Killer Treg cells ameliorate inflammatory insulitis in non-obese diabetic mice through local and systemic immunomodulation

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Abstract

Treg cells endowed with enhanced killing activity through decoration with Fas-ligand (FasL) protein (killer Treg) have been effective in delay of hyperglycemia in prediabetic non-obese diabetic (NOD) mice. In this study, we assessed the therapeutic efficacy of these cells, harvested from age-matched euglycemic NOD donors, on the course of disease in new-onset diabetics. One dose of 4 × 10⁶ killer Treg cells stabilized blood glucose associated with increased insulin levels in 5 of 9 mice and partially reversed the severity of islet inflammation, whereas naive Treg cells did not modulate the course of disease significantly. Killer Treg cells were shown to operate through induction of cell apoptosis within the pancreatic lymph nodes, resulting in reduced efficiency of adoptive disease transfer to NOD/SCID recipients. A second mechanism of action consisted of increased fractions of CD4⁺CD25⁻FoxP3⁺ T cells in the pancreas and all lymphoid organs. Immunomodulation with FasL rather than Treg cells enhanced the expression of CD25 and FoxP3 in the thymus, suggesting a possible contribution of thymic output to prolonged stabilization of the glucose levels. Autologous Treg cells evolve as excellent vehicles for targeted delivery of FasL as an immunomodulatory protein, which delete pathogenic cells at the site of inflammation and induce systemic dominance of suppressor subsets.

Keywords: destructive insulitis, Fas-ligand, killer Treg, new-onset diabetes, NOD mice, regulatory T cell

Introduction

Non-obese diabetic (NOD) mice are prevalent models of human type 1 diabetes (T1D) used to evaluate the capacity of Treg cells to ameliorate or arrest inflammatory insulitis (1–3). Among numerous studies performed with Treg cells in young, prediabetic NOD mice, only few studies have shown effective modulation of the disease after onset of hyperglycemia, at peak activity of inflammation and low residual β-cell mass (10–20%) (4–6). Naturally occurring Treg cells affect autoimmunity through inhibition of effector cell proliferation and IL-2 production, modulation of the activity of antigen-presenting cells and enrichment of the inflammatory environment with suppressive cytokines (7–10). Although physical elimination of pathogenic cells at the site of inflammation has been considered a relatively minor mechanism of Treg cell function, these inhibitory mechanisms and particularly modulation of the cytokine environment often result in apoptosis of pathogenic cells (11). In addition, apoptotic signals are directly delivered by suppressor cells through perforin/granzyme in naturally occurring Treg cells (12, 13) and predominantly by Fas-ligand (FasL) in adaptive Treg cells (14, 15). We reasoned that deletion of pathogenic cells at the site of inflammation is the best approach to definitive suppression of ongoing autoimmune reactivity (16), in particular in late stages of aggressive destructive insulitis (17). Diabetic autoimmunity is a persistent and relapsing disease with multiple and shifting antigenic targets (5, 11, 18), which can be abrogated only by reinstitution of peripheral negative regulation through induction of mixed chimerism (19).

Diabetogenic cells are sensitive to negative regulation by activation-induced cell death (AICD) (20), and adoptive disease transfer to NOD/SCID mice can be prevented by ex vivo exposure...
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of NOD lymphocytes to FasL (21). We, therefore, reasoned that a similar treatment may be effective in vivo using autologous Treg cells as vehicles for targeted delivery of an apoptotic ligand to the inflamed islets. In prior studies, we showed that activated Treg cells display reduced sensitivity to apoptosis mediated by Fas cross-linking (20), and Treg cells from age-matched NOD females migrate to and expand in the mesenteric/pancreatic lymph nodes of prediabetic NOD females (22). Treg cells decorated with short-lived FasL protein (killer Treg cells) induced apoptosis in diabetogenic cells and administration of relatively small doses of these cells to prediabetic NOD females postponed the onset of hyperglycemia by a significant period of 15 weeks (22). Although the diabetogenic potential persisted and disease incidence was only decreased from 83 to 40%, immunomodulation with FasL caused significant variations in regulatory phenotypes that sustained protection over extended periods of time.

In this study, we evaluate the capacity of naive and killer Treg cells to modulate the course of inflammatory insulitis after onset of hyperglycemia in NOD mice. For initial evaluation of the clinical outcome and immune characterization, we used one infusion of CD25+ T cells harvested from age-matched euglycemic NOD females. In contrast to ineffective treatment with naive Treg cells, we found that killer Treg cells stabilize blood glucose levels in ~50% of the mice, ameliorate the histological score of inflammation, reduce pathogenic cell activity and increase significantly the fractions of FoxP3+ regulatory subsets in peripheral lymphoid organs and in the thymus.

Methods

Mice and diabetes monitoring

Mice used in this study were NOD and NOD/SCID mice from and inbred colony housed in a barrier facility. The Institutional Animal Care Committee approved all procedures. Sub-lethal total body irradiation at 650 rad was applied to recipients 6 h prior to cell infusion using an X-ray irradiator (RadSource 2000, Brentwood, TN, USA) at a rate of 106 rad min⁻¹ (19, 23). Blood glucose was monitored between 9 and 11 a.m. using a standard glucometer, and diabetes was defined as two consecutive blood glucose measurements above 200 mg/dl (22). The glucose tolerance test was performed by intra-peritoneal administration of 2 g glucose followed by repeated measurements of blood glucose (19, 24). Serum insulin was assessed using the Insulin ELISA Kit (R&D Systems, Abingdon, UK) from absorbance at 450 nm and 590 nm (BioTeK, Winooski, VT, USA) (22).

Cell isolation, characterization and staining

Single-cell suspensions were prepared by gentle mincing of spleens, lymph nodes, thymus and pancreata on 40 µm nylon mesh (20), the latter after treatment with 20 µg ml⁻¹ of Collagenase-P (Roche Diagnostics) and lymphocytes were isolated by centrifugation over Lympholyte-M (Cedarlane, Burlington, NC, USA) (22). CD4+CD25+ and CD4−CD25− cells were isolated using the Regulatory T cell isolation kit (Miltenyi, Bergisch-Gladbach, Germany) (20). In adoptive transfer experiments, 0.2 ml PBS was injected into the tail vein: 2 x 10⁷ cells from spleens and mesenteric lymph nodes into NOD mice and combinations of 2.5 x 10⁷ CD4+CD25+ and 2.5 x 10⁶ CD4−CD25− into NOD/SCID mice. Cells were labeled with 10 µM CFSE (Molecular Probes, Carlsbad, CA, USA) or PKH membrane linkers (Sigma, St. Louis, MO, USA) (22).

Adsorption of FasL protein on cell surface

Isolated CD4+CD25+ T cells were biotinylated in 5 µM EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) under aseptic conditions, washed thoroughly, and incubated with streptavidin-FasL chimeric protein (50 ng protein per 10⁶ cells in 1 ml PBS) (22). After two additional washes, the efficiency of decoration was evaluated by flow cytometry using anti-streptavidin and anti-FasL antibodies.

Flow cytometry

Cell samples were immunophenotyped using fluorochrome-labeled mAb: CD4 (clone RM 4–5), CD8 (clone 53–6.7), and CD25 (clone PC61.5) (22). FoxP3 was determined following permeabilization and intracellular staining with PE-labeled antibody (buffer set NRRF-30, ebioscience, San Diego, CA, USA). Measurements were performed using a Vantage SE flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Apoptosis and early death were determined using Annexin-V (IQ products, Groningen, The Netherlands) and non-specific membrane permeabilization was assessed using 7-aminomycin-D (7-AAD, Sigma) (20). Positive staining was determined on a log scale, normalized with control cells stained with isotype control antibodies. Proliferation was determined from quantified CFSE dilution using the ModFit software.

Histology and immunohistochemistry

Pancreata were excised from mice euthanized by CO₂ asphyxiation and fixed in ice-cold PBS containing 1.5% paraformaldehyde before overnight immersion in 30% sucrose (22). OCT-embedded tissue (Sakura Finetek, Torrance, CA, USA) was frozen in isopentane suspended in liquid nitrogen, and tissue was sectioned (3–6 µm) using a Cryotome (Thermo Shandon, Runcorn, UK). Islet inflammation was scored following hematoxylin and eosin staining according to the following: 0, no inflammation; 1, peri-insulitis; 2, inflammatory infiltration < 50% of islet area; 3, inflammation > 50% of islet area and islet structure disruption.

Pancreatic cryosections were immunostained with primary antibodies: guinea pig anti-insulin (Dako, Glostrup, Denmark), rat anti-mouse CD4 (BD Pharmingen), biotinylated anti-mouse CD45 (1:100, Biologend, San Diego, CA, USA), and FITC-conjugated rat anti-FoxP3 antibody (ebioscience) and FasL was detected using a FITC-conjugated MFL4 antibody (BD Pharmingen) (22). Primary antibodies against insulin and CD4 were counterstained with AlexaFluor-647 and AlexaFluor-555 secondary antibodies (Dako, eBioscience), respectively, and biotinylated antibodies were conjugated with Cy3-labeled Streptavidin (1:400, Jackson ImmunoResearch, West Grove, PA, USA).

Statistical analysis

Data are presented as mean ± SD for each experimental protocol. Differences among the experimental protocols were estimated with a post hoc Scheffe t-test and significance was considered at P < 0.05. Differences in disease incidence were assessed by Mantel-Cox analysis.
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Results

Immunomodulation attenuates the course of established disease

In our female NOD colony, overt hyperglycemia appears at the age of 14 weeks and 83% of the mice are diabetic at 25 weeks of age (22, 24). Female NOD mice developing new-onset diabetes (blood glucose >200 mg/dl) progress to glucose levels exceeding 500 mg/dl within 2 weeks and subsequently die (Table 1). Treatment consisted of infusion of $4 \times 10^6$ naive and killer Treg cells from age-matched non-diabetic NOD females within 1 week after onset of hyperglycemia. Adoptive transfer of naive Treg cells slowed progression but did not attenuate significantly the gradual increase in to high glucose levels (Fig. 1A). In

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<th>Mean time to &gt;500 mg/dl (days)</th>
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<td>Killer Treg</td>
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<td>21</td>
<td>31.5 ± 4.2</td>
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<tr>
<td>650 rad killer Treg</td>
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<td>2 (26)</td>
<td>29</td>
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<td>650 rad Treg</td>
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<td>1 (14)</td>
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Fig. 1. Immunomodulation of NOD females after onset of diabetes. New-onset diabetic NOD females (spontaneous, >200 mg/dl) were infused with $4 \times 10^6$ naive CD25$^+$ T cells (Treg) harvested from age-matched euglycemic NOD females and with Treg cells decorated with FasL protein (killer Treg). (A) Glucose levels in peripheral blood after onset of spontaneous diabetes ($n = 23$) and following infusion of naive (Treg cells, $n = 9$) and killer Treg cells ($n = 9$). (B) Fasting serum insulin levels in the corresponding groups at 6 weeks after immunomodulation ($n = 4–5$) compared with levels in prediabetic NOD females ($n = 11$). (C) Histological inflammatory score of 30–42 islets in the corresponding treatment groups (3–4 mice) at the experimental end-point. (D) Glucose tolerance tests at the experimental end-point of 6 weeks after treatment of NOD females with naive ($n = 5$) and killer Treg cells ($n = 6$).
contrast, Treg cells decorated with FasL protein (killer Treg) stabilized the blood glucose levels in 5 of 9 NOD females (~350 mg dl⁻¹) for periods exceeding 6 weeks, resulting in slow disease progression in the entire group (P < 0.005 versus untreated mice, Table 1). Stable hyperglycemia in mice treated with killer Treg cells was consistent with higher serum insulin levels at 6 weeks after immunomodulation (Fig. 1B), suggesting arrest of disease progression. This was confirmed by the decrease in inflammatory scores of islets from mice treated with killer Treg cells (P < 0.01 versus untreated mice, Fig. 1C), demonstrating partial reversal of destructive insulitis. Consistently, the responses to the glucose tolerance test remained largely defective in all mice demonstrating insufficiency of residual β-cell mass to meet the insulin demand and very late (>3 h) improvement was observed in mice immunomodulated with killer Treg cells (Fig. 1D). This result was unexpected, as spontaneous endogenous regeneration of β cells does not occur within the time frame of this experiment.

**Immunomodulation with killer Treg cells modulates the local and systemic phenotypes**

The apparent impact of immunomodulation on islet inflammation prompted phenotypic characterization of the infiltrates. Onset of diabetes in NOD females is accompanied by significant decrease in FoxP3⁺ subsets, most significant in the CD4⁺CD25⁺FoxP3⁺ T cells (P < 0.005 versus prediabetic) in the pancreatic infiltrates (Fig. 2A). This feature was accompanied by a marked increase (P < 0.005 versus prediabetic) in contents of FoxP3 expression in the lymph nodes (Fig. 2B), with relatively small but significant decline in the spleen (Fig. 2C) and thymus (Fig. 2D). These variations might reflect either an effort of the regional lymph nodes to counteract aggressive destructive insulitis or redistribution of suppressor cells required to terminate inflammation due to extinction of the target pancreatic islets. All together, these data emphasize dynamic changes in the composition of the inflammatory infiltrates and peripheral lymphoid organs in late stages of destructive insulitis at an early time that is not yet affected by persistent hyperglycemia.

Treatment of NOD females by adoptive transfer of naive Treg cells caused minor variations in composition of the pancreatic infiltrates (Fig. 2A), regional lymph nodes (Fig. 2B), spleen (Fig. 2C) and thymus (Fig. 2D). These minor variations in immune phenotypes corresponded to a marginal impact of naive Treg cells on the course of the disease after onset of hyperglycemia (Table 1). In contrast, stable glucose levels and delayed disease progression in good and poor NOD responders to killer Treg cell infusion showed marked variations in immune phenotypes, most prominent in CD4⁺CD25⁺FoxP3⁺ cells (Fig. 2E) that exert essentially suppressor function (25). Good responders (mean progression to high glucose of 36.3 ± 4.1 days) displayed increased FoxP3 expression in the pancreas, lymph nodes and thymus compared with low levels displayed by the poor responders (mean progression to high glucose of 25.4 ± 4.3 days, P < 0.005 versus good responders). The variations in CD4⁺FoxP3⁺ fractions in the good responders to killer Treg cells were proportionate to the phenotypes of age-matched euglycemic mice, suggesting a causative relationship to slow disease progression and the corresponding partial reversal of islet inflammation (Fig. 1).

**Systemic variations in lymphoid lineages**

Comparative analysis of the variations in immune profiles of the treated groups attributes immunomodulation with FasL the dominant role in delay of disease progression. The composition of the pancreatic infiltrates to enhance the fractions of FoxP3 regulatory subsets might be modulated through various mechanisms: a direct effect of the ligand on all lymphoid organs or redistribution of cell subsets among various lymphoid compartments. We assessed possible redistribution from the contents of T and B cells in lymphoid tissues, which were quite stable in the pancreatic infiltrates under the various treatment protocols (Fig. 3A). Immunomodulation with both naïve and killer Treg cells resulted in increased CD8⁻ T cells with a compensatory decrease in B lymphocytes in the lymph nodes (P < 0.05, Fig. 3B), along with increased CD8⁺ T cells in the thymus (P < 0.01, Fig. 3D). In variance, the spleens of mice treated with killer Treg cells displayed increased CD4⁺ T-cell subsets compensated by decreased B lymphocytes (P < 0.05, Fig. 3C). All variations were observed at 3 weeks after cell infusion and persisted at the end-point analysis (6 weeks), consistent with early immunomodulatory activity of naïve and killer Treg cells. These data disclosed that Treg cells variably modulate the ratios between CD4⁺ and CD8⁺ T cells with a common decrease in B cells in the lymphoid organs, without apparent impact on the course of disease. Furthermore, corresponding changes suggest that the thymus might affect the composition of the mesenteric/pancreatic lymph nodes.

**The lymph nodes buffer and control pancreatic inflammation**

To determine the mechanism of action, labeled Treg cells (harvested from age-matched euglycemic donors) were traced in the mesenteric lymph nodes 1 day after infusion. Detection of these Treg cells in the lymph nodes of the diabetic NOD females demonstrates targeted traffic to the site of inflammation (Fig. 4A). The adoptively transferred Treg cells were found at higher rates than expected from incidental distribution, within the body, indicating targeted traffic to this site. Nevertheless, these cells constitute a minor fraction (~4%) of the FoxP3⁺ subset in the lymph nodes. The significant differences in the clinical and histological course of the disease (Fig. 1) and the variations in suppressor subsets between recipients of naïve and killer Treg cells (Fig. 2) indicate that enrichment of the local inflammatory environment with regulatory cells is a relatively insignificant mechanism of immunomodulation under our experimental conditions.

The proportionate variations in regulatory subsets in the pancreas and draining lymphatics and the stable distribution of lymphoid lineages within the pancreas suggest that the lymph nodes regulate cell traffic to the inflamed tissue. We, therefore, assessed the relative composition of CD4⁺ T cells in the mesenteric and pancreatic lymph nodes compared with the pancreatic infiltrates. A gradient of CD25 and FoxP3 expression between the mesenteric and pancreatic lymph nodes.
Fig. 2. Immune profiles of euglycemic, diabetic and treated NOD females. NOD females with spontaneous diabetes (diabetic, n = 16) were compared with age-matched euglycemic mice (n = 7), and at the experimental end-point of 6 weeks following treatment with naive Treg cells (n = 6) and killer Treg cells, the latter according to stable (n = 5) and progressive glycemic levels (n = 4) in peripheral blood. The CD4^+CD25^-FoxP3^- and CD4^+CD25^+FoxP3^+ fractions are presented in (A) pancreatic infiltrates, (B) mesenteric and pancreatic lymph nodes, (C) spleen, and (D) thymus. (E) Representative flow cytometric plots of CD25 and FoxP3 expression and detection of CD4^+FoxP3^- T cells in pancreata of spontaneously diabetic NOD females (upper) and good responders to treatment with killer Treg cells (lower, scale bar 40 μm). CD4^+FoxP3^- T cells in the right immunohistochemistry panels are stained in yellow by superposition of the red (CD4) and green (FoxP3) pseudocolored layers.
nodes of diabetic NOD females (Fig. 4B) suggests specific migration of these subsets toward the site of inflammation.

**Killer Treg cells deplete pathogenic cells at sites of inflammation**

The rationale of the approach based on enhanced killing activity of Treg cells is their use as vehicles for delivery of apoptotic signals to sites of inflammation. Following demonstration of direct migration, it was questioned whether these cells indeed reduce the pathogenic burden. In first stage, we assessed the presence of apoptotic cells within the mesenteric/pancreatic lymph nodes shortly after infusion of killer Treg cells. Over-expression of FasL protein caused a 3.5-fold increase in apoptotic cells (11.2 ± 2.3% versus 3.3 ± 1.4% with naive Treg cells, Fig. 4C), confirming that deletion of pathogenic cells by the apoptotic ligand is a major mechanism of disease suppression. We also assessed the diabetogenic activity of lymphocytes from the treated mice by adoptive transfer into NOD/SCID mice. Infusion of $2 \times 10^7$ cells (from spleen and lymph nodes) from new-onset NOD induced the disease in all NOD/SCID recipients with a mean onset time of 32 ± 14 days (Fig. 4D). Adoptive transfer from mice treated with naive Treg cells had a marginal effect on disease incidence (6 of 8) with similar onset time, but mice treated with killer Treg cells displayed markedly reduced pathogenic potential with a low incidence of disease transfer (3 of 14). Reduced efficacy of adoptive disease transfer is consistent with physical elimination of pathogenic cells, as a major mechanism of immunomodulation with killer Treg cells.

**Radiation abolishes the therapeutic activity of killer Treg cells**

We questioned whether targeted deletion of pathogenic cells might be enhanced by additional immunosuppression. Sublethal irradiation at 650 rad postponed the progression of blood glucose levels in new-onset NOD females; however,
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only one of 14 mice displayed stable disease after 6 weeks (Table 1). The same dominant impact of irradiation was observed in conjunction with killer Treg cell infusion; however, the slightly delayed disease progression in irradiated mice (26% stable glycemia) was accompanied by a reduced protective effect of killer Treg cells (56% stable glycemia). Therefore, similar to the negative impact of irradiation on naive Treg cells, generalized immunosuppressive therapy also abolishes the immunomodulatory activity of FasL.

Killer Treg cells affect thymic function

Whereas adoptive transfer of Treg cells did not affect thymic composition of regulatory subsets, immunomodulation with FasL caused a significant increase in thymic FoxP3 expression (Fig. 2D) that roughly correlated with the changes in composition of the pancreatic infiltrates and draining lymph nodes (Fig. 2A and B). Considering that attenuation of thymic output of regulatory subsets might participate in modulation of the course of disease at the systemic level, we monitored the impact of killer Treg cells on reconstitution of the thymus of NOD/SCID mice. Adoptive transfer of $2.5 \times 10^7$ CD4+CD25−T cells induced diabetes in 11 of 12 NOD/SCID mice, whereas all recipients of $2.5 \times 10^6$ FasL-coated CD4+CD25+T cells remained euglycemic (Fig. 5A). Co-adoptive transfer of effector and killer Treg cells (10:1 ratio) resulted in disease evolution in 60% of the NOD/SCID recipients with a mean onset time of 7.9 ± 1.5 weeks (Fig. 5A). In contrast, co-adoptive transfer of effector T cells and naive Treg cells ((10:1 ratio) did not prevent disease development. Immunophenotypes of the pancreatic infiltrates, mesenteric and pancreatic lymph nodes, spleen and thymus of the euglycemic mice showed a consistent increase in CD25 ($P < 0.01$ versus diabetic, Fig. 5B) and FoxP3...
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expression (P < 0.05 versus diabetic, Fig. 5C). The subset of naturally occurring CD4+CD25−FoxP3+ Treg cells was least affected in all organs including the thymus (Fig. 5D). Since these tissues are simultaneously reconstituted in NOD/SCID mice by adoptive transfer of effector and Treg cells, these data demonstrate that the apoptotic protein directly modulates the composition of peripheral lymphoid tissues and thymus.

Discussion

Data presented in this study demonstrate that Treg cells endowed with enhanced killing capacity attenuate the course of autoimmune insulitis in NOD females after the onset of hyperglycemia. Adoptive transfer of relatively small numbers of killer Treg cells from age-matched euglycemic NOD mice stabilized the blood glucose levels by increasing insulin levels and reduced the inflammatory scores of islets in approximately 50% of the treated mice. Our findings suggest a causal relationship between suppressor subsets in the pancreas, peripheral lymphoid organs and thymus, and the efficacy of immunomodulation by killer Treg cell therapy. Blood glucose levels are expected to remain high as there was no significant regeneration or neogenesis of β cells within the time frame of these experiments (19, 26).

The two therapeutic ingredients used in this study include Treg cells and FasL protein. Under our experimental conditions, naive Treg cells were consistently ineffective in attenuation of disease progression, inflammatory scores, and adoptive disease transfer into NOD/SCID mice. The only documented common impact of naive and killer Treg cells was modulation of the composition of peripheral lymphoid organs, consisting primarily of an increase in T-cell subsets and a compensatory decrease in B-cell fractions. The small fractions of adoptively transferred Treg cells within the FoxP3+ subset in the lymph nodes indicate that enrichment of the environment by the mere presence of additional Treg cells is not the major mechanism of immunomodulation. Therefore, the main therapeutic activity is attributed to the apoptotic protein, which operates through several mechanisms.

The first mechanism relies on effective migration of Treg cells to sites of inflammation and regional lymphatics, and physical elimination of pathogenic cells. A 3.5-fold increase
in fractional apoptosis within the mesenteric and pancreatic lymph nodes corresponded to slow disease progression in NOD mice treated with killer Treg cells and reduced efficiency of adoptive disease transfer into NOD/SCID mice. Treg cells isolated from NOD mice according to CD25 expression evolve as good vehicles for delivery of the apoptotic protein to the site of inflammation because they are relatively resistant to Fas cross-linking under stimulatory conditions (20). Apoptosis mediated by Fas cross-linking requires physical contact (27), which is characteristic of the mode of engagement of Treg cells (1–7). The activity of killer Treg cells against diabetogenic cells prompts the use of autologous Treg cells, which have already acquired antigen specificity (2, 28), particularly in the face of the frequent shifts in antigenic targets along the course of diabetic autoimmunity (29). However, one infusion of killer Treg cells at doses used in this study (equivalent to 1.6 × 10^6 cells kg^-1) is only partially effective against the autoimmune reaction and does not abolish the diabetogenic potential, as also seen in prediabetic mice (22). This early mechanism is limited in time, considering that FasL protein has a short life time in vivo, which is further shortened by active proliferation of Treg cells within inflammatory environments (22). To further accentuate the mechanism of pathogenic cell depletion, sub-lethal irradiation was applied prior to killer Treg cell infusion. Although radiation postponed the onset of hyperglycemia, it did not prevent delayed progression of destructive insulitis. The detrimental impact of non-selective immunosuppression on immunomodulation with FasL and on naive Treg cell infusion (3) is likely caused by superior recovery of effector cells from lymphopenia (30), particularly in the case of autoimmune insulitis (31). The second significant mechanism is modulation of the immune phenotypes, which is the likely cause of protracted amelioration of disease progression. The compensatory changes in T and B cells detected in the spleen and lymph nodes might reflect local modulation, redistribution of cells among the lymphoid organs or both. Relocation of cells was evident from the gradient of CD25 and FoxP3 subsets of CD4+ T cells from the mesenteric to the pancreatic lymph nodes, which buffer and determine the composition of the pancreatic infiltrates. Consistently, but in the opposite direction, the remarkable decrease in inflammatory scores emphasizes efflux of lymphocytes from the pancreas to the draining lymph nodes. Extending the significant role of the lymph nodes in initiation of the autoimmune reaction against the islets (32), these data delineate continued involvement of the draining lymph nodes in bidirectional traffic of immune cells from and to the pancreas.

Onset of diabetes was associated with an acute reduction in contents of CD4+FoxP3+ T cells in the pancreas and a reciprocal rise in the mesenteric and pancreatic lymph nodes, possibly a reaction of the lymph nodes to peak islet inflammation. Immunomodulation with Treg-targeted FasL protein induced sustained systemic variations with predominant FoxP3+ phenotype. We have previously demonstrated that the CD4+CD25+FoxP3+ subset mediates suppression (26); therefore, increased fractions of these cells in all lymphoid organs of good responders compared with poor responders suggest a causal relationship with disease resolution. Increased fractions of CD25-FoxP3+ Treg cells have been also observed in prediabetic NOD mice (22) and in an experimental model of chronic colitis treated with killer Treg cells (33). This Treg cell subset might be enriched in two ways. Mice immunomodulated with killer Treg cells showed increased contents of CD25-FoxP3+ T cells in the thymus, similar to simultaneous peripheral and thymic reconstitution of NOD/SCID mice after adoptive transfer of T cells. These cells emerging from the thymus are a pool of precursors that can adopt either suppressive (34) or effector functions under different conditions (35). CD25+ Treg cells that egress from the thymus acquire antigen specificity (27) and down-regulate or shed this receptor in the periphery, which is not required to sustain suppressor function (36–40). Alternatively, CD25-FoxP3+ Treg cells develop in the periphery from CD25+ precursors (41–43). Low CD25 expression in both potential mechanisms might be affected by the relative IL-2 deficiency in NOD mice in advanced stages of destructive insulitis (44).

The partial response of the treated mice was likely a result of insufficient numbers of killer Treg cells, an approach chosen for better characterization of the consequences of immunomodulation. Either repeated infusions of autologous Treg cells or their expansion (2, 45–47) might be applied to increase the numbers and enhance the therapeutic efficacy. Antigen-specific regulatory T cells circulate in peripheral blood of human subjects (48) and can be therefore easily harvested and manipulated. Even if this approach does not definitely abrogate the autoimmune reaction and additional measures such as induction of hematopoietic chimerism might be required (19), immediate arrest of the inflammatory reaction is essential to preserve tissue for subsequent regeneration of the β-cell mass (49).

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**References**

T cells prevent transfer of type 1 diabetes in NOD mice only when their antigen is present in vivo. J. Immunol. 181:4516.


