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DAB₃₈₉IL-2 recombinant fusion toxin effect on lymphocyte- and macrophage- producing cytokine subpopulation cells in experimentally induced demyelinating disease in mice

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Abstract

Context—We have reported previously that DAB₃₈₉IL-2 recombinant fusion toxin targets IL-2R bearing CD4⁺ cells, and suppresses demyelinating disease in acute (A) - and chronic (C) - experimental autoimmune encephalomyelitis (EAE) animal models of multiple sclerosis.

Objectives—The present study was undertaken to investigate the effect of DAB₃₈₉IL-2 treatment on various cytokine-secreting cell populations in A-EAE and C-EAE mice.

Materials and methods—The effects of DAB₃₈₉IL-2 at doses of 200-, 800-, or 1600 kU administered i.v. on days 11–13 and 15 on the clinical score and cytokine-secreting cell populations were examined using flow cytometry.

Results—C-EAE mice treated with 1600 kU DAB₃₈₉IL-2, but not A-EAE mice treated with 800 kU had significantly reduced disease. The CD3⁺CD25⁺ sub-population in spleens and spinal cords of A-EAE mice treated with 800 kU DAB₃₈₉IL-2 was increased, whereas in C-EAE mice treated with 1600 kU this population was decreased. DAB₃₈₉IL-2 treatment reduced CD3⁺CD4⁺, CD3⁺CD8⁺, CD4⁺CD8⁺, CD3⁺IL-2⁺, CD3⁺IFN- γ ⁺ and CD3⁺TNF- α ⁺ T cell subpopulations in the spinal cord in A-EAE, and C-EAE mice on day 16. CD11b⁺ macrophages that were IL-2-, IFN- γ -, and TNF- α -positive were reduced in A-EAE mice. DAB₃₈₉IL-2 treatment reduced CD19⁺ B-cells positive for IL-2 or CD11b⁺ in the spinal cord in acute and chronic disease. DAB₃₈₉IL-2 treatment also reduced lymph node CD3⁺CD8⁺, CD4⁺CD8⁺, CD3⁺CD25⁺ populations on day 16, and lymph node CD3⁺IL-10⁺ and peripheral blood CD3⁺CD25⁺ populations on day 24.

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Disclosure statement

The authors have declared that they have no conflicts of interest.

Discussion and conclusions—Our study demonstrates that DAB₃₈₉IL-2 fusion toxin suppresses EAE in a dose-dependent manner, and alters inflammatory cell sub-populations during disease development.

Keywords

DT; diphtheria toxin; IL-2R; interleukin-2 receptor; EAE; experimental autoimmune encephalomyelitis; MS; multiple sclerosis; DAB₃₈₉IL-2

Introduction

Multiple sclerosis (MS) is an inflammatory disorder of the brain and spinal cord in which focal lymphocytic infiltration leads to loss and dysfunction of myelin and axons. Commonly used drugs for treatment of multiple sclerosis (MS) are interferon-beta1b, interferon-beta1a, glatiramer acetate, natalizumab and mitoxantrone. Treatments for MS have different side effects, but the biomarker profile may help to elucidate disease subtypes to therapy at cellular and humoral levels of the immune response¹⁻⁴. Experimental autoimmune encephalomyelitis (EAE) serves as an animal model for the human disease, multiple sclerosis (MS) in which pathological features of inflammation, demyelination, axonal loss and gliosis are studied. The rodent model has been used extensively for the preclinical testing of newer drugs for MS and studies for potential therapeutic interventions⁵.

In acute and relapsing MS patients, the disease response is contingent upon activating effector phenotype CD4⁺ and CD8⁺ T cells. The disease process starts with increased migration of CNS antigen-specific lymphocytes across the blood–brain barrier, leading to an immune response within the brain, and eventually to the formation of pathogenic demyelinated plaques. CD4⁺CD25^{hi} regulatory T cells demonstrated a significant decrease in the peripheral blood in MS patients. CD8⁺CD25⁺ FoxP3⁺ cells have a significant role on CD4⁺ T-cell regulation during the course of MS⁶⁻⁷. Interferon- γ secreting cells and T-lymphocyte subtypes play an important role in activating interleukin-17 under interleukin-23 control. IL-23–dependent CD4⁺ T cells are highly pathogenic and essential for maintaining central nervous system autoimmunity⁸, participating in disruption of the human blood–brain barrier and damaging or killing neurons, however, both IL-17⁺CD4⁺ and CD8⁺ T cells play an important role in MS pathogenesis⁹⁻¹⁰. Myelin-specific T cells isolated from peripheral blood of MS patients are in an enhanced state of activation, expressing the high affinity IL-2R. These IL-2 responsive activated T cells can migrate to the CNS and give rise to pro-inflammatory responses to myelin epitopes.

DAB₃₈₉IL-2, diphtheria toxin IL-2 fusion protein, is an FDA-approved drug for the treatment of cancer and autoimmune diseases¹¹⁻¹⁶. The mode of DAB₃₈₉IL-2 action is to kill IL-2R-expressing CD4⁺ cells. Our previous studies demonstrated the clinical efficacy of DAB₃₈₉IL-2 in acute EAE (A-EAE), relapsing EAE (R-EAE) and chronic EAE (C-EAE), mouse models that correspond to different courses of MS in humans¹⁷. Our previous report has shown that DAB₃₈₉IL-2 indirectly suppresses the activation of macrophage CD169⁺ (ED3⁺) and microglia CD11b/c (OX42⁺) populations in the CNS of mice that with EAE¹⁸. Denileukin diftitox, a diphtheria toxin fragment IL-2 fusion protein, targets and kills CD25

(⁺) cells¹⁹ Recently, a newly constructed diphtheria toxin DT₃₉₀ porcine IL-2 truncated fusion toxin (Bi-NonGly) has also shown great potential for *in vivo* depletion of porcine CD25⁺ cells.²⁰ Bivalent human IL-2 fusion toxin has proved to be more potent than the current, clinically-used monovalent fusion toxin (denileukin diftitox, Ontak®) for *in vivo* depletion of Treg cells^{21–24}.

In this study, we have focused on the effect of the drug DAB₃₈₉IL-2 on the cellular activation of lymphocyte- and macrophage-producing cytokine subpopulation cells in the peripheral immune system and the CNS in mild acute and chronic EAE at the early and late phases of demyelinating disease.

Materials and methods

Animals

Ten week-old SJL and twelve week SJL × PLJ F1 female mice were used for the experiment as shown in the Table 1. These mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained according to the guidelines set forth by the Institutional Animal Care and Use Committee. Two experimental animal models were used: an acute disease form of EAE characterized by spontaneous recovery and low mortality rate, and is the other a chronic disease form of EAE with a recurring clinical course and also low mortality rate.

Acute EAE induction in mice

Acute EAE (A-EAE) was induced in a group of twenty 10-week-old SJL female mice by immunizing them with 400 µg myelin basic protein purified from guinea pig spinal cord (gpMBP)¹⁷.

Chronic EAE induction in mice

Chronic EAE (C-EAE) was induced in a group of forty 12-week-old female (SJL × PLJ) F1 mice with 5 mg mouse spinal cord homogenate (mSCH) as described previously¹⁷.

Antigens were injected into the rear footpads. Mice received 200 ng of *B. pertussis* toxin (Biological Laboratory) (10 µg/kg b.w.), in PBS i.p. 24 h after the first immunization and 48 h later.

Both acute and chronic EAE models showed mild types of clinical disease in our study.

DAB₃₈₉IL-2

DAB₃₈₉IL-2 was stored at −70 °C and freshly diluted before use with Tris buffered saline (20 mM Tris, 150 mM NaCl, and pH 8.2). EAE induced mice were then evenly divided into groups for DAB₃₈₉IL-2 or PBS treatments. DAB₃₈₉IL-2 (IL-2 DAB) in PBS was injected via the tail vein at a dose of 200 kU (equivalent to 40 µg/kg body wt.); 800 kU (equivalent to 160 µg/kg body wt.); 1600 kU (equivalent to 320 µg/kg body wt.) for A-EAE and C-EAE induced mice and phosphate buffered saline (PBS) solution was administered equal volume to EAE mice serving as controls in each experiment as shown below:

*** DAB₃₈₉IL-2 treatment in mice with A-EAE and C-EAE**

EAE model	No animal	Treatment Dose	Study period (Days)	Day animal sacrificed
<i>Clinical Score study</i>				
A-EAE				
PBS	6	1000 µl	12–30	
DAB ₃₈₉ IL-2	6	800 kU	12–30	
C-EAE				
PBS	6	1000 µl	10–35	
DAB ₃₈₉ IL-2	6	200 kU	10–35	
DAB ₃₈₉ IL-2	6	1600 kU	10–35	
<i>Flow cytometry study</i>				
A-EAE				
PBS	10	1000 µl		21
DAB ₃₈₉ IL-2	10	800 kU		21
C-EAE				
PBS	10	1000 µl		16
DAB ₃₈₉ IL-2	10	1600 kU		16
C-EAE				
PBS	10	1000 µl		24
DAB ₃₈₉ IL-2	10	1600 kU		24

* Mice were injected i.v. route with PBS or DAB₃₈₉IL-2 in 1000 µl volume of PBS (20mM Tris, 150mM NaCl and pH 8.2) on days 11, 12, 13 and 15.

Clinical evaluation

A daily clinical score was obtained based on a previously reported scale¹⁷ that included the following parameters: 0 = normal; 1 = tail paralysis or tail paralysis; 2 = hind leg weakness; 3 = hind limb paralysis; 4 = fore and hind limb paralysis; 5 = death. Two trained and blinded researchers made the evaluations. Clinical scores of non-sacrificed mice from acute EAE and chronic EAE continued to be observed until day 30 and 35, respectively.

Isolation and characterization of spleen, lymph nodes, peripheral blood and infiltrating cells from spinal cord, of EAE induced mice

Mice with acute EAE were sacrificed at day 21 to study the long term effect of DAB₃₈₉IL-2. Mice with chronic EAE were sacrificed at day 16 to study a short term effect, and at day 24 post-immunization (p.i.) to study the long term effect of DAB₃₈₉IL-2. Peripheral blood was collected from the mouse heart with a heparinized syringe, and blood samples were pooled in the tube. Spleen and lymph nodes were removed separately and stored in ice-cold RPMI-1640 (Life Technologies, Gaithersburg, MD) containing 2% FCS (Sigma). Mononuclear cells (MNC) from spinal cords were collected in RPMI-1640 medium containing 2% FCS as per the method previously described¹⁷.

Immunostaining and FACS analysis

Fluorescein isothiocyanate (FITC)-labeled anti-rat CD3 (mouse IgG₃, G4.18 clone), CD4 (mouse IgG_{2a}, OX-35), CD8 (mouse IgG₁, OX-8) mAbs, phycoerythrin (PE)-labeled anti-rat $\alpha\beta$ TCR (mouse IgG₁, R73), FITC-labeled anti-rat IL-2, IFN γ , TNF- α , IL-4 and IL-10 from PharMingen (San Diego, CA), and biotinylated anti-rat CD3, CD4, CD8, CD25 (mouse IgG₁, OX39) mAbs were purchased from PharMingen (San Diego, CA). FITC-labeled anti-rat CD11b (mouse IgG_{2a}, OX42) and CD25 mAbs, FITC-fluorochrome tagged antibody IL-2, IFN- γ , TNF- α , IL-4 and IL-10 from PharMingen, San Diego, CA; PE labeled anti-rat CD11b and B cell (mouse IgG_{2a}, RLN-9D3) mAbs were purchased from Caltag Laboratories (Burlingame, CA), and PerCP-labeled anti-rat CD19 from PharMingen (San Diego, CA). Analysis of T cells, mainly CD4⁺, CD8⁺ and CD3⁺, macrophages and B cells with sub-populations were performed on a Becton–Dickinson FACScan instrument. Typically 1×10^6 cells were incubated for 45–60 min on ice with 1 μ g of FITC-, biotin-and PE-Cy5- labeled antibodies in 0.1 ml of staining buffer (PBS+BSA+0.1% sodium azide). Flow cytometer instrument was calibrated with beads to minimize background signals for FL-1, FL-2 and F-3 to align with corresponding unlabelled populations. The cells stained with FITC, PE and PerCP fluorescence antibodies. Cells were stained with isotype antibodies to assess levels of nonspecific staining in the cells and 10,000 data events were analyzed by flow cytometry. Dead cells were excluded by selective gating. Data files were analyzed using CellQuest software (Becton Dickinson, San Jose, CA).

Acquisition and analyses of spleen, lymph node, peripheral blood and spinal cord-derived lymphocytes

Data were acquired using a FACS flow cytometer FACScan (Becton Dickinson, San Jose, CA). During sample data acquisition 30,000 to 50,000 events were acquired in a user-defined gate morphologically tailored for lymphocytes. Data analyses were performed using (CellQuest) FACS software, and different data management procedures were applied depending on sample staining. Mean of percentage of cell population was determined in two individual samples of each, and PBS and DAB₃₈₉IL-2 samples were compared.

Statistics

The sum of total clinical score of PBS and DAB₃₈₉IL-2 treated groups were compared statistically with ANOVA paired parametric t' test of statistical significance two-tailed p value at <0.05 by GraphPad software (La Jolla, CA).

The percentage of each cell population was converted into $\log_2$ from Tables 2–4 where ' r ' value was determined by the Correlation Coefficient Calculator. Statistical formula: $\text{Correlation}(r) = \frac{N\sum XY - (\sum X)(\sum Y)}{\sqrt{[N\sum X^2 - (\sum X)^2][N\sum Y^2 - (\sum Y)^2]}}$ was used to determine the correlation coefficient between the PBS and DAB₃₈₉IL-2 variable values of X and Y. *Correlation coefficient* was used to measure the strength of a linear association between two variables, where the value $r = 1$ means a perfect positive correlation and the value $r = -1$ means a perfect negative correlation.

Statistical t' test (paired samples) was applied using Linear regression (R_2) analysis at $p < 0.05$ in straight-line relationship between individual $\log_2$ value of individual cell

population in PBS and DAB₃₈₉IL-2 treated groups. R₂ statistical relationship between two variable y against that of the independent variable x was determined by GraphPad Software using QuickCalcs by Linear regression calculator.

Results

Effect of DAB₃₈₉IL-2 treatment on the clinical course of acute EAE

There were no statistically significant differences between the mean clinical scores on days 12–30 of the gp-MBP-immunized acute EAE (A-EAE) mice treated with DAB₃₈₉IL-2 at a dose of 800 kU or equivalent volumes of PBS on the same days (PBS-treated: 0.0917 ± 0.0509 vs. DAB₃₈₉IL-2-treated: 0.1194 ± 0.0426, $p = .5747$). Clinical scores, however, were very low in both groups (Table 1).

Effect of DAB₃₈₉IL-2 treatment on the clinical course of chronic EAE

DAB₃₈₉IL-2 significantly suppressed the intensity of clinical score at 1600 kU and delayed disease onset (Table 1; Figure 1). The mean clinical score on days 10–35 of DAB₃₈₉IL-2 treated mice was reduced compared to PBS-treated animals (PBS-treated: 0.9192 ± 0.1551 vs. DAB₃₈₉IL-2 treated: 0.0300 ± 0.0166; $p = 0.0001$), whereas a lower dose of 200 kU DAB₃₈₉IL-2 did not suppress clinical disease (PBS-treated: 0.91920 ± 0.15508 vs. DAB₃₈₉IL-2 treated: 0.87860 ± 0.16041; $p = .7942$).

Effect of DAB₃₈₉IL-2 treatment on percentages and inflammatory cytokine expression of immune cells in the spleen and spinal cord of mice with acute EAE

The aim of this experiment was to investigate the effect of DAB₃₈₉IL-2 treatment on T-, B-, and macrophage cells and sub-populations in the spleen and spinal cord at the onset of disease.

Flow cytometry results showed that there was not a drastic change in CD3⁺ cell percentages in the spleen of PBS-treated (52.10%) and DAB₃₈₉IL-2 (53.36%) treated A-EAE mice. DAB₃₈₉IL-2 treated A-EAE mice showed a decrease in CD3⁺CD8⁺ and CD4⁺CD8⁺ sub-population; however, the CD3⁺CD4⁺ sub-population was not remarkable changed in PBS-treated (38.47%) and DAB₃₈₉IL-2-treated (38.91%) A-EAE mice. On the contrary, the CD3⁺CD25⁺ sub-population was increased from 37.58% to 49.93% in DAB₃₈₉IL-2 treated A-EAE mice. CD3⁺IL-2⁺, CD11b⁺IL-2⁺, CD3⁺IFN γ ⁺, CD11b⁺IFN γ ⁺ and CD3⁺TNF- α ⁺ sub-populations were decreased in DAB₃₈₉IL-2 treated A-EAE mice, while CD11b⁺TNF- α ⁺ and CD11b⁺IL-4⁺ sub-populations were slightly increased in DAB₃₈₉IL-2 treated mice compared to PBS-treated A-EAE mice (Table 2).

Percentage of CD3⁺, CD4⁺ cell populations in total mononuclear cells and Th1 type cytokines IL-2⁺, IFN- γ ⁺ and TNF- α ⁺ cell populations from the spinal cord MNC were decreased in DAB₃₈₉IL-2 treated compared to PBS-treated A-EAE mice (Figure 2). There was a decrease in the CD3⁺CD8⁺ sub-population from 26.89% to 19.50% in DAB₃₈₉IL-2 treated mice compared to PBS-treated A-EAE mice. CD3⁺CD25⁺ sub-population was increased from 2.12 to 5.15% in DAB₃₈₉IL-2 treated A-EAE mice.

The CD4⁺CD8⁺ sub-population was decreased from 28.68% to 21.26%. There were also decreased in CD3⁺CD4⁺, CD3⁺IL-2, CD11b⁺IL-2⁺, CD11b⁺TNF- α ⁺ and CD11b⁺B7.1⁺ sub-populations in the spinal cord due to the effect of DAB₃₈₉IL-2 treatment (Table 2).

The sum of total *Log*₂ converted sub-populations of T-, B-, and macrophage cells in PBS and DAB₃₈₉IL-2 treated mice were compared. Regression analysis showed a significant decrease in cell population in the spleen ($R_2=0.5048$; $r=0.71051$; $p<.0044$), and in the spinal cord ($R_2=0.5281$; $r=0.726$; $p<.0021$) of DAB₃₈₉IL-2 treated A-EAE mice.

Effect of DAB₃₈₉IL-2 treatment on percentages and inflammatory cytokine expression of immune cells in the spleen, lymph nodes and spinal cord of mice with chronic EAE on day 16

The aim of this experiment was to investigate the effect of DAB₃₈₉IL-2 treatment on T-, B-, and macrophage cells and sub-populations in the spleen, lymph nodes and spinal cord at the onset of disease. FACS analysis showed changes in T cell sub-populations and cytokine-secreting cells due to the effect of DAB₃₈₉IL-2 treatment in C-EAE mice compared to PBS-treated mice, as shown in Table 3. CD3⁺ and CD3⁺CD4⁺ and CD3⁺CD8⁺ sub-populations were increased in the spleen of DAB₃₈₉IL-2 treated mice.

CD3⁺, CD25 and CD3⁺CD4⁺ cell populations were not greatly changed in lymph node cells of PBS and DAB₃₈₉IL-2 treated mice. CD3⁺CD8⁺, CD4⁺CD8⁺ and CD3⁺CD25⁺ sub-populations were highly decreased so also CD3⁺IL-2⁺, CD3⁺IFN- γ ⁺ and CD3⁺TNF- α ⁺ sub-populations in DAB₃₈₉IL-2 treated C-EAE mice at day 16 (Table 3).

A comparative flow cytometry analysis study of the CD3⁺CD25⁺ cell population showed a decrease in lymph nodes, spleen and spinal cord on day 16 of C-EAE mice treated with DAB₃₈₉IL-2 (Figure 3).

In the spinal cord, CD19⁺IL-2⁺, CD19⁺IFN- γ ⁺ and CD19⁺TNF- α ⁺ cell sub-populations of MNC cells were decreased in DAB₃₈₉IL-2 treated C-EAE mice on day 16 (Figure 4). CD3⁺CD4⁺, CD3⁺CD8⁺ and CD4⁺CD8⁺, CD3⁺IL-2⁺, CD3⁺IFN- γ ⁺ and CD3⁺TNF- α ⁺ sub-populations were also decreased remarkably in DAB₃₈₉IL-2 treated compared to PBS-treated C-EAE mice (Table 3).

The sum of total T-, B-, and macrophage related cell and sub-populations of PBS and DAB₃₈₉IL-2 treated mice were compared using regression analysis in C-EAE mice groups at day 16. Percent cell population is converted to *Log*₂ in DAB₃₈₉IL-2 treated mice were decreased in spleen ($R_2=0.7542$; $r=0.84374$; $p<.0002$), lymph nodes ($R_2=0.5471$; $r=0.71891$; $p<.0060$) and spinal cord ($R_2=0.5553$; $r=0.74518$; $p<.0054$) of DAB₃₈₉IL-2 treated mice compared to PBS-treated mice.

Effect of DAB₃₈₉IL-2 treatment on percentages and inflammatory cytokine expression of immune cells in the spleen, lymph nodes, spinal cord and peripheral blood of mice with chronic EAE on day 24

The aim of this experiment was to demonstrate an effect of DAB₃₈₉IL-2 treatment on T-, B-, and macrophage related cell and sub-populations in the spleen, lymph nodes, spinal cord and the peripheral blood during the recovery phase (late) of disease.

CD3 and sub-populations CD3⁺CD4⁺ and CD3⁺IL-4⁺ were increased, CD3⁺CD8⁺ were not much changed whereas CD3⁺IL-2⁺ were decreased in the spleen of DAB₃₈₉IL-2 treated C-EAE mice compared to PBS-treated C-EAE mice at day 24 (Table 4).

Remarkably, the CD3⁺IL-10⁺ cell sub-population was also decreased in lymph nodes in DAB₃₈₉IL-2-treated mice on day 24 (Figure 5). Both CD3⁺CD4⁺ and CD3⁺CD8⁺ sub-populations were drastically increased while CD3⁺IFN γ ⁺ and CD11b⁺IFN- γ ⁺ sub-populations were decreased in lymph node cells in DAB₃₈₉IL-2 treated mice (Table 4).

In the spinal cord, CD3 and sub-populations CD3⁺CD4⁺, CD3⁺CD8⁺ and CD4⁺CD8⁺ were decreased in MNC of in DAB₃₈₉IL-2 treated compared to PBS-treated mice (Table 4). On the contrary CD3⁺IFN- γ ⁺, CD3⁺IL-2⁺, CD3⁺TNF- α ⁺ sub-populations were remarkably increased in DAB₃₈₉IL-2 treated compared to PBS treated C-EAE mice (Table 4; Figure 6).

In the peripheral blood samples at the recovery phase of disease in C-EAE mice, CD3⁺ cell population in PBS (56.8%) and DAB₃₈₉IL-2 (58.0%) and also CD3⁺CD4⁺ and CD3⁺CD8⁺ sub-population were not much changed. However, the CD3⁺CD25⁺ sub-population was decreased from 42.2% to 3.5% in DAB₃₈₉IL-2 treated mice. On the contrary, CD3⁺IL-2⁺ subpopulations were increased in DAB₃₈₉IL-2 treated compared to PBS treated mice. CD3⁺IFN γ ⁺, CD11b⁺IFN γ ⁺, CD3⁺TNF- α ⁺, CD11b⁺TNF- α ⁺, CD11b⁺B7.2⁺, and CD3⁺IL-4⁺ sub-populations were decreased. However, CD11b⁺B7.1⁺ sub-population was highly decreased. CD11b⁺IL-2⁺ and CD11b⁺IL-10⁺ sub-populations were increased, whereas CD3⁺IL-10⁺ sub-populations were remarkably decreased in DAB₃₈₉IL-2 treated mice (Table 4).

The sum of total percent converted into Log_2 cell population of T-, B-, and macrophage related cells and sub-populations in peripheral blood of PBS and DAB₃₈₉IL-2 treated C-EAE mice were compared at day 24. Regression analysis revealed a significant decrease in these populations in peripheral blood ($R_2=0.5110$; $r=0.71482$; $p<.0009$) whereas there was no significant decrease in spleen ($R_2=0.1531$; $r=0.39132$; $p<.2340$, lymph nodes ($R_2=0.0576$; $r=0.24115$; $p<.4521$) and spinal cord ($R_2=0.0896$; $r=0.05114$; $p<0.3710$) of DAB₃₈₉IL-2 treated mice compared to PBS treated control mice.

Linear regression (R_2) analysis applied between PBS and DAB₃₈₉IL-2 treated groups at different intervals where DAB₃₈₉IL-2 treatment significantly suppressed the sum of total T, B and macrophage and sub-population and cytokine secreting cells in the spleen and spinal cord at the acute phase of EAE on day 21; in the spleen and spinal cord at the chronic early phase of EAE on day 16; and peripheral blood at the late chronic phase of EAE on day 24 (Figure 7).

4. Discussion

We have reported earlier that DAB₃₈₉IL-2 recombinant fusion toxin targets IL-2R bearing CD4⁺ cells, and suppresses demyelinating disease in both A-EAE and C-EAE animal models of multiple sclerosis (MS) ^{16–18}. We now show the clinical score and the changes occurring in the inflammatory sub-populations in various lymphoid organs and infiltrates in the spinal cord at different phases of the disease during the DAB₃₈₉IL-2 therapy.

In our study, we show that DAB₃₈₉IL-2 treatment on days 11–13 and 15 can suppress clinical scores significantly at the dose of 1600 kU in a chronic (C-EAE) disease mouse model. However, DAB₃₈₉IL-2 did not suppress clinical disease at the dose of 200 kU in chronic and 800 kU in acute EAE mice. There was no mortality in either acute or chronic EAE mice attributable to DAB₃₈₉IL-2 immunotoxin treatment. In our previous studies, we found that DAB₃₈₉IL-2 treatment at 1600 kU dose can effectively suppress clinical score, but mortality occurs in acute, relapsing and chronic EAE induced mice when treatment is started on days 7–11 at disease peak ¹⁷.

Our flow cytometry results show that CD3⁺ and CD4⁺ T cell sub-populations, IL-2⁺, IFN- γ ⁺ and TNF- α ⁺ T cell subsets and CD3⁺CD4⁺, CD3⁺CD8⁺, CD4⁺CD8⁺, CD3⁺IL-2⁺, CD11b⁺IL-2⁺, CD3⁺IFN- γ ⁺, CD11b⁺IFN- γ ⁺, CD11b⁺TNF- α ⁺ sub-populations were all decreased in the spinal cord of DAB₃₈₉IL-2 treated acute EAE mice as a long term effect in acute EAE mice on day 21. In chronic EAE, CD3⁺CD4⁺, CD3⁺CD8⁺, CD4⁺CD8⁺, CD3⁺IL-2⁺, CD19⁺IL-2⁺, CD11b⁺IFN- γ ⁺ sub-populations decreased at day 16, and CD3⁺CD4⁺ and CD3⁺CD8 sub-populations decreased at day 24 in the spinal cord of DAB₃₈₉IL-2 treated mice. However, CD19⁺TNF- α ⁺ increased in the spinal cord of C-EAE mice as a long term effect on day 24. CD3⁺IL-10⁺ cell sub-populations decreased in lymph nodes and the CD3⁺IL-4⁺ cell population increased in the spleen at day 24 in chronic EAE in DAB₃₈₉IL-2 treated mice showing sign of the disease recovery.

In this study we found that DAB₃₈₉IL-2 treatment decreased inflammatory cytokine production by T cells and macrophage cells in the spinal cord of C-EAE mice. We found CD11b⁺IL-2⁺ and CD11b⁺ IFN- γ ⁺ populations decreased in the spinal cords in acute EAE. However, the reason for an increase in CD11b⁺IL-2⁺ cell populations in the chronic phase is not clear; nor is it clear whether this immunotoxin fusion protein plays an immunomodulatory role, or whether macrophage and T cells contribute to spinal cord injury repair.

DAB₃₈₉IL-2 does not affect CD3⁺CD4⁺ and CD3⁺CD8⁺ sub-populations in the systemic blood in chronic disease period on day 24. The CD19⁺IFN- γ ⁺ sub-population decreases in the spinal cord of mice treated with DAB₃₈₉IL-2 on day 16. The accumulation of these cells is apparently contingent upon the prior immigration and activation of Th1 IL-2R⁺ cells.

Our study showed that there was a decrease in CD3⁺CD25⁺ cells in the spleen and spinal cord with a dose regime of 1600 kU DAB₃₈₉IL-2 in C-EAE mice; however; there was an opposite effect in A-EAE at a dose of 800 kU. The increase in the CD3⁺CD25⁺ sub-populations in the spleen and spinal cord likely reflects a preferential targeting Treg cells at the lower dose of DAB₃₈₉IL-2 treatment in A-EAE where Treg cells were not completely

depleted at the sub-acute phase by therapeutic interventions. Diphtheria toxin fusion protein has various therapeutic properties. In previous reports it has been shown that Diphtheria toxin-based recombinant murine IL-2 fusion toxin depleted murine regulatory T cells in spleen by as much as 70% as early as 12 h until day 3 after treatment and returned to control levels by Day 7²³. Denileukin diftitox (DAB (389) IL-2) reduces regulatory T cells and enhances vaccine-mediated T-cell immunity²⁴. In another targeted therapy to the IL-2R, the use of diphtheria toxin and caspase-3 fusion proteins modulated Treg cells and ameliorated inflammation in colitis²⁵. Hence, as shown in our present study, we consider that DAB₃₈₉IL-2 immunotoxin may have a modulatory effect on various lymphocyte- and macrophage-producing cytokine subpopulation cells during the course of demyelinating disease.

In conclusion, our results suggest that DAB₃₈₉IL-2 can ameliorate clinical signs in chronic disease at 1600 kU dose when treatment is given to mice on days 11–13 and 15. CD3⁺CD4⁺, CD3⁺CD8⁺, CD4⁺CD8⁺, CD3⁺IL-2⁺, CD11b⁺IL-2⁺, CD3⁺IFN γ ⁺, CD11b⁺IFN γ ⁺, CD11b⁺TNF- α ⁺ cell populations were decreased in acute-EAE and CD3⁺CD4⁺, CD3⁺CD8⁺, CD4⁺CD8⁺, CD3⁺IL-2⁺, CD19⁺IL-2⁺, on the day 16 and CD3⁺CD4⁺, CD3⁺CD8⁺, CD4⁺CD8⁺, CD3⁺IL-2⁺, CD11b⁺IFN- γ ⁺ sub-populations in chronic-EAE on day 24 are significantly suppressed in the spinal cord. CD3⁺CD25⁺ sub-populations increased in the spleen in acute EAE but decreased in spleen, lymph nodes and peripheral blood in chronic EAE mice. Thus, DAB₃₈₉IL-2 ameliorated clinical signs in chronic demyelinating disease at 1600 kU in an experimental mouse model by decreasing inflammatory cytokine-secreting cells, mainly in the spinal cord, which can be monitored by flow cytometry.

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References

1. Lim SY, Constantinescu CS. Current and future disease-modifying therapies in multiple sclerosis. *Int J Clin Pract.* 2010; 64:637–650. [PubMed: 20456216]
2. Carrithers MD. Update on disease-modifying treatments for multiple sclerosis. *Clin Ther.* 2014; 36:1938–1945. [PubMed: 25218310]
3. Tanasescu R, Ionete C, Chou IJ, Constantinescu CS. Advances in the treatment of relapsing-remitting multiple sclerosis. *Biomed J.* 2014; 37:41–49. [PubMed: 24732658]
4. Constantinescu CS, Gran B. The essential role of T cells in multiple sclerosis: a reappraisal. *Biomed J.* 2014; 37:34–40. [PubMed: 24732657]
5. Constantinescu CS, Farooqi N, O'Brien K, Gran B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol.* 2011; 164:1079–1106. [PubMed: 21371012]
6. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4⁺CD25⁺ regulatory T cells in patients with multiple sclerosis. *J Exp Med.* 2004; 199:971–979. [PubMed: 15067033]

7. Correale J, Villa A. Role of CD8+ CD25+ Foxp3+ regulatory T cells in multiple sclerosis. *Ann Neurol.* 2010; 67:625–638. [PubMed: 20437560]
8. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med.* 2005; 201:233–240. [PubMed: 15657292]
9. Kebir H, Kreymborg K, Ifergan I, Dodelet-Devillers A, Cayrol R, Bernard M, Giuliani F, Arbour N, Becher B, Prat A. Human T(H)17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med.* 2007; 13:1173–1175. [PubMed: 17828272]
10. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, Fugger L. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol.* 2008; 172:146–155. [PubMed: 18156204]
11. Duvic M, Talpur R. Optimizing denileukin diftitox (Ontak) therapy. *Future Oncol.* 2008; 4:457–69. [PubMed: 18684057]
12. Williams DP, Parker K, Bacha P, Bishai W, Borowski M, Genbauffe F, Strom TB, Murphy JR. Diphtheria toxin receptor binding domain substitution with interleukin-2: genetic construction and properties of a diphtheria toxin-related interleukin-2 fusion protein. *Protein Eng.* 1987; 1:493–498. [PubMed: 3334101]
13. Williams DP, Wen Z, Watson RS, Boyd J, Strom TB, Murphy JR. Cellular processing of the interleukin-2 fusion toxin DAB486-IL-2 and efficient delivery of diphtheria fragment A to the cytosol of target cells requires Arg194. *J Biol Chem.* 1990; 265:20673–20677. [PubMed: 2243114]
14. Bacha P, Williams DP, Waters C, Williams JM, Murphy JR, Strom TB. Interleukin 2 receptor-targeted cytotoxicity. Interleukin 2 receptor-mediated action of a diphtheria toxin-related interleukin 2 fusion protein. *J Exp Med.* 1988; 167:612–622. [PubMed: 3126255]
15. Waters CA, Schimke PA, Snider CE, Itoh K, Smith KA, Nichols JC, Strom TB, Murphy JR. Interleukin 2 receptor-targeted cytotoxicity. Receptor binding requirements for entry of a diphtheria toxin-related interleukin 2 fusion protein into cells. *Eur J Immunol.* 1990; 20:785–791. [PubMed: 2140788]
16. Phillips SM, Bhopale MK, Constantinescu CS, Ciric B, Hilliard B, Ventura E, Lavi E, Rostami A. Effect of DAB(389)IL-2 immunotoxin on the course of experimental autoimmune encephalomyelitis in Lewis rats. *J Neurol Sci.* 2007; 263:59–69. [PubMed: 17603081]
17. Phillips SM, Bhopale MK, Hilliard B, Zekavat SA, Ali MA, Rostami A. Suppression of murine experimental autoimmune encephalomyelitis by interleukin-2 receptor targeted fusion toxin, DAB(389)IL-2. *Cell Immunol.* 2010; 261:144–152. [PubMed: 20042183]
18. Bhopale MK, Hilliard B, Constantinescu CS, Fujioka T, Ventura E, Phillips SM, Rostami A. DAB389IL-2 suppresses autoimmune inflammation in the CNS and inhibits T cell-mediated lysis of glial target cells. *Exp Mol Pathol.* 2014; 96:108–117. [PubMed: 23872438]
19. Baur AS, Lutz MB, Schierer S, Beltrame L, Theiner G, Zinser E, Ostalecki C, Heidkamp G, Haendle I, Erdmann M, Wiesinger M, Leisgang W, Gross S, Pommer AJ, Kämpgen E, Dudziak D, Steinkasserer A, Cavalieri D, Schuler-Thurner B, Schuler G. Denileukin diftitox (ONTAK) induces a tolerogenic phenotype in dendritic cells and stimulates survival of resting Treg. *Blood.* 2013; 122:2185–2194. [PubMed: 23958949]
20. Peraino JS, Schenk M, Li G, Zhang H, Farkash EA, Sachs DH, Huang CA, Duran-Struuck R, Wang Z. Development of a diphtheria toxin-based recombinant porcine IL-2 fusion toxin for depleting porcine CD25+ cells. *J Immunol Methods.* 2013; 398–399:33–43.
21. Peraino JS, Zhang H, Rajasekera PV, Wei M, Madsen JC, Sachs DH, Huang CA, Wang Z. Diphtheria toxin-based bivalent human IL-2 fusion toxin with improved efficacy for targeting human CD25 (+) cells. *J Immunol Methods.* 2014; 405:57–66. [PubMed: 24462799]
22. Manoukian G, Hagemester F. Denileukin diftitox: a novel immunotoxin. *Expert Opin Biol Ther.* 2009; 9:1445–1451. [PubMed: 19817678]
23. Wei M, Marino J, Trowell A, Zhang H, Stromp Peraino J, Rajasekera PV, Madsen JC, Sachs DH, Huang CA, Benichou G, Wang Z. Diphtheria toxin-based recombinant murine IL-2 fusion toxin for depleting murine regulatory T cells in vivo. *Protein Eng Des Sel.* 2014; 27:289–295. [PubMed: 25147093]

24. Litzinger MT, Fernando R, Curiel TJ, Grosenbach DW, Schlom J, Palena C. IL-2 immunotoxin denileukin diftitox reduces regulatory T cells and enhances vaccine-mediated T-cell immunity. *Blood*. 2007; 110:3192–3201. [PubMed: 17616639]
25. Yarkoni S, Sagivm Y, Kaminitz A, Farkas DL, Askenasy N. Targeted therapy to the IL-2R using diphtheria toxin and caspase-3 fusion proteins modulates Treg and ameliorates inflammatory colitis. *Eur J Immunol*. 2009; 39:2850–2864. [PubMed: 19735074]

Figure 1. Clinical score of C-EAE treated with PBS or DAB₃₈₉IL-2 at the dose of 200 kU and 1600 kU.

Figure 2. Effect of *in vivo* DAB₃₈₉IL-2 treatment on T cells, and Th1 cytokine-secreting cells, in the spinal cord of mice with acute EAE. A) Percentage of T cell populations in total mononuclear cells. B) Percentage of total mononuclear cells producing Th1 type cytokines.

Figure 3.

Effect of DAB₃₈₉IL-2 treatment on CD25⁺ (IL-2Rα-bearing cells) in the spleen, lymph nodes and spinal cord of mice with chronic EAE at day 16. The T lymphocyte expressing cells expressing marker CD3 and CD25 cell population is shown, and the percentages of T cells expressing CD3⁺CD25⁺ are shown in the upper right quadrant.

Figure 4.

Effect of DAB₃₈₉IL-2 treatment on CD19⁺ B cells in spinal cords of mice with chronic EAE at day 16. The mononuclear cell population was identified by their forward and side light scatter characteristics, and the B lymphocytes within this gate and producing INF- γ , IL-2 and TNF- α cells. CD19⁺INF- γ ⁺, CD19⁺TNF- α ⁺ and CD19⁺IL-2⁺ sub-populations are shown in the upper right quadrant.

Figure 5.

Effect of DAB₃₈₉IL-2 treatment on CD3⁺IL-4-producing cells in the spleen and CD3⁺IL-10-producing cells of the lymph nodes of mice with chronic EAE at day 24 post immunization with mSCH. Graphs represent the total mononuclear cell populations gated by forward and side light scatters in spleen cell and lymph node cell preparations. CD3⁺ T cell producing IL-4 cell populations in the spleen is shown, and the percentage of CD3⁺IL-4⁺ sub-populations in the upper right quadrant of the upper graphs. The CD3⁺ T cell producing IL-10 cell populations in the lymph nodes is shown and CD3⁺IL-10⁺ sub-populations in the upper right quadrant of the lower graph.

Figure 6.

Effect of DAB₃₈₉IL-2 treatment on CD3⁺ Th1 cytokine-producing cells in the spinal cord of mice with chronic EAE on day 24 post immunization with mSCH. Distributions of CD3⁺ and IFN- γ ⁺, IL-2⁺ and TNF- α ⁺ selected by their forward and side light scatter characteristics. CD3⁺IFN- γ ⁺, CD3⁺IL-2⁺ and CD3⁺TNF- α ⁺ sub-population cells are shown in the upper right quadrant.

Figure 7.

Linear regression (R^2) analysis applied between PBS and DAB₃₈₉IL-2 treated groups at different intervals after conversion of percent cell population into Log_2 . DAB₃₈₉IL-2 treatment significantly suppressed the sum of total T, B and macrophage and sub-population and cytokine secreting cells from Table 2 in the spleen and spinal cord at the acute phase of EAE on day 21. DAB₃₈₉IL-2 treatment significantly suppressed the sum of T, B and macrophage and sub-populations and cytokine secreting cells from Table 3 in the spleen, lymph nodes and spinal cord at the chronic early phase of EAE on day 16; and from Table 4 in the peripheral blood at the late chronic phase of EAE on day 24.

Table 1Mean clinical scores^a of mice with A-EAE and C-EAE

Treatment	EAE model study period		
	A-EAE ^b (12–30 days) (n=6)	C-EAE ^c (10–35 days) (n=6)	C-EAE ^d (10–35 days) (n=6)
PBS treated	0.0917±0.0509	0.91920 ± 0.15508	0.9192±0.1551 ^e
DAB389IL-2 treated	0.1194±0.0426 (P=0.5747)	0.87860 ± 0.16041 (P= 0.7942)	0.0300±0.0166 (P=0.0001)

^aThe clinical scores of mice were recorded daily for each mouse in the experiment. The average clinical score for each mouse was calculated by the sum of the clinical scores over the study period by the number of days in the study period and subsequently the mean of each group of mice (six animals in each group, i.e. n=6) was calculated.

^bMice were treated with PBS or DAB389IL-2 800 kU on days following immunization in A-EAE.

^cMice were treated with PBS or DAB389IL-2 200 kU on days following immunization in C-EAE.

^dMice were treated with PBS or DAB389IL-2 1600 kU on days following immunization in C-EAE.

^eDifference between PBS control and DAB389IL-2 1600 kU treated group was statistically significant as P<0.05.

Table 2

FACS analysis * of T cell subpopulations from different organs in acute-EAE mice treated with PBS or DAB₃₈₉IL-2 at day 21.

* T cell subpopulation	PBS treated	DAB ₃₈₉ IL-2 treated
<i>Spleen</i>		
CD3 ⁺	52.10	53.36
CD25 ⁺	2.88	0.02
CD3 ⁺ CD4 ⁺	38.47	38.91
CD3 ⁺ CD8 ⁺	2.10	0.61
CD4 ⁺ CD8 ⁺	4.29	1.48
CD3 ⁺ CD25 ⁺	37.58	49.93
CD3 ⁺ IL-2 ⁺	1.15	0.98
CD11b ⁺ IL-2 ⁺	0.79	0.74
CD3 ⁺ IFN- γ ⁺	1.42	1.26
CD11b ⁺ IFN- γ ⁺	2.78	1.87
CD3 ⁺ TNF- α ⁺	1.78	1.0
CD11b ⁺ TNF- α ⁺	3.87	4.8
CD11b ⁺ IL-4 ⁺	1.03	1.6
CD3 ⁺ IL-10 ⁺	1.03	1.02
CD11b ⁺ IL-10 ⁺	0.96	0.95
<i>Spinal cord</i>		
CD3 ⁺	54.21	29.10
CD25 ⁺	2.86	0.0
CD3 ⁺ CD4 ⁺	17.65	0.0
CD3 ⁺ CD8 ⁺	26.89	19.50
CD4 ⁺ CD8 ⁺	28.68	21.26
CD3 ⁺ CD25 ⁺	2.12	5.15
CD3 ⁺ IL-2 ⁺	11.24	1.43
CD11b ⁺ IL-2 ⁺	12.36	1.43
CD3 ⁺ IFN- γ ⁺	1.96	0.67
CD11b ⁺ IFN- γ ⁺	3.92	0.67
CD3 ⁺ TNF- α ⁺	2.94	0.0
CD11b ⁺ TNF- α ⁺	47.06	18.18
CD19 ⁺ CD11b ⁺	20.0	1.92
CD11b ⁺ B7.1 ⁺	23.33	5.77
CD11b ⁺ B7.2 ⁺	18.33	13.46

* Percent cell population data represent mean of two samples (n=2).

Table 3

FACS analysis * of T cell subpopulations from different organs in chronic-EAE mice treated with PBS or DAB₃₈₉IL-2 at day 16.

T cell subpopulation	PBS treated	DAB ₃₈₉ IL-2 treated
<i>Day 16.</i>		
<i>Spleen</i>		
CD3 ⁺	50.11	62.66
CD25 ⁺	1.11	0.72
CD3 ⁺ CD4 ⁺	27.85	37.66
CD3 ⁺ CD8 ⁺	8.83	14.40
CD4 ⁺ CD8 ⁺	4.20	0.59
CD3 ⁺ CD25 ⁺	11.42	1.23
CD3 ⁺ IL-2 ⁺	7.19	0.74
CD19 ⁺ IL-2 ⁺	0.05	0.04
CD3 ⁺ IFN- γ ⁺	2.01	0.0
CD19 ⁺ IFN- γ ⁺	0.0	0.03
CD3 ⁺ TNF- α ⁺	4.59	0.5
CD19 ⁺ TNF- α ⁺	0.07	0.03
<i>Lymph node</i>		
CD3 ⁺	82.38	79.44
CD25 ⁺	1.65	0.57
CD3 ⁺ CD4 ⁺	53.71	51.19
CD3 ⁺ CD8 ⁺	69.95	9.37
CD4 ⁺ CD8 ⁺	49.57	0.8
CD3 ⁺ CD25 ⁺	11.20	1.88
CD3 ⁺ IL-2 ⁺	5.47	0.53
CD19 ⁺ IL-2 ⁺	3.91	0.05
CD3 ⁺ IFN- γ ⁺	0.1	0.07
CD19 ⁺ IFN- γ ⁺	0.1	0.0
CD3 ⁺ TNF- α ⁺	0.39	0.31
CD19 ⁺ TNF- α ⁺	0.19	0.03
<i>Spinal cord</i>		
CD3 ⁺	53.56	36.9
CD25 ⁺	10.13	4.90
CD3 ⁺ CD4 ⁺	27.77	13.53
CD3 ⁺ CD8 ⁺	37.02	17.47
CD4 ⁺ CD8 ⁺	27.16	14.50
CD3 ⁺ CD25 ⁺	14.50	10.13
CD3 ⁺ IL-2 ⁺	17.23	0.94
CD19 ⁺ IL-2 ⁺	15.65	0.85

T cell subpopulation	PBS treated	DAB ₃₈₉ IL-2 treated
<i>Day 16.</i>		
CD3 ⁺ IFN- γ ⁺	12.85	1.41
CD19 ⁺ IFN- γ ⁺	12.87	1.12
CD3 ⁺ TNF- α ⁺	10.53	2.67
CD19 ⁺ TNF- α ⁺	10.82	2.17

* Percent cell population data represent mean of two samples (n=2).

Table 4

FACS analysis * of T cell subpopulations from different organs in chronic-EAE mice treated with PBS or DAB₃₈₉IL-2 at day 24.

T cell subpopulation	PBS treated	DAB ₃₈₉ IL-2 treated
<i>Spleen</i>		
CD3 ⁺	27.22	35.26
CD3 ⁺ CD4 ⁺	0.48	15.5
CD3 ⁺ CD8 ⁺	11.0	10.2
CD4 ⁺ CD8 ⁺	0.14	3.29
CD3 ⁺ CD25 ⁺	13.09	1.32
CD3 ⁺ IL-2 ⁺	6.16	0.05
CD11b ⁺ IL-2 ⁺	0.02	0.38
CD3 ⁺ IL-4 ⁺	0.28	0.62
CD3 ⁺ IFN γ ⁺	0.20	0.1
CD3 ⁺ TNF- α ⁺	0.20	0.51
CD11b ⁺ TNF- α ⁺	0.33	0.25
<i>Lymph nodes</i>		
CD3 ⁺	16.87	14.41
CD3 ⁺ CD4 ⁺	0.53	25.39
CD3 ⁺ CD8 ⁺	0.05	31.34
CD4 ⁺ CD8 ⁺	0.16	1.09
CD3 ⁺ IL-2 ⁺	0.07	0.16
CD11b ⁺ IL-2 ⁺	0.0	0.02
CD3 ⁺ IL-10 ⁺	34.51	10.33
CD3 ⁺ IFN γ ⁺	0.73	0.09
CD11b ⁺ IFN γ ⁺	0.12	0.02
CD3 ⁺ TNF- α ⁺	0.05	0.79
CD11b ⁺ TNF- α ⁺	0.0	0.64
<i>Spinal cord</i>		
CD3 ⁺	46.64	18.02
CD3 ⁺ CD4 ⁺	25.17	9.01
CD3 ⁺ CD8 ⁺	7.47	6.91
CD4 ⁺ CD8 ⁺	26.12	8.11
CD3 ⁺ CD25 ⁺	13.5	4.14
CD3 ⁺ IL-2 ⁺	0.67	10.70
CD11b ⁺ IL-2 ⁺	0.26	25.85
CD3 ⁺ IFN- γ ⁺	0.92	11.47
CD11b ⁺ IFN- γ ⁺	12.95	0.23
CD3 ⁺ TNF- α ⁺	1.66	9.36
CD19 ⁺ TNF- α ⁺	0.87	5.81

T cell subpopulation	PBS treated	DAB ₃₈₉ IL-2 treated
<i>Peripheral blood</i>		
CD3 ⁺	56.8	58.00
CD3 ⁺ CD4 ⁺	40.2	38.6
CD3 ⁺ CD8 ⁺	25.2	27.3
CD3 ⁺ CD25 ⁺	42.2	3.5
CD3 ⁺ IL-2 ⁺	1.5	6.5
CD11b ⁺ IL-2 ⁺	0.5	2.0
CD3 ⁺ IFN- γ ⁺	2.2	1.5
CD11b ⁺ IFN γ ⁺	2.78	1.87
CD3 ⁺ TNF- α ⁺	2.0	0.5
CD11b ⁺ TNF- α ⁺	0.5	0.2
CD3 ⁺ IL-4 ⁺	0.2	0.15
CD11b ⁺ IL-4 ⁺	0.15	0.1
CD3 ⁺ IL-10 ⁺	26.5	2.0
CD11b ⁺ IL-10 ⁺	1.0	3.0
TCR $\alpha\beta$ +B7.1 ⁺	2.0	2.0
TCR $\alpha\beta$ +B7.2 ⁺	2.0	25.0
CD11b+B7.1 ⁺	32.5	2.0
CD11b+B7.2 ⁺	3.0	2.0

* Percent cell population data represent mean of two samples (n=2).