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## Tissue antigen expression levels fine-tune T cell differentiation decisions *in vivo*

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### Abstract

Immune homeostasis in peripheral tissues is to a large degree maintained by the differentiation and action of regulatory T cells (Treg) specific for tissue antigens. Using a novel mouse model, we have studied the differentiation of naïve CD4<sup>+</sup> T cells into Foxp3<sup>+</sup> Treg in response to a cutaneous antigen (OVA). We found that expression of OVA resulted in fatal autoimmunity and in prevention of peripheral Treg generation. Inhibiting mTOR activity with rapamycin rescued the generation of Foxp3<sup>+</sup> T cells. When we varied the level of antigen expression to modulate TCR signaling, we found that low antigen concentrations promoted the generation of Foxp3<sup>+</sup> T cells whereas high levels expanded effector T cells and caused severe autoimmunity. Our findings indicate that the expression level of tissue antigen is a key determinant of the balance between tissue-reactive effector and peripheral Foxp3<sup>+</sup> T cells, that determines the choice between tolerance and autoimmunity.

### Introduction

Regulatory T cells (Treg) control potentially destructive responses of self-reactive effector T cells (Teff) and thus prevent autoimmunity (1, 2). Treg are classically divided into thymic Treg (tTreg), generated in the thymus, and peripheral Treg (pTreg) that arise from naïve T cells activated in the periphery (3–6). The latter constitute a mechanism to regulate immune responses towards antigens that are not present in the thymus, such as self and microbial

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antigens (7–9). Both tTreg and pTreg are characterized by the expression of the transcription factor Foxp3, which drives Treg identity (10). Mutations in the *FOXP3* gene in mice and humans lead to loss of Treg function, causing a severe autoimmune phenotype called scurfy (in mice) and IPEX syndrome (in humans) (1). Foxp3 induces the expression of molecules related to Treg function, such as CTLA-4, while suppressing expression of inflammatory cytokines (11, 12).

T cell fate, including Treg differentiation, is defined by the local conditions naïve T cells are exposed to during their activation (13–15). Costimulatory signals and the local cytokine milieu regulate Foxp3 expression in activated T cells and their differentiation to pTreg (5, 16, 17). Additionally, the amount of antigen presented to naïve T cells during activation modulates the strength of TCR signaling and plays a major role in T cell differentiation (18, 19). Specifically, low antigen doses favor induction of Foxp3 *in vitro*, and pTreg generation and/or expansion *in vivo* in response to immunization (20, 21). Interestingly, a high affinity peptide administered at a low concentration induced Foxp3 expression in a naïve monoclonal population whereas a low-affinity agonist showed a diminished ability to induce a persistent Foxp3<sup>+</sup> population *in vivo* (21). These studies were performed with administered foreign peptides and it is therefore unknown whether similar mechanisms govern the response to tissue antigen.

In the present study, we utilized a unique mouse model to study pTreg induction, function and stability *in vivo*. We investigated the impact of different expression levels of a transgene-encoded tissue antigen on the activation and differentiation of adoptively transferred antigen-specific naïve T cells. Understanding the mechanisms that regulate pTreg differentiation in response to tissue antigens could promote the development of better clinical interventions in inflammatory and autoimmune settings where induction of tolerance against tissue antigens is desirable.

## Materials and Methods

### Mice

All animal studies were performed in compliance with institutional guidelines in a specific pathogen-free facility and were approved by the Federal Ministry of Science, Research and Economy of Austria.

K5/rTA (22) or INV/rTA (23) and TGO (Tg(TetO-Tfrc/EGFP/OVA)#Sfz (MGI:5503055), TRE-OVA) mice were crossed and designated K5/TGO or INV/TGO mice. The double-transgenic K5/TGO or INV/TGO mouse lines were crossed onto TCR $\alpha^{-/-}$  mice on BALB/c background. DO11.10 TCR-transgenic mice were crossed with Rag2 $^{-/-}$ /CD90.1 $^{+/+}$  or Rag2 $^{-/-}$ /CD45.1 $^{+/+}$  BALB/c mice as previously described (24).

### Adoptive transfer of T cells and doxycycline administration

Single-cell suspensions from skin-draining (sdLNs: auricular, brachial, axillary, inguinal, and popliteal lymph nodes) and mesenteric lymph nodes of DO11.10/Rag2 $^{-/-}$ /CD90.1 $^{+/+}$  or DO11.10/Rag2 $^{-/-}$ /CD45.1 $^{+/+}$  mice were prepared by mechanical disruption of LNs.  $0.5 - 2 \times 10^6$  LN cells were adoptively transferred by i.v. injection into K5/TGO/TCR $\alpha^{-/-}$

or INV/TGO/TCR $\alpha^{-/-}$  recipient mice. Cells were stained with 10  $\mu$ M CFSE where indicated. Doxycycline (dox) (Sigma-Aldrich) was administered in the drinking water in concentrations ranging from 1200 – 1.2  $\mu$ g/mL in a sterile 5% sucrose solution. Dox treatment started a day before the transfer of DO11.10/Rag2 $^{-/-}$  cells and mice were maintained on dox for the duration of the experiments.

### Evaluation of skin disease

A 15-point clinical scoring scale was used to quantify skin disease. The clinical parameters of scaling, alopecia, erythema, level of activity, and periocular inflammation were each given a score of 0–3. Scores for individual clinical parameters were summed for each mouse.

### Rapamycin treatment

Mice received daily i.p. injections of 1  $\mu$ g/g of body weight of rapamycin (Selleckchem; 2 mM stock solution stored in ethanol) dissolved in PBS starting one day before adoptive transfer of DO11.10/Rag2 $^{-/-}$  cells.

### Cell isolation from the skin

Skin from the tail, ears and shaved trunk was minced and digested for 45 min in 4 mL C10 medium with an enzyme mix containing collagenase XI, hyaluronidase, and DNase in supplemented RPMI medium as previously described (7). The cell suspension was filtered, washed, and stained for flow cytometry.

### Restimulation for intracellular cytokine staining

Single-cell suspensions from sDLNs were stimulated with 70 ng/ml phorbol-12-myristate-13-acetate and 700 ng/ml ionomycin for 3 hours in C10 medium, with 20  $\mu$ g/mL brefeldin A added for the last 2 h. Cells were washed with PBS before staining for flow cytometry.

### Flow cytometry staining and evaluation

For flow cytometry staining  $2 \times 10^6$  freshly isolated or *in vitro* restimulated single-cell suspensions from LNs or skin were stained using the Foxp3 staining kit (eBioscience) according to the manufacturer's instructions. For the detection of phosphorylated S6 sDLNs were mechanically disrupted in PBS containing 1.6% paraformaldehyde and fixed for 10 mins at room temperature. The cells were permeabilized by adding 4 volumes of ice-cold methanol and stored at  $-80$  °C. Cells were stained in PBS with 0.5% BSA. Phosphorylated molecules were stained in a two-step staining (using biotin and streptavidin).

Data were acquired on FACSCanto (BD Biosciences) flow cytometer and analyzed using FlowJo software (Tree Star, Inc.). The following gating strategies were applied: cells were gated on live, singlets, and DO11.10 T cells were defined by gating on CD4, KJ1–26, CD90.1 or CD45.1 (as stated in the Figure legends) and Foxp3 $^{-}$  or Foxp3 $^{+}$ , for Teff and Treg cells, respectively. When data was combined from measurements on different days it was normalized to naïve Foxp3 $^{+}$  tTreg isolated from DO11.10 $^{+}$  Rag2-sufficient mice and stained alongside pTreg according to the resolution metric:

$$\frac{\text{MedianFluorescenceIntensity}(\text{sample}) - \text{MedianFluorescenceIntensity}(\text{naiveFoxp3}^+\text{tTreg})}{\text{robustSD}(\text{sample}) + \text{robustSD}(\text{naiveFoxp3}^+\text{tTreg})}$$

## RNA isolation and RT-PCR

Total RNA was isolated from the trunk skin using RNeasy plus mini kit (Qiagen, 74134) combined with a gDNA eliminator column and an on-column DNase treatment. RNA (0.3–1 µg) was used for reverse transcription with the high capacity iScript™ reverse transcription super mix for RT qPCR (Biorad). qPCR assay was performed on a Rotor Gene using the GoTaq qPCR master mix 2X (Promega). HPRT expression was used as internal housekeeping control.

Primer sequences were: Mouse HPRT1 (forward: GTCCCAGCGTCGTGATTAGC reverse: GAGCAAGTCTTTTCAGTCCTGTCC); TGO (forward: TGAAAACTGACTGAATGGACCA, TGO reverse: TGCTGACCCTACCACCTCTC).

## Statistical analysis

Statistical analysis of results was performed using GraphPad Prism Version 8.0. For comparison of two groups two-sample t or Mann Whitney test was used and for more than two groups, one-way ANOVA with Tukey's multiple comparisons test or Kruskal-Wallis test with Dunn's multiple comparisons test was used. Error bars indicate means ± SD. Denotation of asterisks is the following: \* for p < 0.05, \*\* for p < 0.01, \*\*\* for p < 0.001 and \*\*\*\* for p < 0.0001. For Fig. 4C we applied a linear regression model to the curves after the dose shift and asked whether the slopes were different from each other using an ANCOVA equivalent test using GraphPad Prism.

In all plots individual animals are represented by individual symbols.

A detailed list of antibodies and reagents can be found in supplemental table S1.

## Results and Discussion

### Absence of pTreg generation in INV/TGO mice correlates with lethal autoimmunity

We previously demonstrated that an endogenous skin antigen can promote pTreg generation and protect from autoimmune skin disease. For these studies we utilized a tetracycline-inducible system for the expression of a membrane bound GFP-OVA fusion protein, designated TGO, under the control of the keratin 5 promoter (K5) expressed in the basal cell layer of the epidermis of transgenic mice (K5/TGO) and we followed adoptively transferred naïve OVA-specific CD4<sup>+</sup> T cells (DO11.10) *in vivo* (scheme Supplemental Fig. 1A) (7, 24). As previously described, adoptive transfer recipients developed cutaneous inflammation that quickly resolved, and resolution was associated with the development of OVA-specific Foxp3<sup>+</sup> pTreg (7, 24). However, we observed a lethal autoimmune phenotype when we used the same adoptive transfer model but expressed OVA under the control of the involucrin promoter (INV) in the upper layers of the epidermis (INV/TGO). These animals developed progressive inflammation and had to be euthanized within 22–50 days after

adoptive transfer, due to disease progression without resolution (Fig. 1A, B). A significantly higher fraction of DO11.10 cells isolated from the sdLNs of INV/TGO mice expressed the proliferation marker Ki67 compared to K5/TGO recipients and significantly more T cells produced the effector cytokine IFN- $\gamma$  in INV/TGO mice (Fig. 1C, D). In addition, no Foxp3<sup>+</sup>CD25<sup>+</sup> pTreg were generated in INV/TGO recipients whereas a sizeable fraction of DO11.10 cells within K5/TGO mice developed into pTreg (Fig. 1E, and (7)).

### **mTOR signaling regulates *de novo* expression of Foxp3 in peripheral T cells responding to tissue antigen**

It is well known that the PI3K/Akt/mTOR network, activated downstream of the TCR, regulates many metabolic and cell differentiation processes and plays a pivotal role in Teff cell differentiation choices via metabolic adaptation (25–30). Additionally, rapamycin (rapa), an immunosuppressive drug that preferentially inhibits the mTORC1 complex (31, 32), has been utilized to generate Foxp3<sup>+</sup> iTreg from naïve T cells *in vitro* (33) or expand tTreg *in vivo*. However, few studies investigated *de novo* expression of Foxp3 by naïve T cells in the periphery using rapa (34).

Strong T cell proliferation and lack of Foxp3 induction in INV/TGO mice (Fig. 1C, E) would be consistent with high mTOR activity. To test the role of mTOR, we administered rapa to the recipients of naïve DO11.10 cells (scheme Supplemental Fig. 1B). 18 of 23 (78%) rapa treated mice did not show signs of clinical disease and survived long-term beyond the duration of rapa treatment, while 13 of 16 (81%) of the vehicle treated mice succumbed to autoimmunity (Fig. 2A). Rapa treatment inhibited T cell expansion (Fig. 2B), an effect that has been observed before (35) and which may impact clinical disease. However, most notably in rapa treated mice, Foxp3<sup>+</sup> T cells differentiated from naïve T cell precursors upon encountering cutaneous antigen. Foxp3<sup>+</sup> T cells could be detected in the blood (Supplemental Fig. 1C, D), sdLNs, and the skin (Fig. 2C, D and Supplemental Fig. 1E). Importantly, once generated Foxp3<sup>+</sup> T cells were maintained over time and could be readily detected after up to 10 weeks (i.e. 8 weeks after rapamycin was discontinued) in the face of continued exposure to the tissue antigen for the duration of the experiment (Fig 2C, D).

### **Concentration of tissue antigen affects T cell activation and differentiation *in vivo***

We next aimed to study the endogenous regulation of mTOR activity by the dose of cognate antigen. We hypothesized that high levels of antigen sensed by the TCR lead to high mTOR activity and lack of Treg generation in INV/TGO mice. To test this, we modulated the levels of tissue antigen expressed under the control of a tetracycline-responsive element by varying the dose of the tetracycline analog, doxycycline (dox). By RT-PCR analysis we found that INV/TGO mice displayed about 50-fold higher TGO mRNA expression in the skin than K5/TGO mice (Fig. 3A), and the amount of TGO mRNA expressed by the keratinocytes could be reduced in a dose dependent manner with doses of dox ranging from 1200 to 1.2  $\mu\text{g}/\text{mL}$  in the drinking water. We further found that TGO mRNA levels correlated with the activity of mTOR pathway measured by phosphorylation of the ribosomal protein S6, a downstream target of mTORC1 (29, 31), in DO11.10 cells 48–96 hours after adoptive (Fig. 3B and Supplemental Fig. 2A).

We found that DO11.10 T cell proliferation, measured by CFSE dilution 4 days after transfer, correlated with the titrated antigen levels (Fig. 3C, D). The expression of activation markers and IFN- $\gamma$  was similarly dependent on the dose of dox (Supplemental Fig. 2B, C).

Mice that received reduced doses of dox developed little clinical disease (Fig. 3E) and in line with the dose-dependent activation of responding T cells, the absolute numbers of DO11.10 cells isolated from the sDLNs of INV/TGO recipients at low doses of dox were comparable to the K5/TGO model (Fig. 3F). Most importantly, at lower antigen doses in INV/TGO recipients, Foxp3<sup>+</sup> T cells were detectable in the periphery (Fig. 3G, H) similar to animals treated with rapa, which permitted expression of Foxp3 even at high antigen concentration (Fig. 2D). Thus, *de novo* Foxp3 expression in peripheral T cells can be achieved through expression of endogenous tissue antigen at low levels.

### **Foxp3<sup>+</sup> T cells induced by low dose of tissue antigen are stable**

Treg generated from naïve T cells can be insufficiently functional or lose Foxp3 expression upon inflammatory challenge thus converting into Teff cells (36). To investigate whether Foxp3<sup>+</sup> T cells generated at permissive doses of dox (12 and 1.2  $\mu\text{g}/\text{mL}$ ) in the INV/TGO model were functional and stable, we first interrogated their phenotype by flow cytometry. The *in vivo* generated Foxp3<sup>+</sup> T cells expressed the canonical Treg markers GITR and CTLA-4 (Fig. 4A, B). The expression of these molecules was even higher than that of naïve thymic Treg (Fig. 4A).

To functionally test the suppressive activity and stability of Foxp3<sup>+</sup> T cells generated at permissive doses, we challenged these cells by increasing the dose of dox to 1200  $\mu\text{g}/\text{mL}$  after 35 days of low dose exposure. At the same time, we adoptively transferred a second bolus of congenically marked naïve DO11.10 cells (scheme Supplemental Fig. 1F) which cause lethal disease at the high dose of dox (Fig. 3E). Recipients without Foxp3<sup>+</sup> T cells from the first bolus were not protected from autoimmunity (0% survival at initial dose of 120  $\mu\text{g}$  dox/mL), while 33%, 3/9 animals, survived in hosts where the first bolus of DO11.10 cells was primed at 12  $\mu\text{g}$  dox/mL and 100% survived after receiving 1.2  $\mu\text{g}$  dox/mL in the initial dox treatment (Fig. 4C). In line with reduced or absent disease, Foxp3<sup>+</sup> T cells generated from the first bolus of DO11.10 cells in response to low dose tissue antigen (12 and 1.2  $\mu\text{g}$  dox/mL dox) retained Foxp3 expression *in vivo* and could be recovered from the sDLNs as well as from the skin up to 8 weeks after increasing the dose of dox and exposing T cells to high levels of antigen (Fig. 4D, E).

Taken together, using a system in which *de novo* expression of Foxp3 can be studied *in vivo* in the absence of any potentially confounding impact of expanding tTreg (37), we show that reducing antigen-induced signals in T cells is permissive for the development of Foxp3<sup>+</sup> T cells in the periphery. Although similar conclusions have been reached using titrated administration of foreign antigens (with and without rapamycin) and by *in vitro* studies (20, 21, 33), we now show that Foxp3 expression is induced in response to low levels of endogenous tissue antigen *in vivo*. Our results also show that Foxp3<sup>+</sup> T cells induced under conditions of limiting TCR signals are stable *in vivo* long-term. These findings suggest that this approach may be beneficial in protocols for therapeutic tolerance induction to treat autoimmunity.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations used in this article:

<b>dox</b>	doxycycline
<b>Foxp3</b>	forkhead boxP3
<b>GITR</b>	glucocorticoid-induced TNFR-family-related gene
<b>INV</b>	involucrin
<b>IPEX syndrome</b>	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
<b>K5</b>	keratin 5
<b>mTOR</b>	mammalian target of rapamycin
<b>rapa</b>	rapamycin
<b>(sd)LN</b>	(skin-draining) lymph node
<b>Teff cells</b>	effector T cell
<b>TGO</b>	TGO construct encodes a fusion protein linking the transferrin receptor transmembrane domain, GFP, and aa 230–359 of chicken OVA
<b>tTreg or pTreg</b>	thymic or peripheral regulatory T cell

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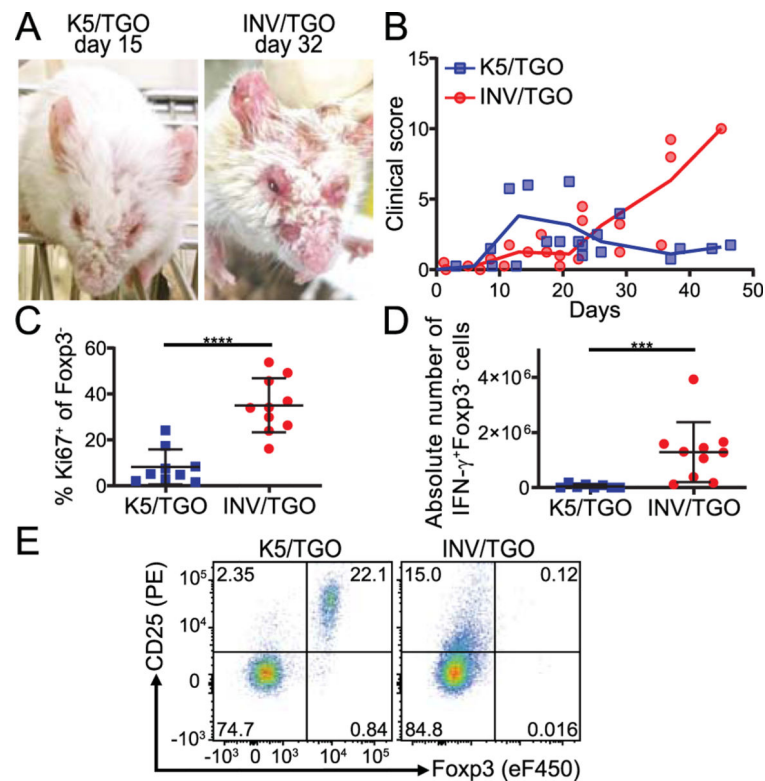
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**Key points:**

Expression level of tissue antigen determines the fate of responding T cells *in vivo*

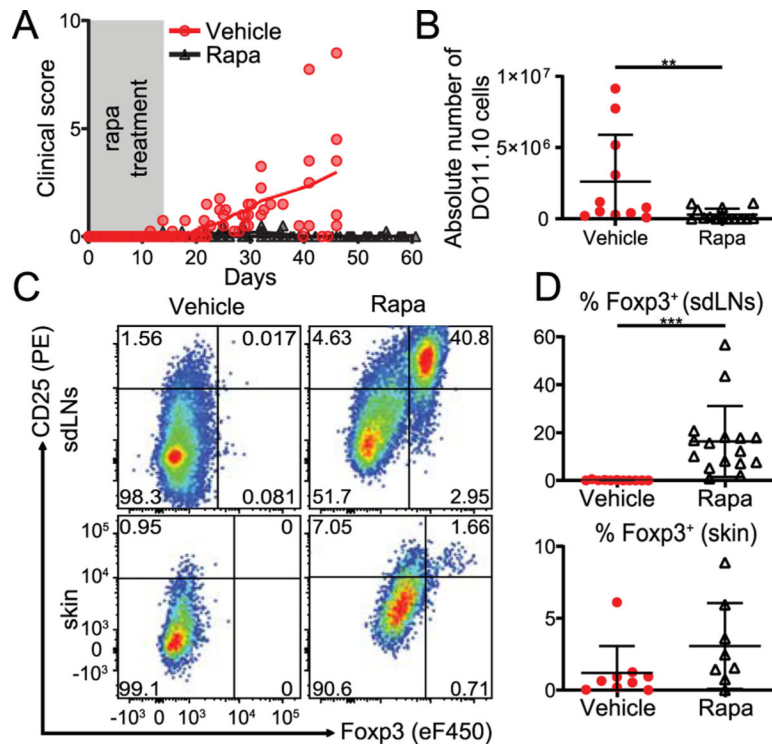
Rapa rescues the generation of peripheral Foxp3<sup>+</sup> T cells even at high antigen dose

Low level of endogenous tissue antigen differentiates stable Foxp3<sup>+</sup> T cells *in vivo*



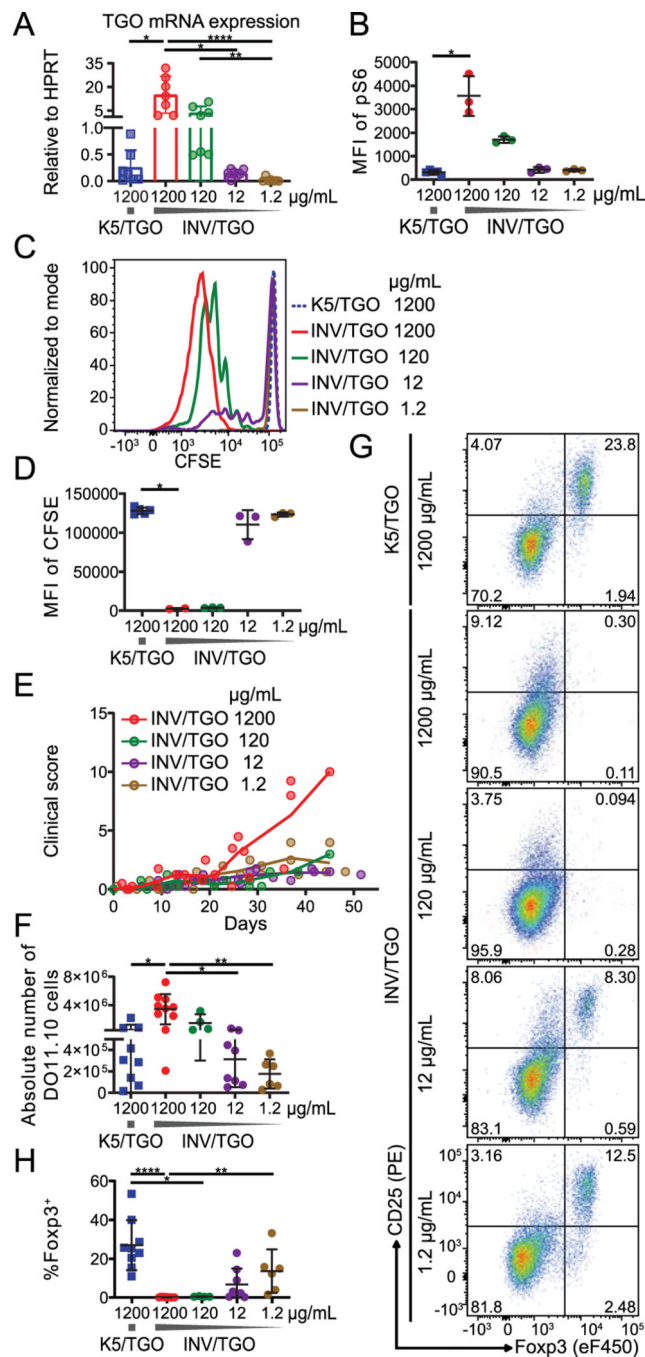
**Figure 1: INV/TGO mice developed a lethal autoimmune disease in contrast to the self-resolving disease in the K5/TGO model.**

OVA expression in the skin was induced by 1200  $\mu\text{g}$  dox/mL and  $0.8\text{--}1 \times 10^6$  LN cells from DO11.10 Rag2<sup>-/-</sup> animals were transferred to K5/TGO or INV/TGO mice. (A) Representative pictures of K5/TGO and INV/TGO mice at the height of disease (15 and 32 days after transfer, respectively). (B) Clinical disease course over the duration of the experiment (representative of 3 experiments, N = 8/group in total). (C, D) sdLNs were analyzed by flow cytometry 22–57 days after transfer. Graphical summary of the Ki67 expression and IFN- $\gamma$  production by live gated CD4<sup>+</sup>Foxp3<sup>-</sup>DO11.10-TCR<sup>+</sup> Teff cells. Cumulative data from 4 experiments, N = 8/group. (E) Representative flow plots of Foxp3 and CD25 expression by live gated CD4<sup>+</sup>DO11.10-TCR<sup>+</sup> T cells in the sdLNs of recipient animals 22 days after transfer. Mean and SD is shown.



**Figure 2: High mTOR activation inhibits pTreg generation in INV/TGO mice.**

OVA expression in the skin was induced with 1200  $\mu\text{g}$  dox/mL and  $0.5\text{--}0.9 \times 10^6$  LN cells from DO11.10 Rag2<sup>-/-</sup> animals were transferred into INV/TGO mice. Starting a day before adoptive transfer, mice were treated with either rapamycin (1  $\mu\text{g}/\text{g}$  of body weight) or vehicle i.p. injections daily for 16 days. (A) Clinical disease course over the duration of the experiment (representative of 4 experiments, N = 16/group in total). (B) Absolute number of recovered DO11.10 cells from sdLNs between 41–89 days. (C) Representative flow plots of DO11.10 cells recovered from sdLNs or skin (67 and 89 days after transfer in vehicle and rapa treated groups, respectively). (D) Percentages of Foxp3<sup>+</sup> DO11.10 cells recovered from the sdLNs (top) and the skin (bottom) between days 41–67 (vehicle) and 71–89 (rapa). B, D show Cumulative data from 4 experiments, N = 8/group. Mean and SD is shown.

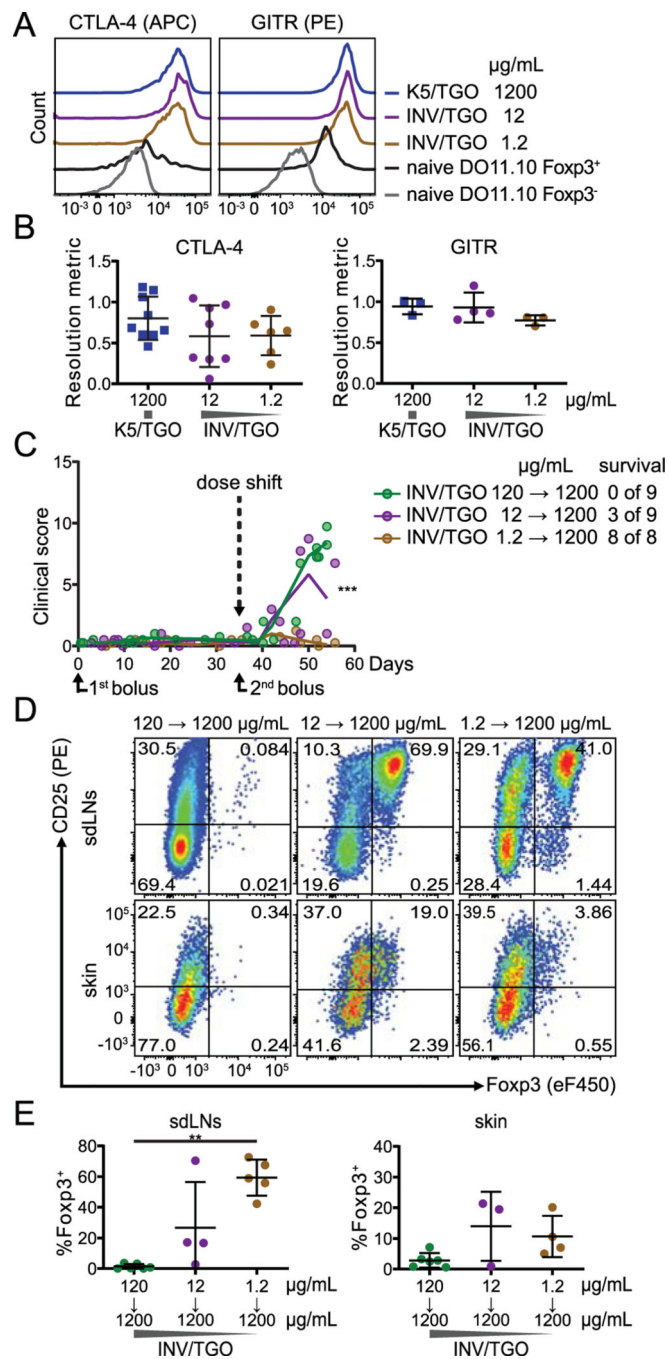


**Figure 3: T cell activation and differentiation depends on dose of tissue antigen in INV/TGO mice.**

OVA expression was induced by the indicated doses of dox (1200 – 1.2 µg/mL) and  $0.9\text{--}2 \times 10^6$  CFSE-labeled (A-D) or  $0.8\text{--}1 \times 10^6$  unlabeled (E-H) LN cells from DO11.10 Rag2<sup>-/-</sup> mice were transferred to K5/TGO or INV/TGO mice. (A) TGO mRNA expression in the skin analyzed by RT-PCR 2–4 days after dox administration. (B) sdLNs were isolated after 2–4 days, immediately fixed *ex vivo* and pS6 stained for flow cytometry. The Median fluorescence intensity (MFI) of pS6 in transferred DO11.10 cells is plotted (representative

of 2 experiments, N = 4/group in total). **(C, D)** Representative overlay of the CFSE dilution and summary graph of the MFI of CFSE in live DO11.10 cells in the sdLNs after 2–4 days (representative of 2 experiments, N = 4/group in total). **(E)** Clinical disease course over the duration of the experiment (representative of 2 experiments, N = 5/group in total) **(F)** Absolute number of live CD4<sup>+</sup> DO11.10 cells in sdLNs after 22–57 days. **(G)** Representative flow plots of the expression of Foxp3 and CD25 by live CD4<sup>+</sup> DO11.10 cells from sdLNs 46 days after transfer. **(H)** Percentages of live CD4<sup>+</sup> Foxp3<sup>+</sup> DO11.10 cells in the sdLNs after 22–57 days. F-H show cumulative data from 4 experiments, N = 4/group. Mean and SD is shown.





**Figure 4: Low dose of tissue antigen favors the generation of stable Foxp3<sup>+</sup> cells.** (A and B) OVA expression was induced by the indicated doses of dox (1200, 12, 1.2 µg/mL) and 0.875–1×10<sup>6</sup> LN cells from DO11.10 Rag2<sup>-/-</sup> mice were transferred to K5/TGO or INV/TGO mice. Cells were isolated from sdLNs between 22–57 days after transfer. (A, B) Representative overlays (on day 50) and summary graphs displaying the intracellular expression of CTLA-4 (left) or the surface expression of GITR (right) by Foxp3<sup>+</sup> DO11.10 cells compared to naïve Foxp3<sup>+</sup> tTreg and Foxp3<sup>-</sup> cells isolated from DO11.10<sup>+</sup> Rag2-sufficient mice (all populations gated on live CD4<sup>+</sup> DO11.10<sup>+</sup>). Cumulative data from

3 experiments, N = 3/group. **(C, D and E)** OVA expression in the skin was induced by different doses of dox (120 – 1.2 µg/mL) and 0.5–0.9×10<sup>6</sup> LN cells from DO11.10 Rag2<sup>-/-</sup>CD90.1<sup>+/+</sup> mice were transferred into INV/TGO mice. On day 35, 0.9 ×10<sup>6</sup> LN cells from DO11.10 Rag2<sup>-/-</sup>CD45.1<sup>+/+</sup> mice were transferred into the mice as a second bolus and the dose of dox was shifted to 1200 µg/mL for all groups. **(C)** Clinical disease course over the duration of the experiment (N = 2/group). The slopes of the curves after the dose shift are significantly different (p= 0.0004). Survival was determined by day 54 after transfer. **(D)** Representative flow plots of the CD90.1<sup>+/+</sup> DO11.10 population (i.e. first bolus) isolated from sdLNs or skin 56–90 days after transfer (from left to right). **(E)** Percentages of Foxp3<sup>+</sup> cells of CD90.1<sup>+/+</sup> DO11.10 cells isolated from the sdLNs (left) and the skin (right) 56–90 days after transfer. Cumulative data from 3 experiments, N = 3/group. Mean and SD is shown.