Peripheral CD4loCD40+ auto-aggressive T cell expansion during insulin-dependent diabetes mellitus

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The generation of auto-aggressive T cells involves failure of central or peripheral tolerance. We previously demonstrated that peripheral CD4loCD40+ T cells give rise to pathogenic T cells in the non-obese diabetic (NOD) model. Here we show that peripheral CD4+CD40+ T cells from diabetic or pre-diabetic NOD mice induce insulin-dependent diabetes mellitus. Consistent with breach of peripheral tolerance, CD4loCD40+ T cells expand with age in NOD mice but not in MHC-matched non-obese resistant (NOR) or BALB/c controls. Suggestive of a causal role for CD40 in autoimmunity, blocking CD40–CD154 interactions early during NOD development prevents autoaggressive T cell expansion while promoting increases in CD4+CD25+ regulatory T cells. Importantly, CD40 signals promote expansion of Vδ3.2+ and Vγ8.3+ T cells. Furthermore, peripheral Vδ3.2+CD40+ T cells induce diabetes in NOD.scid recipients while Vγ8.3+ T cells or Vγ3.2δ-depleted T cell populations do not. This is the first demonstration that primary T cells transfer disease with the kinetics of auto-aggressive T cell clones and that specific TCR Vδ expansion promotes diabetes.

Key words: T cell tolerance / T cell receptor / Auto-aggressive T cells / Regulatory T cells / Autoimmunity

1 Introduction

Numerous cell types are involved in the development of insulin-dependent diabetes mellitus (IDDM). However, auto-aggressive T cells are fundamental in fulmination of the disease [1–6]. Studies involving adoptive transfer of diabetogenic T cell clones into nonobese diabetic (NOD) mice or using diabetogenic TCR-transgenic (TCR-Tg) mice demonstrated that CD4+ T cells infiltrate pancreatic β cells, leading to loss of insulin production [3, 5]. CD8+ TCR-Tg NOD mice develop diabetes, suggesting a preeminent role for CD8+ T cells in disease progression [7]. However, when primary CD8+ T cells are used, CD4+ T cell help is required [8].

While diabetogenic T cell clones and TCR-Tg mice provide important information about the disease process, it is essential to address primary T cells as disease culprits. It was recently suggested that NOD mice suffer a failure of central tolerance [9]. We have proposed that auto-aggressive T cells in IDDM arise from a peripheral subset of T cells characterized as CD4loCD40+ [1]. These findings are not mutually exclusive. CD4loCD40+ T cells are induced through CD40 to transcribe, translate and translocate the RAG1 and RAG2 proteins to the nucleus [10]. Because RAG function alters TCR expression, this suggests that CD40 signals contribute to altered TCR expression after thymic selection, perhaps leading to the generation of autoaggressive T cells in the periphery. In fact, CD40 signals promote alteration in Vα expression exclusively in the peripheral CD4loCD40+ T cell population [10].

Blocking CD40–CD154 interaction prevents rejection of islet transplants [11, 12]. T cell infiltration into the pancreas occurs at 3 to 4 weeks of age in NOD mice, with extensive insulitis at 12 weeks of age [13]. Injecting 3-week-old NOD mice with CD154 blocking antibody prevented IDDM, but injecting mice at 9 weeks of age had no effect [14]. This suggests an important developmental framework with regard to CD40 and diabetes that potentially involves T cells. It was demonstrated that CD154 signals are required for pancreatic infiltration [7] and that CD154−/− NOD mice do not develop diabetes or insulitis.

Abbreviations: IDDM: Insulin-dependent diabetes mellitus TCR-Tg: TCR-transgenic NOR: Non-obese resistant

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Here we show that CD4\(^+\)CD40\(^+\) T cells, including T cells purified from pre-diabetic animals, rapidly transfer diabetes to NOD.scid recipients. Importantly, these T cells expand as NOD mice develop diabetes. Furthermore, CD40 promotes expansion of V\(\alpha\)3.2\(^+\) and V\(\alpha\)8.3\(^+\) T cells only within the auto-aggressive T cell population. However, while primary CD40\(^+\)V\(\alpha\)3.2\(^+\) T cells induce diabetes with kinetics established by diabeticogenic T cell clones, V\(\alpha\)8.3\(^+\) T cells do not. This study demonstrates that blocking CD40–CD154 interaction in NOD mice prevents auto-aggressive T cell expansion and prevents V\(\alpha\)3.2\(^+\) T cell expansion, while promoting accumulation of CD25\(^+\) regulatory T cells. These data suggest that specific V\(\alpha\)\(^+\) T cells can be predictive of diabetes onset.

2 Results

2.1 Purified CD4\(^+\)CD40\(^+\) T cells isolated from diabetic and pre-diabetic NOD donors are highly diabetogenic

We previously showed that an effector subset of T cells in NOD mice characterized as CD4\(^+\)CD40\(^+\) transfers IDDM to NOD.scid recipients in a dose-dependent manner, resulting in peri-insulitis (at low transfer numbers), severe insulitis (at moderate transfer numbers) and eventually diabetes (at high transfer numbers) [1], thus suggesting that these T cells are causal in diabetes. Here we clarify the pathogenic potential of peripheral CD4\(^+\)CD40\(^+\) T cells by transferring these T cells from diabetic (18–20-week-old) or pre-diabetic (9-week-old) NOD donors. Transfer of relatively low numbers (1.5\(\times\)10\(^6\)) of CD4\(^+\)CD40\(^+\) T cells rapidly induced severe insulitis and diabetes in NOD.scid recipients (Fig. 1A–C). Importantly, CD40-depleted T cells from diabetic NOD animals did not transfer diabetes (Fig. 1A) or insulitis (Fig. 1D). It has been stated that CD25\(^+\) T cells are pathogenic, but in that study both CD25\(^+\) and CD25\(^-\) T cells caused diabetes [16]. Our data demonstrate that auto-aggressive T cells...
can be designated by CD40 expression. Crucial to this hypothesis, very low numbers of CD40+ T cells were highly diabetogenic, while CD40-depleted cells did not induce disease at any level (Fig. 1A).

Previous reports demonstrated that a point of no return with regard to CD40–CD154 interaction occurs by 9 weeks of age: inhibition of CD40–CD154 interaction at 9 weeks of age cannot prevent diabetes onset [14]. Here we show that highly purified CD4loCD40+ T cells isolated from 9- to 12-week-old pre-diabetic NOD animals successfully transferred diabetes to NOD.scid recipients (Fig. 1B). None of the CD40-depleted T cell recipients were diabetic after 60 days (Fig. 1B), confirming that CD40+ T cells are not pathogenic.

Histology of the pancreata revealed severe insulitis in recipients of CD40+ T cells by 15 days (Fig. 1C), while pancreata from recipients of CD4+CD40+ cells demonstrated no insulitis through 60 days post-transfer (Fig. 1D). CD4loCD40+ T cells were also isolated with increasing frequency from the pancreata of NOD mice during development of IDDM (data not shown). With regard to the contribution of CD8+ T cells, while CD8+ TCR-Tg NOD mice develop diabetes, that process is independent of CD40–CD154 interaction [7]. As a precaution, injected T cells were CD8 depleted. Examination of T cells after diabetes onset demonstrated no detectable CD8+ T cells (data not shown). Collectively these data confirm the pathogenic potential of CD40+ T cells and demonstrate that these T cells have diabetogenic potential in the periphery well before diabetes onset.

2.2 Expansion of CD4+CD40+ T cells in diabetes-prone NOD mice

Auto-aggressive T cells respond to self-antigens, leading to expansion. Because primary CD4loCD40+ T cells are diabetogenic, we examined levels of CD4+CD40+ T cells as autoimmune-prone NOD mice age. We compared levels of CD4loCD40+ T cells in NOD, diabetes-resistant (NOR) and non-autoimmune BALB/c mice. NOR mice serve as an important control because these animals contain the same unique MHC configuration (IAg7) as NOD mice but are congenic at other loci and do not develop diabetes [17].

Pancreatic infiltration in NOD mice occurs at 3 weeks of age, with progressive insulitis at 12 weeks, fulminating in diabetes onset by 16 to 20 weeks [13, 18, 19]. In 3-week-old NOD females, there were low levels (4%) of CD4loCD40+ T cells (Fig. 2A). The percentage of CD4loCD40+ T cells had tripled at 6 weeks of age, and by 12 weeks had increased to 24% of the T cell compartment (data not shown). With regard to the contribution of CD8+ T cells, while CD8+ TCR-Tg NOD mice develop diabetes, that process is independent of CD40–CD154 interaction [7]. As a precaution, injected T cells were CD8 depleted. Examination of T cells after diabetes onset demonstrated no detectable CD8+ T cells (data not shown). Collectively these data confirm the pathogenic potential of CD40+ T cells and demonstrate that these T cells have diabetogenic potential in the periphery well before diabetes onset.
Another important observation is that as CD4loCD40+ T cells increased in frequency with age in NOD mice, there was no concomitant decrease in CD4hi T cells (Fig. 2A), further suggesting that CD4loCD40+ T cells constitute a unique subpopulation of T cells. If the CD4loCD40+ phenotype was a consequence of activation, concomitant losses of CD4hi T cells in conjunction with the demonstrated CD4lo increases would be expected. Consistent with the hypothesis that CD4loCD40+ T cells are a unique subpopulation is the report that CD40+ thymocytes are readily detected in thymus of NOD animals [1]. CD4loCD40+ T cells express CD69 or CD154 when engaged by TCR/CD3 (data not shown).

In the NOR strain, which contains an MHC component identical to NOD but does not develop diabetes [21], 15% of the T cell population was CD4loCD40+ at 6 weeks of age, dropping to 10% later in development (Fig. 2B). Interestingly, levels of CD4loCD40+ T cells in non-autoimmune prone BALB/c mice were highest at 3 weeks of age (16%), decreasing to 5% by 18 weeks of age (Fig. 2C). Unlike NOD mice, the percentage of CD4loCD40+ T cells increased in NOR and BALB/c mice during development.

### 2.3 Blocking CD40–CD154 interaction early during NOD development prevents autoaggressive T cell expansion

Administration of anti-CD154 antibody to NOD mice at 3 weeks of age prevents diabetes, while administration of that antibody at 9 weeks of age has no effect [14]. CD154−/− NOD mice experience no CD4+ pancreatic infiltration [7] and do not develop diabetes. These data suggest the importance of CD40–CD154 interactions in diabetes [15]. Because autoaggressive CD4loCD40+ T cells expand as NOD mice age, the effect of blocking CD40–CD154 interaction on the development of these T cells was determined.

NOD mice were injected with anti-CD154 at 3 weeks of age and monitored for diabetes. Within the experimental period (24 weeks) post injection, none of the MR1-injected animals (28 of 28) developed diabetes, while isotype control-injected animals (6 of 8) developed diabetes with expected kinetics (Fig. 3A). This is similar to the report by Balasa et al. [14]. At 12 weeks post-injection, the CD4loCD40+ T cell population was reduced to 2.2% of total T cells in MR1-injected NOD mice (Fig. 3B), compared to 25% in control animals (data not shown). At 18 weeks post-injection, CD4loCD40+ T cells had expanded to only 10% of the CD4 T cell compartment in MR1-injected animals (data not shown). Thus, CD40–CD154 blockade substantially delays expansion of autoaggressive T cells.

Another important aspect of diabetes control is T cell regulation. In the diabetes model, CD25+ T cells are instrumental in prevention or progression of diabetes [22–24]. Thus, homeostasis would be maintained through a balance of autoaggressive T cells and regulatory T cells. We examined the effect of blocking CD40–CD154 interaction on levels of CD25+ regulatory T cells. Total CD25+ T cells comprised 5% of the CD4 T cell compartment in isotype control-injected NOD mice 12 weeks post-injection (Fig. 3C). In these studies, CD25+ T cells were detected in both CD4lo and CD4hi T cell populations. Interestingly, CD25+ T cells from older NOD mice successfully transfer diabetes [16]. It has been reported that different populations of CD25+ T cells occur in NOD mice relative to CD62L expression, but all CD25+ T cells were equally effective in prevention of diabetes onset [19]. Interestingly, blockage of CD40–CD154 interaction resulted in increased numbers of CD25+ T cells in both the CD4lo and CD4hi subpopulations 12 weeks post-injection, totaling 15.8% of the CD4+ compartment (Fig. 3D).

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These findings help explain mechanistically how CD40–CD154 blockage prevents or substantially delays diabetes onset. While the effects of blocking CD40–CD154 interaction could be direct or indirect, the functional consequences (limiting expansion of autoreactive T cells and promoting expansion of regulatory T cells) are the same. In either case diabetes onset is forestalled (Fig. 3A).

2.4 Expansion of \( V_\alpha \) subsets among CD40+CD4+ T cells in autoimmune NOD mice

T cell studies in diabetes have focused largely on diabetogenic T cell clones such as BDC2.5 [5, 13]. It has been suggested that a restricted TCR repertoire is instrumental in disease progression [19, 25]. However, the majority of diabetogenic TCR studies have concentrated on TCR \( V_\beta \) expression; for instance, it was reported that specific \( V_\beta \)+ T cells occur early during NOD development [18]. We have reported that CD40 signals preferentially induce altered \( V_\alpha \) expression [1, 20]. This finding suggests that TCR restriction in certain autoimmune diseases may be defined by specific \( V_\alpha \)+ T cell subsets.

Altered TCR \( V_\alpha \) expression, or TCR revision, in the \( V_\alpha \)+CD4+ T cell population was examined during the development of IDDM. We previously determined that RAG1 and RAG2, the recombinase proteins responsible for generating the T cell repertoire, are activated in peripheral T cells through CD40 signals [10]. Furthermore, this activation led to alteration of TCR \( V_\alpha \) molecules on diabetogenic T cell clones [10]. We therefore addressed how CD40 signals affect \( V_\alpha \) expression during the development of CD4+CD40+ T cells in IDDM. \textit{Ex vivo}, CD4+CD40+ T cells from 3-week-old NOD animals demonstrated low levels of the examined \( V_\alpha \) molecules, and no statistically significant change in \( V_\alpha \) expression was seen after overnight exposure to anti-CD40 antibody (Fig. 4A). At 12 weeks of age, isotype control-treated T cells showed no significant change in \( V_\alpha \) T cells, but \textit{in vitro} CD40 cross-linking of T cells from NOD mice induced substantial increases (almost four-fold) in \( V_\alpha 3.2^+ \) and \( V_\alpha 8.3^+ \) T cells (Fig. 4A). At 18 weeks of age, \textit{in vivo} expansion (when compared to \( V_\alpha \) levels of 3-week-old animals) of \( V_\alpha 2^+ \) and \( V_\alpha 3.2^+ \) T cells were evident; thus, these particular T cells have expand as NOD mice age. \textit{In vitro} CD40 cross-linking of CD4+CD40+ T cells induced further increases in percentages of \( V_\alpha 2^- \) and \( V_\alpha 8.3^- \)-expressing T cells (Fig. 4A). In contrast, the percentage of \( V_\alpha 3.2^- \) T cells was reduced after CD40 cross-linking; thus, later in development these T cells can be induced to alter TCR expression upon CD40 engagement [10], effectively reducing their numbers. Importantly, CD40 cross-linking did not result in T cell death (data not shown).

NOR mice have the same unique MHC class II component (I-A\(^ \gamma \)), and therefore have an identical T cell selective environment, as NOD mice. However, congenic differences at the gene loci that render these animals resistant to diabetes [17] may affect T cell development. As demonstrated in Fig. 2, CD4+CD40+ T cells are increased in NOR mice. At 12 and 18 weeks of age, NOR animals had higher \textit{in vivo} levels of \( V_\alpha 3.2^+ \) T cells relative to the other \( V_\alpha \)+ cells examined (Fig. 4A). The levels were lower than in NOD. Unlike in NOD animals, CD40 cross-linking of T cells induced reduction in the frequency of \( V_\alpha 3.2^- \)

Fig. 4. CD40-driven expansion of specific \( V_\alpha \)+ T cells in NOD mice. (A) The percentage of \( V_\alpha \)+ T cells within the CD4+CD40+ population (x-axis) from 3-, 12-, or 20-week-old NOD and NOR mice was determined after treatment of the T cells with isotype control (light bars) or crosslinking with CD40 for 18 h (dark bars). Gating was based on the isotype controls. Data are an average of three experiments with three animals in each experiment. CD40 treatment did not induce cell proliferation in (B) CD4+CD40+ or (C) CD4+CD40+ column-purified splenic T cells, while CD3 treatment induced proliferation in both T cell populations.
T cells, which was not due to induced cell death (data not shown). The likely explanation is that CD40 induced TCR revision, consistent with our recent report [10].

The mechanisms for altered levels of Vα+ T cells are induction of proliferation, cell death or alteration of TCR expression. As mentioned, CD40 signals do not induce cell death (data not shown and [10]). Induced proliferation was addressed by CFSE labeling of total purified CD4+ T cells. CD40 cross-linking did not induce either CD40+ or CD40- T cells to proliferate. Importantly, CD3 engagement induced both CD40+ (Fig. 4B) and CD40- (Fig. 4C) T cell populations to proliferate. As reported previously, CD40 signals induce the RAG1/RAG2 TCR recombination complex, while CD3 engagement does not [10], suggesting that CD40 signals induce expansion of specific Vα+ T cells by altering peripheral TCR expression.

2.5 Vα3.2+ CD4+CD40+ T cells are increased in pancreata of pre-diabetic and recently diabetic NOD mice

If a specific Vα+ T cell is pathogenic in IDDM, it should be present in pancreata. Pancreata from 12-week-old pre-diabetic as well as >18-week-old diabetic NOD mice showed higher percentages of Vα3.2+ T cells within the CD4+CD40+ auto-aggressive T cell population (Fig. 5A). Anti-CD154 injection inhibited specific Vα+ T cell expansion within pancreata of NOD mice (Fig. 5B). This suggests that T cell CD40 signals are determinant for Vα3.2+ and Vα8.3+ T cells. As reported for NOD-CD154–/– mice [7], MR1-injected animals had no islet infiltration (260 islets from 6 different animals examined).

After diabetes onset within NOD.scid recipients that received CD4+CD40+ T cells, analysis revealed expansion of Vα3.2+ cells, comprising 25% of the CD4+CD40+ T cell population (Fig. 5C). T cells from recipients of CD4+CD40+ cells demonstrated levels of the specific Vα+ T cells at <4% (Fig. 5C). These data cumulatively suggest that CD40-promoted expansion of specific Vα+ T cells is associated with, if not directly responsible for, IDDM onset.

Fig. 5. Expansion of Vα3.2+ T cells in pancreata of pre-diabetic and diabetic NOD mice. T cells directly purified from pancreata of (A) 12-week-old pre-diabetic (n=4) and >18-week-old diabetic NOD (n=4) mice show expansion of Vα3.2+ and Vα8.3+ T cells within the gated CD4+CD40+ population. (B) MR1 (anti-CD154) injection substantially inhibited levels of detectable Vα+ T cells in the pancreata. (C) Splenic T cells isolated from NOD.scid recipients of CD4+CD40+ T cells demonstrate expansion of Vα3.2+ and Vα8.3+ T cells 15 days post-transfer, while no significant Vα+ expansion was seen in recipients of CD4+CD40- cells. Data represent three separate experiments (n=12 for each treatment).
2.6 \( \nu \alpha 3.2^+ \) T cells are highly diabetogenic while \( \nu \alpha 8.3^+ \) T cells are not

In these studies we demonstrate that CD4\(^{lo}\)CD40\(^+\) T cells are directly pathogenic and that specific \( \nu \alpha \) T cells correlate with diabetogenicity. That is, \( \nu \alpha \) expression may define a particular subset of auto-aggressive T cells. We compared the pathogenicity of \( \nu \alpha 3.2^+ \) and \( \nu \alpha 8.3^+ \) T cells through adoptive transfers into NOD.scid recipients. Recipients of \( \nu \alpha 3.2^+ \) T cells became diabetic with the same kinetics as recipients of purified CD40\(^+\) T cells: 50\% (3 of 6) were diabetic 10 days after injection, with the remaining animals becoming diabetic at day 12 post-injection (Fig. 6A). These T cells were determined to be CD8\(^-\). After 45 days, none of the recipients of \( \nu \alpha 8.3^+ \) cells (6 of 6) had become diabetic (Fig. 6A). Further suggesting that \( \nu \alpha 3.2^+ \) T cells are instrumental in diabetogenesis, none of the NOD.scid recipients of \( \nu \alpha 3.2^-\) depleted became diabetic (8 of 8). While it is not possible to call these primary T cells a true clonal expansion since they may express different V\( \beta \) molecules, the kinetics of disease transfer is similar to that of established diabetogenic T cell clones [5].

Histology of pancreata from recipients of \( \nu \alpha 3.2^+ \) and \( \nu \alpha 8.3^+ \) T cells confirmed that \( \nu \alpha 3.2^+ \) T cells migrate to the pancreas, infiltrate islets and diminish insulin production (Fig. 6B). Conversely, \( \nu \alpha 8.3^+ \) T cells, examined at 15 days, do not infiltrate the pancreas and therefore insulin granules remain intact (Fig. 6C).

3 Discussion

CD40 involvement in IDDM has been established. Blocking CD40–CD154 interaction prevents rapid rejection of transplanted islets [26, 27]. Blocking CD40–CD154 interaction early (3 weeks) during NOD development but not later (9 weeks) prevents diabetes [14]. Another study demonstrated that NOD-CD154\(^-\)\(^-\) mice fail to exhibit CD4\(^+\) T cell pancreatic infiltration, suggesting that CD154 may be critical for T cell trafficking [7]. Furthermore, NOD-CD154\(^-\)\(^-\) animals do not develop spontaneous diabetes [15]. These studies prompted the current study of the newly described CD4\(^{lo}\)CD40\(^+\) auto-aggressive T cell population.

![Fig. 6. \( \nu \alpha 3.2^+ \) T cells rapidly transfer diabetes. (A) Transfer of \( \nu \alpha 3.2^+ \) T cells (diamonds, \( n=6 \)), but not \( \nu \alpha 8.3^+ \) T cells (squares, \( n=6 \)), results in diabetes (blood glucose levels >250 mg/100 ml). As controls, CD40-depleted (triangles, \( n=10 \)) and \( \nu \alpha 3.2^-\) depleted (X, \( n=8 \)) T cells did not transfer diabetes. (B) Histology (aldehyde/fuchsin staining) of pancreatic islets from recipients of \( \nu \alpha 3.2^+ \) T cells demonstrate extensive infiltration and loss of insulin granules. (C) Histology (aldehyde/fuchsin staining) of pancreas from recipients of \( \nu \alpha 8.3^+ \) T cells demonstrate no infiltration and intact insulin granules. Greater than 200 islets from each treatment group were examined.](image-url)
CD40 is expressed on a wide variety of tissues, including B and T cells [20, 28]. CD40 is expressed on several diabetogenic T cell clones, and a subpopulation of T cells characterized as CD40CD40- occur in high number in diabetic NOD mice [1]. Furthermore, CD40CD40 T cells transfer diabetes to NOD.scid recipients [1]. CD40 T cells occur in greater numbers in autoimmune-prone strains [20]. CD40 signals induce transcription, translation, and nuclear translocation of the RAG1 and RAG2 recombinase proteins in peripheral T cells [10]. The RAG proteins are responsible for V(D)J recombination of the TCR and subsequent antigen diversity of the T cell repertoire. This finding suggests a mechanism by which auto-aggressive T cells could be generated in the periphery, escaping thymic negative selection. It is important, however, to recognize CD40 T cells as a subpopulation of the T cell compartment. CD40 mice develop T cells, although their adaptive immune response including T cell antigen recall is highly impaired [29, 30]. With regard to diabetes, it was demonstrated using a well-described CD8 TCR-Tg model that CD40–CD154 interactions are not involved in the onset of CD8 T cell-mediated diabetes [7].

Until now it has been difficult to assess primary T cells as disease culprits in diabetes. It was demonstrated that transfer of highly purified primary CD8 T cells from diabetic NOD mice to NOD.scid recipients did not induce diabetes until primary CD4 T cells were transferred [8]. There are likely to be multiple ways of inducing diabetes, involving several different cellular mechanisms.Complicating this picture, there are highly successful diabetogenic CD8 T cell clones and subsequent TCR-Tg animals that do not appear to require CD4 help [31, 32].

The involvement of CD4 T cells in IDDM has focused largely on diabetogenic T cell clones, e.g. BDC2.5 and the corresponding BDC2.5 TCR-Tg animal [6]. Although BDC2.5 was isolated from a diabetic NOD mouse and rapidly transfers disease, it has been reported that the clonally defined TCR, V3.2/Vα1, is grossly underrepresented within NOD mice, including the BDC2.5 TCR-Tg animal [33]. Another study demonstrated that within BDC2.5 TCR-Tg mice there is substantial drift within Vα usage, but animals nevertheless become diabetic [13]. The current report demonstrates that auto-aggressive T cells expand as NOD mice age. Importantly, there were not concomitant decreases in CD4 T cells, suggesting that CD4CD4 T cells are an independent effector T cell subset. Additionally, there is CD40-driven expansion of V3.2 T cells among NOD T cells. In the NOR control, there was early expansion of V3.2 T cells, but this population decreased as animals aged, and CD40 signals caused reductions in that population. Because the CD4CD4 T cell population does not expand in NOR mice, the numbers of Vα3.2 T cells potentially do not reach a critical mass to induce disease. Nevertheless, these data suggest that changes in TCR relative to Vα expression are intrinsic to diabetogenesis. The finding that blocking CD40–CD154 interaction prevents expansion of the auto-aggressive T cell population is also consistent with CD40 signals driving the expansion of auto-aggressive T cells. By preventing CD40–CD154 interaction, expansion of Vα3.2, highly pathogenic T cells was prevented, lending further evidence to the pathogenic potential of these specific T cells.

The initial report that blocking CD40–CD154 interaction prevents diabetes when administered early during NOD development proposed that the block affects cellular trafficking [14]. This prediction is not in dispute considering later reports that NOD-CD154 T cells do not experience pancreatic infiltration [7]. The current studies, however, provide an additional picture of CD40 involvement in IDDM. Not only does blocking CD40–CD154 interaction early during NOD development prevent expansion of auto-aggressive T cells, it also allows the expansion of accepted regulatory (CD4CD25) T cells. The likely explanation is that when CD40 signals to peripheral CD4CD4 T cells are prevented, those T cells do not activate RAG and therefore do not alter TCR expression [10]: these T cells remain non-responsive to ‘self-antigens’ and do not expand. The expansion of regulatory T cells is likely a reflection of the ratio between auto-aggressive T cells and regulatory T cells. Homeostasis must rely on a balance between auto-aggressive and regulatory T cells, and by blocking CD40 signals to auto-aggressive T cells, the balance is tipped to favor regulatory T cells.

There are two possible scenarios to explain the Vα increases within the periphery: 1) proliferation or 2) alteration in Vα expression. CD40 signals do not promote T cells to proliferate. CD40 signals auto-aggressive T cells to increase RAG1 and RAG2 expression, and importantly, CD40 signals induce translocation of the RAG proteins to the nucleus [10]. CD40 signals also induced changes in TCR Vα expression within the auto-aggressive T cell population. The most likely explanation is therefore that CD40 signals induce altered Vα expression, explaining the expansion of Vα3.2 and Vα8.3 T cells. The clonal nature of these cells is indeterminate because the Vβ repertoire of these cells is as yet unknown. It has been demonstrated that diabetogenic T cell clones become heterogeneous with respect to antigen specificity [3], suggesting that several β cell antigens are involved in the diabetogenic process. Thus, Vα3.2 T cells may express several different Vβ molecules but nonetheless rapidly induce diabetes.

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4 Materials and methods

4.1 Mice

NOD, NOR and BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained under pathogen-free conditions in the IACUC-approved facility at the University of Colorado Health Sciences Center (Denver, CO).

4.2 Staining

T cells were purified from excised spleens and lymph nodes (mesenteric and pancreatic) from NOD, NOR or BALB/c mice at the ages indicated and incubated on nylon wool-columns wetted with PBS/5% BSA for 45 min. Purified T cells (>87% CD3+) were washed, treated with anti-Fc-receptor (2.4.G2) and then stained with directly conjugated FITC-anti-CD40 (1C10, produced in-house [34]) or FITC-anti-CD25 (PharMingen, San Diego, CA), PE-anti-TCR-β (H57.597) or PE-anti-CD3 (145.2C11, PharMingen,) and CyChrome™-anti-CD4, (H129.19, PharMingen). Cells were run on a FACS Calibur (Becton Dickinson) and assayed using CellQuest™ software.

For Vα staining, purified T cells were treated with an IgG2A isotype control antibody or cross-linked with biotin anti-CD40 followed by avidin for 18 h. T cells were incubated with 2.4.G2 and then stained with FITC-anti-Vα2, anti-Vα3.2 or anti-Vα8.3 (all from PharMingen), biotinylated anti-CD40 (1C10) with PE-avidin (PharMingen) and CyChrome™-anti-CD4 for analysis.

4.3 Adoptive transfer

T cells were nylon wool-purified from the spleens of diabetic and pre-diabetic NOD females and incubated with biotinylated anti-CD40 (1C10), anti-Vα3.2 or anti-Vα8.3 (both from PharMingen). T cells were washed with PBS, incubated with magnetic avidin beads (Miltenyi, Auburn, CA) and passed over magnetic purification columns (Miltenyi). Purified T cells were eluted and determined to be >98% pure by flow cytometry. CD8+ T cells were removed by incubating T cells with magnetic beads conjugated with anti-CD8 antibody (Miltenyi), followed by passage over a magnetic column (Miltenyi). Purified CD4+CD40+ T cells (1.5×10⁶) were injected i.p. into 9-day-old NOD.scid recipients. Control animals received an equivalent number of CD40-depleted T cells. Animals were monitored for diabetes onset by blood glucose determination.

Highly purified Vα3.2+ and Vα8.3+ T cells (1.5×10⁶) were injected i.p. into 9-day-old NOD.scid recipients that were monitored for diabetes. Controls received an equivalent number of CD40-depleted T cells or Vα3.2+–depleted T cells. Experiments were repeated three times.

4.4 Histology

Pancreata from NOD.scid recipients of CD4+CD40+ or CD4+CD40− T cells were fixed in formalin and paraffin-embedded, and slides were generated. Slides were stained with Hematoxylin and Eosin (H&E) or Aldehyde Fuchsin as described [1] and scored for infiltration and insulin production [1].

4.5 Antibody injections

NOD mice were injected i.p. with 100 µg purified anti-CD154 (MR1 [35]) in 100 µl PBS as described [14] at 3 weeks of age. Control animals were injected with an isotype control. A booster was administered at 4 weeks of age. Animals were monitored weekly by urine glucose analysis and checked for blood glucose levels 1 week prior to termination.

4.6 CFSE labeling

Purified T cells were incubated with CFSE (2.5 µM) in PBS for 15 min in the dark and washed with PBS/5% FCS. T cells were then treated with biotinylated anti-CD3 (5 µg/ml) or anti-CD40 (10 µg/ml) or were left untreated. Cells were incubated with avidin (2.5 µg/ml), washed with PBS and incubated 18 h in DMEM/5% FCS. Data were analyzed using CellQuest software following flow cytometry.

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References


defects manifest by antigen presenting cells. Autoimmune diabetes results from genetic changes in the peripheral T cell receptor repertoire. Peripheral T cells in autoimmunity: A mechanism for acquiring high levels of CCR7. The subpopulation of CD4(+)CD25(+) splenocytes that islet-cell reactive T cell repertoire of early pancreatic islet infiltration permits subsequent engraftment of donor strain islet or heart allografts. Transplant Proc. 1999. 31: 627–628.


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