RESEARCH PAPER

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Long-term treatment of NZB mice with anti-CD4 results in wasting disease, lymphoid atrophy and chronic diarrhea

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In this paper, we have shown that long-term treatment of NZB mice with anti-CD4 antibody results in four major pathological effects: firstly the development of a severe wasting disease; secondly lymphoid atrophy of the thymus, spleen, mesenteric lymph node and Peyers patches (PP); thirdly, severe chronic ulcerative colitis and fourthly a neutrophilia with neutrophil infiltration in the spleen, liver and mesenteric lymph nodes. At the same time, mice subjected to anti-CD4 treatment showed a reduction in the microbial diversity in ileal walls and contents, as well as in colonic contents, together with overgrowth of *E. coli* in the intestinal lumen and wall. In addition, there was the appearance of large numbers of spiral shaped bacteria on the mucosal surface often associated with colonic ulceration.

Introduction

The gastrointestinal tract is heavily colonized by a wide range of bacteria and of necessity has an effective and multi-layered defence system, regulating a controlled tolerogenic response at the mucosal surface. Indeed, the commensal intestinal microflora is necessary both for the full development of the hosts' immune defences and for the functional and dynamic development of the mucosal epithelial surface,[1,2] as well as acting a source of potential pathogens. Additionally the commensal microbial flora provide what is known as "colonization resistance", which, by a variety of mechanisms, including the secretion of biocidel compounds, occupation of potential binding sites and sequestration of available nutrients, prevent the establishment of acquired potential pathogens.[3]

Mucosal innate defence mechanisms include peristalsis, the topological physical integrity of the epithelial layer, the protective mucous layer covering the epithelial cells and the secretion of α and β defensins, calithicidins and other protective agents from epithelial cells. During invasion of a potential pathogen neutrophils are recruited to the site of invasion following a concentration gradient of CXCL8 (IL-8) secreted by the epithelial cells. Pro-inflammatory cytokines including IL-1, TNF and IFN are secreted by epithelial and immune cells.[4]

Adaptive immune defences are initiated by dendritic cells (DC) [which are antigen presenting cells (APC)], principally at Peyers patches, by sampling of antigens taken up by specialized epithelial cells called M cells. Dendritic cells also sample the luminal antigens at sites other than Peyers patches by pseudopodia extended into the lumen. After antigen processing CD4 helper cells are primed and provide signals for the activation of CD8 T cells and B cells, the latter producing antigen specific immunoglobulins, IgM, IgG and IgA. Immunoglobulin A is secreted through the epithelial barrier into the intestinal lumen and is one of the local effector mechanisms binding the specific pathogen.[5]

Under normal conditions lamina propria T cells include γδ T cells, CD4 memory T cells, CD4 helper T cells (Th) and regulatory CD4 T cells, Tr1 (secreting TGFβ and IL-10), Th3 (secreting TGFβ and Treg ($CD4^+$ $CD25^+$ FoxP3, secreting IL-10), which maintain the tolerogenic response in the absence of invasion. During gastrointestinal inflammation either Th1, Th2 or Th17 T cells are produced depending on the stimulus and the level of IL-12 and secrete varying constellations of cytokines.[6,7]

Animal models have been used widely to investigate these normal physiological responses as well as investigating pathophysiological events in specific infections or diseases. The importance of the intestinal microflora has been demonstrated in some animal models of IBD. Severe combined immunodeficiency disease (SCID) mice and IL-10 knockout mice develop IBD following infection with *Helicobacter hepaticus*.[8,9] The former also develop IBD after experimentally infected with *Helicobacter bilis*.[10] In addition, some animal strains that develop IBD spontaneously

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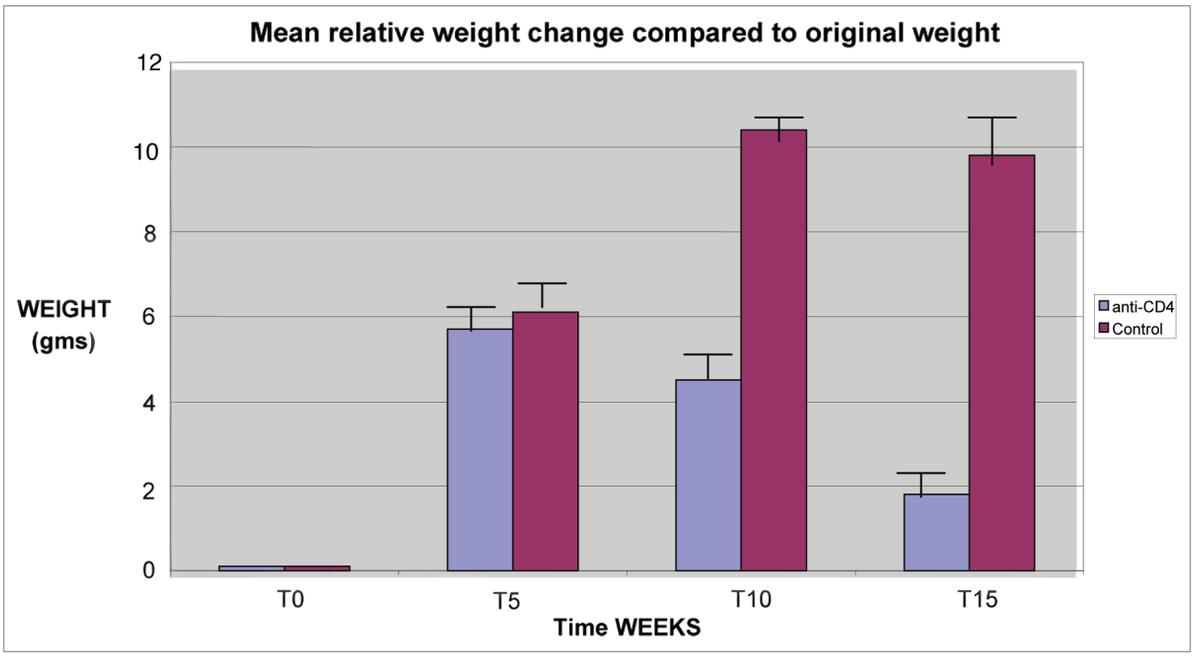

Figure 1. Histogram of change in body weight of control and anti-CD4 mice. Mice were injected with 1 mg of a rat IgG2a or anti-CD4 three times weekly from 1.5 months of age onwards. Body weight and survival were determined (mice were sacrificed after onset of severe wasting disease). *Student's t-test: $p < 0.05$ for average of body weight.

or after appropriate manipulation when housed in conventional conditions, and accordingly exhibit an intestinal flora, fail to do so if kept in gnotobiotic conditions.

Each separate animal model does not perfectly represent the whole human condition. However, in combination they cover the majority of immunological, clinical and pathological manifestations of a disease and provide a relevant tool for the development of new therapeutic approaches.[11-16]

New Zealand Black (NZB) mice spontaneously develop autoimmune diseases including lupus erythematosis, autoimmune haemolytic anemia (AHA) and immune thrombocytopenia as a result of production of auto-antibodies to single stranded DNA, erythrocytes and platelets respectively. The genetics of lupus is polygenic and complex leading to the expression of different phenotypes and for AHA 2 major loci on chromosomes 1 and 7 have been identified.[17,18] These mice develop auto-antibodies to erythrocyte proteins from about three months of age and this leads (by nine months) to AHA.[19] Several functional differences have been demonstrated in NZB animals compared to other mice relating to the levels of suppressor and helper T cell activity and CD4 subsets.[20-22]

Preliminary studies by the authors demonstrated that anti-CD4 treatment of NZB mice starting by three months of age prevents the development of anti-RBC auto-antibodies.[23] Furthermore, treatment of six-month old Coombs' positive NZB mice with anti-CD4 antibody abrogates erythrocyte auto-antibodies production. During these studies, we noticed that although the anti-CD4 treatment was effective in suppressing RBC autoantibody production, the NZB mice developed a non-autoimmune-related anemia and began to lose weight.[24] BALB/c and NZB/NZW F1 (prone to develop lupus-like disease) mice have not shown any apparent detrimental effects of long-term treatment with anti-CD4 antibody (unpublished results). Further studies, herein described, showed that after long-term treatment with anti-CD4, NZB mice developed ulcerative colitis-like histology accompanied by persistent diarrhea, together with a reduction in the diversity of the microbial flora, and most strikingly, scanning electron studies of the surface of the colon revealed large numbers of helical-shaped bacteria.

Results

Weight loss and diarrhea. During the experiment, control mice, treated with a rat IgG2a antibody, progressively gained weight. During the first six weeks of treatment, the mice administered with anti-CD4 antibody looked healthy, then, one by one they started to lose weight. Eventually, virtually all anti-CD4 treated mice developed a severe weight loss. Around 7 weeks of the treatment, the anti-CD4 treated mice showed a statistically significant decrease of mean body weight (mean ± SD, 22.2 ± 3.1 g, (**Fig. 1**), compared to the control mice (25.0 ± 1.7 g, Student's t test, $p < 0.05$). Comparison at the time of sacrifice showed even a more significant decrease in body weight in the anti-CD4 treated mice (18.9 ± 1.4 g), in comparison with the controls (26.3 ± 2.3 g, $p < 0.001$). This represents an average loss of 28% of body weight. The experimental mice developed diarrhea approximately one week before the development of the wasting disease as judged by the presence of non-formed feces in the cages. None of the control mice developed diarrhea.

Hematology and histopathology. *Blood.* In the peripheral blood, significant higher absolute numbers of neutrophils were

seen in the experimental anti-CD4 treated group as compared with the controls (**Table 1**). However, the absolute numbers of lymphocytes, T cells, CD4 T cells and B cells were significantly lower in the anti-CD4 treated mice compared to the controls. There was a reduction of 59% of T cells, 86% of CD4 T cells and 82% of B cells.

Thymus. The thymus of all 9 anti-CD4-treated mice was markedly reduced in size (**Fig. 2**). Both the cortex and the medulla were reduced and the boundaries between the two areas blurred (**Fig. 3k and l**). Sections of the thymus of the control mice showed normal features.

Spleen. Spleen sections of anti-CD4-treated mice showed the white pulp occupying approximately 20–40% of the tissue. Four out of nine mice had a reduced area of the periarterial sheaths. Follicles with germinal centers could be recognized in only one out of nine mice. The red pulp was infiltrated with neutrophils. The white pulp in control animals represented about 40–60% of the total area. It was well supplied with peri-arterial sheaths. Inside the sheaths there were a moderate number of follicles with germinal centers (**Fig. 4**).

Mesenteric lymph nodes. Of seven anti-CD4 antibody treated mice, the most consistent finding was infiltration of the medullary lymphoid cords with neutrophils. Generally follicles with germinal centers were not seen and the para-cortical area had low numbers of lymphocytes. In contrast the control mice had lymphoid follicles with medium to large germinal centers and the para-cortical area was relatively more cellular.

Small intestine. In the control mice, the lymphoid tissue in the ileum was mainly composed of large germinal centers with little diffuse lymphoid tissue. The anti-CD4-treated mice showed normal features, apart from the reduction in lymphoid mass which was generally composed of small aggregations of lymphocytes localised in the sub-mucosa without germinal centers (**Fig. 5**).

Liver. Sections of liver from eight out of nine mice in the anti-CD4 treated group showed mild to moderate neutrophil infiltration in some portal and peri-portal spaces. In addition the parenchyma showed small infiltrations with neutrophils. Control mice had normal histology with no neutrophil infiltrates.

Other organs. Sections of lungs, kidney and adrenal were not significantly different between the two groups and showed essentially normal features.

Large intestine and colitis. The anti-CD4-treated mice all had a moderately dilated cecum and colon wall thickening. The contents were soft and non-formed.

Histological examination of the large intestine showed intense diffuse chronic colitis in all nine mice treated with anti-CD4 (**Fig. 6**). The colonic wall of these mice showed mixed inflammatory infiltrations in the lamina propria and sometimes in the sub-mucosa. The inflammation was sometimes accompanied by edema or fibrosis. The crypts were sometimes deformed, either tortuous or dilated and were covered by pseudo-stratified epithelium exhibiting loss of goblet cells. In some areas the epithelium showed features of dysplasia. Some crypts showed accumulations of neutrophils (crypt micro-abscess) and sometimes bacteria were associated with them. The muscular layers were essentially preserved, rarely exhibiting peri-vascular inflammatory infiltration and edema. The serosa was intact in most of the mice. In six

Table 1. Results of heamatological analysis

Cell population	Control mice cells/mm³	Anti CD4 mice cells/mm³	p
Leucocyte	4744 ± 784	8083 ± 3408	none
Neutrophil	1131 ± 291	6607 ± 3209	$p < 0.01$
Lymphocyte	3255 ± 593	1276 ± 351	$p < 0.001$
B cell	1069 ± 54	192 ± 120	$p < 0.001$
T cell	2166 ± 132	864 ± 152	$p < 0.001$
CD4 T cell	1691 ± 329	229 ± 42	$p < 0.001$
CD8 T cell	551 ± 64	552 ± 102	none

out of nine mice areas of ulceration covered with fibro-purulent exudates were seen. These ulcers were either superficial compromising the mucosa and sub-mucosa or deep affecting the whole gut wall. Two mice showed deep ulcers that were elliptical in shape and measured about 6 x 4 mm. In these circumstances the serosa developed granulation tissue and showed dense infiltration with neutrophils and mononuclear cells. The control mice had a normal histological appearance (**Fig. 6**).

Ultrastructural features. Samples from each of the control mice showed a normal appearance detected by scanning electron microscopy. Examination of samples from the anti-CD4 treated mice showed many of the features of severe colitis. The mucosal surface showed loss of folds. There were large areas coated with inflammatory exudates. In some areas there were irregular features of epithelial proliferation with grooves or irregular-shaped areas of epithelial destruction in which large numbers of a variety of bacteria were observed. In some ulcerated areas the predominant organism was a helical-shaped bacillus (**Fig. 7**).

Bacteriology of the intestine. Estimates of the diversity of bacterial types were performed by analysis of morphotypes, based on the Gram stain (Gram-positive, Gram-negative); colonial morphotypes based upon inspection of the different colony-types after culture; the total viable counts of aerobic and anaerobic bacteria and identification of the predominant colonial types. The data is shown in **Tables 2 and 3**.

There was a significant increase in the number of Gram-negative bacteria in the anti-CD4-treated mice from the colon wall (median 2.35×10^7 compared to 1.0×10^5 for the controls, Mann Whitney test, $p < 0.003$). Principally a significant increase in the number of *E. coli* in the colonic walls (1.0×10^5 compared to 4.0×10^1, $p < 0.003$), colonic contents (5.0×10^5 compared to 5.0×10^1, $p < 0.01$), ileal walls (7.0×10^3 compared to 1.0×10^1, $p < 0.02$) and ileal contents (3.0×10^3 compared to 0×10^1, $p < 0.01$) of anti-CD4-treated mice, compared to controls.

There were no significant differences between the anti-CD4-treated and control groups regarding the number of either Gram-positive, Gram-negative, aerobic and anaerobic bacteria per gram of intestinal contents from either ileal wall, ileal contents or colonic contents, nor was there any significant difference for both gram positive, aerobic and anaerobic bacteria from the colon wall.

The diversity of bacterial species was significantly reduced (as judged by colonial morphology and identification) in both ileal contents and wall and in the colon contents of the anti-CD4 treated mice. The median of the number of different species

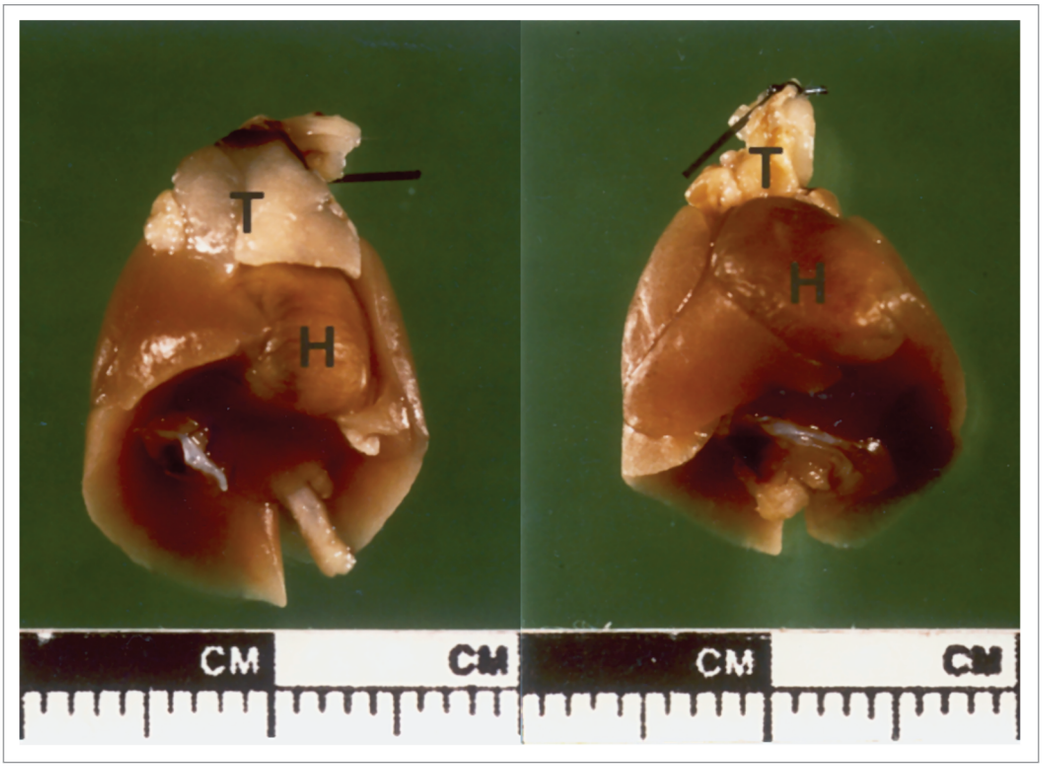

Figure 2. Photograph illustrating atrophy of thymus in anti-CD4-treated mice. Representative thoracic blocks of both groups showing the heart (H), lungs and thymus (T). Note the atrophied thymus of the anti-CD4-treated mouse (right), compared with that of a control mouse (left).

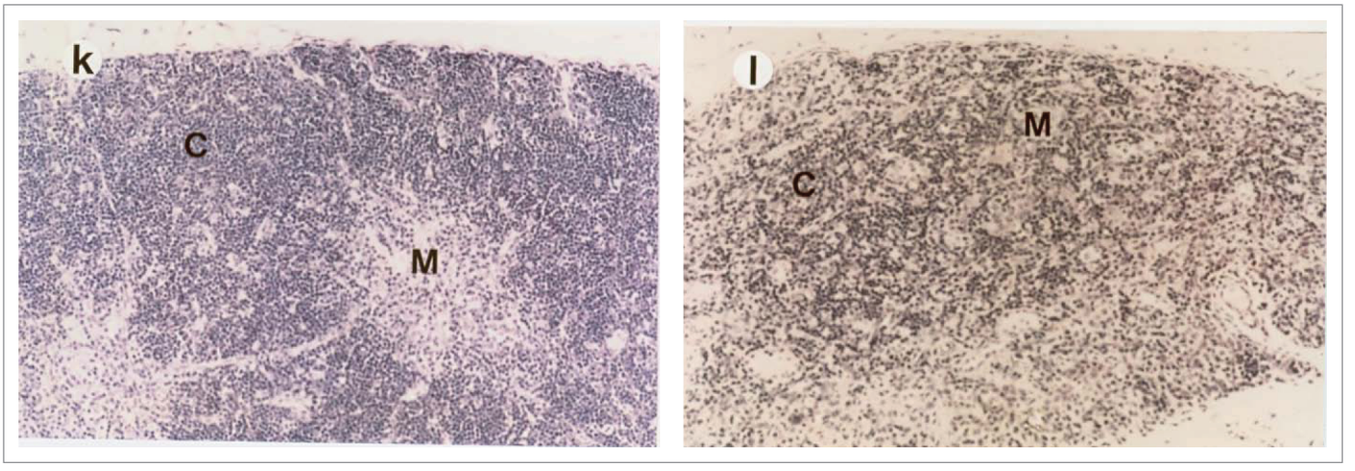

Figure 3. Histological changes in thymus (k and l). Control group. (k) Section shows an area of the thymus with cortex (C) and medulla (M) revealing normal histological aspects. x100. Anti-CD4 group. (l) Section shows the whole width of the thymus with severe atrophy. The cortex (C) exhibits a great decrease in the number of lymphocytes and the medulla (M) is virtually reduced to Hassal's bodies and no lymphocytes. x100.

of bacteria in ileum contents (2 compared to 8, $p < 0.002$), ileum wall (3 compared to 6, $p < 0.03$) and colon contents (4 compared to 8, $p < 0.004$) of the anti-CD4-treated group was significantly less than in the controls respectively. There was no statistical difference between the median number of different bacterial species found in the colon wall of the two groups (2 compared to 4).

Discussion

Overview of main results. In this paper, we have shown that long-term treatment of NZB mice with anti-CD4 antibody results in the development of a severe wasting disease accompanied by lymphoid atrophy of the thymus, spleen, mesenteric lymph node and Peyer's patches (PP); diarrhea and severe chronic

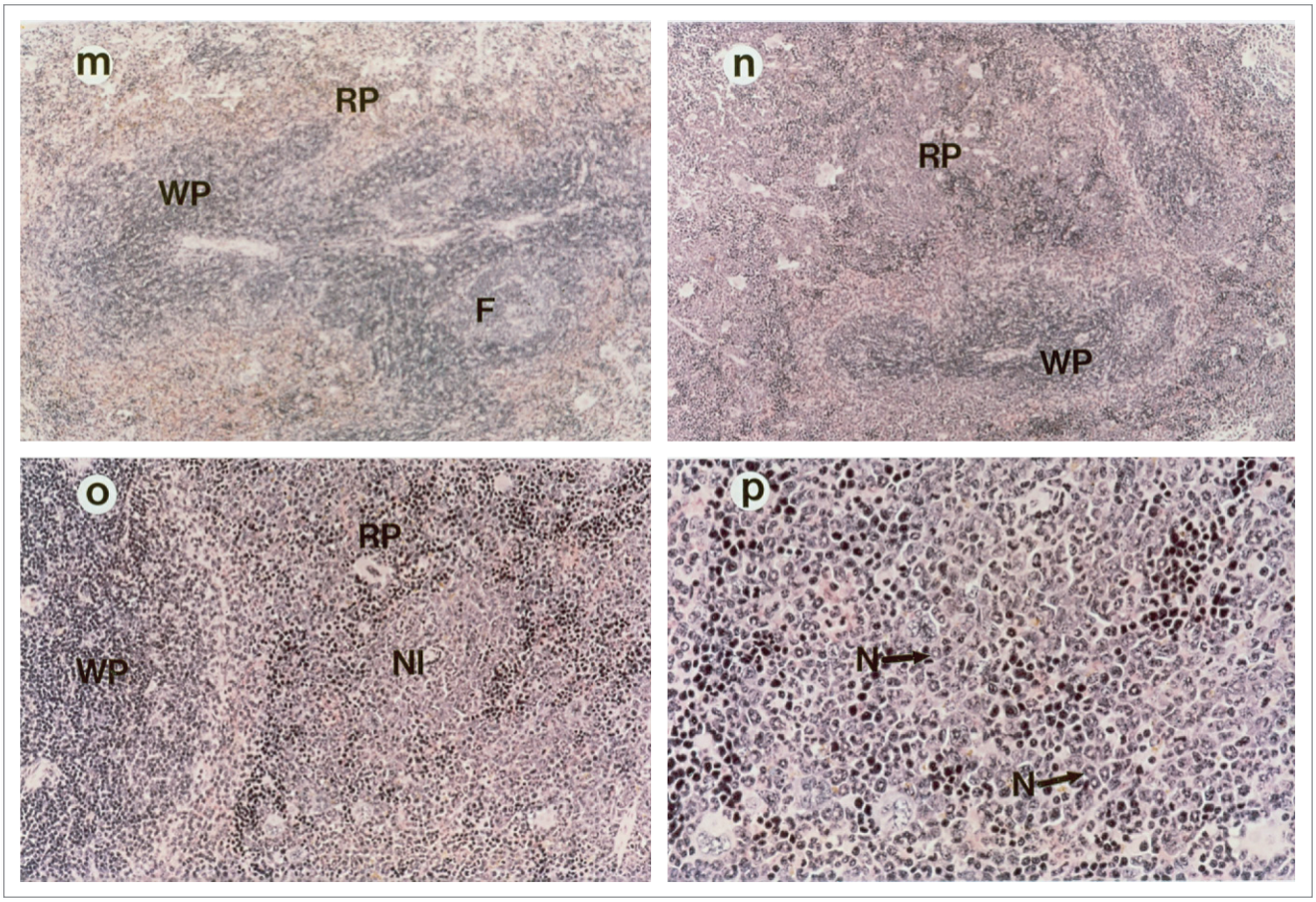

Figure 4. Changes in splenic histology. Control group. (m) Section shows white pulp (WP) with periarteriolar lymphoid sheath and follicle (F) with germinal center. The red pulp (RP) is also normal. x100. Anti-CD4 group. (n) Section shows atrophy of the white pulp (WP). There is severe reduction in the lymphocyte population of the periphery of the periarteriolar lymphoid sheaths and a lack of follicles. The red pulp (RP) shows extensive polymorphonuclear neutrophil infiltration. x100. (o) Intermediate magnification showing neutrophil infiltration (NI) in the red pulp (RP). White pulp (WP). x200. (p) High magnification showing neutrophils (N) in the red pulp. x400.

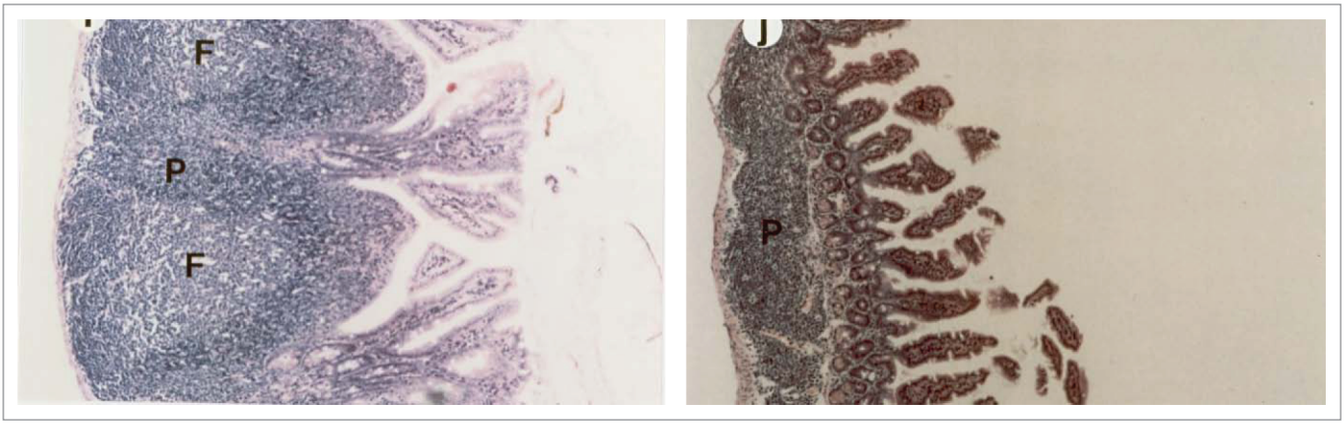

Figure 5. Control group. (I) Section of the ileum showing Peyer's patches (P) with large follicles containing germinal centers (F). x100. Anti-CD4 group. (j) Section of the ileum showing Peyer's patches (P) greatly reduced in size and a lack of follicles. x100.

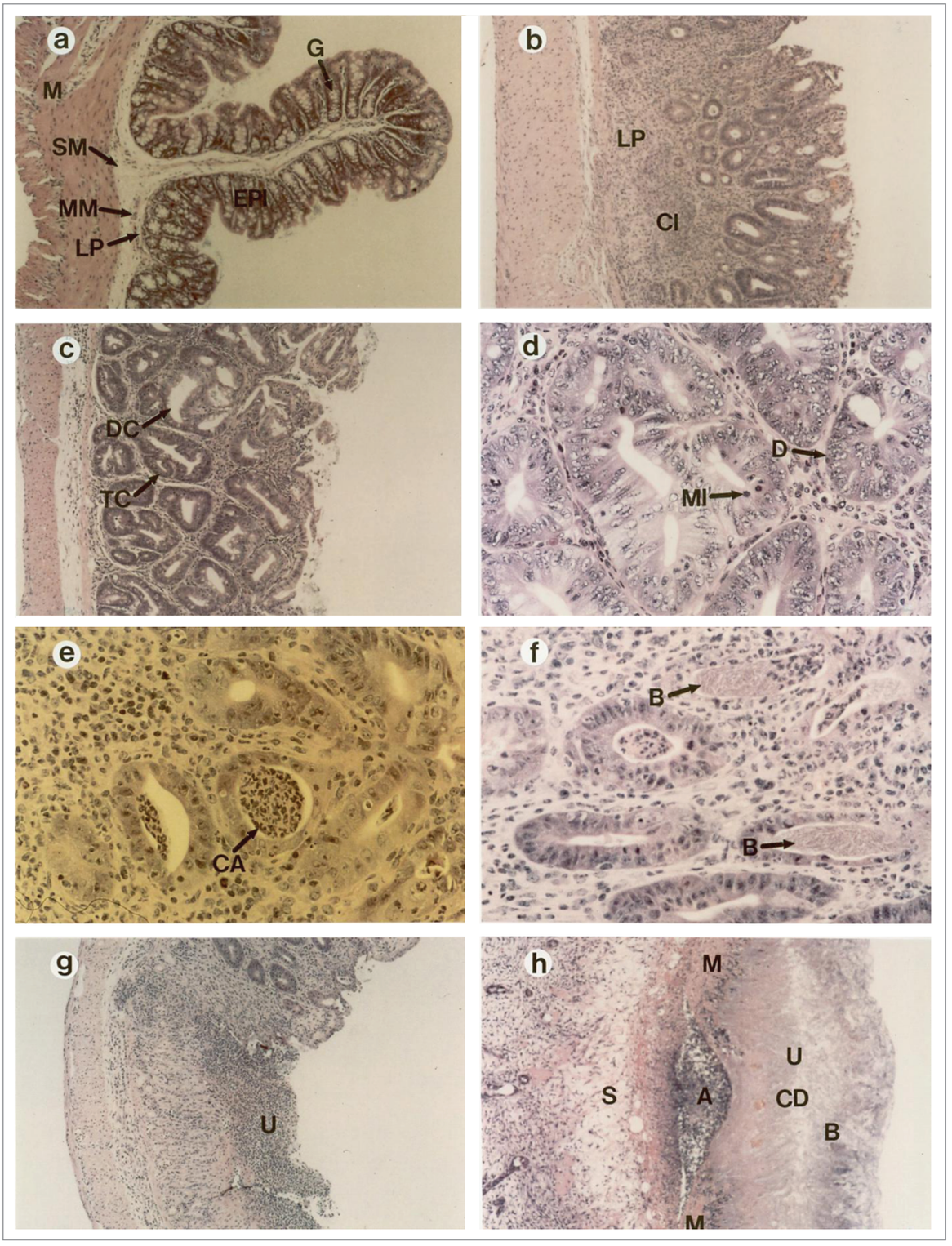

Figure 6. Histopathology of NZB mice treated with an irrelevant antibody (a) or anti-CD4 (a–h). Tissues fixed in 10% formaldehyde PBS, embedded in paraffin, sectioned 6 μm thick and stained with H&E. Control group: (a) Section of the colon showing normal structures: goblet cells (G) epithelium (EPI), lamina propria (LP), muscularis mucosa (MM), submucosa (SM) and muscular layer (M). x100. Anti-CD4 group: (b) Section of the colon showing flattened and enlarged epithelial layer with intense chronic inflammatory infiltrates (CI) predominantly in the lamina propria (LP). x100. (c) Colitis with crypts tortuous (TC) and dilated (DC). The epithelium shows proliferative aspects and lack of goblet cells. x100. (d) Colitis with areas of intense crypt proliferation showing crypts packed side by side and epithelium with mitotic cells (MI) and dysplastic changes (D). x400. (e) Colitis with cryptic abscesses (CA). x400. (f) Colitis with cryptic abscesses sometimes in association with bacteria (B). x400. (g) Colitis with superficial ulcer (U). x100. (h) Colitis with deep ulcer (U). The ulcer is covered with cell debris (CD) associated with bacteria (B), the muscular layers were replaced by an abscess. The serosa (S) is thickened, shows edema, congested blood vessels and mononuclear cell infiltrates. x100.

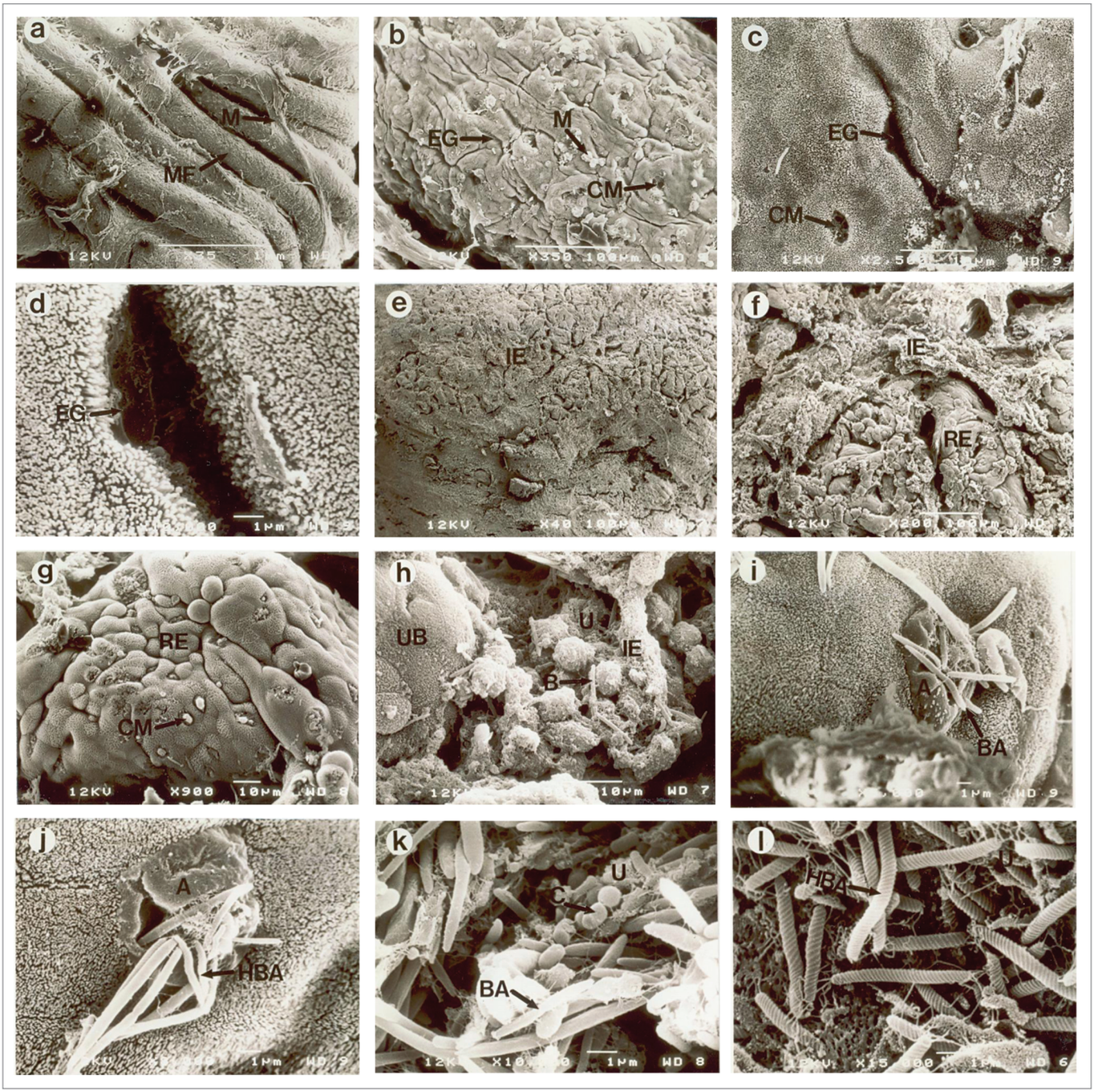

Figure 7. Scanning microscopy analysis of the colon of NZB mice treated with anti-cd4 or irrelevant isotype-matched antibody. Control group: (a) Normal epithelial surface showing folds (MF) coated with mucus (M). x35. (b) A mucosal fold showing the mouth of Lieberkühn crypts (CM), epithelial grooves (EP) and pieces of mucus (M). x350. (c) A high magnification of the epithelium showing the mouth of Lieberkühn crypts (CM) and epithelial grooves (EP). x2,500. (d) A high magnification of the epithelium showing epithelial grooves (EP) and on each side, well preserved microvilli. x10,000. Anti-CD4 group (e). Epithelial surface flattened and extensively covered by inflammatory exudate (IE). x35. (f) Areas of bulging regenerative epithelium (RE) surrounded by deep grooves filled in with inflammatory exudate (IE). x200. (g) Areas of mucosa of irregular profile, compatible with regenerative epithelium (RE). Mouth of Lieberkühn crypts (CM). x900. (h) Area of the mucosa showing ulcer (U) coated by inflammatory exudate (IE) mixed with bacteria (B). Ulcer border (UB). x2,000. (i) Area (A) of the epithelium showing effacement of microvilli in close association with bacilli (BA). x5,000. (j) Area (A) of the epithelium showing effacement of microvilli in close association with helical-shaped bacillus (HBA). x8,000. (k) Bottom of an ulcer (U) showing mixed cocci (C) and bacilli (BA). x10,000. (l) Bottom of an ulcer covered with a helical-shaped bacillus (HBA). x15,000.

Table 2. Viable counts of main bacterial types

Location	Bacteria	Control mice cfu/mg tissue	Anti CD4 mice cfu/mg tissue	p
Ileum lumen	GP	5×10^6	5×10^6	
	GN	8.5×10^7	1.9×10^8	
	O	1.6×10^6	4.6×10^6	
	ANO	4×10^5	1.4×10^5	
	E. coli	0	3.0×10^3	$p < 0.01$
Ileum wall	GP	6.5×10^5	5.0×10^5	
	GN	1.0×10^1	1.0×10^2	
	O	4.0×10^5	2.2×10^5	
	ANO	6.5×10^4	5.7×10^4	
	E. coli	1.0×10^1	7.0×10^3	$p < 0.02$
Colon lumen	GP	5×10^8	5.0×10^7	
	GN	8.5×10^7	1.9×10^8	
	O	4.7×10^6	5.0×10^6	
	ANO	6.2×10^6	4.3×10^6	
	E. coli	5 x 10	5.0×10^5	$p < 0.01$
Colon wall	GP	5.5×10^5	1.5×10^4	
	GN	2.3×10^7	1.0×10^5	$p < 0.003$
	O	4.5×10^5	7.1×10^7	$p < 0.003$
	ANO	2.0×10^4	2.5×10^5	
	E. coli	4.0 x 10	1.0×10^5	$p < 0.003$

GP, Gram positive; GN, Gram negative-determined by Breed slide method; O, aerobic; ANO, anaerobic-determined by culture.

ulcerative colitis. Additionally we have demonstrated a systemic neutrophilia with neutrophil infiltration in the spleen, liver and mesenteric lymph nodes. At the same time, mice subjected to anti-CD4 treatment showed a reduction in the microbial diversity in the gastrointestinal tract together with overgrowth of *E. coli*. In addition, there was the appearance of large numbers of spiral-shaped bacteria on the mucosal surface often associated with colonic ulceration.

Clinical, histological and immunological changes associated with anti-CD4. Decreases in the amount of lymphoid tissue in the thymus, spleen, MLN and PP argue that the NZB mice at the time of sacrifice had an overall reduction in lymphoid cells. It is interesting that thymectomy of young NZB mice alone or anti-lymphocytic treatment of NZB mice has also been shown to result in wasting disease and that thymectomy induces severe lymphopenia and atrophy of T cell areas of MLN, although the intestinal microbiology was not reported in this study.[25] Intestinal bacteria were essential for the development of wasting disease in these animals as it did not occur in germ-free animals. Interestingly thymectomy, although abrogating systemic disease, can lead to organ-specific autoimmunity.[26]

The fact that there was a decrease in thymus size and a decrease in both cortex and medulla argues that there is a direct effect on thymic CD4 T cells. Furthermore it is unlikely that the injected anti-CD4 antibody was simply blocking the detection of CD4 T cells since a different monoclonal antibody (YTS 191.1) with only partial cross reactivity was used for analysis. A significant decrease in absolute numbers of T cells without a change in CD8 cells would also argue that we were detecting all the CD4 T cells present.

Chronic CD4 deficiency leads to a reduced synthesis of IgA. A reduction in the production of TGFβ, IL-2, IL-5 and IL-6, secreted by CD4 cells, also occurs, which are important for isotype switching and terminal differentiation of IgA secreting plasma cells.[27] This would explain the decrease in B cells noted in this study.

By seven weeks after commencing treatment with anti-CD4 the NZB mice already showed significant lower body weights and anemia than those receiving irrelevant antibody. At sacrifice Coombs-positive antibodies were absent in the anti-CD4-treated mice compared to controls. This is consistent with our previous observation of the inhibition of erythrocyte auto-antibodies with concomitant development of anemia.[24] NZB CD4 knockout mice have been produced which have challenged the requirements of CD4 T cells for the production of erythrocyte auto-antibodies[28] although our results would argue that CD4 cell function is important as inhibition of CD4 cells results in loss of antibodies. To our knowledge these knockout mice have not been investigated for wasting disease or colitis.

Wasting disease and premature death was also observed following long-term anti-CD4 treatment and shown to be specific for NZB mice since neither BW/F1 nor BalbC mice showed weight loss and premature death, although BalbC mice did show similar changes in the cell profile (results not shown).

It is highly likely that the reduction in the CD4 count included regulatory T cells and thus in some respects mimicked the situation in AIDS patients or in patients with IBD leading to lack of control of some component of the normal flora with outgrowth of a specific morphotype that resembles a species of Helicobacter.

Diarrhea, wasting and CD4 suppression in HIV. The onset of diarrhea following depletion of CD4 cells has parallels to the development of disease in patients infected with HIV.

Pathological depletion of CD4 cells in AIDS induces a number of opportunistic infections of which one clinical presentation is diarrhea and which remains etiologically undiagnosed in 60% of cases.[29] However, this study was based on microscopy and culture and did not include electron microscopy of the intestine. In a further histopathological study of chronic diarrhea from HIV infected individuals 62% had an indeterminate diagnosis with nine histopathogically suggestive of inflammatory bowel disease.[30] Helicobacter-like bacteria in the colon were not identified in this study as the study only used the Gram and Giemsa stain on paraffin sections of colon.

Apart from opportunist infection chronic colitis may also be caused directly by HIV[31] or an immune imbalance in the gut mucosa resulting in infiltration by CD8 cells.[32] In these circumstances there is a diffuse colitis with superficial ulceration/exudates and loss of vasculature pattern but preserved crypt architecture.[33] In our study, however, on examination of the histopathology of the colon the appearance was reminiscent of ulcerative colitis with distorted crypt architecture and crypt abscesses-hallmarks of IBD.

Table 3. The principle species of bacteria isolated

	Ileum wall	Ileum lumen	Colon wall	Colon lumen
Control	Clostridium Enterococcus Leuconostoc Campylobacter Rothia	Listeria Leuconostoc Enterococcus Clostridium Rothia Listeria Bacteroides *E. coli*	Listeria Clostridium Bacteroides *E. coli*	Clostridium Enterococcus Leuconostoc Agrobacterium Pseudomonas Campylobacter Capnocytophaga Bacteroides *E. coli*
Anti-CD4	*E. coli* Clostridium Listeria	Clostridium *E. coli*	Pseudomonas *E. coli*	Clostridium Bacteroides Pseudomonas *E. coli*

Inflammatory bowel disease. The second possibility that this study could represent is the development of inflammatory bowel disease (IBD) following on from the disruption of the regulatory T cell caused by the depletion of CD4 cells, which allowed the overgrowth of certain bacterial groups that are normally dependent on CD4 cells for their regulation. This change may be related to a decrease in IgA levels but this requires confirmation.

One study has shown that the use a synthetic mimetic of CD4 or anti-CD4 antibodies are able to suppress inflammation in a mouse colitis model using immunization with the hapten 2,4,6-trinitrobenzene sulfonic acid.[34] The mechanisms by which the synthetic mimetic of CD4 and anti-CD4 antibodies suppress colonic inflammation are different. Also the mouse strain used in this study was BALB/c, whereas our model uses the NZB mouse. Thus in our model, a separate effect of anti-CD4 seems to be implicated, emphasizing the complexity of the role of CD4 T cells in the regulation of immune responses. Anti-CD4 antibodies have been used to inhibit autoimmune diseases in mice, rodents and humans but with regard to our model, anti-CD4 antibodies may be not appropriated for all autoimmune diseases and more studies are necessary.

Changes in the colonic microflora are recognized in ulcerative colitis in animals and humans. After induction of colitis in rats by instillation of TNB an increase in Gram-negative bacilli has been recorded with a decrease in Gram-positive cocci. In our study several anti-CD4-treated mice developed colonic ulcers and in all anti-CD4-treated mice there was the classical features of colitis. Concomitantly with CD4 suppression there was a marked increase in *E. coli,* which has been linked to pathogenesis of colitis in previous studies. Also in this regard it is interesting that both patients with IBD and HIV-infected individuals with low CD4 counts often suffer wasting disease and diarrhea, and that certain types of *E. coli* have been implicated in the pathogenesis of disease.[35,36]

Scanning electron microscopy revealed intensive ulcerative colitis with appearance of Helicobacter-like bacteria at the site of ulceration. In addition in regions where the mucosa was better preserved there were small areas of epithelial damage in close association with spiral shaped bacteria. These organisms may represent Helicobacter species although our cultures would not have detected them, as they were only incubated for 48 hours. From previous work with SCID mice and IL-10 deficient mice it is possible that these bacteria could be *H. bilis*, *H. hepaticus* but morphologically more closely resemble *H. heilmanii* Type I and the *H. felis* group. This is an area that requires further study. There appeared to be little histological change in the ileum of the anti-CD4-treated NZB mice.

It has been shown that Long Evan Cinnamon rats spontaneously develop IBD associated with a reduction in Tregs and a pronounced Th-1 cytokines response,[37] which was prevented by injecting CD45RB^{low}, IL-10 or anti_TNF and INF, suggesting the colitis is Th-1 mediated. Unfortunately, this study did not include examination of the microbiota of the intestine.

In our study the bacteriology showed significant changes to the intestinal flora. Although the total number of flora in the ileum and colon luminal contents was not different between the two groups, there was a significant reduction in the diversity and a shift in the type of different species of bacteria found in the antiCD4 treated group. This indicates that there was an increase in a few species of bacteria at the expense of others. These findings are consistent with finding in animals and patients with IBD.

It may well be that this NZB model may be useful as a model to better understand the relationship between the mucosal immune system and the bacterial intestinal microflora and may represent an in vivo model to study AIDS-related gastrointestinal pathology or IBD.

Materials and Methods

Animals. NZB mice were purchased from Harlan Ltd., (Oxford, UK). The mice were maintained in the animal care facility of the Immunology Department of UCL Medical School.

Ethical approval for this study was obtained from the Ethics Committee of University College London.

Monoclonal antibody. Mice were treated with a putative non-depleting rat IgG2a monoclonal antibody to mouse CD4 (YTS 177.9) or an isotype-matched antibody control (YTS3.45.6) a kindly gift from Professor Herman Waldmann, Cambridge, UK.

Mice and experimental procedure. Two groups of 1.5 month old female NZB mice were used. One group of 9 mice received 1 mg anti-CD4 and the other group of 8 mice received 1 mg

of an irrelevant isotype-matched antibody at each injection. The initial injection was intravenous, followed by three intraperitoneal injections weekly for about 9 weeks. The mice were observed daily for weight loss and diarrhea. The body weight was measured approximately every ten days until 5 weeks of treatment and from then on daily. Cages were examined for non-formed feces weekly. Experimental mice were sacrificed, with paired controls, when they developed signs of advanced wasting disease, which occurred between 7 and 13 weeks of treatment.

White blood cell counts. Peripheral blood samples were collected at the day of sacrifice of the last 11 mice (six anti-CD4 and 5 control mice) between 9 and 13 weeks of treatment. Absolute white cell counts were determined with a Neubauer chamber and blood films were air-dried onto glass slides, fixed with methanol and then stained with Wright-Giemsa (Sigma WG-16) for a differential count.

Fluorescence analysis of lymphocyte subpopulations. The flow cytometry analysis was carried out as previously reported.[23] Briefly, blood samples were dispensed in 30 μL aliquots into tubes (LP3, Luckman) and washed once with PBS. Cells were incubated for 30 minutes with 10 μL anti-CD4-PE conjugate (Coulter), anti-Ly5-PE (Coulter), or a control rat MoAb IgG-2b-PE, of irrelevant specificity (R-PE; Bradsure). They were then washed once with PBS and further incubated for 30 minutes with 10 μL of anti-Ly2-FITC-conjugate (Coulter), anti-CD3-FITC or control rat IgG-2b-FITC of irrelevant specificity (Bradsure), respectively. Becton Dickinson FACS lysis solution was added for 5 minutes to lyse the erythrocytes. The cells were washed, fixed with 1% formaldehyde in PBS and analyzed for two-color fluorescence using a Becton Dickinson FACScan 30. Lymphocytes were gated using forward angle and side scatter and at least 3,000 cells analyzed.

Post-mortem examination. At the time of sacrifice the mice were examined externally and both the abdominal and thoracic cavities opened. Samples of the lungs, thymus, liver, spleen, mesenteric lymph nodes, kidney, adrenal gland and intestines were taken. Small fragments of all the organs collected were fixed with 10% formaldehyde in phosphate-buffered saline (PBS). The specimens were embedded in wax, cut into six micron sections stained with hematoxylin and Eosin and examined with a Zeiss optical microscope. Small segments of the colon were fixed with 2.5% glutaraldehyde for scanning electron microscopy.

Microbiology examination. Samples of terminal ileum and ileal contents, the colon contents and colon wall were collected and intestinal samples washed three times in PBS to remove loosely adherent matter. Mesenteric lymph nodes were separately collected from each mouse at the time of sacrifice. Each specimen was placed in a pre-weighed Eppendorf tube containing 500 μl of anaerobic transport medium [45 mL of 0.3% K_2HPO_4 + 45 mL 0.3% KH_2PO_4 + 0.6% $(NH_4)_2SO_4$ + 0.6% NaCl + 0.06% $MgSO_4$ + 0.06% $CaCl_2$ + 0.5% cysteine + 0.3% $NaCO_3$ pH 7.2 + distilled water to 300 mL] and re-weighed. Each sample was processed within half an hour of collection. Fecal matter was vortexed prior to culture and intestinal walls and lymph nodes homogenized.

Breed slide. Ten microliters of diluted (1:100) suspension of vortexed fecal matter or homogenized tissue was spread over a one centimeter area on a microscope slide. The slide was fixed and Gram-stained. Gram-positive and Gram-negative morphotypes were counted in six fields. The number of micro-organisms in the original sample was calculated from the count, the dilution and the sample weight and expressed as organisms per milligram of specimen.

Culture. Serial ten-fold dilutions from 10^0–10^{-8} of each specimen were prepared. One hundred microliters of each dilution was spread onto the following media: 5% horse blood Colombia agar base (BA) (Oxoid); Campylobacter agar (CA) (Oxoid); Schaedles agar (SA) (Oxoid). The BA was incubated aerobically for 24 hours, SA was incubated anaerobically for 48 hours and CA incubated micro-aerobically for 3 days, all at 37°C. After incubation the total number of colonies and the different colonial morphotypes were recorded. Each recognizable colony type was identified using the appropriate API (BioMerieux, France).

Statistics. The parametric Student's t-test was used to compare two independent groups and Mann-Whitney test was used to compare several independent groups of data that have not shown normal distribution.

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