Letter

DOI: 10.5582/bst.2020.03291

Half depletion of Foxp3+ regulatory T cells by diphtheria toxin for long-term study *in vivo*

Xuemin Qiu[1,2,3,§], Wing Ting Leung[1,2,3,§], Hans-Jürgen Gober[4], Lisha Li[1,2,3], Na Zhang[1,2,3], Nan Chu[1,2,3], Ling Wang[1,2,3,*]

[1] Laboratory for Reproductive Immunology, Hospital & Institute of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai, China;
[2] The Academy of Integrative Medicine of Fudan University, Shanghai, China;
[3] Shanghai Key Laboratory of Female Reproductive Endocrine-related Diseases, Shanghai, China;
[4] Department of Pharmacy, Neuromed Campus, Kepler University Hospital, Linz, Austria (Present address: Pharmaceutical Outcomes Programme, British Columbia Children's Hospital, 938 West 28th Avenue, Vancouver BC, Canada).

SUMMARY Depletion of regulatory T cells (Tregs) is an appropriate approach to study the function of Tregs *in vivo*, and most previous studies have focused on complete depletion. The purpose of the current study was to determine an appropriate dose and timing for half depletion of Tregs *in vivo*. DETREG (DEpletion of REGulatory T cells) mice were produced and injected with different doses of diphtheria toxin (DT) for 7 days and 14 days. The mice were then sacrificed to collect the spleen and mesenteric lymph nodes (MLN) for analysis using flow cytometry. Foxp3+eGFP+ cells were significantly reduced by DT injection. A dose of 5 ug/kg DT led to half depletion and no deaths. A DT dose of 25, 50, or 100 ug/kg led to a progressively higher depletion rate but also a higher mortality rate. In conclusion, a low dose of DT is effective for half depletion of Tregs and long-term study. Half depletion of Tregs may become a new method for the future study of Tregs *in vivo*.

Keywords half depletion of Foxp3+ regulatory T cells, DETREG mice, Diphtheria toxin (DT)

Researchers have focused on the modulation of regulatory T cells (Tregs) for years because of the important role of Tregs *in vivo* (*1*). However, specific *in vivo* targeting of Tregs is precluded because of the lack of appropriate markers. Now that Foxp3 has been discovered, the development of mouse models of Treg-specific depletion is feasible (*2*). DETREG (DEpletion of REGulatory T cells) mice carry a diphtheria toxin receptor - enhanced green fluorescent protein (DTR-eGFP) transgene under the control of an additional Foxp3 promoter, thereby allowing specific depletion of Tregs by diphtheria toxin (DT) at any desired point of time during any ongoing immune responses (*3*). Most previous studies have focused on complete depletion of Tregs, which facilitates the study of Tregs *in vivo*. However, half depletion of Tregs may be a new method for studying the function of Tregs *in vivo*.

The aim of the current study was to explore how to use DT to half deplete Tregs *in vivo* and to determine a proper dose and timing in order to facilitate the long-term study of Tregs.

DT was purchased from Sigma-Aldrich. Flow cytometry antibodies including fluorescein isothiocyanate (FITC)-conjugated anti-CD4, anti-Foxp3-PE, anti-CD25-APC, and their corresponding isotype controls were purchased from eBioscience (San Diego, CA, USA). RT-PCR reagents were purchased from Applied Biosystems (Darmstadt, Germany).

To create a DETREG (Foxp3DTR-EGFP) mouse model, two transgene mouse models were used, pCAG-STOP-DTR-2A-EGFP mice (denoted as DTR-EGFP mice, product number: BCG-TO-0001) were purchased from Beijing Biocytogen Co., Ltd (Beijing, China), and B6.129(cg)-Foxp3$^{tm4(YFP/cre)Ayr}$/J mice (denoted as Foxp3-Ycre mice) were obtained from the Chinese Academy of Sciences (Dr. Qi-bin Leng's Lab, Shanghai, China). All mice were bred and reproduced in an animal facility of the Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University under specific pathogen-free conditions. This study was approved by the Animal Care and Ethics Committee of the Department of Laboratory Science, Fudan University (approval no. 2020 Obstetrics & Gynecology Hospital JS020). DETREG mice genotyping Primers (5'-3'): Rosa-GT-F: AGTCGCTCTGAGTTGTTATCAG, Rosa-GT-R: TGAGCATGTCTTTAATCTACCTCGATG; WPRE-F: GCATCGATACCGTCGACCTC, WPRE-R: GCTGTCCATCTGCACGAGAC; Ycre-WT-F:

CCTAGCCCCTAGTTCCAACC, Ycre-WT-R: AAGGTTCCAGTG CTGTTGCT; Ycre-MU-F: AGGATGTGAGGGACTACCTCCTGTA, Ycre-MU-R: TCCTTCACTCTGATTCTGGCAATTT.

After mice were sacrificed, spleen and mesenteric lymph node (MLN) cells were analyzed using a flow cytometer (Becton Dickinson, Palo Alto, CA, USA).

Continuous variables were expressed as the mean ± SEM. Continuous variables were analyzed using a Student's *t*-test for two groups and a one-way ANOVA for multiple groups. All analyses were performed using the SPSS 19.0 Statistical Package for the Social Sciences. $P < 0.05$ was considered statistically significant.

Genotyping of DETREG mice with RT-PCR is shown in Figure 1. The GFP+ cell and Foxp3+ cell populations in the spleen and MLNs were analyzed using FCM. The number of GFP+ Tregs was much higher in DETREG mice compared to that in wild-type (WT) mice (13.3% *vs*. 3.01% in the spleen, 16.1% *vs*. 0.87% in MLNs, $p < 0.05$). However, the number of Foxp3+ cells did not differ, suggesting that a DETREG mouse model was successfully created.

In order to achieve half depletion, DETREG and WT mice were injected i.p. with DT (5, 25, 50, or 100 ug/kg) every 48 h. After mice were sacrificed, Foxp3+ and GFP+ cells in the spleen and MLNs were analyzed. As shown in Figure 2, both GFP+ cells in the spleen and MLNs decreased after DT injection. There was a severe drop in Foxp3+ cell as the dose increased, suggesting a higher DT dose led to a higher depletion rate. However, a high dose led to severe weight loss and a high mortality rate in the 50 ug/kg and 100 ug/kg groups (data not shown).

On the day of the eighth DT injection (day 14), the number of Foxp3+ cells did not differ in the DT and WT groups, suggesting a rebound of Tregs in DETREG mice (data not shown).

DT injection allows for a highly specific depletion of Tregs at any desired time point during an ongoing immune response. Most researchers use a large dose of DT to completely deplete Tregs in order to study their function *in vivo* (*4*). However, complete depletion of Tregs *in vivo* causes a fatal autoimmune pathology in mice (*5,6*). The aim of the current study was to determine an appropriate dose and timing for half depletion of Tregs *in vivo*, while previous studies only achieved complete depletion for a short period of time.

In conclusion, previous studies of Tregs using DETREG mice focused on complete depletion for a short period of time. In contrast, the current study achieved a half depletion of Tregs for a prolonged period of time. This approach is potentially a new way to study Tregs *in vivo* in the future, offering new possibilities for research on Tregs and especially for long-term study of Tregs.

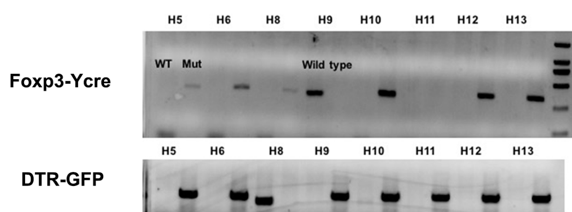

Figure 1. A DEREG mice model has successfully been created and verified using RT-PCR. DETREG mice were genotyped using RT-PCR. For DTR mice: WT=Rosa-GT-F/R: 469 bp; Mutant=WPRE-F/R: 561 bp; For Ycre mice: Mutant = ~346 bp; Heterozygote = 322 bp and ~346 bp; Wild type = 322 bp.

Funding: This work was supported by grants from the Program to Guide Medicine ("Yixueyindao") of the Shanghai Municipal Science and Technology Commission (no. 18401902200 to Ling Wang) and the Shanghai Program for Support of Leading Disciplines-Integrative Medicine (no. 20180101).

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Sharabi A, Tsokos MG, Ding Y, Malek TR, Klatzmann D,

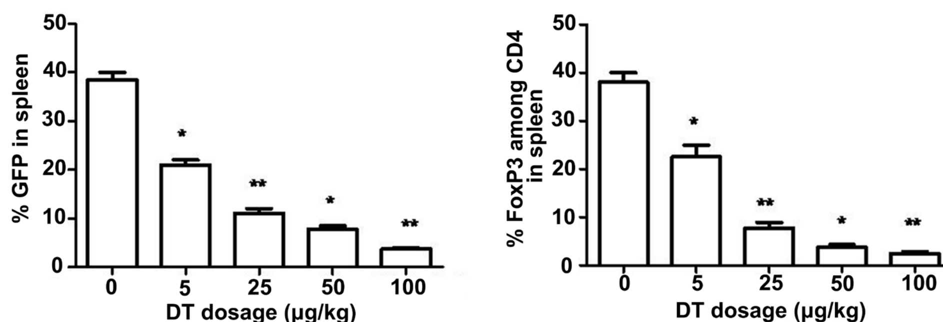

Figure 2. Depletion of Tregs by DT was dose-dependent. DEREG and WT mice were injected i.p. with different doses of DT (0, 5, 25, 50, 100 ug/kg) every 48 h. Mice were sacrificed on the day following the fourth DT injection because of the significant mortality rate in the DT high-dose groups. GFP+ and Foxp3+ cells were counted using FCM. (*$P < 0.05$, **$P < 0.01$)

Tsokos GC. Regulatory T cells in the treatment of disease. Nat Rev Drug Discov. 2018; 17: 823-844.
2. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003; 4: 330-336.
3. Lin W, Haribhai D, Relland LM, Truong N, Carlson MR, Williams CB, Chatila TA. Regulatory T cell development in the absence of functional Foxp3. Nat Immunol. 2007; 8: 359-368.
4. Kim J, Lahl K, Hori S, Loddenkemper C, Chaudhry A, deRoos P, Rudensky A, Sparwasser T. Cutting edge: depletion of Foxp3+ cells leads to induction of autoimmunity by specific ablation of regulatory T cells in genetically targeted mice. J Immunol. 2009; 183: 7631-7634.
5. Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature. 2007; 445: 766-770.
6. Chen GY, Chen C, Wang L, Chang X, Zheng P, Liu Y. Cutting edge: Broad expression of the FoxP3 locus in epithelial cells: A caution against early interpretation of fatal inflammatory diseases following *in vivo* depletion of FoxP3-expressing cells. J Immunol. 2008; 180: 5163-5166.

Received August 5, 2020; Revised October 25, 2020; Accepted November 30, 2020.

[§]These authors contributed equally to this work.
**Address correspondence to:*
Ling Wang, Obstetrics & Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai 200011, China.
E-mail: Dr.wangling@fudan.edu.cn

Released online in J-STAGE as advance publication December 16, 2020.