Animals in a Time-Dependent Manner and Breaks Tolerance in Male Nonobese Diabetic Animals

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The IL-2 Diphtheria Toxin Fusion Protein Denileukin Diftitox Modulates the Onset of Diabetes in Female Nonobese Diabetic Animals in a Time-Dependent Manner and Breaks Tolerance in Male Nonobese Diabetic Animals

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Denileukin diftitox, also known as DAB_389IL-2 or Ontak, is a fusion protein toxin consisting of the full-length sequence of the IL-2 protein and as toxophore the truncated diphtheria toxin. As a consequence, it delivers the toxic agent to CD25-bearing cells, whereby CD25 represents the high-affinity α-subunit of the IL-2 receptor. Initially it was developed for the treatment of patients with cutaneous T cell lymphoma. Meanwhile, denileukin diftitox is also used as an adjuvant in other tumor therapies and neoplastic disorders. In this study, to our knowledge we report for the first time that denileukin diftitox has also dramatic effects regarding the pathology of type 1 diabetes using the NOD mouse model.Repeated injections of denileukin diftitox into female NOD mice at 12 wk of age led to a clear acceleration of disease onset, whereas injection at 7 wk of age did not. Using male NOD mice, which are much less susceptible to diabetes, we demonstrate that the injection of denileukin diftitox leads to a dramatic development of type 1 diabetes within days after injection, thereby obviously breaking pre-existing tolerance mechanisms. This is accompanied by an increased IFN-γ production of autoreactive splenic cells and a decreased presence of regulatory CD4+CD25+ Foxp3 T cells. In contrast, transfer of CD4+CD25+Foxp3 T cells could correct the defect after denileukin diftitox treatment. Furthermore, whereas IFN-γ production was increased in the pancreata of treated animals, insulin expression was strongly reduced. These findings should be considered when denileukin diftitox is used for the treatment of patients suffering from tumors and/or autoimmune disorders. The Journal of Immunology, 2012, 189: 000–000.

The NOD mouse model is a spontaneous model for type 1 diabetes (T1D) (1). In humans as well as in NOD animals the insulin-producing β cells of the pancreas are destroyed during the course of the disease (2–5). In both male and female animals, islet Ags appear in the draining lymph nodes around 2–4 wk of age and sensitize autoreactive T effector cells, which subsequently infiltrate the islets. This is associated with a decrease of regulatory T cells (Tregs) in the pancreatic islets and draining pancreatic lymph nodes (6). Nonpathogenic autoantibodies against insulin are responsible for insulitis (7), but diabetes outcome is mainly provoked by T cell-mediated β cell islet destruction (8–10), accompanied by increased IFN-γ production of pathogenic CD4+ and CD8+ T cells. Presentation of autoantigens by APCs, such as dendritic cells, and the role of Tregs play an important role in the initiation and prevention of autoimmune insulitis (11, 12). Fatal influence on the outcome/control of the autoimmune T1D is attributed to CD4+CD25+Foxp3 Tregs (13–15). Mutations in Foxp3 (e.g., in X-linked syndrome patients) led to the development of T1D (16). Depletion of Tregs enhanced the development of T1D in mice (14). In contrast, an increase of Tregs by an IL-2 treatment reversed T1D in NOD mice (17). In addition to their CD4 and Foxp3 expression, Tregs are characterized by their highly increased CD25 expression, the high-affinity α-subunit of the IL-2 receptor.

Denileukin diftitox (DAB_389IL-2) is a recombinant fusion protein consisting of peptide sequences for the enzymatically active and membrane translocation domain of diphtheria toxin linked to human IL-2 (18). Denileukin diftitox specifically binds to CD25 on cell surface-expressed IL-2 receptor molecules, is then internalized via receptor-mediated endocytosis and inhibits protein synthesis by ADP ribosylation of elongation factor 2, finally resulting in cell death (19, 20).

In 1999, the U.S. Food and Drug Administration approved the denileukin diftitox treatment of cutaneous T cell lymphoma in humans. Additional oncologic use includes recurrent and refractory chronic lymphocytic leukemia, non-Hodgkin’s B cell lymphoma, and human T cell lymphotropic virus-1–associated adult T cell leukemia/lymphoma (21). Potential additional uses of denileukin diftitox include the therapy of graft-versus-host disease and autoimmune disorders such as psoriasis, rheumatoid arthritis, and systemic lupus (22, 23).

Previously it has been reported that denileukin diftitox leads to the depletion of human and murine Tregs in vitro and in vivo (24–26). Furthermore, it has been shown that administration of denileukin diftitox in combination with tumor vaccination decreased the number of Foxp3 Tregs in cancer patients, thereby increasing the antitumoral immune responses (27, 28). However, other investigators did not observe an elimination/reduction of regulatory

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Abbreviations used in this article: NOD.SCID, NOD.CB17-Prkdc[−/−]; TID, type 1 diabetes; Treg, regulatory T cell.

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T lymphocytes in melanoma patients (29). Thus, the precise immunomodulatory activity of denileukin diftitox especially in connection to vaccination strategies is currently not fully understood.

To investigate denileukin diftitox-mediated effects in more detail in vivo, the TID NOD model has been used in the present study. Female as well as male animals were treated with denileukin diftitox and the outcome of TID was assessed over a period of 30 wk. In parallel, immunological parameters including cytokine profiles as well as modulation of Tregs were assessed. In summary, this study led to the identification of new effects of denileukin diftitox that should be taken into account when this compound is used for the treatment of patients suffering from malignant or autoimmune disorders.

Materials and Methods

Animals

NOD/LtJ mice were housed and bred under specific pathogen-free conditions in the animal facilities of the Franz Penzoldt Center in Erlangen, Germany. For cotransfer experiments of splenocytes, NOD.CB17-Prkdcre/+/J (NOD.SCID) mice were purchased from Charles River. Treatment groups were established and compared with mock-treated control groups. Female animals were either treated at 7 wk of age with a dose of 5 × 3 μg denileukin diftitox on consecutive days or at 12 wk of age with a dose of 5 × 3 μg. The group of male animals received 5 × 3 μg denileukin diftitox at 7 wk of age and a second course of treatment (5 × 3 μg) at 12 wk of age. Injections were administered on consecutive days. Denileukin diftitox was diluted in PBS to a final volume of 200 μl and injected i.p. Mice were screened once a week for glucosuria to detect diabetes onset until the 30th wk of age (Diabur 5000; Roche Diagnostics, Mannheim, Germany). Male animals were screened from the 10th wk onward three times a week.

Denileukin diftitox

Denileukin diftitox is a recombinant cytotoxic protein composed of the amino acid sequences for diphteria toxin fragments A and B (Met.-Thr133)-His and the sequences for human IL-2 (Ala1-Thr133) and was purchased from Eisai Medical Research (Frankfurt, Germany) as a sterile solution (300 μg/ml).

ELISPOT analyses

ELISPOT assays were used to quantify Ag-specific IFN-γ release from activated spleen cells derived from denileukin diftitox-treated or mock-treated animals. Briefly, spleen cells were prepared from individual mice in ice-cold PBS. RBCs were lysed with 1.6% NH4-chloride solution for 5 min at 37˚C; splenocytes were washed with PBS and resuspended in ELISPOT medium (RPMI 1640; Lonza, Walkersville, MD). Duplicates of 3 × 10^5 cells/well in 200 μl ELISPOT medium were then plated out on anti-mouse IFN-γ mAb (AN18)-precoated, transparent ELISPOT plates purchased from Mabtech (Stockholm, Sweden). In advance, plates were washed four times with sterile PBS and blocked with ELISPOT medium containing 5% heat-inactivated FCS (PAA, Colbe, Germany) for a minimum of 1 h at room temperature. Plates were then incubated for 24 h at 37˚C in 5.5% CO2. After washing (5 × with PBS), biotinylated anti-mouse IFN-γ mAb (1 μg/ml; mAb R4-6A2-biotin [Mabtech]; diluted in PBS:0.5% FCS) was added, incubated for 2 h at room temperature, and immediately afterward washed 5 × with PBS. Then, streptavidin-alkaline phosphatase (Mabtech; diluted in PBS:0.5% FCS) was added at a 1:1000 dilution for 1 h at room temperature and plates were washed 5 × with PBS. For development, 0.1 ml ml developing solution 5-bromo-4-chloro-3-indolyl phosphate/NBT (BCIP/NBT Plus; Mos, Pasadena, MD) was added per well. The reaction was stopped by rinsing the wells in double distilled H2O. Spots were evaluated and counted using a computer-assisted video imaging system (Carl Zeiss Vision, Jena, Germany).

Autoantigens

Spleen cells (5 × 10^5) were used for the ELISPOT assays. Cells were stimulated with the NRP-A7 peptide (KYNKANAFL) (30, 31) and the MHC class I-restricted epitope as 15–23 of the murine insulin B chain (LYLVCGERG). The peptides were generated by simultaneous multiple synthesis with a Syro automated peptide synthesizer (MultiSynTec, Bochum, Germany) and added to the cultures at a final concentration of 40 μg/ml.

Immunofluorescence

Mice were sacrificed at diabetes onset, and cryostat sections (5 μm) were made from pancreata, fixed in ice-cold acetone for 10 min, and blocked using 1% BSA/PBS and subsequently using an avidin–biotin blocking kit (Vector Laboratories, Burlingame, CA). Slides were stained with a polyclonal anti-insulin antisemur (DakoCytomation, Glostrup, Denmark) followed by a polyclonal tetramethylrhodamine isothiocyanate-labeled rabbit anti-guinea pig antisemur (Sigma-Aldrich, Taufkirchen, Germany). To detect T cell subsets, an anti-CD4 mAb (clone GK1.5; BD Biosciences, Heidelberg, Germany) and an anti-CD8α mAb (clone 53-6.7; eBioscience, Frankfurt, Germany) were used as primary Abs. To visualize the bound Abs, slides were incubated with a biotinylated polyclonal goat antirat antisemur (Caltag-MedSystems, Buckingham, U.K.) followed by streptavidin-Alexa 488 (Molecular Probes/Invitrogen, Karlsruhe, Germany). Slides were analyzed using a fluorescence microscope (Zeiss Axiosvert 200M with AxiosCam MRC and the software AxioVision 4.7.1; Carl Zeiss, Oberkochen, Germany).

T cell purification

For adoptive transfer of CD4+CD25− and CD4+CD25+ T cells, cells were purified from healthy male NOD mice using a mouse CD4+CD25+ magnetic cell separation kit (Miltenyi Biotech) according to the manufacturer’s protocol. Cells were analyzed by flow cytometry for purity (>90%).

Adoptive cell transfers

To investigate whether splenocytes derived from diabetic male animals, which received denileukin diftitox (two times for 5 × 3 μg), are able to induce diabetes in healthy male NOD mice, spleens were removed after sacrifice and single-cell suspensions were prepared from individual mice in ice-cold PBS. RBCs were lysed with 1.6% NH4-chloride solution for 5 min at 37˚C. Splenocytes were washed with PBS and resuspended in cold PBS. For transfer experiments, 1 × 10^7 splenic cells were injected i.v. into the tail vein of healthy untreated, nonirradiated, 9-wk-old male NOD animals. At 16 wk of age one additional dose of denileukin diftitox (3 μg/mouse) was injected i.p. Additionally, cotransfer experiments of splenocytes derived from diabetic denileukin diftitox-treated (two times for 5 × 3 μg) and control mice with diabeticogenic splenocytes were performed. NOD.SCID recipients received either 1 × 10^7 diabetogenic splenic cells i.v. into the tail vein or splenocytes from control mice and diabeticogenic splenocytes at a ratio of 1:1 at 12 wk of age. Mice were scored weekly until manifestation of diabetes.

To investigate whether male NOD animals could be protected from diabetes after the application of DAB389IL-2, purified CD4+CD25− or CD4+CD25+ T cells (2 × 10^5) were transferred to diabetic, denileukin diftitox-treated animals (two times for 5 × 3 μg) 1 d after the last application of DAB389IL-2. All mice were screened for TID as indicated in the individual figures.

RT-PCR

Tissue samples were snap-frozen and stored at −80˚C until further use. Tissue was disrupted by two runs with a Bio101 Thermo Savant in a Precellys Keramik-Kit (PeQlab, Erlangen, Germany) containing 600 μl RLT lysis buffer from an RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). Total RNA was isolated using the RNeasy Mini Kit and QIAshredder spin columns (Qiagen). The cDNA synthesis was performed using a first-strand cDNA synthesis kit (Fermentas, St.-Leon-Rot, Germany) according to the manufacturer’s instructions. Real-time PCR using the LightCycler system (Roche Diagnostics) was performed in glass capillaries at a total volume of 20 μl in the presence of 10 μl 2X reaction buffer (Dynamo Capillary SYBR Green quantitative PCR Kit (Finnzymes, Espoo, Finland)) and 1 μl cDNA. The following primer pairs were used: insulin (sense, 5'-AGC ACT GGA GCT GGG AGG AA-3'; anti-sense, 5'-AAA TCG TGG TGG TGC AGT ACT GA-3'), hypoxanthine phosphoribosyltransferase (sense, 5'-GTT GGA TAC CGG AGC TAT GGT-3'; anti-sense, 5'-GAT TCA AGT TGC CAT CCT AGG-3'), IFN-γ (sense, 5'-AGC TCA GAT TGC ACG TGA CTC AG-3'; anti-sense, 5'-GTC ACA GTG TTC AGT GGT AAA G-3'), Foxp3 (sense, 5'-CCC AGG AAA GAC AGC AAC CTF-3'; anti-sense, 5'-CCT TGC TCT CAT CCA GGA-3'). The exact copy numbers were estimated using control plasmids harboring the corresponding inserts. The copy numbers were relativized by the copy numbers of the housekeeping gene hypoxanthine phosphoribosyltransferase.
**Isolation and FACS analyses of spleen and pancreatic lymph nodes**

Spleen and pancreatic lymph nodes from mock- and denileukin diftitox-treated animals (two times for $5 \times 3 \, \mu g$) were removed after the animals had been sacrificed and single-cells suspension were generated. Organs were disrupted with glass slides and resuspended in PBS (Lonza). Additionally, spleen cells were incubated for 5 min with 1.6% NH$_4$Cl to obtain complete depletion of erythrocytes. Cells were filtered using a cell strainer (BD Falcon), washed twice with PBS, and then used for FACS analyses. Directly fluorochrome-conjugated mAbs for the specific surface staining of CD4 (clone RM 4-5 FITC or PerCP) and CD25 (clone PC61 PerCP-Cy5.5 or 7D4 FITC) were purchased from BD Pharmingen. Cell surface staining was performed for 30 min on ice using a standard method previously described by Lutz et al. (32). Intracellular Foxp3 staining was performed according to the manufacturer’s instructions (Foxp3 anti-mouse/rat Foxp3 staining set; eBioscience).

**Statistical analysis**

Statistical calculation was performed with GraphPad Prism. The log-rank (Mantel–Cox) test was used for diabetes-free survival of the investigated groups. ELISPOT and FACS data were analyzed using the Student t test as well as the Mann–Whitney U test. RT-PCR experiments were analyzed with the Mann–Whitney U test. A p value of <0.05 was considered statistically significant.

**Results**

**Denileukin diftitox accelerates disease onset and progress in prediabetic female animals**

A good documented model to investigate T1D is the NOD mouse model, where insulin-producing β cells of Langerhans islets are destroyed during the course of disease. Approximately 60–80% of female animals develop hyperglycemia by 30 wk of age, whereas only 10–35% of male animals develop T1D. Because immune responses including Tregs play a crucial role in the control and development of T1D, we first assessed the effect of denileukin diftitox in female NOD animals. As shown in Fig. 1A, administration of denileukin diftitox at 7 wk of age had no major influence regarding disease onset and severity when compared with mock-treated animals. However, when animals were treated with denileukin diftitox at 12 wk of age a clearly accelerated disease onset could be observed (Fig. 1B). Additionally, all animals (100%) became diabetic within 10 wk after treatment (i.e., week 22). In contrast, mock-treated animals show slower disease progression and only 90% became diabetic until week 30.

**Denileukin diftitox induces severe T1D in male NOD animals and decreases the frequency of Tregs in spleen and pancreatic lymph nodes**

Next, the effect of denileukin diftitox was investigated in male NOD animals, which are more resistant to disease development. Thus, 3 µg denileukin diftitox was injected (i.p.) on 5 consecutive days twice at 7 and 12 wk of age. As shown in Fig. 2A, 22 of 29 male animals developed diabetes within 3 wk after the last denileukin diftitox injection (i.e., week 15). Three additional animals became diabetic until 22 wk of age and only four animals did not develop disease symptoms. Thus, 86% of male animals developed T1D. In sharp contrast, in the mock-treated control group ($n = 27$) a strongly reduced onset and disease severity was observed. Only 9 animals developed diabetes at all whereas 18 were free of disease symptoms at week 30. Thus, only 33% of animals developed T1D. As previously reported, administration of DAB$_{389}$IL-2 induces depletion of Tregs. Therefore, we were interested to investigate the possible effects of DAB$_{389}$IL-2 using the T1D animal model and analyzed the frequency of Tregs in the spleen and pancreatic lymph nodes by FACS (Fig. 2B). A significantly lower percentage of CD4$^+$CD25$^+$Foxp3$^+$ T cells could be detected in the spleen from DAB$_{389}$IL-2-treated animals in comparison with mock-treated nondiabetic animals. A similar but less pronounced effect could also be observed in the pancreatic lymph nodes.

**Denileukin diftitox induces a strong increase of IFN-γ-secreting splenocytes and reduced insulin and Foxp3 levels in the pancreas**

Th1-mediated immune responses play a crucial role in the development of T1D. Thus, we next investigated whether denileukin diftitox treatment modulates Th1-associated cytokine expression levels (i.e., IFN-γ) after stimulation with the T1D-specific auto-antigens NRP-A7 and insulin aas 15–23. ELISPOT analyses were performed showing a strongly increased frequency of IFN-γ-secreting splenocytes in denileukin diftitox-treated and diseased animals, whereas mock-treated healthy age-matched animals show no or very little IFN-γ secretion levels in response to the auto-antigens. As shown in Fig. 3A, an increased frequency of IFN-γ-secreting splenocytes could be observed after the incubation with NRP-A7 (upper panel) and to a lesser extent, but also significant, to insulin aas 15–23 (lower panel). These data clearly highlight the diabetogenic effect of denileukin diftitox. Furthermore, IL-4 production, one of the major cytokines inducing Th2 immune responses, was also analyzed. However, no IL-4 secretion was detectable after treatment with denileukin diftitox (data not shown).

To confirm and further extend these findings, real-time PCR analyses were performed to assess expression levels of genes that play an important role during the development of T1D. Thus, pancreata were removed 1) from healthy age-matched mock-treated animals, and 2) from denileukin diftitox (two times for...
that coincides with fewer Foxp3 + Tregs in denileukin diftitox-an immune deviation as a potential mechanism for denileukin (data not shown). Thus, from these data we have no evidence forferences between treated and untreated animals could be observedtranscripts were detectable only at very low levels, but no dif-IL-4 transcripts were not detectable in any of the animal groups. IL-10 transcripts were strongly reduced in denileukin diftitox-treated animals. Also, insulin-specific transcripts were detected in denileukin diftitox-treated animals in comparison to mock-treated animals. In contrast, insulin-specific transcripts were significantly reduced in the spleen of diabetogenic denileukin diftitox animals \( (n = 9) \) compared with the age-matched nondiabetic mock animals \( (n = 7) \). A less pronounced effect could also be observed in the pancreatic lymph nodes of diabetogenic denileukin diftitox animals \( (n = 6) \) compared with mock animals \( (n = 5) \). For statistical analyses the Mann–Whitney \( U \) test was used \( (p < 0.05) \). The experiment was performed three times, and data present represent a typical experiment. **\( p < 0.005 \), ***\( p < 0.0005 \).

5 \( \times \) 3 \( \mu \)g-treated animals with diabetes. Subsequently, mRNA copy numbers specific for IFN-\( \gamma \), insulin, and Foxp3 mRNA were determined (Fig. 3B). Statistically significant higher IFN-\( \gamma \) levels were detected in denileukin diftitox-treated animals in comparison to mock-treated animals. In contrast, insulin-specific transcripts were strongly reduced in denileukin diftitox-treated animals. Also, Foxp3-specific transcripts were significantly decreased in denileukin diftitox-treated animals when compared with the mock-treated group. Additionally, IL-4 as well as IL-10 transcripts were assayed by RT-PCR. Confirming the ELISPOT data, IL-4 transcripts were not detectable in any of the animal groups. IL-10 transcripts were detectable only at very low levels, but no differences between treated and untreated animals could be observed (data not shown). Thus, from these data we have no evidence for an immune deviation as a potential mechanism for denileukin diftitox.

RT-PCR data from the pancreas indicate an inflammatory milieu that coincides with fewer Foxp3 + Treg in denileukin diftitox-treated animals correlating with decreased insulin transcription levels. To extend these findings, serial sections of pancreatic tissue from denileukin diftitox-treated diabetic animals (two times for 5 \( \times \) 3 \( \mu \)g) and healthy mock-treated animals were stained with Abs specific for CD4, CD8, or insulin (Fig. 3C). Denileukin diftitox-treated animals were examined at the time of disease onset (i.e., 13 wk of age). Healthy mock-treated male animals (13 wk of age) show unaffected islets with strong insulin expression (Fig. 3C, rows 1 and 3). In contrast, denileukin diftitox-treated animals with diagnosed diabetes show strong infiltration of CD4 + and CD8 + T cells, accompanied by a decreased insulin expression and modulated islet architecture (Fig. 3C, rows 2 and 4). Thus, denileukin diftitox-treated animals show marked signs of insulitis.

Splenocytes from diabetogenic denileukin diftitox-treated mice transfer diabetes to NOD mice and NOD.SCID mice

To investigate whether splenocytes are able to transfer the disease, transfer experiments were performed: as donor cells, splenocytes derived from 13-wk-old diabetic denileukin diftitox-treated male mice (two times for 5 \( \times \) 3 \( \mu \)g at weeks 7 and 12) or splenocytes from mock-treated animals were used and injected into 9-wk-old male diabetes-free NOD animals (Fig. 4A). Splenic cells (\( 1 \times 10^7 \)) were transferred into recipient mice and, additionally, these animals were given a single dose of denileukin diftitox (3 \( \mu \)g) at 16 wk of age. The control group received spleen cells from healthy animals. Recipients that received diabetogenic spleen cells developed severe diabetes. By week 18, five of six animals were diabetic. The second group receiving spleen cells from healthy animals in combination with one dose of denileukin diftitox also developed disease symptoms, but onset and severity were clearly reduced. By week 21, three of six animals were diabetic. The control group receiving splenocytes from healthy animals only did not develop diabetes. These data clearly show that denileukin diftitox-induced diabetogenic spleen cells can, in combination with one dose of denileukin diftitox, induce diabetes in healthy male NOD mice.

To further investigate these effects and to support the hypothesis that Treg depletion is part of the mechanism, cotransfer experiments of splenocytes were performed using NOD.SCID animals. Thus, donor splenocytes derived from 13-wk-old diabetic deni-

**FIGURE 2.** DAB\(_{389}\)IL-2 induces severe T1D in male NOD animals and reduces the frequency of the CD4 +CD25 +Foxp3 + cell population. (A) DAB\(_{389}\)IL-2 (3 \( \mu \)g) was injected on 5 consecutive days twice a week at 7 and 12 wk of age. Twenty-two of 29 male animals developed diabetes within 3 wk after the last injection (i.e., week 15). Three additional animals became diabetic until week 22 of age. Thus, only 4 animals of 29 did not develop disease symptoms. In the mock-treated control group \( (n = 27) \) a strongly reduced onset and disease severity were observed. Only 9 animals developed diabetes at all, and 18 were free of disease symptoms at week 30. The log-rank (Mantel–Cox) test was used for statistical analyses. A \( p \) value of <0.05 was considered statistically significant. (B) Splenocytes and pancreatic lymph nodes from DAB\(_{389}\)IL-2–treated animals were harvested at the time of T1D onset and used for FACS analyses. Age-matched male animals were used as mock controls. Cells were gated on CD4 + cells. Representative flow cytometry dot plots from three in-
leukin diftitox-treated male mice (two times for 5 × 3 μg at weeks 7 and 12) were injected alone or in combination with splenocytes from mock-treated animals (ratio 1:1) into 12-wk-old male diabetes-free NOD.SCID mice. As expected, no evidence of suppression of diabetes could be observed (Fig. 4B).

Adoptive transfer of male CD4+CD25+ T cells suppresses T1D induction after treatment with denileukin diftitox

As shown above, the treatment with denileukin diftitox leads to a significant onset of T1D in male NOD mice accompanied by a reduction of CD4+CD25+Foxp3+ T cells in the spleen, pancreatic lymph nodes, as well as the pancreas. Therefore, we addressed the question of whether purified CD4+CD25+ Tregs of nondiabetic age-matched male NOD donors are able to correct the defect when transferred 24 h after the last denileukin diftitox treatment. This time point was chosen to exclude the damage of transferred cells by denileukin diftitox, as the biological half-life time of the drug is ~70–80 min. The control group received CD4+CD25− T effector cells from healthy donors. As shown in Fig. 5, CD4+CD25+ Tregs delayed and clearly reduced the onset of diabetes in denileukin diftitox-treated male NOD mice. In comparison, animals receiving CD4+CD25− T cells showed an increased diabetes incidence.

Repeated denileukin diftitox treatment leads to an increased reduction of Tregs and increased IFN-γ expression levels

It has been reported that denileukin diftitox treatment leads to reduced Treg numbers in cancer patients, thereby increasing an-
titumoral immune responses (27, 28). Thus, one possible explanation for the above-described severe autoimmunity might be the imbalance of Treg activity. Therefore, different animal groups were either treated with denileukin diftitox for 1, 3, or 5 d (3 mg each dose) or were mock treated. Subsequently, the percentage of Tregs present in the spleen was analyzed by FACS. These analyses revealed that the CD4+CD25+Foxp3+ cell population in the spleen was clearly reduced in a dose- and time-dependent manner when compared with mock-treated healthy animals (Fig. 6A, 6B).

To further investigate whether the reduction of Tregs influences the autoimmune response in the pancreas, real-time PCR analyses were performed. Thus, pancreata were removed 1) from healthy male mice at 12 wk of age, 2) from 1 mg denileukin diftitox-treated animals, 3) from 3 mg denileukin diftitox-treated animals, and 4) from 5 mg denileukin diftitox-treated animals. mRNA copy numbers specific for insulin and IFN-γ were determined (Fig. 6C). Concentration-dependent higher IFN-γ levels were detected in denileukin diftitox-treated animals in comparison with mock-treated animals. Insulin-specific transcripts were decreased in denileukin diftitox-treated animals. Thus, there is a clear correlation between decreased Treg numbers and enhanced autoimmune responses.

**Discussion**

The aim of the study was to investigate the immunomodulatory effect of denileukin diftitox in the NOD mouse model. Denileukin diftitox was developed to target CD25-expressing T cell in lymphoma. However, also in tumor vaccination trials denileukin diftitox has been reported to have beneficial effects in patients suffering from renal cell carcinoma and melanoma by switching...
off immune suppression and thereby increasing antitumoral immune response (27, 28, 33). However, other investigators could not observe such beneficial effect by denileukin diftitox (29). Furthermore, because denileukin diftitox could also deplete CD25+ tumor-reactive effector T cells, the precise mode of action, especially in connection to tumor vaccination, is still controversially discussed (25).

In the NOD model the imbalance between T effector cells and Tregs is a major mechanism for disease development (34, 35). In previous studies it has been reported that Treg depletion by anti-CD25 mAbs at different time points led to diabetes in NOD mice (36, 37). Other studies demonstrated diabetes induction after cyclophosphamid treatment (38, 39). Thus, immunomodulation is crucial for the development of T1D in NOD animals.

In the present study, we could clearly show that denileukin diftitox effects disease development in female as well as in male animals. Interestingly, when female animals were treated with denileukin diftitox during week 12 of age a much faster and more severe increased disease development could be observed. This could be explained by the depletion of Tregs at this later stage of disease and thus lack of control of T effector cells. However, when NOD female animals were treated during week 7 of age no major differences were observed between treated and mock-treated animals. Thus, at this earlier stage, Tregs do not seem to play the decisive role for T1D development under our experimental settings or, alternatively, are not amenable to denileukin diftitox-mediated depletion. At this time point other cells, such as APCs, may have a major impact. Thus, the modulation of individual immune cells at a particular time point of disease by denileukin diftitox seems to be very critical and may explain some of the differences observed in clinical tumor vaccination trials where denileukin diftitox treatment was included (27–29, 33).

In comparison with female NOD animals, in which at least 80% have clinically overt diabetes within 12–30 wk of age, male animals are innately more resistant to T1D and only 10–35% of them develop the disease. To investigate the effects of denileukin diftitox in male animals the drug was applied at 7 and 12 wk of age. A very fast onset of diabetes paralleled by a dramatic increase of disease severity could be observed in denileukin diftitox-treated male animals. Because male animals are more resistant and under normal physiological conditions tolerance mechanisms are able to control T1D in most animals, denileukin diftitox is obviously able to break these tolerogenic mechanisms by depleting CD25+ Tregs. These results correlate with data published by Litzinger et al. (24) reporting that depletion of Tregs in denileukin diftitox-treated C57BL/6 animals led to an enhanced vaccination-mediated T cell immunity. In the present study, an increased immune response against autoantigens could be observed. The augmented autoantigen-specific IFN-γ production correlated with decreased insulin expression levels in the pancreas of denileukin diftitox-treated animals.

**FIGURE 6.** Repeated DAB389IL-2 treatments led to the reduction of Tregs and increased IFN-γ expression levels. (A and B) Animal groups were either treated with DAB389IL-2 for 1, 3, or 5 days (3 μg/d) or were mock treated. Subsequently, the percentage of Tregs in spleen was analyzed by FACS, revealing that the CD4+CD25+Foxp3+ cell population in the spleen was clearly reduced in a dose- and time-dependent manner when compared with mock-treated healthy animals. (C) Real-time PCR analyses: pancreata were removed from healthy male mice at 12 wk of age, from 1 × 3 μg-treated animals, from 3 × 3 μg-treated animals, and from 5 × 3 μg-treated animals. Concentration-dependent higher IFN-γ levels were detected in DAB389IL-2-treated animals in comparison with mock-treated animals. Insulin-specific transcripts were also decreased in DAB389IL-2-treated animals.
However, these effects could only be observed when animals were treated with denileukin diftitox at 7 and 12 wk of age. Single treatment of male NOD mice during week 7 induced diabetes only in a minor percentage of the animals (<15%; data not shown). In contrast, single treatment during week 12 induced diabetes in ~50% of the animals. Thus, from these data we conclude that both time points are needed to induce diabetes at high rates. Double treatment, however, did not induce signs of peripheral neuropathy.

Interestingly, in a study reported by Billiard et al. (36) where T cell depletion was performed using an anti-CD25 mAb, an exacerbation of diabetes could only be observed when depletion was performed at an early stage of the disease (i.e., week 3 or 4). Among other experimental details, the observed differences may be due to the use of an anti-CD25 mAb in comparison with denileukin diftitox, which is an IL-2 diphtheria toxin fusion protein, with different pharmacological properties. However, the importance of timing by Treg-depleting substances for immunomodulation is again highlighted by these findings and should be considered in the design of clinical studies.

Islet-specific T cells are necessary to induce diabetes. Thus, spleen cells from denileukin diftitox-induced diabetic male mice were injected into healthy male animals, demonstrating that diabetogenic spleen cells can transfer diabetes in combination with one additional dose of denileukin diftitox. This single dose of denileukin diftitox is probably necessary since whole spleen cell populations were transferred whereby islet-specific T cells are present among other cell types. Additionally, when spleen cells derived from denileukin diftitox-induced diabetic male mice were injected into healthy age-matched male NOD.SCID mice, a rapid incidence of diabetic onset starting at 5 wk after cotransfer could be observed, indicating that Tregs play a mechanistic role.

In a former study it has been reported that a variant of denileukin diftitox, that is, DAB489IL-2, blocks diabetogenic autoimmunity in NOD mice (40). However, to induce diabetes the authors adoptively transferred diabetogenic spleen cells into irradiated recipients. Treatment with DAB489IL-2 started at the day of cell transfer and continued for 4 wk. Thus, adoptively transferred diabetogenic spleen cells are most likely deleted by this treatment, and therefore animals were protected from diabetes for a limited time period. The obvious experimental differences explain the diverging results observed by these authors.

Time of treatment and dosing of denileukin diftitox are very crucial. In this respect we could show that deletion of Foxp3+ Tregs is more efficient when five doses are applied in comparison with three or one. This correlates also with the induction of islet-specific immune responses, because animals receiving five injections showed a dose-dependent increase of insulin-specific IFN-γ production that correlated with reduced insulin expression levels. This indicates that at later time points denileukin diftitox affects mainly Tregs expressing CD25 at high levels and not primarily T effector cells. At earlier time points, however, APCs such as dendritic cells also expressing CD25 and T effector cells could also be affected.

Taken together, in this study we could show that denileukin diftitox treatment has different effects in female NOD animals depending on the time of application; that is, at early time points it does not greatly modulate disease development, whereas applied at a later time point it clearly increases diabetes, indicating that at later time points Tregs play a major role in controlling diabetogenic T cells. Furthermore, we report that denileukin diftitox induces fast and severe disease progression in male NOD animals, which normally are more resistant to T1D. This is also most likely due to the loss of Tregs, which under physiological conditions orchestrate tolerogenic mechanisms inhibiting autoimmunity. In summary, it became clear that time of administration and dosing are two very important factors that should be taken into consideration for the future design of clinical studies including denileukin diftitox treatments.

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References


