Role of T lymphocytes in rat 2,4,6-trinitrobenzene sulphonic acid (TNBS) induced colitis: increased mortality after γδ T cell depletion and no effect of αβ T cell depletion

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Abstract

***Background and aim*—Indirect evidence suggests that CD4⁺ T cells have a pathogenic while γδ T cells have a protective role in the initiation and perpetuation of inflammatory bowel disease. To define the role of T cell subsets in a rat colitis model (2,4,6-trinitrobenzene sulphonic acid (TNBS)) we analysed colitis severity after effective depletion of T helper cells, αβ T cells, or γδ T cells.**

***Methods*—T helper cells, αβ T cells, or γδ T cells were depleted using previously described monoclonal antibodies directed at the CD4 molecule (OX38), the CD2 molecule (OX34, both depleting CD4⁺ T cells), the αβ T cell receptor (R73), and the γδ T cell receptor (V65). Depletion was verified by flow cytometry and/or immunohistology. Colitis was induced using intracolonic application of TNBS.**

***Results*—Surprisingly, depletion of T helper cells or αβ T cells had no influence on survival, macroscopic or microscopic scores, or myeloperoxidase activity following colitis induction. In contrast, depletion of γδ T cells resulted in significantly increased mortality (V65: 73%, n=15) compared with controls (30%, n=13; p<0.03). In addition, colitis was histologically more severe in the γδ T cell depleted group compared with controls (p<0.05).**

***Conclusions*—T helper cells or αβ T cells did not influence the initiation or perpetuation of rat TNBS colitis. In contrast, γδ T cells had a protective role in rat TNBS colitis as depletion caused increased mortality.**

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Keywords: αβ T cells; γδ T cells; experimental colitis; inflammatory bowel disease; rat

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A subpopulation of T lymphocytes expresses a T cell receptor (TCR) that is distinct from the conventional αβ TCR (that is, it is characterised by a γ and a δ chain). Several important immune functions have been reported for intestinal γδ T cells, namely T cell help for immunoglobulin class switching,[1] production of epithelial growth factors,[2] first line defence against various pathogens,[3 4] an immunodownregulatory role during infection (for example, coccidiosis),[5] and requirement for oral tolerance induction.[6] In addition, the percentage of γδ T cells in the intraepithelial lymphocyte compartment is several fold higher compared with peripheral blood lymphocytes, particularly in mice but also in rats and humans.[7–9] In spite of these important aspects, little is known of the role of γδ T cells in inflammatory bowel disease (IBD). As γδ T cells can downregulate contact sensitivity responses to skin haptens and prevent diabetes in non-obese diabetic mice, a regulatory function of γδ T cells in IBD seems likely.[10 11]

The hapten 2,4,6 trinitrobenzene sulphonic acid (TNBS) in 50% ethanol induces in susceptible rodents a subacute or even chronic form of colitis (TNBS colitis).[12 13] Histologically, the inflammatory response includes mucosal and submucosal infiltration of, initially, neutrophils, macrophages, and mast cells, and later lymphocytes and fibroblasts.[12] Granulomas and even Langhans'-type giant cells can be detected. Because of these similarities with IBD (particularly Crohn's disease) this model has been used by many investigators since its first description in 1989. Indirect evidence suggests that CD4⁺ T cells play a major role in the initiation and perpetuation of TNBS colitis, particularly a T helper 1 (Th1) cytokine response as seen after CD3/CD28 stimulation of lamina propria T lymphocytes[14] and the attenuating effects of T cell directed immunotherapies (anti-IL-12 monoclonal antibody (mAb), anti-CD40L mAb, anti-interferon-γ mAb, or OX40-Ig).[14–16] However, it has never conclusively been shown that CD4⁺ T cells have a pathogenic role in TNBS colitis. The role of other T cell subsets—for example, γδ T cells—has not been investigated in this model.

We therefore examined if depletion of γδ T cells, CD4⁺ T cells, or αβ T cells influenced the initiation and/or perpetuation of rat TNBS colitis.

Materials and methods

ANIMALS

Inbred female Lewis rats were obtained from Charles River, Germany, at the age of 10–12 weeks and weighing 160–180 g. The animals

Abbreviations used in this paper: IBD, inflammatory bowel disease; ko, knock out; PALS, periarteriolar lymphocyte sheath; TCR, T cell receptor; TNBS, 2,4,6-trinitrobenzene sulphonic acid; Th1, T helper 1; mAb, monoclonal antibody.

were kept in polycarbonate cages and had free access to standard chow and tap water.

mAb

The following depleting mAbs were used in vivo: OX34 (IgG2a), directed at CD2 (depletes T helper cells),[17 18] OX38 (IgG2a), directed at CD4 (depletes T helper cells),[19] both kindly provided by AN Barclay (University of Oxford, UK), R73 (IgG1), directed at the rat αβ TCR (depletes αβ T cells),[20 21] and V65 (IgG1), directed at the rat γδ TCR,[21] both kindly donated by T Hünig (University of Würzburg, Germany). Mouse mAbs directed at epitope 1 of human CD58 AICD58.6 (epitope 1 of domain 1, IgG2a) and TS2/9 (epitope 1 of domain 1, IgG1) served as controls.[22] The AICD58.6 mAb was a gift from SC Meuer (University of Heidelberg, Germany); the hybridoma TS2/9 was obtained from ATCC. mAb were purified from supernatants of hybridoma cell lines using spinner flasks followed by affinity chromatography employing protein A Sepharose beads (Pharmacia, Germany). Purified mAb was filtered (0.25 μm) and administered intraperitoneally. All depleting mAb were used in regimens that have previously been demonstrated to strongly deplete indicated T cell subsets.[17 19 21] Effective depletion was confirmed by either flow cytometry or immunohistology at the end of the study when depletion was least pronounced (see below).

mAb TREATMENT

Treatment with mAb was performed in two treatment blocks:

(1) *Anti-CD4 treatment*: 37 animals were blindly assigned to one of three mAb treatment groups: (a) $CD4^+$ T cell depleting anti-CD2 mAb (OX34, n=14), (b) $CD4^+$ T cell depleting anti-CD4 mAb (OX38, n=10), or (c) isotype matched control mAb (anti-LFA-3, AICD58.6, n=13). mAbs were given over three consecutive days starting with 2 mg followed by 1 mg each. On day 3, TNBS was administered (see below). These studies were performed in three separate experiments.

(2) *Anti-TCR αβ or γδ treatment*: 40 animals were blindly assigned to one of three treatment groups: (a) αβ T cell depleting mAb (R73, n=12), (b) γδ T cell depleting mAb (V65, n=15), or (c) isotype matched control mAb (anti-LFA-3, TS2/9, n=13). TNBS was injected into the colon on day 0. The αβ T cell depleting mAb (R73) and the control mAb (TS2/9) were started two days prior to colitis induction at 200 μg, followed by 100 μg on days −1, 0, 2, 4, 6, 8, and 10. The γδ T cell depleting mAb V65 was given seven days prior to colitis induction and at the same time as colitis induction at a dose of 500 μg each. These previously reported treatment regimens were used to obtain depletion of either αβ or γδ T cells over the whole observation period of 13 days.[21] This treatment block was performed in three separate experiments.

INDUCTION OF TNBS COLITIS

Animals were randomised into treatment or control groups in a blinded fashion by BH, as stated above. After mAb pretreatment rats were lightly anaesthetised with ether and a central venous catheter (OD 1.7 mm) was inserted intrarectally such that the tip was 8 cm proximal to the anus, as previously described.[12] TNBS 40 mg (Fluka, Munich, Germany) in 0.5 ml of 50% ethanol (vol/vol) was injected intrarectally. Thereafter, the catheter was slowly removed and the animals were kept in a vertical position for 30 seconds.

Clinical assessment included daily determination of weight and general well being. Animals were killed after a weight loss of greater than 20% of original weight (n=5) or other clinical signs of severe colitis (for example, massive abdominal distention due to megacolon (n=2) or severe lethargy (n=1)). These animals were counted as fatal cases in the mortality analysis with maximal macroscopic and microscopic colitis scores.

ASSESSMENT OF COLITIS

Macroscopic scoring

At the end of the study animals were exsanguinated under ether anaesthesia and the small and large intestine as well as the spleen were excised. Samples of the terminal ileum and spleen were quickly frozen in liquid nitrogen for immunohistology (see below) and samples of the descending colon were obtained for paraffin section and measurement of myeloperoxidase activity (see below). The remaining descending, sigmoid colon, and rectum were opened longitudinally, pinned out on a board, and the macroscopic appearance was scored by two blinded investigators on a scale of 0–4, similar to the original scoring system of Morris and colleagues[12]: briefly, 0, no evidence of inflammation; 1, erythema only; 2, erythema, slight oedema, and small erosions; 3, two or more bleeding ulcers and/or inflammation and/or moderate adhesions; 4, severe ulceration and/or stenosis with prestenotic dilations and/or severe adhesions.

Microscopic scoring

Tissues were fixed in phosphate buffered saline containing 4% formalin and embedded in paraffin. Sections (5 μm) were stained with haematoxylin and eosin. The degree of inflammation was blindly assessed by two investigators using the scoring system described by Neurath *et al* from 0–4 (0, no signs of inflammation; 1, low level of leucocyte infiltration; 2, moderate level of leucocyte infiltration; 3, high level of leucocyte infiltration, high vascular density, thickening of bowel wall; 4, transmural infiltrations, loss of goblet cells, high vascular density, strong bowel wall thickening).[14]

DETERMINATION OF MYELOPEROXIDASE ACTIVITY

Colonic myeloperoxidase activity was measured as previously described by Grisham *et al* and Suzuki and colleagues.[23 24] Briefly, tissue was homogenised in approximately 10 vol of 50 mM KPO_4, pH 7.4, and centrifuged for

20 minutes at 20000 *g*. The pellet was homogenised in 10 vol 50 mM KPO_4, 10 mM EDTA, pH 6, containing 0.5% hexadecyltrimethylammonium bromide (wt/vol). After thaw-freezing once, the homogenate was sonicated and myeloperoxidase activity was determined by measuring H_2O_2 dependent oxidation of 3,3',5,5'-tetramethylbenzidine and expressed as units per gram of tissue.

IMMUNOHISTOLOGY

Cryostat sections were made (thickness 5 mm), air dried, wrapped in aluminum foil, and stored at −20°C. To determine localisation and phenotype of different cell types a immunohistochemical staining technique was used as previously described employing the APAAP method[17] using the following antibodies: R73 (αβ T cells), V65 (γδ T cells), and OX8 ($CD8^+$ T cells and NK cells) (kindly provided by AN Barclay, Oxford, UK). The slides were counterstained and mounted in glyceryl (Dakopatts, Hamburg, Germany). The number of stained cells/mm^2 mucosa was determined in four different sections. Splenic lymphocytes were counted as previously described on two sections per animal and are expressed as number of lymphocytes within the periarteriolar lymphocyte sheath (PALS)/mm^2 of the spleen.[17] Percentages of lamina propria lymphocytes or lymphocytes expressing αβ or γδ T cells were calculated using these values.

FLOW CYTOMETRY

Splenocytes and peripheral blood lymphocytes were obtained by intracardial puncture or splenectomy and homogenisation as previously described.[17] Single cell suspensions were exposed to hypotonic solution to lyse erythrocytes. Cell viability was greater than 95% as assessed by trypan blue exclusion. For flow cytometry staining R73 was biotinylated and OX8 was FITC labelled using standard techniques. T helper cells were defined as $R73^+$ $OX8^-$ cells using streptavidin PE. Only viable cells, as determined by forward and side scatter characteristics, were analysed using a FACS Vantage cell sorter (Becton/Dickonson, Heidelberg, Germany).

STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS for Windows. Survival was analysed using Kaplan-Meier analysis. Comparison of macroscopic and microscopic scores, and myeloperoxidase activity was carried out using the Mann-Whitney U test. Values are expressed as mean (95% confidence intervals) (colitis scores, myeloperoxidase activity) or mean (SD) (cell counts).

Results

DEPLETION OF T HELPER CELLS DOES NOT INFLUENCE THE INITIATION OR PERPETUATION OF RAT TNBS COLITIS

Previously, we have shown that the anti-CD2 mAb OX34 ameliorates chronic adjuvant arthritis and selectively depletes T helper cells.[17 18] To test the potential benefit of this anti-CD2 mAb in hapten induced colitis and to clarify the role of T helper cells in the initiation of hapten induced colitis, we randomly treated 37 female Lewis rats over three days with either anti-CD2 (OX34, CD4 depleting, n=14), anti-CD4 (OX38, CD4 depleting, n=10), or an isotype matched control mAb (AICD58.6, n=13). On the third treatment day animals received 40 mg of TNBS in 50% ethanol (day 0 after TNBS colitis induction). As anti-CD2 mAb treatment over three days was previously shown to result in long term depletion which is most pronounced on day 1, depletion by anti-CD2 or anti-CD4 was only confirmed at the end of the observation period—that is, day 13 after colitis induction. As shown in table 1, αβ T cells of the spleen and mucosa of the ileum were strongly depleted on day 13, as demonstrated by immunohistology (mean reduction of 95% in the spleen and >80% in the lamina propria). Depletion of αβ T cells was due to reduced numbers of T helper cells, as assessed by two colour flow cytometry (CD8/TCR αβ) and immunohistology showing unchanged numbers of CD8 positive cells in the spleen and ileum (data not shown). However, no difference was found with regard to mortality for both anti-CD2 and anti-CD4 mAb compared with isotype matched control mAb treated animals. In addition, neither anti-CD2 nor anti-CD4 mAb treatment resulted in differences in macroscopic or microscopic scores, myeloperoxidase activity, or weight (data not shown).

NO INFLUENCE OF αβ T CELL DEPLETION ON THE CLINICAL COURSE OF RAT TNBS COLITIS

As depletion of T helper cells had no influence on the development of TNBS colitis, we next examined if other T cell subsets (for example, $CD4^-/CD8^+$ T cells) might be involved in the initiation of TNBS colitis. After treatment with R73 on days −2, −1, 0, 2, 4, 6, 8, and 10

Table 1 Depletion of αβ T cells or γδ T cells in the spleen and the ileum after anti-CD2 monoclonal antibody (mAb), anti-CD4 mAb, anti-αβ T cell receptor (TCR), or anti-γδ TCR treatment. Values are mean (SD) of absolute numbers per PALS/mm^2 of spleen or absolute numbers/mm^2 mucosa of the ileum

mAb treatment	*No αβ T cells in PALS/mm^2 spleen*	*No γδ T cells in PALS/mm^2 spleen*	*No αβ T cells/mm^2 ileum*	*No γδ T cells/mm^2 ileum*
Control (IgG2a)	829 (65)	—	676 (32)	—
Anti-CD2 (IgG2a)	43 (11)	27.1 (3.5)	128 (22)	28.1 (3.1)
Anti-CD4 (IgG2a)	47 (10)	—	136 (25)	—
Control (IgG1)	774 (46)	29.5 (3.9)	661 (36)	26.7 (3)
Anti-αβ TCR (IgG1)	48 (12)	—	88.5	—
Anti-γδ TCR (IgG1)	—	2.7 (1.1)	—	3.7 (1.3)

PALS, periarteriolar lymphocyte sheath.

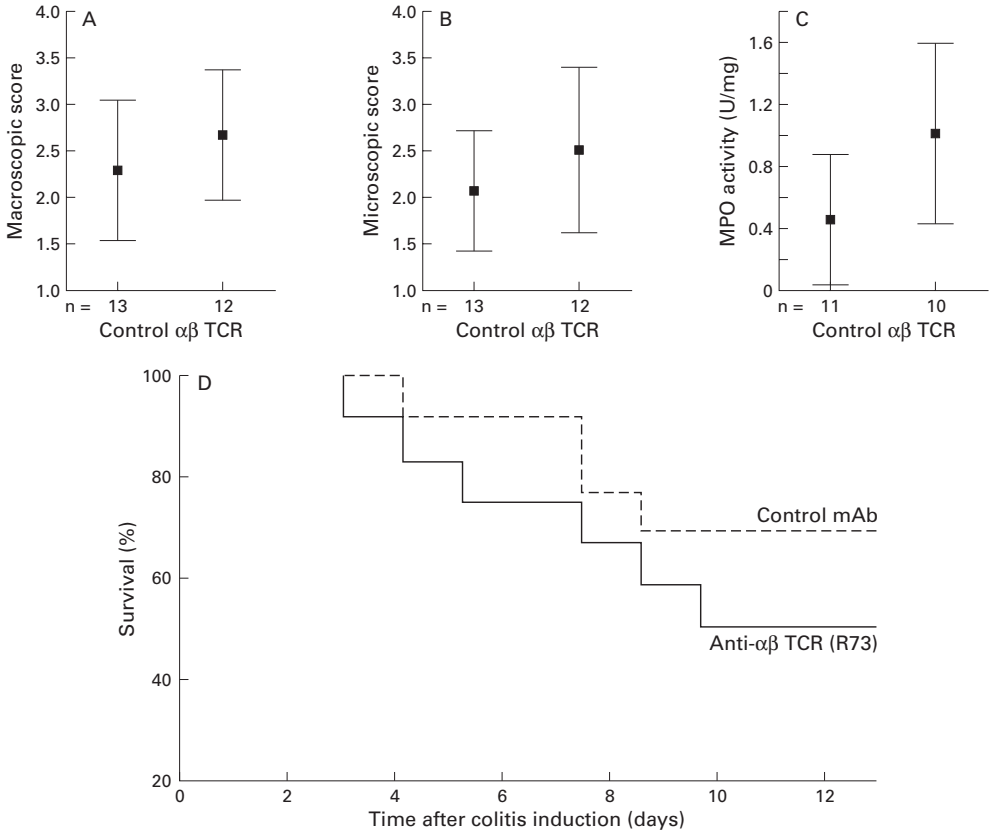

Figure 1 No significant effect of αβ T cell depletion on colitis severity. Mean (95% confidence interval) values for (A) macroscopic score, (B) microscopic score, (C) myeloperoxidase (MPO) activity, and (D) survival (Kaplan-Meier analysis) comparing isotype matched control monoclonal antibody (mAb) (TS2/9, n=13) and the anti-αβ T cell receptor (TCR) mAb (R73, n=12). A dose of 200 μg followed by 100 μg of each mAb was started two days prior to colitis induction and continued on days −1, 0, 2, 4, 6, 8, and 10 after colitis induction.

(n=12), effective depletion of αβ T cells was confirmed by immunohistology in the spleen and mucosa of the ileum (table 1): 94% of αβ T cells/mm^2 PALS and greater than 85% of αβ T cells/mm^2 lamina propria were depleted compared with controls 13 days after TNBS colitis induction. Although microscopic and macroscopic scores, and myeloperoxidase activity were higher in the αβ T cell depleted group this difference was not significant, as demonstrated in fig 1(A–C). In addition, αβ T cell depleted animals showed only slightly increased mortality compared with controls (anti-αβ TCR: 50% *v* control: 30%), which was again not significant (fig 1D). Therefore, αβ T cell depletion does not affect the initiation of TNBS colitis and has no influence on the perpetuation of this type of colitis.

DEPLETION OF γδ T CELLS AGGRAVATES RAT TNBS COLITIS

Conflicting evidence exists as to the pathogenic or protective role of γδ T cells in colitis. We therefore used a long lasting γδ T cell depletion protocol prior to TNBS colitis induction. Effective depletion of γδ T cells by anti-γδ TCR mAb treatment was confirmed by immunohistology showing greater than 85% depletion of intestinal γδ T cells and greater than 90% depletion of splenic γδ T cells, even 13 days after colitis induction (table 1). Interestingly, no reduction in γδ T cells was found after the anti-CD2 mAb treatment although γδ T cells were shown to be CD2 positive (table 1).

The most dramatic finding after depletion of γδ T cells was the markedly increased mortality (anti-γδ TCR: 73% *v* control: 30%) as shown in fig 2D. Using Kaplan-Meier analysis this difference was significant at a p value of 0.026. The differences in microscopic scores were also significant at $p<0.05$ (mean: 3.1 (anti-γδ TCR) *v* 2.1 (control)) while macroscopic scores were not significantly increased (mean 3.0 (anti-γδ TCR) *v* 2.3 (control); p=0.23) (fig 2A–C). No difference was seen with regard to myeloperoxidase activity.

Discussion

The present study provides the first direct evidence that depletion of γδ T cells aggravates rat TNBS colitis resulting in significant mortality. In addition, no evidence was found that αβ T cells or particularly T helper cells play a role in the initiation or perpetuation of rat TNBS colitis as effective depletion of these cell types had no effect on the clinical course of this IBD animal model.

There are conflicting data on the role of γδ T lymphocytes in IBD. This T cell subset is involved in important regulatory immune functions in the intestine, such as IgA production, oral tolerance induction, and epithelial growth factor production.[1 2 6] Also, γδ T cells have a role in first line defence against

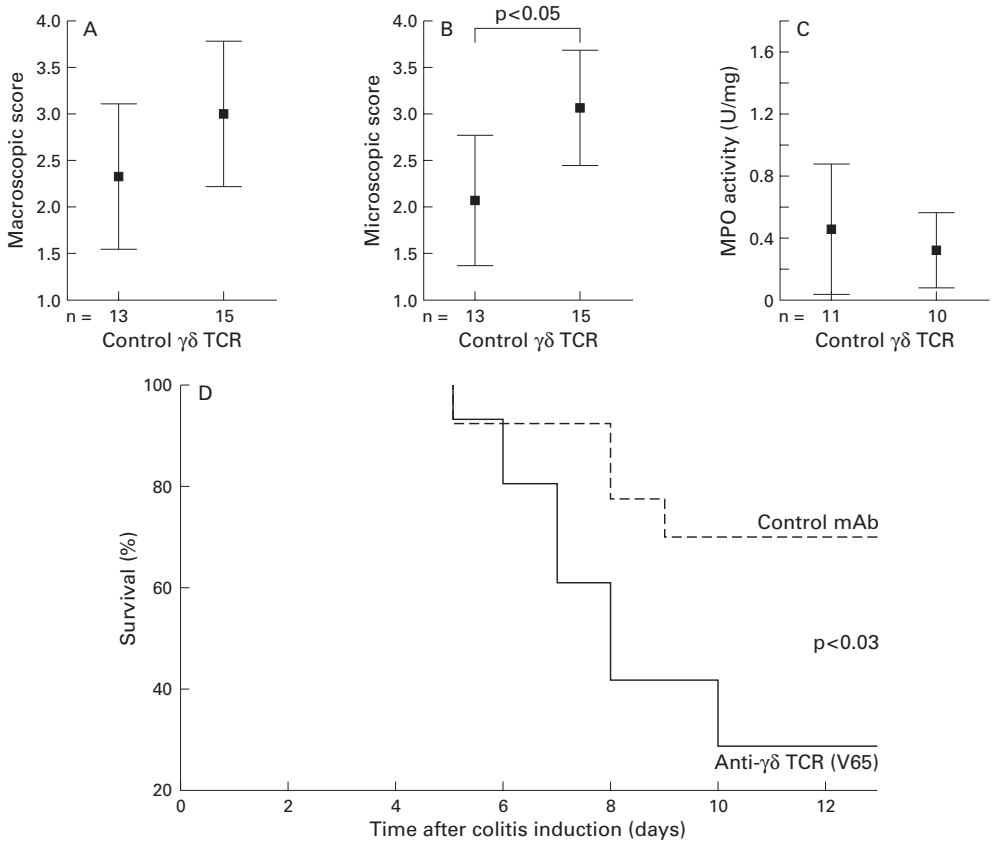

Figure 2 Increased mortality of γδ T cell depleted rats with TNBS colitis. Mean (95% confidence interval) values for (A) macroscopic score, (B) microscopic score, (C) myeloperoxidase (MPO) activity, and (D) survival (Kaplan-Meier analysis) comparing isotype matched control monoclonal antibody (mAb) (TS2/9, n=13) and the anti-γδ T cell receptor (TCR) mAb (V65, n=15).

pathogens[3 4] and a immuno-downregulatory role during infection (for example, coccidiosis).[5] Therefore, γδ T cell deficient mice develop severe epithelial damage on infection with *Eimeria vermiformis* (coccidiosis) due to failure to regulate the consequences of the αβ T cell response. Recently, γδ T cells at another mucosal surface (the respiratory tract) were shown to ensure clearance of damaged epithelial cells and to prevent airway inflammation/hyperresponsiveness.[25 26] Therefore, a protective role for γδ T cells during bowel inflammation seems likely. This notion is supported by a recent report in abstract form on γδ T cell deficient mice (truncated common cytokine receptor γ chain) who spontaneously develop colitis at eight weeks of age.[27] Further, three reports have described decreased numbers of γδ T cells in involved areas of Crohn's colitis[28–30] and one group reported increased numbers of γδ T cells.[31] However, γδ T cells can also secrete proinflammatory cytokines such as interferon γ. More importantly, T cells from αβ deficient mice can induce colitis after transfer into immunocompromised tgε26 mice.[32] Indirect evidence suggests that γδ T cells may be involved in genetic models of ulcerative colitis, namely the TCR α knockout (ko) and interleukin 2 ko mice.[33 34] While TCR-α ko mice have only $CD4^+$ α^-/β^+ T cells and γδ T cells left to induce colitis, interleukin 2 ko mice do not develop colitis in a germfree environment and at the same time show decreased numbers of intestinal γδ T cells. However, the germfree environment also leads to a reduction in αβ T cells which have been shown to induce colitis in this model. Therefore, the role of γδ T cells in both interleukin 2 ko and TCR-α ko mice is presently unclear. In contrast, γδ T cells in TNBS colitis seem to play a protective role which might be exerted either by clearance of damaged epithelial cells, secretion of epithelial growth factors, or by first line defence against enteric pathogens. As the hapten TNBS primarily leads to epithelial damage it is tempting to speculate that depletion of γδ T cells leads to defective clearance of damaged epithelial cells and therefore to more severe inflammation in TNBS colitis. On the other hand, the significance of epithelial growth factor production is exemplified by attenuating effects of keratinocyte growth factor for example, in rat TNBS colitis.[35] Finally, γδ T cells can function as regulatory T cells in autoimmune diseases (for example, preventing murine insulin dependent diabetes).[11] Ultimately, studies using T cell subset targeted and inducible ko mice of mediators involved in mucosal immunoregulation or epithelial integrity have to be done to discriminate between these possibilities.

Crohn's disease is generally believed to be a T cell dependent illness. To this end, most patients with Crohn's disease improve during progression of human immunodeficiency virus infection due to progressive depletion of $CD4^+$

T cells.[36] As a model for Crohn's disease, TNBS colitis has also been proposed to be controlled by $CD4^+$ T cell driven illness, namely by T helper 1 (Th1) type cells.[14] Our data however suggest that $CD4^+$ T cells are not required for the induction and maintenance of rat TNBS colitis as effective depletion of $CD4^+$ T cells or αβ T cells had no influence on the initiation or perpetuation of bowel inflammation. These results confirm previous observations that immunodeficient athymic rats develop even more severe TNBS colitis than immunocompetent (T cell bearing) controls.[37] One might assume that production of Th1 type cytokines (interferon γ and interleukin 2) in TNBS colitis might be a secondary phenomenon as TNBS colitis has recently been reported to occur in both interferon γ and interferon γ receptor ko mice.[38 39] These recent data may also indicate that the beneficial effect of anti-interleukin 12 mAb therapy is not necessarily dependent on interferon γ producing T helper cells. Further, other supposedly T cell directed immunotherapies could also target other cell types in TNBS colitis—for example, CD40L mast cells or anti-interleukin 12 natural killer cells.

In favour of a role of T helper cells in TNBS colitis are two reports demonstrating beneficial effects of CD4 T cell directed immunotherapies.[40 41] At first glance these data seem to be in contrast with our observation that depletion of T helper cells or even αβ T cells have no beneficial effect on the course of TNBS colitis. However, both studies induced colitis in a slightly different way—that is, after systemic sensitisation employing adjuvant in rats and coupling to BSA in mice. As TNBS is a hapten this may be important to induce a real hypersensitivity reaction rather than an immune reaction to a single colonic application of TNBS. Importantly, the first study was only able to induce amelioration of colitis using a synthetic mimetic of CD4 which binds to and possibly modulates MHC class II positive cells. However, a depleting anti-CD4 mAb had no effect. Similarly, the anti-CD4 mAb therapy (a non-depleting mAb) in the second study might modulate $CD4^+$ T cells by induction of anti-inflammatory cytokines without showing the involvement of $CD4^+$ T cells in rat TNBS colitis. With regard to the "classical" rat TNBS colitis (without prior systemic sensitisation) we can virtually exclude a role for $CD45RC^+CD4^+$ T cells in the initiation of colitis because the anti-CD2 mAb OX34 depletes this subset by more than 95% in the whole body.[23] It is presently unknown why OX34 depletes only this subset while also binding to other T cell subsets—for example, γδ T cells or $CD8^+$ T cells. As gene targeted models are not available in the rat there remains a theoretical possibility that the remaining $CD4^+$ T cells might suffice to mediate colitis. Finally, it cannot be excluded that the same substance (TNBS) induces different forms of disease in rats and mice. In favour of this hypothesis are certain differences—for example, the higher susceptibility of rats to TNBS colitis or the different disease distribution (pancolitis in mice and distal colitis in rats).

In conclusion, the present data demonstrate a protective role of γδ T cells in rat TNBS colitis. In addition, our results indicate that αβ T cells, particularly $CD4^+$ T cells, are not involved in the initiation or perpetuation of "classical" rat TNBS colitis. Future studies should investigate if γδ T cells are further examples of regulatory T cells in other colitis models and how they mediate protection.

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