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×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 3×10^5 $CD4^+CD25^+$ 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.[1 2] There is compelling evidence from both human and animal models that this controlled homeostatic immune response is lost in genetically susceptible hosts that develop chronic immune mediated colitis.[3 4] 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.[5–7] 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).[8] Transfer of regulatory $CD4^+CD25^+$ cells in different experimental settings demonstrated their pivotal role in the maintenance of self tolerance,[9] in regulating peripheral T cell homeostasis,[10 11] in transplantation tolerance,[12] and in graft versus host protection after bone marrow (BM) transplantation.[13 14] The thymus has been identified as the place where regulatory T cells (Treg cells) develop[15 16] and a normal thymic architecture is necessary for adequate selection of Treg cells.[17]

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 $CD4^+CD45RB^{hi}$ T cells and can be prevented by co-transfer of the naturally activated $CD45RB^{low}$ subset.[18 19] Because colitis in SCID mice can only be induced by transfer of adult T lymphocytes but not by BM transplantation,[20 21] 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 the human CD3ε gene. This results in very early arrest of T cell development which prevents the induction of a normal thymic microenvironment.[22] BM transplantation from syngenic 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.[22] $CD4^+$ cells are characterised by low $CD45RB^{high}$ 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).[23] These cells cause severe colitis.[23–26]

To date, the mechanisms underlying the development of aggressive T cells in the abnormal thymus in Tgε26 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⇒Tgε26 mice with a persistent abnormal thymus prevented the development of colitis.[23] However, direct evidence for a key role of absent Treg cells in the pathogenesis of colitis in Tgε26 mice is missing and such cells need to be further characterised.

The purpose of the present study was to investigate whether defective selection of $CD4^+CD25^+$ Treg cells in the compromised thymic environment of Tgε26 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⇒Tgε26 mice can be corrected by regulatory $CD4^+CD25^+$ 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). Tgε26 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 tibias and fibulas of (C57BL/6 × CBA/J) F1 mice. To avoid allorecognition of the Tgε26 recipients, donor BM cells were depleted of mature T cells. This was achieved by two rounds of complement mediated lysis using anti-Thy1.2 monoclonal antibody (clone 30-H12; BD PharMingen, San Diego, California, USA) on ice for 30 minutes followed by rabbit complement (Cedarlane) at 37℃ for 45 minutes. Thereafter, less than 0.1% $CD4^+$ and 0.2% $CD8^+$ 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) intraperitoneally 48 hours before engraftment with 5×10^6 BM cells resuspended in sterile phosphate buffered saline and injected intraperitoneally into SPF Tgε26 recipients.

Antibodies

The following antibodies were used for cell purification: antimouse CD45R (B220) Microbeads (clone RA3-6B2) and antimouse CD8a (Ly-2) Microbeads (clone 53-6.7) (Miltenyi Biotec, Bergisch Gladbach, Germany); PE conjugated antimouse CD4 (clone GK1.5) and fluorescein isothiocyanate (FITC) conjugated antimouse CD25 (IL-2 receptor α chain, p55) (clone 7D4) were used for FACS sorting. FITC conjugated antimouse CD4 (clone GK1.5) and PE conjugated antimouse CD8a (Ly-2) (clone 53–6.7) were used for FACS analysis after T cell depletion in BM and for T cell analysis in sacrificed mice. FITC conjugated antimouse IL-10 (clone JES5-16E3) and FITC conjugated antimouse TNF-α (clone MP6-XT22) were used for intracellular cytokine staining. For cell stimulation, antimouse CD3 (CD3ε chain, clone 145-2C11), and for IL-10 staining antimouse CD28 (clone 37.51), were used.

Purification of T cell subsets

For $CD4^+CD25^+$ and $CD4^+CD25^-$ cell transfer, cells were isolated from mesenteric lymph nodes (MLN). Cells were washed in RPMI l640 (Gibco, Grand Island, New York, USA), supplemented with 5% heat inactivated fetal bovine serum (Greiner, Germany) and antibiotics/antimycotics (penicillin G, streptomycin, and amphotericin B) (Sigma Chemical Co., St Louis, Missouri, USA), and resuspended in MACS buffer. Cells were depleted of $CD8^+$ and $B220^+$ cells by negative selection using antimouse coated Microbeads (Miltenyi Biotec). The resulting $CD4^+$ enriched cells were stained with PE conjugated anti-CD4 and FITC conjugated anti-CD25 monoclonal antibodies. Subsets of $CD4^+$ cells were generated by two colour sorting on a BD FACS Vantage SE/DiVa Option. All populations were >98% pure on reanalysis.

T cell transfer experiments

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were separately resuspended in phosphate buffered saline and injected intraperitoneally into BM⇒Tgε26 recipients. The injections were done one week after BM transplantation. One group of mice received $CD4^+CD25^+$ cells on the day of BM transplantation.

Mice were observed daily and weighed at least twice a week. Mice showing clinical signs of severe disease were sacrificed (4–5 weeks after BM transplantation). Healthy recipients were sacrificed six weeks after 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 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 FACscan 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 described[5] 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.

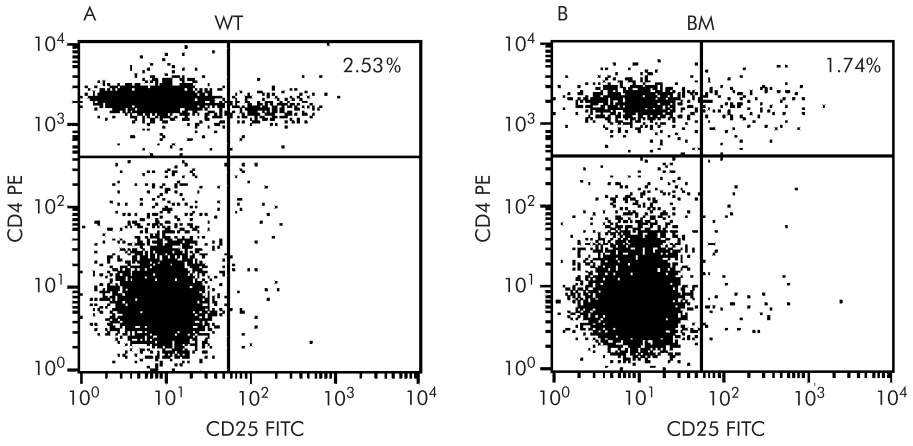

Figure 1 Flow cytometric analysis of expression of $CD4^+CD25^+$ in mesenteric lymph node (MLN) cells of Tgε26 mice transplanted with wild-type bone marrow (BM⇒Tgε26) (B) and of syngenic wild-type mice (A). Dot plots showing representative proportions of $CD4^+CD25^+$ MLN cells in Tgε26 mice 4–6 weeks after bone marrow (BM) transplantation and in syngenic wild-type (WT) mice.

RESULTS

Regulatory $CD4^+CD25^+$ cells are reduced in BM transplanted Tgε26 mice

We first investigated whether regulatory $CD4^+CD25^+$ cells were reduced in BM⇒Tgε26 mice. Therefore, MLN cells of BM⇒Tgε26 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 Tgε26 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 found in transplanted mice confirms previous results (table 1).[5 23] Interestingly, the percentage of $CD8^+$ cells in MLN increased significantly in response to $CD4^+CD25^+$ cell transfer (table 1). In contrast, $CD4^+CD25^-$ cells did not change the $CD8^+$ population (table 1) which remained as low as in the BM⇒Tgε26 mice. Thus $CD4^+CD25^+$ cell transfer normalised the ratio of $CD4^+$ cells to $CD8^+$ cells while transfer of $CD4^+CD25^-$ cells substantially increased the CD4/CD8 MLN cell ratio (table 1). Taken together, these data suggest that regulatory $CD4^+CD25^+$ cells reduce the pathogenic $CD4^+$ T cells in BM⇒Tgε26 mice and correct imbalances of peripheral T cell differentiation.

$CD25^+$ regulatory cells reduce TNF-α producing cells dose dependently

In the BM⇒Tgε26 mouse model, $CD4^+$ cells operate via a Th1 cytokine profile with a predominance of TNF-α and IFN-γ production.[26 27] Therefore, we studied the effect of $CD4^+CD25^+$ cells on MLN Th1 T cells in transplanted mice. Transfer of 3×10^5 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 (1.5×10^5 cells) or $CD4^+CD25^-$ cells (3.0×10^5 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⇒Tgε26 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 Th1$CD4^+$ 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 Tgε26 mice after bone marrow (BM) transplant alone, or after bone marrow transplant plus $CD4^+CD25^+$ or $CD4^+CD25^-$ cell transfer

Mouse group	No of MLN cells $\times10^6$	No of $CD4^+$ cells $\times10^6$ (% gated)	No of $CD8^+$ cells $\times10^6$ (% gated)	Ratio CD4/CD8 cells (%gated)
WT	16.4 (8.27)*†	5.6 (1.9)‡ (39.9 (7.9))§	2.7 (1.6)† (17.5 (1.6))†	2.07 (2.28)
BM	34.0 (22.0)	4.1 (1.4)‡ (16.4 (4.4))‡	0.4 (0.3)¶ (1.7 (0.8))§	10.25 (9.65)
BM+$CD25^+$	29.0 (14.4)	2.8 (1.8) (10.8 (5.6))§	0.8 (0.5)§ (3.9 (2.5))§	3.5 (2.77)
BM+$CD25^-$	23.0 (15.6)	4.4 (2.2) (20.0 (5.8))	0.2 (0.3) (1.0 (0.92))	22.0 (20.0)

*Mean (SEM) number of MLN cells, number (percentages) of MLN $CD4^+$ and $CD8^+$ T cells obtained from Tgε26 mice transplanted with 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).
†$p<0.01$ versus BM-Tgε26mice, versus BM+$CD4^+CD25^+$ Tgε26 mice, and versus BM+$CD4^+CD25^-$ Tgε26 mice.
‡$p<0.005$ versus BM+$CD4^+CD25^+$ Tgε26 mice.
§$p<0.005$ versus BM+$CD4^+CD25^-$ Tgε26 mice.
¶$p<0.05$ versus BM+$CD4^+CD25^+$ Tgε26 mice and versus BM+$CD4^+CD25^-$ Tgε26 mice.

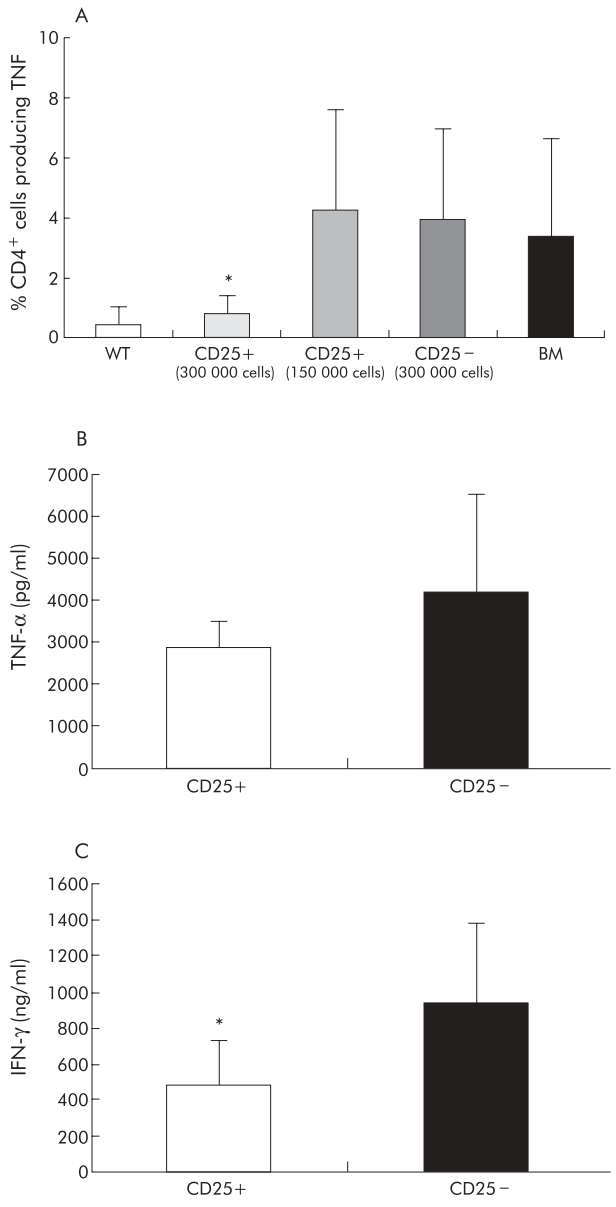

Figure 2 Intracytoplasmic staining of tumour necrosis factor α (TNF-α) (A) and production of TNF-α (B) and interferon γ (IFN-γ) (C) measured by ELISA. (A) Summation of intracytoplasmic staining of TNF-α is shown on gated $CD4^+$ mesenteric lymph node (MLN) cells from wild-type (WT) mice and from Tgε26 mice transplanted with wild-type bone marrow (BM⇒Tgε26) that received 3×10^5 $CD4^+CD25^+$ cells, 1.5×10^5 $CD4^+CD25^+$ cells, 3×10^5 $CD4^+CD25^-$ cells, or no T cells, analysed by flow cytometry five hours after stimulation with immobilised anti-CD3 antibody. Mean (SEM) percentage of cytokine positive cells are shown. *$p<0.05$ versus BM⇒Tgε26 mice that received 1.5×10^5 $CD4^+CD25^+$ cells, versus BM⇒Tgε26 mice that received 3×10^5 $CD4^+CD25^-$ cells, and versus BM⇒Tgε26 mice. (B) TNF-α and (C) IFN-γ in supernatants of MLN cell cultures three days after stimulation with anti-CD3 antibody. Values represent means (SEM) in supernatants of MLN cell cultures from BM⇒Tgε26 mice that received 3×10^5 $CD4^+CD25^+$ cells or 3×10^5 $CD4^+CD25^-$ cells.

$CD4^+CD25^+$ cells prevent colitis in BM transplanted Tgε26 mice

To investigate the regulatory activity of $CD4^+CD25^+$ cells in the prevention of colitis in Tgε26 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, piloerection, hunching, bloody diarrhoea). Within this group, two mice died two weeks after BM transplantation. The control group of only BM⇒Tgε26 mice started to show clinical signs of inflammation, including weight loss four weeks after transplantation, confirming previous results (fig 3).[23]

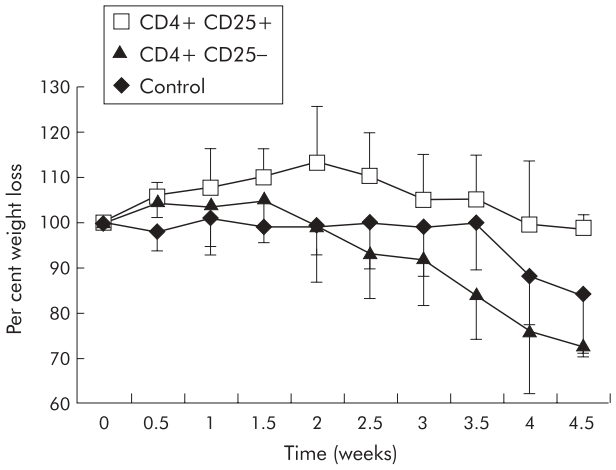

Figure 3 Weight loss. Body weight of Tgε26 mice transplanted with wild-type bone marrow (BM⇒Tgε26) that received 3×10^5 $CD4^+CD25^+$ cells, 3×10^5 $CD4^+CD25^-$ cells, or no cells (control group) was measured twice per week and was divided by starting body weight (on the day of bone marrow transplantation) to calculate the percentage of body weight at each time point. Body weights were plotted as mean (SEM).

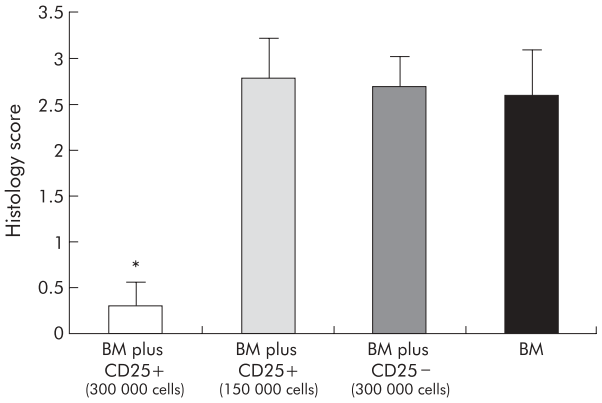

Figure 4 Blinded histological scores of the colon of Tgε26 mice. Tgε26 recipients were transplanted with bone marrow from normal mice and one week later injected with 3×10^5 $CD4^+CD25^+$ cells, 1.5×10^5 $CD4^+CD25^+$ cells, or 3×10^5 $CD4^+CD25^-$ cells or did not receive T cells. Mice were killed 5–6 weeks later. Inflammation was scored on a scale of 0–4 (see materials and methods) and results are expressed as mean (SEM). *$p<0.05$ versus all other groups.

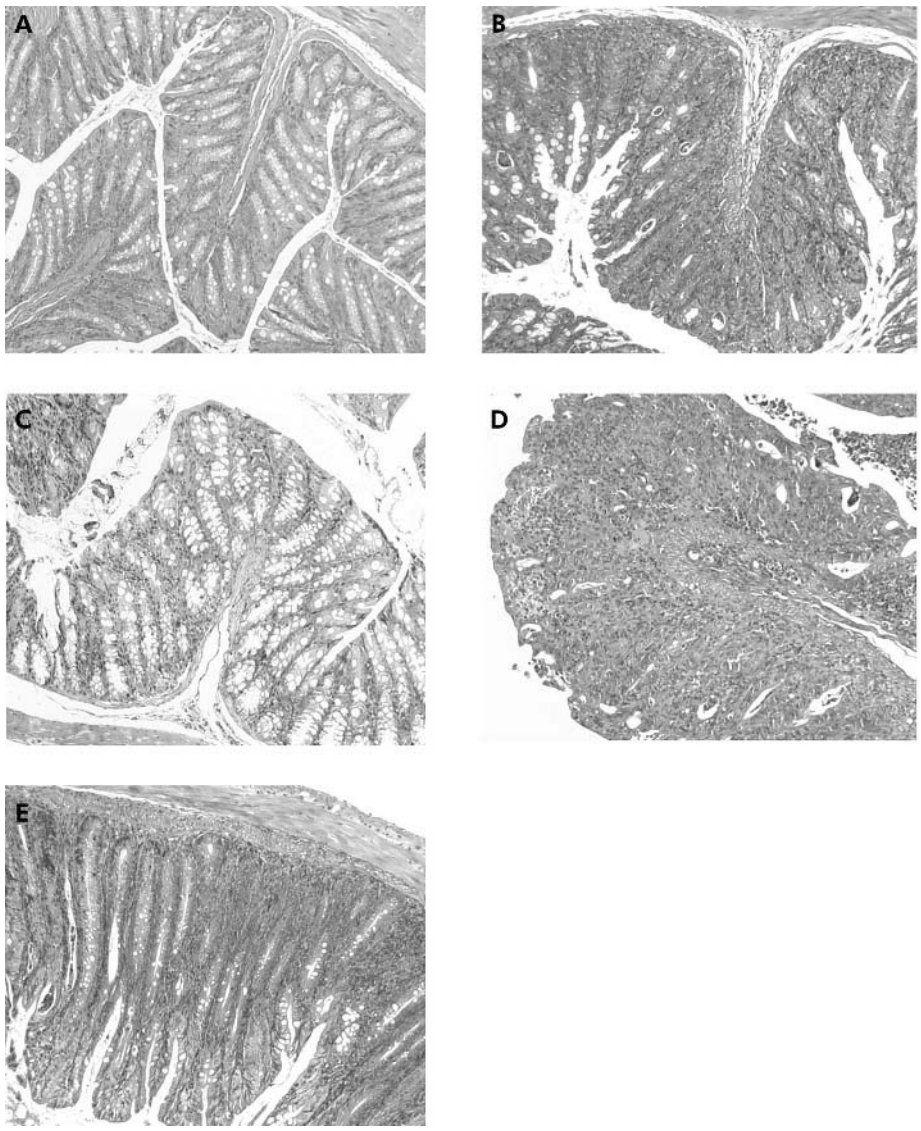

Figure 5 Photomicrographs of the colons of Tgε26 mice five weeks after bone marrow (BM) transplant or after transplantation of BM plus transfer of different T cell subsets. Representative haematoxylin-eosin stained sections from the distal colon are shown (magnification 200×). (A) Normal colon from a non-transplanted specific pathogen free (SPF) Tgε26 mouse. (B) Severe colitis in a SPF BM transplanted Tgε26 mouse demonstrating crypt hyperplasia, decreased goblet cells, pronounced lamina propria lymphocytic infiltration, and crypt abscesses. (C) No signs of inflammation in a Tgε26 mouse that received BM plus 3×10^5 $CD4^+CD25^+$ cells. (D) Active colitis in a BM transplanted Tgε26 mouse which was reconstituted with only 1.5×10^5 $CD4^+CD25^+$ cells. (E) Severe colitis in a SPF Tgε26 mouse that was injected with BM plus 3×10^5 $CD4^+CD25^-$ cells. Extensive lamina propria cellular infiltration, crypt hyperplasia, and decreased goblet cells are evident.

Histopathological analysis of the colon from untreated BM⇒Tgε26 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⇒Tgε26 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.[28–30] To gain further inside into the mechanism by which $CD25^+$ cells exert their regulatory potential in the Tgε26 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⇒Tgε26 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 Tgε26 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

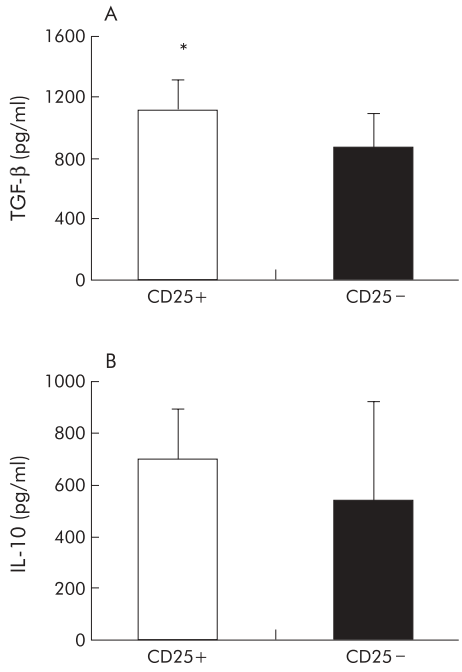

Figure 6 Cytokine production detected in supernatants of mesenteric lymph node (MLN) cell cultures three days after stimulation with anti-CD3 antibody for transforming growth factor β (TGF-β) (A) and anti-CD28 antibody for interleukin 10 (IL-10) (B). TGF-β and IL-10 were measured by ELISA. Values represent means (SEM) per $CD4^+$ MLN cells in supernatants of MLN cell cultures from Tgε26 mice reconstituted with bone marrow (BM) plus 3×10^5 $CD4^+CD25^+$ cells or BM plus 3×10^5 $CD4^+CD25^-$ cells. *$p<0.05$ versus Tgε26 mice reconstituted with BM plus $CD4^+CD25^-$ cells.

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⇒Tgε26 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⇒Tgε26 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.[31] Moreover, co-administration of $CD4^+CD25^+$ Treg cells from MLN of normal mice clearly protected against wasting and colitis in the BM⇒Tgε26 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.[32–34] However, the immunological mechanisms of colitis in these models differ from the BM⇒Tgε26 colitis model. Transfer of the naïve $CD4^+CD45RB^{high}$ 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 $CD4^+CD45RB^{low}$ T cells.[18] The $CD25^+$ population comprises the inhibitory subset of $CD4^+CD45RB^{low}$ T cells.[10] In contrast, colitis in Tgε26 mice is driven by $CD4^+CD45RB^{low}$ cells,[5 23] and $CD45RB^{low}$ cells from colitic Tgε26 mice could transfer severe colitis to untransplanted Tgε26 recipients.[23] Prevention of colitis by $CD4^+CD25^+$ cells in BM⇒Tgε26 mice demonstrates that pathogenic responses by not only naïve $CD4^+CD45RB^{high}$ cells as in the SCID mouse model but also by activated colitis conferring $CD4^+CD45RB^{low}$ cells are suppressed by $CD4^+CD25^+$ T cells. Adding to the complexity, Asseman *et al* reported that transfer of $CD4^+CD45RB^{low}$ cells into SCID mice can also induce colitis.[35] However, colitis development was still different from the Tgε26 model because it developed only when anti-IL-10R monoclonal antibody was co-administered. Furthermore, the ability of $CD4^+CD45RB^{low}$ cells to induce inflammation was significantly reduced when these cells were isolated from germ free mice. In contrast, in the Tgε26 model, the colitis inducing $CD4^+CD45RB^{low}$ cells are bacteria specific without further manipulation and have the functional capacity to induce colitis when transferred to specific pathogenic free recipients even when isolated from germ free BM⇒Tgε26 mice.[5] 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 Tgε26 mouse is also visible in the number of Treg cells necessary to prevent inflammation. While Read and colleagues[32] reported that 5×10^4 $CD4^+CD25^+$ cells were sufficient to prevent colitis in SCID mice, we had to transfer a minimum of 3×10^5 cells into BM⇒Tgε26 mice for protection. In the Tgε26 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.[33] Delayed protection in SCID mice was interpreted as a sign that Treg cells suppress the inflammatory response via an indirect mechanism.[33] Foussat *et al* proposed that Treg cells function indirectly by enhancing differentiation of IL-10 secreting T cells.[36] Asseman *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 inhibited colitis.[35] We detected only minimal and almost identical IL-10 on intracellular staining of MLN $CD4^+$ cells from BM⇒Tgε26 mice and BM⇒Tgε26 reconstituted with either $CD4^+25^+$ or $CD4^+25^-$ cells. There was no convincing trend towards higher IL-10 levels in the supernatants of MLN cell cultures from BM⇒Tgε26 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 deficient 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.[29 37 38] Recently, it was demonstrated that TGF-β also converted $CD4^+CD25^-$ naïve T cells to $CD4^+CD25^+$ Treg cells.[39] These cells expressed the transcription factor Foxp3 which is associated with the development of Treg cells.[40 41] In vivo data demonstrated that Foxp3 expressing Treg cells prevented colitis in SCID mice.[40] In our experiments, TGF-β was

secreted in significantly higher amounts by MLN cells from BM⇒Tgε26 mice that had received $CD4^+CD25^+$ cells compared with those receiving either BM or BM plus $CD4^+CD25^-$ cells. While this suggests that TGF-β is involved in the $CD4^+CD25^+$ cell mediated prevention of colitis in the Tgε26 colitis model, we did not perform experiments with TGF-β blocking antibodies.

Protection against colitis by Treg cells was dose dependent in our model. Transfer of at least 3×10^5 Treg cells was necessary to prevent intestinal inflammation. Lower Treg cell transfer numbers led to a higher proportion of activated TNF-α producing T cells in recipients. Moreover, MLN T cells from Treg cell reconstituted mice secreted lower amounts of TNF-α and IFN-γ on stimulation than T cells from $CD4^+CD25^-$ reconstituted mice. Taken together, this suggests that down-regulation of activated T cells is one of the immunosuppressive mechanisms of Treg cells in the Tgε26 colitis model.

In accordance with previous reports,[23] analysis of MLN cells in BM⇒Tgε26 mice demonstrated that most of the T cells were $CD4^+$ while $CD8^+$ T cells were very rare, thereby increasing the ratio of $CD4^+$ to $CD8^+$ T cells several fold in comparison with wild-type mice. Flow cytometry of MLN from BM⇒Tgε26 mice reconstituted with $CD4^+CD25^+$ cells revealed a much lower number and percentage of $CD4^+$ cells in comparison with mice reconstituted with $CD4^+CD25^-$ cells and with BM⇒Tgε26 mice without additional cell transfer. The number and percentage of $CD8^+$ cells in MLN increased in response to transfer of $CD4^+CD25^+$ Treg cells while it remained low in $CD4^+CD25^-$ treated mice. The effect on the peripheral T cell pool was also expressed in the ratio $CD4^+$ to $CD8^+$ cells, which was similar in Treg cell reconstituted and wild-type mice but remained largely increased in mice reconstituted with $CD4^+CD25^-$ cells. Thus our results provide evidence that the aberrant proportion of peripheral T cells in BM⇒Tgε26 mice is corrected by $CD4^+CD25^+$ cells.

Our observation that Treg cells regulate the development of $CD8^+$ cells indicates an as yet undefined role for Treg cells in thymus dependent T cell development. Pettersson and colleagues[42] showed that $CD8^+$ T cells induced $CD4^+CD8^+CD25^+$ thymocytes. These double positive thymocytes are absent in BM⇒Tgε26 mice.[22] We speculate that Treg cell induced $CD8^+$ cells act in a positive loop inducing the development of double positive thymocytes, thereby promoting the normalisation of the Tgε26 thymic architecture and T cell development.

In summary, our experiments show that $CD4^+CD25^+$ T cells play an important role in the prevention of intestinal inflammation in the Tgε26 colitis model. $CD4^+CD25^+$ T cell transfer resulted in a substantial modification of the MLN T lymphocyte subsets. This may identify a possible new mechanism by which Treg cells induce immune suppression in experimental colitis.

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