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Limitations of Foxp3⁺ Treg depletion following viral infection in DEREg mice

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Abstract

Regulatory T cells (Tregs) play a critical role in maintaining tissue homeostasis and preventing the development of immunopathology. Depletion of REGulatory T cell (DEREG) mice express a diphtheria toxin receptor (DTR)-eGFP transgene under the control of the Foxp3 promoter allowing for Treg depletion following diphtheria toxin (DT) administration. DEREg mice have been utilized to investigate the role of Tregs in a wide range of disease settings. Administration of DT to naïve DEREg mice resulted in the rapid depletion of Foxp3⁺ Tregs from the peripheral blood. However, by day 4 post-DT administration, a GFP[−] Foxp3⁺ Treg population emerged that lacked expression of the DTR transgene and was resistant to further depletion by additional DT treatment. We further evaluated the impact of Treg depletion during both acute and chronic viral infections. Similar to naïve mice, Treg numbers rapidly rebounded during an inflammatory setting following an acute viral infection. DT treatment of both WT and DEREg mice following both acute and chronic viral infections induced exacerbated disease as compared to PBS-treated controls. Furthermore, following a chronic systemic viral infection, DT treatment resulted in nearly 100 percent mortality in both WT and DEREg mice while the PBS-treated controls survived. Our results demonstrate that Treg depletion in DEREg mice is transient and that DT administration can have adverse effects during virus-induced inflammation and highlights the critical need to include DT-treated WT mice when using DTR models to control for DT-mediated toxicity.

Keywords

T cell; Viral; Regulatory T cell; Lung; Spleen

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Introduction

Regulatory T cells (Tregs) represent a subset of CD4⁺ T cells defined by expression of the forkhead transcription factor (Foxp3), which is encoded by the X-linked gene *Foxp3*^{1,2}. Deficiencies in Foxp3 function result in autoimmunity very early in life in both mice and humans due to a lack of proper immune regulation^{3,4}. In addition, Tregs have been shown to limit pathogen-specific T cell responses during both acute^{5–8} and chronic infections^{9,10}.

The vital role of Tregs in immune homeostasis has led to the development of novel tools to further dissect the functions of Tregs. Depletion of REGulatory T cell (DEREG) mice were created to allow for the specific depletion of Foxp3 expressing Tregs^{11,12}. DEREG mice express a diphtheria toxin receptor (DTR)-enhanced (e)GFP transgene controlled by a Foxp3 promoter that is inserted via a bacterial artificial chromosome (BAC). Following DT treatment, Foxp3 expressing cells are specifically depleted. These mice have been utilized to study a wide range of immune disorders including cancer^{13–15}, allergy^{16–18}, and various infections^{7,10,19}.

Our studies were aimed at gaining a better understanding of the impact of Treg depletion on the adaptive immune response following an acute respiratory infection as well as during a chronic systemic infection. Following DT treatment of naïve DEREG mice, Treg depletion was transient with the frequency of Foxp3⁺ Tregs returning to WT levels within 6 days. In addition, the newly emerged Foxp3⁺ Tregs no longer expressed the DTR-eGFP transgene, thus preventing prolonged depletion. Similar results were observed in mice undergoing either an acute or chronic viral infection. Furthermore, DT treatment in both transgenic DEREG mice as well as wild-type (WT) mice resulted in enhanced morbidity and mortality. These findings limit the potential versatility of the DEREG mouse model and identify adverse effects of DT treatment on WT mice during inflammation associated with an active viral infection.

Materials and Methods

Mice and Infection

BALB/c and C57BL/6 female mice were bred in the University of Iowa animal facility and were utilized between 6–8 weeks of age. BALB/c and C57BL/6 DEREG female mice expressing DTR-eGFP under the control of the Foxp3 promoter were obtained from Dr. Katharina Lahl and Dr. Tim Sparwasser (Twincore, Hanover, Germany). The A2 strain of respiratory syncytial virus (RSV) was a gift from Dr. Barney Graham (National Institutes of Health; Bethesda, MD) and was propagated on HEp-2 cells (American Type Culture Collections; ATCC, Manassas, VA). The clone-13 (CL-13) strain of lymphocytic choriomeningitis virus (LCMV) was a gift from Dr. Raymond Welsh (University of Massachusetts Medical School, Worcester, MA) and was propagated in BHK-21 cells (ATCC). For RSV infections, mice were anesthetized with isoflurane and infected intranasally with $2–3 \times 10^6$ PFU. For LCMV CL-13 infection, mice were infected i.v. with 1.8×10^6 PFU. Disease was assessed by monitoring weightloss and pulmonary function using an unrestrained whole-body plethysmograph (Buxco Electronics, Wilmington, NC) as

previously described^{6,20,21}. All experimental procedures involving mice were approved by the University of Iowa Animal Care and Use Committee.

DT Treatment

DEREG and WT controls were administered 1 µg DT (Calbiochem, San Diego, CA and Sigma-Aldrich, St. Louis, MO) diluted in endotoxin-free PBS on the indicated days.

Blood collection and staining

Peripheral blood was collected at indicated time points and red blood cells were lysed using NH₄Cl. Cells were fixed and permeabilized using the mouse regulatory T cell staining buffer kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. Cells were stained with mAbs against Foxp3 (clone FJK-16s; eBioscience), CD4 (clone RM4-5; Biolegend, San Diego, CA), CD90.2 (clone 53-2.1; eBioscience). After staining cells were resuspended in FACS buffer prior to analysis on a BD LSRFortessa. Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Graphical and statistical analysis was performed using Prism software (Graphpad Software Inc., San Diego, CA), with error bars representing the SEM. Two-way ANOVAs with a Dunnett's post test were calculated to determine the statistical significance. *P* values were considered significant when *p*<0.05.

Results

Foxp3⁺ Treg depletion in DEREG mice is transient

To determine the longevity of Foxp3⁺ Treg depletion, mice were treated with 1 µg DT for two consecutive days to deplete Tregs as previously reported¹¹. The frequencies of Foxp3⁺ and GFP⁺ CD4 T cells in the peripheral blood were monitored every other day for 6 days (Fig. 1). Less than 1 percent of CD4 T cells expressed Foxp3 on day 2 after 2 days of DT treatment. However, by day 6 post-treatment, the frequency of Foxp3⁺ Tregs in the peripheral blood had returned to levels similar to the controls, indicating that Foxp3⁺ Treg depletion was transient (Fig. 1A, 1C). In addition, eGFP expression, a marker for the continued expression of the DTR-eGFP transgene, was observed on a very low frequency of Tregs appearing on days 4 and 6 post-treatment (Fig. 1B, 1C). Similar results were observed using DT from different sources (data not shown). The lack of eGFP expression suggests that the newly emerging Foxp3⁺ Tregs no longer express the DTR-eGFP transgene.

To verify that the newly emerging eGFP negative Foxp3⁺ Tregs were resistant to further depletion, a third DT treatment was administered on day 3 post-initial treatment (Fig. 2). This additional DT treatment did not prevent the rapid return of Foxp3⁺ Tregs in the peripheral blood by day 4 (Fig. 2A, 2C). Furthermore, the majority of Tregs appearing on days 2–8 post-treatment did not express eGFP (Fig. 2B, 2C). This data indicates that additional DT treatment on day 3 does not alter the longevity of Foxp3⁺ Treg depletion in DEREG mice. Thus, Treg depletion cannot be maintained in naïve DEREG mice due to the emergence of a Treg population that no longer expresses the DTR-eGFP transgene.

DT administration induces disease in both DEREK and WT naïve mice

To determine the physiological impact of DT treatment and transient Foxp3⁺ Treg depletion, weight loss was monitored daily following DT administration (Fig. 3). DT-treated DEREK mice expressed significant ($p<0.01$) weight loss compared to the PBS-DEREK and DT-WT controls (Fig. 3A). In addition, a third treatment of DT on day 4 post-initial treatment induced significant weight loss in both DT-treated DEREK ($p<0.001$) and WT ($p<0.01$) mice (Fig. 3B). Similar results were observed using two independent sources of DT (data not shown). Thus, DT administration induces toxicity in both DEREK and WT naïve mice.

Foxp3⁺ Treg depletion is not maintained following acute RSV infection

Respiratory pathogens such as RSV have been shown to induce acute inflammation in the lung resulting in respiratory disease ^{6,22}. We sought to determine if virus-induced inflammation would alter the kinetics of Treg depletion. Mice were treated with 1 µg DT on days 3 and 4 post-RSV infection (Fig. 4). We have previously reported that the total number of Tregs in the lung begins to increase by day 4 post-RSV infection ⁶, thus Tregs were depleted prior to this expansion. Similar to naïve DEREK mice, administration of 2 consecutive injections of DT resulted in effective Treg depletion in RSV-infected mice (Fig. 4A, 4C). Similar results were observed using DT from 2 different sources (ie. DT1 and DT2). However, by day 9 post-infection (i.e. 6 days post-treatment), Foxp3⁺ Treg frequencies in the PBL had returned to control levels. In addition, the returning Foxp3⁺ Tregs lacked eGFP expression, similar to naïve DT-treated DEREK mice (Fig. 4B, 4C, 1B). Thus, similar to naïve DEREK mice, Treg depletion is not maintained following acute RSV infection.

DT treatment enhances the severity of RSV-induced disease

Given the enhanced weight loss observed in naïve DEREK and WT mice following DT administration, we sought to examine the impact of DT treatment and Treg depletion on RSV-induced disease severity. Weight loss and Penh, as a measure of pulmonary function, were monitored daily in DEREK and WT mice administered DT on days 3 and 4 post-infection (Fig. 5). Treatment with DT1 (Calbiochem) induced significantly enhanced disease as measured by increased Penh compared to both PBS-treated and WT controls (Fig. 5A). However, administration of either DT1 or DT2 (Sigma-Aldrich) induced increased weight loss in both WT and DEREK mice following RSV infection (Fig. 5A, 5B). Slight variations in DT activity can occur in DT obtained from various vendors due to preparation differences affecting DT activity and purity. However, with both vendors tested, DT-treated WT mice exhibited enhanced disease compared to the PBS-DEREK controls. Thus, depletion of Tregs in DEREK mice further enhanced disease. However, DT-treated WT mice also exhibited increased morbidity following acute RSV infection.

DT treatment induces morbidity and mortality following LCMV CI-13 infection

To determine the efficacy of the DEREK model during a chronic viral infection, the impact of DT treatment during chronic LCMV CL-13 infection was examined. WT and DEREK mice were treated with DT on days 10 and 11 following CL-13 infection. Recent work has indicated that the number of Tregs increase after day 10 post-infection ²³, thus day 10 was

chosen for depletion. Weight loss and survival were monitored daily following infection (Fig. 6). Both DT-treated WT and DERE mice exhibited severe morbidity compared to PBS-DERE controls (Fig. 6). In addition, DT treatment induced 95% mortality in WT mice and 100% mortality in DERE mice following LCMV CL-13 infection while PBS-treated DERE controls survived (Fig. 6). DT obtained from two different vendors (DT1 Calbiochem and DT2 Sigma-Aldrich) induced similar weight loss and mortality (Fig. 6A, 6B). Furthermore, two separate lots of DT within one vendor (Calbiochem) were tested with similar results (data not shown). Taken together these results demonstrate a role for DT-induced toxicity during a chronic viral infection in WT mice as well as DERE mice.

Discussion

This study examined the kinetics of Treg depletion in naïve and virus-infected DERE mice. Following two consecutive days of DT treatment, Tregs were 95% depleted in both naïve and RSV-infected mice. However, by day 4 post-depletion approximately 50% of the Foxp3⁺ Treg population had returned in the peripheral blood (Figs. 1 and 4). Furthermore, by day 6 post-treatment the frequency of Foxp3⁺ Tregs in DT-treated DERE mice had returned to levels similar to controls. In addition, the emerging Treg population following depletion no longer expressed the DTR-eGFP transgene and was resistant to further depletion with additional DT treatment (Fig. 2). The emergence of an eGFP⁻ population in DERE mice following DT administration has been previously reported^{10,11,24}. However, to our knowledge, this is the first report detailing the kinetics of the eGFP⁻ Treg population that emerges in DERE mice following depletion. It is unclear what causes the emergence of this eGFP⁻ Foxp3⁺ population, it has been hypothesized that the BAC transgene has delayed expression in newly generated Tregs¹¹, however this has not been formally proven. Our data indicates that Treg depletion in DERE mice is transient, preventing its use to evaluate the impact of prolonged Treg depletion. Moreover, the transient nature of Treg depletion must be taken into account when interpreting results using DERE mice.

We observed exacerbated morbidity in naïve and virally infected DERE and WT mice following DT administration. A recent study by zu Hörste and colleagues noted similar morbidity in C57BL/6 mice following DT treatment from multiple vendors²⁵. However the mechanism by which DT induces disease in WT mice remains unclear. Mice have been shown to be less susceptible to DT-induced cell death but they are not completely resistant^{26,27}. A recent study found that DT administration induced proteinuria and podocyte injury, indicating a need for caution when utilizing DTR transgenic models to study either hypertension or renal disease²⁸. These studies in naïve mice, in addition to our study, indicate a need for caution when utilizing DTR transgenic systems due to DT-mediated toxicity.

In this study we observed that DT treatment enhanced morbidity following both acute and chronic viral infections. Following RSV infection, DT-treated DERE mice exhibited the greatest signs of disease as measured by increased weight loss and Penh (Fig. 5). This data is consistent with a role for Tregs in limiting RSV-induced disease as has been previously reported following either anti-CD25 depletion or DT depletion using DERE mice^{6,7}. However, the intermediate disease exhibited by DT-treated WT mice indicates that not all of

the disease measured can be attributed to the elimination of Tregs. A recent study by Loebbermann *et al.* utilized DERE mice to examine the role of Tregs following RSV infection ⁷. While they found significant differences following RSV infection between untreated WT and DT-treated DERE mice, DT-treated WT controls were not included in the study. Further analysis of Tregs following RSV infection using Foxp3^{DTR} mice ²⁹ has recently been reported, however DT-treated WT mice were not included as controls ³⁰. Thus, it is currently unclear to what extent the enhanced disease severity observed following Treg depletion during acute RSV infection is caused by elimination of the Tregs versus the toxicity induced by DT administration.

We observed enhanced mortality following chronic LCMV CL-13 infection and DT treatment in both DERE and WT mice whereas PBS-treated mice all survived (Fig. 6). This data indicates that following prolonged inflammation induced by a chronic viral infection such as CL-13, DT-induced disease is indistinguishable from disease resulting from a lack of Tregs. This data further highlights the importance of including DT-treated WT control mice when utilizing DTR transgenic systems in order to distinguish the disease contribution caused by cellular depletion from DT-induced toxicity.

DERE mice have been utilized to extensively study Treg functions in a number of disease settings. However, our results highlight the limitations of this model to examine Treg responses during a viral infection. The limited number of days in which Tregs are depleted restricts the versatility of the DERE model and complicates the interpretations of the data at later time points. In addition, the DT-induced morbidity and mortality in infected WT mice introduces the additional complication of DT-induced toxicity. While DT-induced mortality in WT mice may only be observed in a highly inflammatory environment such as during chronic LCMV CL-13 infection, further studies will be necessary to determine the full extent of this toxic side effect. Our results highlight a complication in all DTR models that must be considered when utilizing these model systems.

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Abbreviations

BAC	bacterial artificial chromosome
CL-13	LCMV CL-13
DERE	depletion of regulatory T cell
DT	diphtheria toxin
DTR	diphtheria toxin receptor
Foxp3	forkhead transcription factor 3
LCMV	lymphocytic choriomeningitis virus
RSV	respiratory syncytial virus

Treg regulatory T cell
WT wild-type

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Figure 1.

Foxp3⁺ Treg depletion is not maintained in naïve DERE mice. Naïve DERE and WT mice were treated with either 1 µg DT or PBS i.p. on days 0 and 1. The frequency of Foxp3⁺ (A) CD4 T cells were monitored in the peripheral blood. The frequency of eGFP⁺ and eGFP⁻ cells in DT-treated DERE mice is shown in a stacked bar graph where the total Foxp3⁺ Treg population is set to 100% (B). Gated Thy1.2⁺ CD4⁺ cells depicting Foxp3 expression (x-axis) and eGFP expression (y-axis) are shown (C). Plots are concatenated from one of three independent experiments ($n=4-5$). Data in A and B depict cumulative data from two to three independent experiments ($n=8-12$). Statistical significance was determined by a two-way ANOVA with a Dunnett's post-test comparing DT-DEREG to PBS-DEREG (B) or to both DT-WT and PBS-DEREG (A). * $p < 0.05$, *** $p < 0.001$.

Figure 2.

Additional DT treatment does not extend the duration of Foxp3⁺ Treg depletion. Naïve DEREG and WT mice were treated with either 1 µg DT or PBS i.p. on days 0, 1 and 3. The frequency of Foxp3⁺ CD4 T cells was monitored in the peripheral blood (A). The frequency of eGFP⁺ and eGFP⁻ cells in DT-treated DEREG mice is shown in a stacked bar graph where the total Foxp3⁺ Treg population is set to 100% (B). Gated Thy1.2⁺ CD4⁺ cells depicting Foxp3 expression (x-axis) and eGFP expression (y-axis) are shown (C). Plots are concatenated from one of two independent experiments (n=3-4). Data in A and B depict

cumulative data from two independent experiments ($n=7-8$). Statistical significance was determined by a two-way ANOVA with a Dunnett's post-test comparing DT-DEREG to PBS-DEREG (B) or to both DT-WT and PBS-DEREG (A). $**p < 0.01$, $***p < 0.001$.

Figure 3.

DT treatment induces disease in naïve WT and DERE mice. Naïve DERE and WT mice were treated with either 1 µg DT or PBS i.p. on days 0 and 1 (A) or days 0,1, and 3 (B). Weight loss was monitored daily following treatment. Data in A and B depict cumulative data from 2–3 independent experiments ($n=7-12$). Statistical significance was determined by a two-way ANOVA with a Dunnett's post-test comparing PBS-DEREG to DT-DEREG (*) or DT-WT (+). * $p < 0.05$, *** $p < 0.001$, ++ $p < 0.01$.

Figure 4.

Foxp3⁺ Treg depletion is not maintained following RSV infection. Following i.n. RSV infection DEREG and WT mice were treated with either 1 µg DT or PBS i.p. on days 3 and 4 post-infection. The frequency of Foxp3⁺ CD4⁺ T cells was monitored in the peripheral blood (A). The frequency of eGFP⁺ and eGFP⁻ cells in DT1 or DT2 treated DEREG mice is shown in a stacked bar graph where the total Foxp3⁺ Treg population is set to 100% (B). Gated Thy1.2⁺ CD4⁺ cells depicting Foxp3 expression (x-axis) and GFP expression (y-axis) are shown (C). Plots are concatenated from one of two independent experiments ($n=3-4$). Data in A and B depict cumulative data from two independent experiments ($n=7-8$). Statistical significance was determined by a two-way ANOVA with a Dunnett's post-test comparing both DT1- and DT2-DEREG to PBS-DEREG (B) or to DT1/2-WT and PBS-DEREG (A). *** $p < 0.001$.

Figure 5.

DT treatment enhances disease in both WT and DERE mice following RSV infection. Following i.n. RSV infection DERE and WT mice were treated with either 1 μ g DT1 (A), DT2 (B) or PBS i.p. on days 3 and 4 post-infection. Weight loss was monitored daily following infection. Data in A and B depict cumulative data from 2–5 independent experiments ($n=8-22$). Statistical significance was determined by a two-way ANOVA with a Dunnett's post-test comparing each group to PBS-DEREG. Data from (A) and (B) were collected simultaneously and PBS-DEREG values are repeated on each graph.

Figure 6.

DT treatment enhances disease and mortality in WT and DEREG mice following CL-13 infection. Following i.v. CL-13 infection DEREG and WT mice were treated with either 1 μ g DT1 (A), DT2 (B) or PBS i.p. on days 10 and 11 post-infection. Weight loss and survival were monitored daily following infection. Data in A and B depict cumulative data from 3 independent experiments ($n=3-11$). Statistical significance was determined by a two-way ANOVA with a Dunnett's post-test comparing each group to PBS-DEREG. Data from (A) and (B) were collected simultaneously and PBS-DEREG values are repeated on each graph.