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Defective positive selection results in T cell lymphopenia and increased autoimmune diabetes in ADAP deficient BDC2.5-BI/6 mice

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Abstract

Adhesion and Degranulation Promoting Adapter protein (ADAP), a positive regulator of T cell receptor (TCR) signaling, is required for thymocyte development and T cell homeostasis. To investigate the role of ADAP in a T cell-driven autoimmune response, we generated ADAP-deficient, BDC2.5 TCR transgenic, diabetes-prone (C57Bl/6) mice (BDC/B6). We observed a striking enhancement of diabetes incidence in ADAP-deficient mice, both in animals homozygous for I-A^{g7}, and in mice carrying one I-A^b allele (BDC/B6^{g7/b}). Increased disease correlates with significantly reduced numbers of pathologic CD4⁺ T cells in the mice. Consistent with a state of functional lymphopenia in ADAP-deficient BDC/B6^{g7/b} mice, T cells display increased homeostatic proliferation. Transfer of syngeneic lymphocytes or T cells both blocks ADAP-dependent diabetes and relieves exaggerated homeostatic T cell proliferation observed in ADAP-deficient mice. Marked attenuation in cellularity of the CD4⁺ single-positive (SP) thymocyte compartment in ADAP-deficient BDC/B6^{g7/b} animals suggests a mechanism for induction of the lymphopenia. We conclude that inefficient positive selection in ADAP deficiency results in lymphopenia that leads to enhanced autoimmune diabetes in the BDC/B6^{g7/b} model. Our findings support the notion that ineffective thymic T cell output can be a powerful causative factor in lymphopenia-driven autoimmune diabetes.

Keywords

ADAP; Autoimmune Diabetes; BDC2.5; Lymphopenia; Thymocyte development

INTRODUCTION

Type 1 diabetes (T1D) develops due to an organ-specific autoimmune reaction mediated by T lymphocytes responsive to β -islet cell self-antigen(s) [1,2]. In part, failure to maintain self-tolerance in T1D may be ascribed to dysregulation of peripheral T cell homeostasis. Alterations in both the quality (reduced repertoire diversity) and the quantity (T cell lymphopenia) of the T cell compartment have been causally linked with increased diabetes incidence in animals [3-5]. In non-obese diabetic (NOD) mice, T cell lymphopenia has been strongly implicated in diabetes development [6]. A prevailing model of T lymphocyte homeostasis holds that in a lymphopenic animal, decreased competition for growth-

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regulating resources and for antigen/Major Histocompatibility Complex (MHC)[7] results in enhanced proliferation of the remaining cells. Selective expansion and activation of autoreactive clones induced by T cell lymphopenia may result in augmented destructive T cell potential at the site of autoimmune attack and exacerbation of disease [8].

Causes of lymphopenia may include the actions of environmental toxins (e.g. drugs, irradiation) and impaired regulation of T cell homeostasis determined by either decreased T cell survival or T cell production [8]. A causal link between increased turnover and susceptibility of autoreactive NOD T cells to apoptosis and lymphopenia-induced diabetes was recently established [6]. Ablation of thymic output by thymectomy results in lymphopenia associated with exaggerated diabetes incidence [9,10]. However, a causal relationship between abnormalities in T cell receptor-dependent positive selection of autoreactive T cells and lymphopenia leading to disease has not been demonstrated.

ADAP (previously designated SLAP-130 or Fyb) is a recently-characterized positive regulator of both thymocyte development and of TCR signaling[11,12]. ADAP is a cytoplasmic adapter protein with expression limited to hematopoietic cells [13,14]. Loss of ADAP results in impaired positive and negative thymocyte selection, as well as inefficient population of the peripheral lymphoid organs[15-17]. However, the role of ADAP in regulating autoimmune responses *in vivo* has not been characterized. We set out to determine whether defects in thymic selection and/or T cell homeostasis engendered by ADAP deficiency could modulate the course of T1D.

We bred ADAP-deficient mice to BDC2.5 (on the C57Bl/6 background and congenic for MHC H-2^{g7}) TCR transgenic mice (hereafter designated “BDC/B6”) [18], a well-characterized model for T1D[18]. Disease features in BDC/B6 mice closely mimic those observed in humans and NOD mice. TCR transgenic, CD4⁺ T cells bearing a rearranged islet-antigen specific TCR (V β 4⁺) are both required and sufficient to cause highly-penetrant and synchronized disease in BDC2.5 mice. However, development of frank hyperglycemia in BDC2.5 mice occurs within weeks—as opposed to the delay of many months observed in the NOD strain—allowing relatively rapid evaluation of genetic influences on the autoimmune process [19]. BDC/B6 heterozygous for MHC II molecule H-2 ^{g7/b} are protected from the disease because of the influence of non-diabetogenic clones selected by H-2b molecules (3).

We observed that ADAP deficiency results in a dramatically higher incidence of diabetes in both diabetes prone (BDC/B6 g7/g7) and resistant (BDC/B6 g7/b) mice. Further, we found that the enhanced disease correlates with both decreased numbers of diabetogenic T cells in the periphery and with augmented T cell homeostatic proliferation in ADAP-deficient BDC/B6 mice. Upon intravenous transfer of syngeneic leukocytes, ADAP^{-/-} mice are protected from disease, strongly suggesting a causal influence of lymphopenia on the enhanced rate of diabetes. Pre-diabetic ADAP-deficient BDC/B6 mice display markedly reduced numbers of mature CD4⁺ SP thymocytes. Our data reveal that loss of ADAP results in a defect in peripheral T cell homeostasis that is causally linked to enhanced autoimmune diabetes in the BDC2.5 model. Further, our findings strongly suggest that ADAP promotes efficient thymocyte positive selection and thymic output, processes important for support of peripheral T cell numbers in BDC2.5 mice.

RESULTS

ADAP-deficient BDC2.5 mice display increased incidence of autoimmune diabetes

To evaluate the function of ADAP in T-cell dependent autoimmune disease, we crossed ADAP-deficient mice with diabetes-prone BDC2.5 (C57Bl/6 background) TCR Tg mice

[18]. Animals homozygous for I-A^{g7} (BDC/B6^{g7/g7}), the Class II MHC selecting element for the diabetogenic TCR, were monitored for diabetes from age 4 to 20 weeks. As previously reported [3], we observed that wild type BDC/B6^{g7/g7} mice develop diabetes between 4 and 10 weeks of age with an incidence of 60% (Fig. 1). Greater numbers of ADAP-deficient BDC/B6^{g7/g7} mice (94%) become diabetic over the same age range. We observed an even greater difference between diabetes incidence rates for control (26%) and ADAP-deficient mice (78%) when we examined BDC2.5 transgenic mice heterozygous for I-A^b and I-A^{g7} (BDC/B6^{g7/b}). Expression of I-A^b exerts a protective effect against disease in wild-type BDC2.5 mice [3], but this effect was ablated by ADAP deficiency. Taken together, the diabetes incidence data indicate that ADAP-deficient BDC/B6 mice exhibit enhanced incidence of autoimmune diabetes.

To search for a mechanism of enhanced diabetes resulting from ADAP deficiency, we elected to focus further investigation upon the BDC/B6^{g7/b} mice, given the wide disease incidence gap between ADAP-deficient and control animals of this strain. Prior to development of overt hyperglycemia, BDC2.5 mice display progressive pancreatic islet lymphocyte infiltration [19]. To determine whether ADAP deficiency regulates islet immune cell infiltration, we evaluated hematoxylin and eosin-stained pancreata [4,20] from age-matched, 5.5 week-old control and ADAP-deficient, prediabetic BDC/B6^{g7/b} mice (Fig. 1C). We found no significant difference in the fraction of ADAP-deficient islets exhibiting either non-aggressive (“level 1”; $p = 0.15$) or aggressive (“level 2”; $p = 0.27$) insulitis. These findings suggested that the difference in diabetes incidence between control and ADAP-deficient BDC/B6^{g7/b} mice could not be explained by altered kinetics of islet infiltration by inflammatory cells.

ADAP deficient BDC/B6^{g7/b} mice show reduced diabetogenic T cell numbers

Disordered T lymphocyte homeostasis resulting in lymphopenia has been causally linked to autoimmunity [8]. ADAP-deficient lymph node and spleen display modestly decreased T cell numbers in non-transgenic mice [16,17]. To test whether ADAP deficiency results in altered T cell homeostasis in BDC/B6^{g7/b} mice, we enumerated peripheral T cells expressing TCRV β 4 and CD4, markers expressed by diabetogenic, transgenic cells [3]. In both draining (pancreatic, P-LN) and non-draining (inguinal/axillary, N-LN) lymph nodes, as well as in spleen, we observed a 50–66% reduction in the percentages (Fig. 2A) and numbers (Fig. 2B) of V β 4⁺CD4⁺ T cells. Total cellularity of spleen and lymph nodes was not significantly affected by ADAP deficiency. However, total T cell (combined CD4⁺ and CD8⁺ cells; wild-type = $12 \times 10^6 \pm 2$ versus ADAP-deficient = $5.8 \times 10^6 \pm 1.2$; $p < 0.01$) and total CD4⁺ cell numbers (wild-type = $11 \times 10^6 \pm 1.6$ versus ADAP-deficient = $4.4 \times 10^6 \pm 0.9$; $p < 0.01$) in the lymph nodes are also significantly decreased in the ADAP-deficient animals. We concluded from these data that enhanced diabetes incidence is associated with reduction in the numbers of CD4⁺ diabetogenic T cells in ADAP-deficient, BDC/B6^{g7/b} peripheral lymphoid tissues.

ADAP is dispensable for development and in vivo regulatory function of Tregs

Thymus-derived regulatory T cells (CD4⁺foxP3⁺; Tregs) play an essential disease-suppressive role in the BDC2.5 model [21]. As ADAP is required for efficient selection of CD4⁺ T cells [15], we asked whether loss of ADAP regulates Treg number or function in BDC/B6^{g7/b} mice. We observed increased (approximately 2-fold) fractions of the CD4⁺ T cell subset expressing Foxp3 (Fig. 3A) and CD25 (data not shown) in spleen and lymph nodes from pre-diabetic, 5.5 week-old, ADAP-deficient BDC/B6^{g7/b} mice. However, absolute numbers of Tregs are not significantly altered by ADAP deficiency (data not shown). These observations strongly suggest that the enhanced diabetes incidence in ADAP-deficient mice cannot be explained by altered development of Tregs.

To evaluate cell-intrinsic Treg function *in vivo*, we co-transferred purified CD25⁺ BDC/B6^{g7/b} splenocytes (Tregs) in combination with CD25^{lo} BDC/B6^{g7/b} (effector) T cells into NOD/SCID mice and monitored recipients for diabetes [22]. We observed that co-transfer of Tregs derived from control mice results in cell dose-dependent protection of recipients from effector-induced diabetes for up to 30 days (Fig. 3B). ADAP-null Tregs show a non-significant trend toward enhancement of disease-suppressive capacity when co-transferred in low numbers. ADAP-deficient Tregs also mediate suppression of TCR-stimulated effector proliferation *in vitro* at wild type levels (data not shown). These data strongly suggest that the increased diabetes in ADAP-deficient mice is not caused by defects in BDC/B6^{g7/b} Treg development or function.

Lymphopenia associated with loss of ADAP promotes homeostatic proliferation

Current models of lymphoid homeostasis hold that T cells in a lymphopenic environment display increased propensity to proliferate due to decreased interclonal competition for finite antigen/MHC and cytokine resources [23]. Given the CD4⁺ T cell lymphopenia in ADAP-deficient BDC/B6^{g7/b} mice, we hypothesized that ADAP-deficient animals would support enhanced homeostatic T cell expansion. To test this idea, we injected CFSE-labeled, congenic (CD45.1⁺), non-transgenic "reporter" lymphocytes into wild type or ADAP-null (CD45.2⁺) BDC/B6^{g7/b} mice. Transferred lymphocytes, distinguished by congenic marker expression, were recovered from inguinal lymph nodes and spleen 4 days after injection. We observed that significantly higher percentages of reporter CD4⁺ and CD8⁺ T cells recovered from ADAP-deficient recipients show evidence of CFSE dilution (indicating cell division) compared to cells from control BDC/B6^{g7/b} recipients (Fig. 4A). These data indicate that diminished T cell numbers correlate with the presence of an ADAP-deficient BDC/B6^{g7/b} tissue environment that supports enhanced T cell proliferation.

To examine basal proliferation of endogenous, autoreactive T cells, we fed BrdU to pre-diabetic BDC/B6^{g7/b} mice. FACS analysis revealed BrdU uptake in a greater percentage of splenic and lymph node CD4⁺Vβ4⁺ cells from ADAP-deficient animals than in cells from wild type mice (Fig. 4B). Increased T cell "turnover" was also suggested by the finding, in pre-diabetic mice, of higher proportions of ADAP-deficient, CD4⁺Vβ4⁺ lymph node T cells that express the "memory" and activation markers CD44 [6] and CD69 (data not shown). Together, these data strongly suggest that ADAP-deficient BDC/B6^{g7/b} mice support enhanced homeostatic T cell proliferation.

Syngeneic lymphocytes rescue ADAP-deficient BDC/B6^{g7/b} mice from diabetes and block endogenous T cell proliferation

T cell lymphopenia can be relieved, and diabetes prevented, by transfer of syngeneic splenocytes and T cells into NOD mice [6]. We used a similar lymphocyte replacement approach to investigate whether lymphopenia plays a causal role in ADAP-dependent diabetes. We transferred ADAP-deficient BDC/B6^{g7/b} leukocytes into 4-5 week-old, pre-diabetic syngeneic recipients. We studied the effect of a "replacement" cell dose (240×10^6 splenocytes) calculated to restore total numbers of CD4⁺Vβ4⁺ T cells in prediabetic, ADAP-deficient mice to levels observed in age-matched controls (not shown). A dosage of 80×10^6 cells was chosen for an alternative "low dose" cell infusion.

We observed that transfer of a low dose of syngeneic leukocytes into ADAP-deficient, transgenic BDC/B6^{g7/b} mice results in delayed onset and partial prevention of disease (Fig. 5A). Strikingly, replacement dose leukocyte transfer reduces diabetes incidence in ADAP-deficient mice to the rate observed in wild-type animals. Transfer of purified splenic T cells results in a similar dramatic reduction in diabetes incidence (Fig. 5A), strongly suggesting that the protective effects of cell transfer are determined by the T cell compartment. Taken

together, these data suggest that reconstitution of T cell lymphopenic BDC/B6^{g7/b} mice with either unfractionated leukocytes or with purified T cells is sufficient to protect animals from ADAP-dependent enhanced diabetes.

To investigate the mechanism of protection from disease conferred by leukocyte transfers, we examined basal T cell proliferation in transfer recipients. 7 days after replacement dose transfer as in Fig. 5A, Brdu was fed to transfer recipients. Spleen and lymph node cells were then analyzed for Brdu uptake. We observed that after transfer of syngeneic cells, the percentage of Brdu⁺ cells in ADAP-deficient recipients is reduced to levels observed in unmanipulated BDC/B6 wild-type mice (Fig. 5B). We concluded that disease-preventing transfer of syngeneic leukocytes also reduces CD4⁺ T cell homeostatic expansion in ADAP-deficient BDC/B6^{g7/b} mice.

ADAP is required for efficient positive selection of diabetogenic T cells, but is dispensable for their survival

The T cell lymphopenia observed in ADAP-deficient BDC/B6^{g7/b} mice might be caused by decreased thymic output or by reduced T cell survival in the periphery. To address the first possibility, we counted thymocytes. While overall thymic cellularity of young, pre-diabetic BDC/B6^{g7/b} mice is unaffected by ADAP deficiency, we found a marked reduction in both the percentage and absolute numbers of ADAP-deficient CD4⁺ SP thymocytes (6A). Similar impaired production of CD4 SP thymocytes has also been noted in other ADAP-deficient TCR transgenic mice [15]. The deficit in ADAP-deficient CD4⁺ SP correlates with increased numbers of CD4⁺CD8⁺ double positive (DP) thymocytes (not shown). These data suggest a developmental block in ADAP-deficient BDC/B6^{g7/b} mice at the DP to SP transition, a stage that correlates with positive selection of CD4⁺ thymocytes prior to migration into peripheral tissues.

We next addressed the possibility that altered survival capacity of ADAP-deficient BDC2.5/B6 T cells contributes to lymphopenia (Fig. 6). Mixtures of congenically-marked control or ADAP-deficient CD4⁺Vββ⁺ T cells (harvested from pre-diabetic BDC/B6^{g7/b} donors) were transferred into non-transgenic hosts (see “Input” percentages, left hand panel, Fig. 6B). We then determined the contributions of either control or ADAP-deficient T cells to the donor T cell population in peripheral lymphoid organs after 9 days. We found no difference between control and ADAP-deficient T cells in their abilities to survive (Fig. 6B) or to proliferate (Fig. 6C) in either non-draining or draining lymph nodes. Together, these data strongly suggest that a thymic production deficit, and not reduced T cell survival, is the underlying cause of T cell lymphopenia in ADAP-deficient BDC/B6^{g7/b} mice.

DISCUSSION

Results presented here corroborate recent findings that lymphopenia is a causative influence in T-cell dependent diabetes [6]. In ADAP-deficient BDC/B6^{g7/b} mice, reduced numbers of diabetogenic T cells and increased homeostatic proliferation of both transferred and endogenous T cells suggest the presence of functional lymphopenia. That such lymphopenia is causally linked to disease is strongly suggested by the finding that simple enhancement of peripheral T cell numbers normalizes both increased diabetes incidence and T cell turnover in ADAP-deficient BDC/B6^{g7/b} mice. Our data implicate a marked defect in thymic output of CD4⁺ T cells as the source of lymphopenia in ADAP-deficient mice.

T lymphocyte homeostatic proliferation is critical to restoration of host immune competence [23]. However, in the genetically-predisposed host, the lymphopenia-driven lymphocyte expansion may translate into destructive autoimmune responses [8]. For example, recent work showed that transferred, islet antigen-specific T cells are normally tolerized in NOD

recipients. However, the same cells proliferate and cause severe disease when transferred into NOD/SCID lymphopenic recipients [24]. Furthermore, transfer of diabetogenic leukocytes into modestly-lymphopenic NOD recipients protects mice from disease, suggesting that relief of lymphopenia can restrain the diabetogenic potential of T cells [6].

A key novel finding arising from our studies is that defects in thymic auto-reactive cell productive capacity can result in T1D-inducing lymphopenia. Others have shown that total ablation of thymic output through thymectomy accelerates murine diabetes [9,10]. However, for these studies, numerous thymus-dependent populations known to regulate the course of diabetes were removed by experimental manipulation. For example, numbers of CD4⁺CD25⁺ Treg cells, recently shown capable of suppressing diabetes in the BDC2.5 model [21], are reduced in thymectomized animals [25]. Others have shown disease regulatory properties for thymus-derived NK-T cells and CD8 α intraepithelial lymphocytes in T1D [9,26]. However, we found no differences between control and ADAP-deficient BDC/B6 lymphoid tissues in Tregs, in numbers of NK-T cells (as detected by CD1d tetramers) or of CD8 α -expressing IEL (data not shown). Finally, enhanced usage of alternative (non-transgenic) TCR α chains by transgenic V β 4⁺ expressing cells has been associated with reduced diabetes in BDC2.5^{g7/b}/B1/6 mice [3]. Surprisingly, we observed a modest increase in the percentage of peripheral CD4⁺ T cells expressing alternative TCR α in ADAP-deficient BDC/B6^{g7/b} mice (not shown). There is no literature precedent to support the notion that such alternative TCR α -bearing cells could account for enhanced diabetes, however. Thus, the T cell lymphopenia driving enhanced diabetes in ADAP-deficient BDC/B6 mice is likely not attributable to loss of important regulatory populations.

Although Treg defects do not account for enhanced diabetes in ADAP-deficient BDC/B6 mice (Fig. 3), Tregs may be a contributing factor in the observed amelioration of ADAP-dependent diabetes by syngeneic leukocyte transfer (Fig. 5). Recent studies have suggested that Tregs can control both CD4⁺ homeostatic proliferation and disease expression in transfer models of autoimmunity [27,28]. Further, Herman et al. showed that co-transfer of CD25⁺ Treg could block BDC2.5 T cell-transfer induced diabetes in NOD-Scid recipients in a dose-dependent manner [21]. However, unlike Herman et al., we have transferred Treg (in the context of leukocytes or T cells) into ADAP-deficient mice harboring an already-intact, homeostatically-proliferating, autoreactive repertoire and displaying pancreatic infiltration. Because we transferred ADAP-deficient BDC/B6 leukocytes cells, the ratio of Treg to effector T cells in the recipients was not changed. Thus, if Tregs are playing a role in disease suppression by transferred ADAP-deficient cells, enhanced Treg absolute number in recipient mice is likely the major determinant of their effect. Quantification of the role of Tregs in suppression of disease and/or suppression of homeostatic expansion in our model must await completion of experiments achieving Treg depletion by physical or genetic means.

Severity of the T cell lymphopenia in ADAP-deficient BDC/B6^{g7/b} mice is dependent upon expression of the BDC2.5 TCR transgene. Indeed, thymi from ADAP-deficient H-Y, DO11.10 and AND TCR transgenic mice all display reduced numbers of SP thymocytes, similar to observations in the BDC2.5 mice [15]. However, non-transgenic, ADAP-deficient mice show only mild deficits in peripheral CD3⁺ and CD4⁺ lymphocyte numbers [16,17], and do not support increased spontaneous proliferation of transferred reporter T cells (not shown). The possibility that ADAP regulates development of T cell-dependent, but TCR transgene-independent, autoimmune responses such as diabetes (in the NOD model) or systemic lupus (exemplified by MRL/*lpr* mice) remains under study.

The molecular mechanism whereby ADAP regulates positive selection of CD4 SP thymocytes remains incompletely understood. ADAP is a known modulator of TCR

signaling, which is critical for efficient thymocyte selection [29]. ADAP is dispensable for TCR-mediated ERK activation and calcium flux in thymocytes [15]. However, through association with the adapter SKAP55, ADAP regulates Rap1-dependent TCR-stimulated integrin activation and thymocyte conjugate formation [30,31]. Recent work has also established a requirement for ADAP in TCR-dependent formation of a CARMA-1-containing complex and subsequent activation of the NF- κ B pathway [32]. Experiments to determine whether immature thymocytes also require ADAP to activate NF- κ B upon receipt of a selecting TCR stimulus are underway.

MATERIALS AND METHODS

Mice and diabetes diagnosis

BDC2.5 TCR transgenic (C57Bl/6) mice were generous gifts from D. Mathis and C. Benoist, Harvard University. ADAP-deficient mice (x7 cross to C57Bl/6) [16] were interbred with BDC2.5 mice and housed in conventional facilities. MHC haplotype was determined by blood cell expression of I-A^b and I-A^{g7}. NOD/SCID mice were purchased from the Jackson laboratory. Detection of glycosuria with Diastix strips (Bayer Corporation, Elkhart, IN) during weekly screening was confirmed by blood glucose measurement (glucometer from Ascensia Elite XL, Bayer). Diabetes was defined by the occurrence of two consecutive weekly blood glucose measurements greater than 200mg/dl. Animal work was done in compliance with regulations of the IACUC at the University of Minnesota.

Histology

Pancreata isolated from 5.5 week-old, prediabetic mice were fixed in 10% formaldehyde, paraffin-embedded, and sectioned for hematoxylin and eosin staining per established protocol [4]. At least 100 β -islets from each pancreas were categorized as “aggressive”, “non-aggressive”, “peri-insulitis”, or “no infiltration” in a blinded fashion by a single trained observer (N. O-M.) using light microscopy [20].

Antibodies, Flow cytometry

CD44-FITC (IM7), CD45.2-FITC (104), Foxp3-PE (FJK-16s), V α 2-FITC (B20.1) CD4-APC (GK1.5), CD45.1-APC (A20), and CD25-biotin (PC61) were purchased from eBioscience (San Diego, CA). CD8b.2-FITC (Ly-3.2), I-A^k-FITC (A β k 10-3.6), V β 4-PE (KT4), and I-A^b-PE (AF60120.1) were purchased from BD PharMingen (San Diego, CA). Cells were stained in FACS buffer (2% FBS in PBS with FcBlock (2.4G2)) and subjected to flow cytometric analysis using a Becton-Dickinson FACScaliber.

T cell purification

$\sim 100 \times 10^6$ unfractionated splenocytes and lymph node cells were negatively selected after incubation with FITC-conjugated mAb to B220, CD11b, and NK1.1 (1.5 μ g/ 10×10^6 cells). After washing twice, cells were resuspended in 500 μ l FACS buffer containing BioMag anti-FITC conjugated magnetic beads (Polyscience Inc. Warrington, PA) and rocked at RT for 30 minutes. Magnetic depletion then yielded >90% pure CD3⁺ T cells.

T cell proliferation and survival assays

To measure endogenous DNA synthesis, mice were fed Brdu (Sigma, St. Louis, MO) dissolved in drinking water (0.8 mg/mL). Intracellular staining for Brdu in harvested lymphocytes was performed according to kit manufacturer protocol (BD PharMingen). To measure proliferation of adoptively transferred cells, splenocytes were labeled with CFSE (5 μ M in PBS) for 10 minutes at 37 °C. 3×10^6 CFSE-labeled cells were transferred via tail vein. Proliferation was determined by FACS-detected CFSE dilution amongst transferred

cells. To examine T cell survival, pooled, RBC-depleted “reporter” splenocytes and lymph node cells from wild-type CD45.1⁺ BDC/B6^{g7/b} mice were labeled with CFSE. 10×10^6 labeled cells were injected into CD45.2⁺, non-transgenic recipients. After 4 days, lymph node cells were recovered for FACS analysis.

Diabetes and lymphopenia suppression by leukocyte transfer

Pooled, ADAP^{-/-} BDC/B6^{g7/b} splenocytes and lymph node T cells were transferred into 4-5 week-old ADAP^{-/-} BDC/B6^{g7/b} recipients and mice were monitored up to age 11 weeks. To detect suppression of lymphopenia, Brdu was fed to recipients for 4 days starting 7 days after injection of 240 million cells into ADAP^{-/-} BDC/B6^{g7/b} mice; Brdu-positive splenocytes and lymph node cells were detected by flow cytometric analysis.

Treg purification and functional assessment

BDC/B6^{g7/b} Splenocytes were stained with biotin-anti-CD25 mAb and then positively selected using streptavidin-conjugated magnetic beads and LS midiMACS columns (Miltenyi Biotec, Auburn, CA). Diabetogenic effectors (column-non-adherent CD25⁻ cells) were mixed with CD25⁺ cells (“Tregs”) at indicated ratios and were injected by tail vein into NOD/SCID recipients. Recipients were followed for diabetes 7-28 days after injection.

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Abbreviations used in this paper

ADAP	Adhesion and Degranulation-promoting Adapter Protein
APC	antigen presenting cell
BDC/B6^{g7/b}	BDC2.5 TCR transgenic mice, C57Bl/6 background, bearing I-A ^{g7/b}
Ctl	control
DP	Double Positive
IL	Interleukin
NOD	Non-Obese Diabetic
NK	Natural Killer
NKT	Natural Killer T cells
N-LN	Non-draining Lymph Node
MHC	Major Histocompatibility Complex
P-LN	Pancreatic Lymph Node
SP	Single Positive
TCR	T cell receptor
T1D	Type 1 Diabetes

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Figure 1. ADAP deficient BDC/B6 mice show increased incidence of autoimmune diabetes
 Diabetes incidence curves for control or ADAP^{-/-} BDC/B6 mice (A) homozygous for MHC class II I-A^{g7} (g7/g7), or (B) heterozygous for I-A^{g7} and I-A^b (g7/b) are shown. C) Pancreata from 5.5 week-old, prediabetic, BDC/B6^{g7/b} mice were sectioned and examined with hematoxylin/eosin staining under light microscopy. Islet pathology was scored as “clean” (no infiltrates), “peri-insulinitis”, insulinitis level 1 (leukocyte infiltrates covering <50% of the islet) or level 2 (infiltrates >50 % of the islet and spread to the peripheral tissues). Each pie chart represents average percentages of islets from 5 mice. 40 to 50 islets were counted per animal.

Figure 2. ADAP^{-/-} BDC/B6^{g7/b} peripheral lymphoid tissues contain reduced numbers of CD4⁺ T cells

A) Percentages of live cells in indicated tissues (P-LN = pancreatic lymph nodes) from control or ADAP^{-/-}, 5 week-old BDC/B6^{g7/b} mice staining positive for CD4 and Vβ4 are shown. B) Comparison of control and ADAP^{-/-} BDC/B6^{g7/b} absolute CD4⁺Vβ4⁺ T cell counts in spleen and inguinal, non-draining (N-LN) lymph nodes.

Figure 3. Treg development and function are normal in ADAP^{-/-} BDC/B6^{g7/b} mice
 A) Mean (+/- s.d.) percentage of FoxP3⁺ staining among CD4⁺ cells from prediabetic BDC/B6^{g7/b} non-draining inguinal (N-LN) or pancreatic (P-LN) lymph nodes is shown. B) Indicated numbers of CD25⁺ BDC/B6^{g7/b} splenocytes (Tregs) from ADAP^{+/+} (Ctl) or ADAP-deficient (-/-) were co-transferred with 0.6×10^6 wild type, CD25^{lo} BDC/B6^{g7/b} “effector” spleen cells into NOD/SCID mice (n=5 mice per group). Recipients were monitored for 4 weeks.

Figure 4. Enhanced homeostatic and antigen-specific T cell proliferation in ADAP^{-/-} BDC/B6^{g7/b} mice

A) 3×10^6 purified, CFSE-labeled, wild type CD45.1⁺ nontransgenic B6^{g7/b} T cells were transferred into either ADAP^{+/-} (Ctl) or ADAP-deficient (^{-/-}), CD45.2⁺ transgenic BDC/B6^{g7/b} 4 week-old, pre-diabetic recipients. Representative histograms (left) show CFSE dilution by donor (CD45.1⁺) CD4⁺ or CD8⁺ lymphocytes harvested 4 days after transfer. Graphs (right) display mean (+ s.d.) percentage of CFSE-diluting donor cells from 3 individual recipients. B) 5 week-old, pre-diabetic BDC/B6^{g7/b} mice were fed Brdu for 5 days. CD4⁺V_β4⁺ lymphocytes harvested from indicated tissues were stained for Brdu uptake. Representative histograms (top) and graphs (bottom) showing mean % Brdu⁺ were derived from analysis of 3-5 individual control or ADAP-deficient mice.

Figure 5. Transfer of syngeneic leukocytes inhibits ADAP-dependent diabetes and reduces spontaneous T cell proliferation

A) 4-5 week-old ADAP-deficient mice were injected (age range at time of injection indicated by black bar) with indicated numbers of pooled ADAP-deficient BDC/B6^{g7/b} spleen and lymph node cells or purified (> 90% pure CD3⁺) T cells harvested from age-matched mice. Graph (left) shows diabetes incidence curves for transfer recipients and for unmanipulated (no transfer) control or ADAP^{-/-} mice. B) 4 week-old BDC/B6^{g7/b} mice were injected with pooled ADAP^{-/-} BDC/B6^{g7/b} spleen and lymph nodes cells or were left unmanipulated. One week after injection, mice were fed Brdu for 4 days. The fraction of Brdu⁺ CD4⁺Vβ4⁺ T lymphocytes among live cells from indicated tissues is shown in representative histograms (top). Mean (+ s.d.) Brdu⁺ cell fraction (bottom graph) was calculated from analysis of 3-5 individual animals in each group. Legends indicate subject genotypes and the number of injected cells.

Figure 6. ADAP-deficient BDC/B6^{g7/b} mice show reduced CD4⁺-thymocyte numbers, but intact T cell survival capacity

A) Thymocytes from 5-week old prediabetic, ADAP^{+/-} (Ctl) or ADAP-deficient (-/-) BDC/B6^{g7/b} mice were analyzed for co-receptor expression. Percentages of CD4⁺CD8⁻ (CD4 SP) among total live thymocytes are shown. Graphs show cell counts (mean indicated by bars) from BDC/B6^{g7/b} total thymus and CD4 SP. B) CFSE-labeled, ADAP-deficient (CD45.1⁺CD45.2⁺) and control (CD45.1⁺CD45.2⁻) CD4⁺ T cells, in the context of unfractionated splenocytes, were mixed at a 2 to 1 ratio ("Day 0" histogram at left) and injected into nontransgenic (CD45.1⁺CD45.2⁻) mice. 9 days after injection, inguinal (N-LN) and pancreatic (P-LN) lymph nodes cells were analyzed. Representative histograms show percent CD4⁺Vβ4⁺ T cell contribution by each genotype. Graph shows mean (+/- s.d.) ratios derived by dividing Ctl contribution (%) by ADAP-deficient contribution (%), normalized to input ratio. Results represent analysis of 4 individual recipients. C) BDC/B6^{g7/b} CD4⁺Vβ4⁺ T cells described in (B) were analyzed for CFSE dilution. Representative histograms (left), and graph (right) show mean (+s.d.) fraction of CFSE¹⁰ (divided) donor cells in indicated tissues.