These data suggest a definitive role for IFN-γ in diabetes progression and support other reports that IL-17 is not instrumental in diabetes development in the BDC2.5 model (23). These data further suggest an alternative role for Foxp3 as suppressing effector T cell function and that suppressor function is eventually lost in CD40-competent mice but not in CD40-deficient mice.

Materials and Methods

Mice

NOD.BDC2.5 mice were purchased from The Jackson Laboratory, and NOD mice were from Taconic Laboratories. All mice were confirmed to be pathogen-free by University of Colorado Denver Veterinarian staff. NOD.BDC2.5.CD40−/− mice were generated using established speed congenic methods. NOD.CD154−/− mice were generated by breeding NOD mice to B6.CD154−/− mice through 10 generations. NOD.CD154−/− was confirmed by screening for the neo cassette insert. The CD40 knockout is a functional knockout, with an insertion in the exon 3 region interrupting the CD154 binding site (24). CD40 protein is still detected on cells. Experiments were conducted under University of Colorado Denver Aimschu1l Medical Campus–approved Institutional Animal Care and Use Committee protocols. Procedures to alleviate pain and suffering were done according to University of Colorado Denver Institutional Animal Care and Use Committee guidelines.

Diabetes incidence and histology

Mice were monitored for blood glucose by tail vein bleeds. Diabetes was considered blood glucose ≥ 250 mg/dl for three consecutive readings in a 1-wk period. Pancreata were taken from mice and embedded in paraffin. Sections were stained with H&E. Islets were scored on the following scale: 0 = no infiltrates; 1 = unipolar insulin; 2 = bipolar insulin; 3 = complete infiltration.

Abs, staining, and flow cytometry

Abs with directly conjugated flouors included anti-CD4 (clone H29.19), anti-CD8 (clone 53.6.7), anti-CD5, anti-CD8, anti-Foxp3, anti-CD44, anti-CD62L, anti-IFN-γ, anti–IL-17, and anti–IL-2, as well as isotype controls (eBioscience, San Diego, CA and Miltenyi Biotec, Auburn, CA). Anti-CD40 (hybridomas 1C10 and 4F11) and anti-CD25 (hybridoma PC6.5.3) were produced in-house. Intracellular staining for Foxp3 or cytokines was performed using eBioscience permeabilization/fixation buffers, as per the manufacturer’s instructions. Cells from PLNs, peripheral lymph nodes, or spleen were depleted using anti–MHC II magnetic beads and sorted on an autoMACS cell separator (Miltenyi Biotec). Cells were stained at 4°C, and FcR-blocking Ab 2.4G2 was added to each sample.

Cell sorting

Th40 cells were purified by sorting CD4+ cells, as previously described (7). Th40 cells were isolated by removing MHC-II+ cells, followed by CD40 selection using an autoMACS. In both cases, CD25+ cells were presorted to remove potential Tregs. In studies involving Tregs, CD40+ cells were removed prior to isolation of Tregs. Cells were cultured in DMEM with 10% FCS.

Adaptive transfers

Sorted Th40 cells from BDC2.5 or BDC2.5.CD40−/− mice were injected i.p. into NOD.scid-recipient mice at ≤3 wk of age. Foxp3 levels in donors were confirmed by flow cytometry and Western blots. BDC2.5 T cell clones were maintained, as described (25), and purified T cells were injected. Cells (5 × 106) were transferred in a single injection in all studies.

Results

We compared the kinetics of diabetes onset in NOD.BDC2.5 (BDC2.5 wild type), BDC2.5.CD40−/−, NOD, and NOD.CD154−/− mice. In our colony, 80% of female NOD mice become diabetic by 21 wk of age (Fig. 1A). BDC2.5 mice on a NOD background develop diabetes slowly, but with 100% penetrance over a prolonged period, in this case 48 wk (Fig. 1A). Histology of pancreata from diabetic BDC2.5 mice demonstrates extensive T cell infiltrates (Fig. 1B, 1E). The CD40/CD154 inflammatory dyad is tightly associated with autoimmunity (11, 26, 27), and blocking CD40/CD154 interaction prevents numerous autoimmune diseases, including TID (10, 28). We generated BDC2.5.TCR.Tg mice on a CD40−/− background (BDC2.5.CD40−/−), which completely prevented diabetes onset through 60 wk (Fig. 1A). Furthermore, in a CD40-deficient environment, pancreata do not develop infiltrates (Fig. 1C, 1E). CD40 expression on APCs directly impacts T cell development; therefore, we examined diabetes incidence in NOD.CD154−/− mice that have normal CD40 expression, including normal development of Th40 cells (data not shown). These mice also do not develop diabetes (Fig. 1A), and no islet infiltrates are detected (Fig. 1D, 1E). These data suggest that CD40 expression contributes to islet infiltration and is required for diabetes development.

We compared lymphocytes from pancreatic (draining) lymph nodes and peripheral (nondraining) lymph nodes between BDC2.5 and BDC2.5.CD40−/− mice. Although the BDC2.5 TCR mouse is I-A/I-β restricted, favoring CD4 T cell development, CD8 cells arise in those mice nonetheless (Fig 2A–C). In both draining and non-draining lymph nodes, substantially more CD4+ cells than CD8+ cells were detected in the CD40-deficient environment (Fig. 2A–C). There were significantly more CD4+ cells in peripheral lymph nodes and PLNs in BDC2.5 mice than in BDC2.5.CD40−/− mice, suggesting that CD40 promotes or sustains CD4 expansion in those.
mice. Interestingly, there were significantly more CD8+ cells in PLNs of BDC2.5.CD40−/− mice. A population of CD4+CD8+ cells was detected in the PLNs of BDC2.5 mice (Fig. 2A, 2D); this population was much less prominent in peripheral (non-draining) lymph nodes. In BDC2.5.CD40−/− mice, there were significantly fewer CD4+CD8+ cells (Fig. 2A, 2D).

**Figure 1.** Diabetes incidence in NOD.BDC2.5 and NOD.BDC2.5.CD40−/− mice. (A) Mice with blood glucose > 250 mg/dl for three consecutive readings over 7 d were considered diabetic. Mice included NOD mice (positive control, 40 mice total), NOD.BDC2.5 mice (24 total), NOD.BDC2.5, CD40−/− mice (24 total), and NOD.CD154−/− mice (20 total) that were monitored over 60 wk. Representative histology of pancreata from age-matched BDC2.5 mice (B), BDC2.5.CD40−/− mice (C), and NOD.CD154−/− mice (D). Original magnification ×10. (E) Histology scores of pancreata. Data represent >100 islets examined from each cohort. Scale is 0 = no insulitis, 1 = unipolar insulitis, 2 = bipolar insulitis, and 3 = complete infiltration. BDC2.5 scores are postdiabetes onset, and BDC2.5.CD40−/− and NOD.CD154−/− scores are at 45 wk of age. A one-way ANOVA analysis, using Graph Pad Prism, showed highly significant (p < 0.001) differences among BDC2.5, NOD, BDC2.5.CD40−/−, and NOD.CD154−/− curves.

**Figure 2.** CD4 and CD8 levels in lymph nodes of BDC2.5 and BDC2.5.CD40−/− mice. (A) Peripheral lymph nodes, including axial, brachial, inguinal, and submaxillary, and PLNs were excised from BDC2.5 and BDC2.5.CD40−/− mice at 12 wk of age. Single-cell suspensions were stained for CD4 versus CD8. Representative dot plots from eight mice from each cohort are shown. Gates were set from isotype controls (data not shown). Plots of differences in CD4 and CD8 percentages in peripheral lymph nodes (B) and PLNs (C) of BDC2.5 and BDC2.5.CD40−/− mice. (D) Plots of percentages of CD4+CD8+ double-positive cells in PLNs of BDC2.5 and BDC2.5.CD40−/− mice (n = 8 total). Statistics were determined by the unpaired Student t test using GraphPad Prism software.
CD40 and memory phenotype

Given that CD40 signals are critical for memory T cell development (29), we examined the effect of CD40 on memory versus naive CD4+ cells in this TCR.Tg model. BDC2.5 mice have a substantial proportion of effector memory cells (CD44+CD62L−) and central memory cells (CD44+CD62L+), with very few naive cells (CD44−CD62L+) (Fig. 3A). T cells from BDC2.5.CD40−/− mice have significantly more (p < 0.001) naive phenotype cells and a significantly reduced number of effector memory cells (Fig. 3B). The number of central memory cells (CD44+CD62L+) is unaltered compared with BDC2.5 mice (Fig. 3). Both congenic strains have identical self-reactive TCR molecules, demonstrating an important role for CD40 in memory T cell development, irrespective of TCR expression. It is true that APCs are affected by the lack of CD40 expression in BDC2.5.CD40−/− mice, yet the role of CD40 affecting memory T cell development in these TCR transgenic mice remains evident.

Pathogenic effector T cells

BDC2.5.TCR.Tg mice present a model in which potential Tregs and potential T AAs express the same TCR and, thus, respond to the same autoantigen. The CD40-knockout mouse has a neocassette insertion in exon 3 of CD40 that contains the CD154 binding site and, thus, is a “functional” knockout (24); therefore, the CD40 protein can be expressed but should not receive CD154-mediated signals. BDC2.5 mice have a substantial Th40 cell population in PLNs (Fig. 4A). BDC2.5.CD40−/− mice have Th40 cells, but at significantly reduced numbers (p < 0.0001) (Fig. 4B). A CD4lo and CD4hi population is detectable within the CD4 population (Fig. 4B). In previous work, we showed that phenotypically CD4hi cells have as much CD4 as do phenotypically CD4lo cells when examined by Western blot (7). We compared levels of Th40 cells in PLNs and spleens of mice at 6 and 21 wk of age (Fig. 4C, 4D). In BDC2.5 mice, there was a significant increase in Th40 cells in PLNs and spleen at 21 wk of age compared with 6 wk of age (Fig. 4C). In the BDC2.5.CD40−/− mice, levels of Th40 cells remained contained (Fig. 4D) and were significantly (p < 0.00001) lower than in wild-type mice (compare Fig. 4D and 4C).

Tregs and CD40

BDC2.5 mice eventually breach tolerance to develop diabetes (Fig. 1). Previous studies described a population of CD4+ CD25+Foxp3+CD69− T cells in BDC2.5 mice that could be regulatory (30). Because of the delayed kinetics in diabetes development in BDC2.5 mice and the lack of diabetes development in BDC2.5.CD40−/− mice, we inspected Tregs in each of these mice over time. We examined CD4+Foxp3+ cells in PLNs and spleens at 6 and 21 wk of age. In BDC2.5 mice there were significantly more Tregs in spleen than in PLNs at 6 wk of age (p < 0.001) (Fig. 5A). At 21 wk of age, there was a significant increase...
in Tregs \((p = 0.0019)\) in PLNs, with no significant change in spleens \((p = 0.0019)\) in BDC2.5.CD40 \(^{-/-}\) mice at 6 and 21 wk of age. Cells were stained for CD4 and CD40, with gates set from isotype controls. Representative dot plots from BDC2.5 \((A)\) and BDC2.5.CD40 \(^{-/-}\) \((B)\) mice are shown. \((C)\) The percentage of Th40 cells in PLNs and spleens from nine individual mice of age-matched BDC2.5 and BDC2.5.CD40 \(^{-/-}\) mice \((n = 8\) total). Statistics were determined by unpaired ANOVA using GraphPad Prism software.

**Foxp3 expression in pathogenic effector cells**

Given that Treg numbers are normal in BDC2.5.TCR.Tg mice and because a substantial population of CD4 cells in BDC2.5 and BDC2.5.CD40 \(^{-/-}\) mice is Foxp3\(^+\) but only a portion of those cells is CD25\(^+\), we examined Foxp3 expression within Th40 cells over time as BDC2.5 mice develop diabetes. In young BDC2.5 mice, a subset \((36.6\%)\) of Th40 cells expresses low levels of Foxp3 (Fig. 6A). As mice age to 21 wk, the level of Foxp3\(^+\) cells within the Th40 population and in CD4\(^+\)CD25\(^+\) cells increases substantially (Fig. 6A–C). Furthermore, the expression level of Foxp3 within cells also increases, as measured by mean fluorescent intensity. Once diabetes begins, both the percentage of Foxp3\(^+\) cells and the cellular expression level of Foxp3 diminish (Fig. 6A, 6C) in Th40 cells. By comparison, Foxp3 levels within classic Tregs does not diminish over the same time period (Fig. 6C).

We considered that Foxp3 expression in effector cells, independent of Tregs, affects diabetogenicity. We sorted Th40 cells from prediabetic BDC2.5 mice, with 57\% of the cells being Foxp3\(^+\) (Fig. 7A). Cells were adoptively transferred to NOD.scid recipients that were monitored for disease (Fig. 7B). At diabetes onset, T cells were extracted from spleens of recipient mice that received Foxp3\(^+\) BDC2.5 cells and examined for CD40 and Foxp3 expression. Foxp3 expression in Th40 cells was undetectable (Fig. 7C). Cells from BDC2.5.CD40 \(^{-/-}\) mice did not transfer disease through 60 wk and, importantly, did not lose Foxp3 expression in vivo.

**Foxp3\(^+\) cells in the BDC2.5 diabetes model produce IL-17, but not Th1, cytokines**

Diabetogenesis is driven predominantly by Th1 cytokines; in fact, a substantial role for IFN-\(\gamma\) as the major contributor to disease is suggested (23, 31). Th17 cells produce IL-17 and IL-22; although they have been associated with autoimmunity, their role in T1D is disputed (23). Th17-polarized BDC2.5 cells transfer diabetes to NOD.scid-recipient mice but convert to Th1, producing IFN-\(\gamma\) (23). We previously showed that Th40 cells are capable of producing IFN-\(\gamma\) and IL-17; in fact, they can express both cytokines concomitantly (11). Interestingly, Th40 cells that are Foxp3\(^+\) express ROR\(\gamma\), the transcription factor responsible for IL-17 production (Fig. 8A). Foxp3\(^+\) Th40 cells from BDC2.5 mice and CD4\(^+\) T cells from BDC2.5.CD40 \(^{-/-}\) mice express IL-17 but do not express IFN-\(\gamma\) or IL-2 (Fig. 8B). IL-17 production in CD40-
deficient conditions is significantly \((p < 0.001)\) greater than in BDC2.5 mice. In contrast, Foxp3\(^2\) cells express all three cytokines. Foxp3\(^2\) cells from BDC2.5 mice that are CD40 sufficient express significantly \((p = 0.0003)\) more IFN-\(\gamma\). IL-2 production is not significantly \((p = 0.2586)\) different in BDC2.5 or CD40-deficient conditions (Fig. 8C). As in the case with Foxp3\(^+\) cells, Foxp3\(^2\) cells from CD40-deficient conditions produce significantly \((p < 0.0001)\) more IL-17 (Fig. 8C).

Discussion

A significant role for CD40 in autoimmunity is clear. The role for CD40 on T cells is more controversial, although numerous studies describing CD40-expressing T cells now exist (6–12, 32–34). Understanding the role of Th40 cells is important to understanding how CD40 affects the immune system and the critical role that it plays in autoimmunity. CD40 engagement was first described on B cells and was reported as essential for Ab class-switching (35). Expression of CD40 on macrophages was described later, where engagement of CD40 induced proinflammatory cytokines, including TNF-\(\alpha\), IL-1, and IL-6 (36). On T cells, CD40 serves to induce prosurvival molecules, including Bcl-X\(_L\) and c-FLIP (7), and, importantly, CD40 serves as a functional costimulus, even eclipsing CD28 for proinflammatory cytokine production (37, 38). An interesting aspect is that Th40 cells can simultaneously produce IFN-\(\gamma\), a Th1 cytokine, and IL-17A, the definitive Th17 cytokine (11). This suggests an overlooked link between Th1 and Th17 cells relative to their autoaggressive potential.

The BDC2.5 T cell clone and subsequent TCR.Tg mouse have proved useful in understanding diabetogenesis. The BDC2.5 TCR recognizes chromogranin A (39), and the clone is highly diabetogenic, whereas the TCR.Tg mouse has surprisingly slow disease kinetics. However, given sufficient time, BDC2.5 mice become diabetic in a CD40-dependent process. BDC2.5 mice that do not express CD40 do not become diabetic, but because CD40 is expressed on APCs the precise cellular contribution remains unknown. An important observation made in this study is that T cells isolated from BDC2.5.CD40\(^-\) mice do not transfer disease to a background in which APCs are CD40 sufficient. This is true even over a long time period, demonstrating that CD40 expression directly on T cells is involved in breach of tolerance. In addition, only a small portion of T cells from BDC2.5.CD40\(^-\) mice progresses to memory phenotype. These data suggest that a CD40-
competent background is required for pathogenic/autoaggressive T cell development. Furthermore, by doing these experiments in the BDC2.5 system, in which a highly pathogenic TCR is expressed, it can be concluded that factors intrinsic to T cell development, independent of TCR expression, contribute to pathogenesis of T cells, and these factors are directly influenced by CD40.

It was shown that Tregs develop normally in BDC2.5 mice (22). It was shown in the NOD model of T1D that Treg numbers are normal early, but their qualitative function diminishes as mice develop diabetes (40). However, in those studies, “pathogenic” effector cells were not removed prior to isolation of CD25+ Tregs. CD25 is activation induced and, thus, the true Treg population was qualitatively underestimated. When CD40+ T cells are removed...
and then CD25+ cells are counted or isolated for functional assays, the numbers of Tregs and the function of Tregs in NOD mice are identical to nonautoimmune BALB/c mice (9). Furthermore, different subsets of Tregs were reported within the CD4+ population, including CD25+, CD62L-, and CD45RB+ (41). Depletion of each subset separately resulted in different autoimmune outcomes in NOD mice; however, only CD4+CD25+ cells were capable of suppressing effector T cell proliferation (41). In this article, we further report that, although pathogenic effector T cells require a CD40-dependent background for development, Tregs do not.

Given the finding that BDC2.5 mice eventually develop diabetes with 100% penetrance, but do so with notably longer kinetics, we suspected a temporal peripheral tolerance dysfunction independent of Tregs. As stated, Tregs appear normal in diabetic mice; however, only CD4+CD25+ cells were capable of suppressing effector T cell proliferation (41). In this article, we further report that, although pathogenic effector T cells require a CD40-dependent background for development, Tregs do not.

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