INFLAMMATION

Regulatory CD4+CD25+ cells reverse imbalances in the T cell pool of bone marrow transplanted TGε26 mice leading to the prevention of colitis

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Background and aims: Erroneous thymic selection of developing T lymphocytes may be responsible for the expansion of self reactive T cells or may contribute to the absence of regulatory T cells important in controlling peripheral inflammatory processes. Colitis in bone marrow (BM) transplanted Tgε26 mice is induced by abnormally activated T cells developing in an aberrant thymic microenvironment. We investigated the protective role of regulatory CD4+CD25+ T cells in this model.

Methods: BM from (C57BL/6 x CBA/J) F1 mice was transplanted into specific pathogen free Tgε26 mice (BM→Tgε26). Transplanted mice received no cells (control), sorted CD4+CD25−, or CD4+CD25+ cells from mesenteric lymph nodes (MLN) of normal mice. MLN cell subsets were analysed using membrane markers. Cytokine secretion of MLN cells was measured using intracellular cytokine staining and cytokine secretion in anti-CD3 stimulated cell cultures. Colitis was measured by histological scores.

Results: CD4+CD25+ cells were reduced in the MLNs of BM→Tgε26 mice. Transfer of regulatory CD4+CD25+ but not of CD4+CD25− cells reduced the number of MLN CD4+ T cells in BM→Tgε26 recipients and increased the number of MLN CD8+ cells, thereby normalising the CD4+/CD8+ ratio. CD4+CD25− but not CD4+CD25+ cell transfer into BM→Tgε26 mice reduced the number of tumour necrosis factor α+ CD4+ cells and increased the secretion of transforming growth factor β by MLN cells. Transfer of 3 x 10⁴ CD4+CD25+ cells after BM transplantation into Tgε26 mice prevented colitis whereas CD4+CD25− cells had no protective effect.

Conclusions: These results suggest that defective selection or induction of regulatory T cells in the abnormal thymus is responsible for the development of colitis in BM→Tgε26 mice. Transfer of CD4+CD25+ cells can control intestinal inflammation in BM→Tgε26 mice by normalising the number and function of the MLN T cell pool.

The immune system of the distal intestine must discriminate between harmful foreign antigens and innumerable antigens from the complex resident bacterial flora. Under normal circumstances, immunological tolerance toward the commensal enteric bacterial flora prevents continuous intestinal inflammation. There is compelling evidence from both human and animal models that this controlled homeostasis between harmful foreign antigens and innumerable antigens from the complex resident bacterial flora prevents continuous intestinal inflammation. There is compelling evidence from both human and animal models that this controlled homeostasis between harmful foreign antigens and innumerable antigens from the complex resident bacterial flora prevents continuous intestinal inflammation. We and others have shown that activation of T cells reacting against the resident bacterial flora is a key pathogenic mechanism in rodent models of chronic colitis and in humans. The factors leading to the unrestrained activation of T cells have been a focus of recent investigations.

The normal CD4+ T cell population contains both T cell subsets responsible for the induction of inflammation and other T cells that confer suppression. The immunosuppressive activity is contained predominantly within the CD4+ T cell population that constitutively expresses the interleukin (IL)-2R α-chain (CD25). Transfer of regulatory CD4+CD25+ cells in different experimental settings demonstrated their pivotal role in the maintenance of self tolerance, in regulating peripheral T cell homeostasis, in transplantation tolerance, and in graft versus host protection after bone marrow (BM) transplantation. The thymus has been identified as the place where regulatory T cells (Treg cells) develop and a normal thymic architecture is necessary for adequate selection of Treg cells. By allowing selective transfer of T cell subsets, spontaneously mutated and transgenic mice lacking adult T lymphocytes (SCID, Rag, and Tgε26, respectively) can provide insights into the pathogenic role of different T cell subsets in the intestinal inflammatory process. A growing body of evidence suggests that regulatory CD4+CD25+ T cells play an important role in the prevention and treatment of intestinal inflammation in SCID mice. In this model, colitis develops after transfer of naïve CD4+CD45RB+ T cells and can be prevented by co-transfer of the naturally activated CD45RB− subset. Because colitis in SCID mice can only be induced by transfer of adult T lymphocytes but not by BM transplantation, interaction of thymus dependent T cell development and Treg cells cannot be studied in the context of colitis.

The immunopathogenesis of experimental colitis in Tgε26 mice is substantially different. Tgε26 mice are transgenic for a human CD3ε gene. This results in very early arrest of T cell development which prevents the induction of a normal thymic microenvironment. BM transplantation from syngeneic wild-type mice into Tgε26 mice (BM→Tgε26 mice) restores the T cell compartment. However, T cell development

Abbreviations: BM, bone marrow; BM→Tgε26, Tgε26 mice transplanted with wild-type bone marrow; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; MLN, mesenteric lymph node; SPF, specific pathogen free; Th1, T helper 1; TNF, tumour necrosis factor; Treg cells, regulatory T cells; TGF-β, transforming growth factor β
and selection in the abnormally structured thymus are profoundly compromised. Consequently, aggressive CD4+ T cells develop while peripheral CD8+ cells are almost absent.20 CD4+ cells are characterised by low CD45RB<sup>high</sup> expression and by secretion of a T helper 1(Th1) cytokine profile consisting of interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α).21 These cells cause severe colitis.22,23

To date, the mechanisms underlying the development of aggressive T cells in the abnormal thymus in Tg<sub>e</sub> mice have only been partially elucidated. Failure of the thymus to negatively select aggressive T cells is one possibility. Alternatively, a defect of positive selection of Treg cells in the thymus may cause loss of control. The latter hypothesis is more compatible with experiments in which transplantation of a normal thymus into BM→Tg<sub>e</sub> mice with a persistent abnormal thymus prevented the development of colitis.24 However, direct evidence for a key role of absent Treg cells in the pathogenesis of colitis in Tg<sub>e</sub> mice is missing and such cells need to be further characterised.

The purpose of the present study was to investigate whether defective selection of CD4+CD25<sup>+</sup> Treg cells in the compromised thymic environment of Tg<sub>e</sub> mice is responsible for the development of colitis after BM transplantation. Specifically, we sought to determine whether the aberrant activity of T cells derived from the thymus of BM→Tg<sub>e</sub> mice can be corrected by regulatory CD4+CD25<sup>+</sup> cells and to assess the mechanisms by which this correction is mediated.

**MATERIALS AND METHODS**

**Mice**

Normal (C57BL/6×CBA/J) F1 mice were purchased from Taconic M&B (Bomholtvej, Denmark), Tg<sub>e</sub> mice, generated by overexpression (>30 copies) of the full length human CD3ε gene, were established by sibling breeding of animals on the C57BL/6×CBA/J background under specific pathogen free (SPF) conditions at our local animal facility. All mice were 8–16 weeks old.

**BM purification and transplantation**

BM cells were harvested from ilia and fibulas of (C57BL/6×CBA/J) F1 mice. To avoid allorecognition of the Tg<sub>e</sub> recipients, donor BM cells were depleted of mature T cells. This was achieved by two rounds of complement mediated lysis using anti-Thyl.2 monoclonal antibody (clone 30–H12; BD PharMingen, San Diego, California, USA) on ice for 30 minutes followed by rabbit complement (Cedarlane) at 37°C for 45 minutes. Thereafter, less than 0.1% CD4<sup>+</sup> and 0.2% CD8<sup>+</sup> T cells were present in the BM inoculum, as determined by flow cytometry. BM recipients were pretreated with 150 mg/kg 5-fluorouracil (Gry-Pharma, Germany) and peritoneally into BM→Tg<sub>e</sub> recipients. The injections were done one week after BM transplantation. One group of mice received CD4<sub>+</sub>CD25<sup>+</sup> cells on the day of BM transplantation.

**Histological examination**

Colons were fixed in buffered 5% formalin. Thick paraffin embedded sections (2 μm) were stained with haematoxylin and eosin. Inflammation was scored in a blinded fashion on a scale from 0 to 4, representing no change to severe changes, as previously described.24

**Flow cytometry**

Isolated unseparated MLN cells were stained with FITC labelled antimouse CD4 and PE labelled antimouse CD8. Intracellular staining for TNF-α and IL-10 was performed using MLN cells five hours after stimulation with immobilised anti-CD3 for TNF-α and in addition anti-CD28 for IL-10. Brefeldin A (Sigma) was added two hours after culture initiation. Cells were first stained with antimouse PE. Brefeldin A (Sigma) was added two hours after culture initiation. Cells were then stained with antimouse CD4 PE. Before staining, cells were fixed with 2% paraformaldehyde (Riedel-de Haen, Seelze, Germany) and permeabilised using 0.2% saponin (Sigma). T cells were analysed on a FACSscan flow cytometer (Becton Dickinson, Heidelberg, Germany) using CellQuest software. Isotype matched control antibody staining served as the zero value.

**Cytokine assays**

Transforming growth factor β (TGF-β), IL-10, IFN-γ, and TNF-α were measured in MLN cell culture supernatants prepared as previously described5 and analysed using standard ELISA techniques (R&D Systems, Germany).25 Concentrations of cytokines were established in duplicated culture supernatants by comparison with standard curves. Limits of detection are 7 pg/ml of TGF-β, 4.0 pg/ml of IL-10, 2 pg/ml of IFN-γ, and 5.1 pg/ml of TNF-α.

**Statistical analysis**

Parametric data were analysed by the Student t test and non-parametric data by the Mann-Whitney test. A p value of <0.05 was considered significant.
RESULTS
Regulatory CD4⁺CD25⁺ cells are reduced in BM transplanted Tg26 mice
We first investigated whether regulatory CD4⁺CD25⁺ cells were reduced in BM⇒Tg26 mice. Therefore, MLN cells of BM⇒Tg26 mice were analysed by flow cytometry in comparison with wild-type mice 4–6 weeks after BM transplantation. The percentage of MLN cells that expressed both CD4 and CD25 was lower in Tg26 mice with colitis than in healthy wild-type mice (colitic 1.5 (1.1)%; wild-type 2.6 (1.0)%; p<0.05) (fig 1).

CD4⁺CD25⁺ transfer partially normalises the proportion of MLN CD4⁺ and CD8⁺ cells
Analysis of the effect of Treg cells on the proportion of MLN cells revealed that the regulatory CD25⁺ cell fraction reduced the percentage of CD4⁺ cells in the MLN of reconstituted mice in comparison with transplanted mice, with no additional cell transfer (table 1). In contrast, CD4⁺CD25⁺ cell transfer slightly increased the fraction of CD4⁺ cells (table 1).

The result of 1.7 (0.8)% for CD8⁺ cells did not change the CD8⁺ cell ratio (table 1). Taken together, these data suggest that regulatory CD4⁺CD25⁺ cells reduce the pathogenic CD4⁺ T cells in BM⇒Tg26 mice and correct imbalances of peripheral T cell differentiation.

CD25⁺ regulatory cells reduce TNF-α producing cells dose dependently
In the BM⇒Tg26 mouse model, CD4⁺ cells operate via a Th1 cytokine profile with a predominance of TNF-α and IFN-γ production. Therefore, we studied the effect of CD4⁺CD25⁺ cells on MLN Th1 T cells in transplanted mice. Transfer of 3×10⁵ MLN Treg cells significantly reduced TNF-α producing CD4⁺ MLN cells measured by FACS to as low as in wild-type mice (fig 2A). In contrast, transfer of lower numbers of CD4⁺CD25⁺ cells (1.5×10⁵ cells) or CD4⁺CD25⁻ cells (3.0×10⁵ cells) was unable to reduce TNF-α producing CD4⁺ T cells (fig 2A). After stimulation of MLN cells with anti-CD3, MLN cells from CD4⁺CD25⁺ T cell reconstituted BM⇒Tg26 recipients tended to produce lower amounts of TNF-α and secreted significantly lower amounts of IFN-γ than MLN cells from BM transplanted CD4⁺CD25⁻ cell reconstituted mice (fig 2B, C). These results indicate that Treg cells control intestinal inflammatory responses by reducing Th1CD4⁺ T cells. The regulatory effect of Treg cells was dose dependent.

Table 1 Analysis of mesenteric lymph node (MLN) cells derived from wild-type (WT) mice, from Tg26 mice after bone marrow (BM) transplant alone, or after bone marrow transplant plus CD4⁺CD25⁺ or CD4⁺CD25⁻ cell transfer

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>No of MLN cells x10⁶</th>
<th>No of CD4⁺ cells x10⁶ (% gated)</th>
<th>No of CD8⁺ cells x10⁶ (% gated)</th>
<th>Ratio CD4⁺/CD8⁺ cells (% gated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>16.4 (8.27)†</td>
<td>5.6 (1.9) (39.9 (7.9))</td>
<td>2.7 (1.6) (17.5 (1.6))</td>
<td>2.07 (2.28)</td>
</tr>
<tr>
<td>BM</td>
<td>34.0 (22.0)</td>
<td>4.1 (1.4) (16.4 (4.4))</td>
<td>4.0 (0.3) (1.7 (0.8))</td>
<td>10.25 (9.65)</td>
</tr>
<tr>
<td>BM⇒CD25⁺</td>
<td>29.0 (14.4)</td>
<td>2.8 (1.8) (10.8 (5.6))</td>
<td>0.8 (0.5) (3.9 (2.5))</td>
<td>3.5 (2.77)</td>
</tr>
<tr>
<td>BM⇒CD25⁻</td>
<td>23.0 (16.0)</td>
<td>4.4 (2.2) (20.0 (8.8))</td>
<td>0.2 (0.3) (1.0 (0.9))</td>
<td>22.0 (20.0)</td>
</tr>
</tbody>
</table>

*p<0.01 versus BM⇒Tg26 mice, versus BM⇒CD4⁺CD25⁺ Tg26 mice, and versus BM⇒CD4⁺CD25⁻ Tg26 mice.
\[p<0.005 versus BM⇒CD4⁺CD25⁺ Tg26 mice.
\[p<0.005 versus BM⇒CD4⁺CD25⁻ Tg26 mice.
\[p<0.05 versus BM⇒CD4⁺CD25⁺ Tg26 mice and versus BM⇒CD4⁺CD25⁻ Tg26 mice.

*Mean (SEM) number of MLN cells, number (percentages) of MLN CD4⁺ and CD8⁺ T cells obtained from Tg26 mice transplanted BM alone (n = 12) or with CD4⁺CD25⁺ cells (n = 8) or CD4⁺CD25⁻ cells (n = 12), from C57 Bl/6×CBA/J(F1) mice (n = 20).

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CD4+CD25+ cells prevent colitis in BM transplanted Tg26 mice

To investigate the regulatory activity of CD4+CD25+ cells in the prevention of colitis in Tg26 mice, we analysed the colon of BM transplanted mice (control) in comparison with three groups of mice which in addition to BM received 3×10^5 CD4+CD25+ cells, 1.5×10^5 CD4+CD25+ cells, or 3×10^5 CD4+CD25+ cells one week after transplantation. This time point was chosen to eliminate the chance that Treg cells given at the same time as BM transplant prevented engraftment of BM in the recipient. Transfer of 3×10^5 CD4+CD25+ cells inhibited weight loss (fig 3) and clinical signs of colitis in recipients while 1.5×10^5 CD4+CD25+ cells did not prevent weight loss (data not shown). Results were identical when CD4+CD25+ cells were transferred on the same day as BM (data not shown). In contrast, mice reconstituted with CD4+CD25− cells started to lose weight two weeks after BM transplantation (fig 3) and showed clinical signs of wasting and colitis (for example, piloeruption, hunching, bloody diarrhoea). Within this group, two mice died two weeks after BM transplantation. The control group of only BM⇒Tg26 mice started to show clinical signs of inflammation, including weight loss four weeks after transplantation, confirming previous results (fig 3). 23

Figure 2 Intracytoplasmic staining of tumour necrosis factor α (TNF-α) (A) and production of TNF-α (B) and interferon γ (IFN-γ) (C) measured by ELISA. (A) Summation of intracytoplasmic staining of TNF-α by ELISA. (A) Summation of intracytoplasmic staining of TNF-α by ELISA. Values represent means (SEM) in supernatants of MLN cell cultures from (A) and production of TNF-α antibody. Mean (SEM) percentage of cytokine positive cells are shown.

Figure 3 Weight loss. Body weight of Tg26 mice transplanted with wild-type bone marrow (BM⇒Tg26) that received 3×10^5 CD4+CD25+ cells, 3×10^5 CD4+CD25− cells, or no cells (control group) was measured twice per week and was divided by starting body weight (on the day of bone marrow transplantation) to calculate the percentage of body weight at each time point. Body weights were plotted as mean (SEM).

CD4+CD25+ cells, 1.5×10^5 CD4+CD25+ cells, or 3×10^5 CD4+CD25+ cells one week after transplantation. This time point was chosen to eliminate the chance that Treg cells given at the same time as BM transplant prevented engraftment of BM in the recipient. Transfer of 3×10^5 CD4+CD25+ cells inhibited weight loss (fig 3) and clinical signs of colitis in recipients while 1.5×10^5 CD4+CD25+ cells did not prevent weight loss (data not shown). Results were identical when CD4+CD25+ cells were transferred on the same day as BM (data not shown). In contrast, mice reconstituted with CD4+CD25− cells started to lose weight two weeks after BM transplantation (fig 3) and showed clinical signs of wasting and colitis (for example, piloeruption, hunching, bloody diarrhoea). Within this group, two mice died two weeks after BM transplantation. The control group of only BM⇒Tg26 mice started to show clinical signs of inflammation, including weight loss four weeks after transplantation, confirming previous results (fig 3). 23
Histopathological analysis of the colon from untreated BM>Tg mice showed inflammation in all parts, with the most severe inflammation in the distal colon and caecum. This was characterised by leucocyte infiltration in the mucosa and submucosa, prominent epithelial hyperplasia, loss of goblet cells, occasional crypt abscesses, and ulcerations (distal colon score 2.6 (0.5)) (fig 4, 5B). Transfer of 3×10^5 CD4^+CD25^+ cells led to an almost normal colon architecture with only a mild increase in leucocyte infiltration but no other signs of colitis (distal colon score 0.3 (0.3)) (fig 4, 5C). Lower transfer numbers of CD4^+CD25^+ cells failed to prevent colitis (distal colon score 2.8 (0.4)) (fig 4, 5D). CD4^+CD25^- cell transfer did not prevent colitis (distal colon score 2.7 (0.3)) (fig 4, 5E). Thus CD4^+CD25^- cells prevented colitis in the BM>Tg model while CD4^+CD25^- cells had no such regulatory function.

**CD25^+ Treg cells increase the secretion of TGF-β by MLN cells**

Others have shown that Treg cell action depends at least in part on the secretion of anti-inflammatory cytokines. To gain further insight into the mechanism by which CD25^+ cells exert their regulatory potential in the Tg26 intestine, we examined the production of TGF-β and IL-10 in MLN cells after stimulation with anti-CD3 and anti-CD28, respectively. Significantly higher levels of TGF-β were detected in supernatants of MLN cell cultures from CD4^+CD25^+ cell reconstituted BM>Tg mice in comparison with CD4^+CD25^- cell recipients (fig 6A). Intracytoplasmic staining showed that the frequency of IL-10 producing MLN CD4^+ cells in Treg cell transferred Tg26 recipients was not increased (CD4^+CD25^- mice: 0.2 (0.29)%; CD4^+CD25^- mice: 0.19 (0.14)%; wild-type mice: 0.2 (0.56)%). There was no
convincing trend towards higher IL-10 secretion by MLN cells from CD4+CD25+ mice in comparison with CD4+CD25- mice (fig 6B). These results suggest that CD4+CD25+ cells down-regulate the immune response predominantly by production of TGF-β.

**DISCUSSION**

It is unclear whether the development of colitis in BM→Tg26 mice results from failure of negative selection of aggressive T cells in the abnormal thymus or from insufficient production of thymic dependent regulatory T cells. Our findings provide strong evidence for defective function of regulatory CD4+CD25+ T cells in the pathogenesis of bowel inflammation in this model. Firstly, CD25+ expression on CD4+ cells in the MLN was significantly lower in BM→Tg26 mice with colitis than in healthy wild-type animals. Interestingly, our findings correspond to recent results in IBD patients in whom intestinal CD4+CD25+ cells were decreased in active disease. Moreover, co-administration of CD4+CD25+ Treg cells from MLN of normal mice clearly protected against wasting and colitis in the BM→Tg26 model. In contrast, CD4+CD25- cells had no immunosuppressive effect, indicating that the regulatory function lies within the CD4+CD25+ T cell subset.

Protection of colitis by CD4+CD25+ cells has been demonstrated in SCID and Rag mice. However, the immunological mechanisms of colitis in these models differ from the BM→Tg26 colitis model. Transfer of the naïve CD4+CD45RBhigh T cell subset from wild-type mice into SCID/Rag mice induces colitis in the recipients. This can be prevented by co-injection of the antigen experienced subset of CD4+CD45RBlow T cells. The CD25+ population comprises the inhibitory subset of CD4+CD45RBlow T cells. In contrast, colitis in Tg26 mice is driven by CD4+CD45RBhigh cells and CD45RBlow cells from colitic Tg26 mice could transfer severe colitis to untransplanted Tg26 recipients. Prevention of colitis by CD4+CD25+ cells in BM→Tg26 mice demonstrates that pathogenic responses by not only naïve CD4+CD45RBhigh cells as in the SCID mouse model but also by activated colitis conferring CD4+CD45RBlow cells are suppressed by CD4+CD25+ T cells. Adding to the complexity, Assemann et al reported that transfer of CD4+CD45RBlow cells into SCID mice can also induce colitis. However, colitis development was still different from the Tg26 model because it developed only when anti-IL-10R monoclonal antibody was co-administered. Furthermore, the ability of CD4+CD45RBlow cells to induce inflammation was significantly reduced when these cells were isolated from germ free mice. In contrast, in the Tg26 model, the colitis inducing CD4+CD45RBlow cells are bacteria specific without further manipulation and have the functional capacity to induce colitis when transferred to specific pathogen free recipients even when isolated from germ free BM→Tg26 mice. Based on our present study we speculate that the development of bacteria specific T cells may be due to the aberrant thymic selection of regulatory cells.

Differences in Treg cell mediated protection of colitis in the SCID mouse versus the Tg26 mouse is also visible in the number of Treg cells necessary to prevent inflammation. While Read and colleagues reported that Tg26 mice were sufficient to prevent colitis in SCID mice, we had to transfer a minimum of 5×10^5 Tg26 cells into BM→Tg26 mice for protection. In the Tg26 mouse a high number of very activated T cells develop after bone marrow transplantation. Thus differences in the number and also in the proinflammatory potential of colitic T cells in the two models might be responsible for the different Treg cell numbers necessary for the effects observed.

Our studies examined the mechanisms by which CD4+CD25+ T cells prevented colitis. Protection in our experiments was seen as early as four weeks after cell transfer in contrast with the SCID mouse model in which complete protection became manifest 10 weeks after cell transfer. Delayed protection in SCID mice was interpreted as a sign that Treg cells suppress the inflammatory response via an indirect mechanism. Foussat et al proposed that Treg cells function indirectly by enhancing differentiation of IL-10 secreting T cells. Assemann et al suggested that IL-10 secretion by CD4+CD25+ T cells is not an absolute requirement for inhibition of colitis in the SCID model because CD4+CD25+ cells from IL-10−/− mice induced colitis.

We detected only minimal and almost identical IL-10 on intracellular staining of MLN CD4+ cells from BM→Tg26 mice and BM→Tg26 reconstituted with either CD4+CD25+ or CD4+CD25- cells. There was no convincing trend towards higher IL-10 levels in the supernatants of MLN cell cultures from BM→Tg26 mice after reconstitution with CD4+CD25+ T cells in comparison with mice that received CD4+CD25- cells. A limitation of our study is that we did not transfer CD4+CD25+ cells from IL-10−/− mice or neutralised IL-10 by a blocking antibody. Nevertheless, our data do not suggest a major role for IL-10 as the effector mechanism of protection conferred by CD4+CD25+ cells in this model.

TGF-β has been shown to be the key immunosuppressive cytokine in CD4+CD25+ regulatory cells in various disease models. Recently, it was demonstrated that TGF-β also converted CD4+CD25− naïve T cells to CD4+CD25+ Treg cells. These cells expressed the transcription factor Foxp3 which is associated with the development of Treg cells. In vivo data demonstrated that Foxp3 expressing Treg cells prevented colitis in SCID mice. In our experiments, TGF-β was
Regulatory T cells prevent colitis in Tg26 mice

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Conflict of interest: None declared.

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