Reduced ratio of protective *versus* proinflammatory cytokine responses to commensal bacteria in HLA-B27 transgenic rats

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SUMMARY

Germ-free HLA-B27 transgenic (TG) rats do not develop colitis, but colonization with specific pathogen-free (SPF) bacteria induces colitis accompanied by immune activation. To study host-dependent immune responses to commensal caecal bacteria we investigated cytokine profiles in mesenteric lymph node (MLN) cells from HLA-B27 TG *versus* nontransgenic (non-TG) littermates after *in vitro* stimulation with caecal bacterial lysates (CBL). Supernatants from CBL-stimulated unseparated T- or B- cell-depleted MLN cells from HLA-B27 TG and non-TG littermates were analysed for IFN-γ, IL-12, TNF, IL-10 and TGF-β production. Our results show that unfractionated TG MLN cells stimulated with CBL produced more IFN-γ, IL-12 and TNF than did non-TG MLN cells**.** In contrast, CBL-stimulated non-TG MLN cells produced more IL-10 and TGF-β. T cell depletion abolished IFN-γ and decreased IL-12 production, but did not affect IL-10 and TGF-β production. Conversely, neither IL-10 nor TGF-β was produced in cultures of B cell-depleted MLN. In addition, $CD4^+$ T cells enriched from MLN of HLA-B27 TG but not from non-TG rats produced IFN-γ when cocultured with CBL-pulsed antigen presenting cells from non-TG rats. Interestingly, IL-10 and TGF-β, but not IFN-γ, IL-12 and TNF were produced by MLN cells from germ-free TG rats. These results indicate that the colitis that develops in SPF HLA-B27 TG rats is accompanied by activation of IFN-γ-producing $CD4^+$ T cells that respond to commensal bacteria. However, B cell cytokine production in response to components of commensal intestinal microorganisms occurs in the absence of intestinal inflammation.

Keywords cytokines HLA-B27 transgenic rats commensal bacteria

INTRODUCTION

In recent years several studies have emphasized the role of commensal intestinal bacteria in the pathogenesis of experimental chronic immune-mediated intestinal inflammation and human inflammatory bowel diseases. This is most clearly demonstrated in a wide variety of genetically engineered and induced rodent models in which the susceptible host develops spontaneous colitis in the presence of nonpathogenic resident intestinal organisms [1]. In most rodent models chronic intestinal inflammation is mediated by the Th1 cytokines interleukin-12 (IL-12) and interferon-γ (IFN-γ), while suppression of inflammation is mediated by the regulatory cytokines interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). The influence of resident flora on the initiation and perpetuation of spontaneous colitis, gastritis and arthritis has been well characterized in HLA-B27/β2 microglobulin transgenic (TG) rats, which develop disease by 3 months of age when raised under specific pathogen-free (SPF) conditions [2]. When raised in a germ-free environment these rats fail to develop gastritis, colitis and arthritis [3,4]. However, these animals develop colitis and gastritis within one month after transfer to a SPF environment [3]. In this model *Bacteroides vulgatus* preferentially induces colitis after monoassociation for one month, with no inflammation resulting from *Escherichia coli* monoassociation [5]. The importance of host genetic susceptibility was illustrated by the lack of colitis or activation of immune responses in wild type (non-TG) littermates colonized with the same SPF bacteria or *B. vulgatus* [3,5]. The role of resident intestinal flora in the pathogenesis of colitis in TG rats is further emphasized by an

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increase of caecal inflammation after the creation of a blind caecal loop which results in an increased bacterial load, including *Bacteroides species* [6], and studies showing that broad spectrum antibiotics can prevent as well as treat colitis [7]. In other experimental models of colitis, similar findings have been reported [8,9].

Although these observations suggest a central role for normal luminal bacteria in the induction and perpetuation of immune-mediated colitis in this model, the mechanisms by which bacteria activate immune cells responsible for the development of colitis remain unclear. The aim of our study was to investigate cytokine responses induced in HLA-B27 TG rats and their non-TG littermates by commensal enteric bacteria and their products. We show that lysates of caecal contents induce mesenteric lymph node (MLN) cells to produce an array of cytokines. MLN $CD4^+$ cells from TG rats, but not from non-TG rats, produce IFN-γ, which is dependent on *in vivo* bacterial colonization. Both TG and non-TG MLN B cells produce IL-10 and TGF-β. The production of these two regulatory cytokines is independent of prior bacterial colonization.

MATERIALS AND METHODS

Animals

HLA-B27 TG rats (the 33-3 line on the F344 background) and their non-TG littermates, originally obtained from Dr Joel D. Taurog, University of Texas Southwestern Medical Center were maintained in SPF housing conditions at the University of North Carolina, Chapel Hill or in germ-free conditions in the Gnotobiotic Animal Core of the Center for Gastrointestinal Biology and Disease at the College of Veterinary Medicine, NC State University in Raleigh, NC, USA. Presence or absence of the HLA-B27 transgene was determined by PCR using DNA isolated from tail clippings. Rats between the ages of 4 and 6 months were used for our studies. All studies were approved by University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Histology

Colons and caeca were fixed and stained as previously described [3]. A validated histological inflammatory score ranging from 0 to 4 was used for blinded evaluation [3].

Preparation of MLN cells and enrichment of lymphoid cell subpopulations

Mesenteric lymph nodes were removed from HLA-B27 TG and non-TG rats, and single cell suspensions were prepared by gentle teasing. Magnetic bead separation, using antibody coated microbeads and columns designed for cell depletion (Miltenyi Biotec, Auburn, CA, USA) was performed according to the manufacturer's instructions using the following reagents: LD columns; Rat Pan T Cell Microbeads, clone OX-52, for T cell depletion; Rat CD45RA, clone OX-33, for B cell depletion; Rat CD4 Microbeads, clone OX-38, for $CD4^+$ cell depletion; Rat CD8a Microbeads, clone G38, for $CD8^+$ cell depletion; and Rat CD45RA plus Rat CD8a Microbeads to enrich $CD4^+$ cells.

Preparation of caecal bacterial lysates

Caecal bacterial lysates (CBL) were prepared as described by Cong *et al*. [10]. Briefly, caecal contents from several non-TG or TG rats were solubilized by vortexing in RPMI, and incubated with 10 μg/ml DNA-ase, 0·01 M MgCl and then homogenized for 3 min using 0·1 mm glass beads in a Mini-Bead Beater (Biospec Products, Bartlesville, OK). After centrifuging at 10 000 *g* for 10 min the supernatant was filtered through a 0·45 μM syringe filter. Sterility was confirmed by aerobic and anaerobic culture.

Mesenteric lymph node cell cultures

Either unseparated MLN cells, or cell subpopulations obtained after antibody-coated magnetic bead depletion were washed and 4×10^5 cells were cultured in 96 well flat bottom microplates (Costar 3595), in 0·2 ml complete medium (RPMI 1640 plus 5% heat inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 5×10^{-5} M 2-mecaptoethanol, and 50 μg/ml gentamicin) for 3 days, which we found to be optimal for detection of all of the cytokines measured with the exception of TGF-β. Similar relative differences in cytokines produced by TG *versus* non-TG MLN cells were present at each time point between 12 h and 6 days. For analysis of TGF-β production, 8×10^5 cells were cultured for 5 days in serum-free RPMI which was supplemented with 1% Nutridoma-SP (Roche Molecular Biochemicals, Mannheim, Germany). Higher levels of TGF-β were consistently found in supernatants of day 5 compared to day 3 cultures. Cells were stimulated with different concentrations of caecal bacterial lysate as indicated for each experiment. Culture supernatants were collected and stored at −20°C.

Preparation of APC and cocultures with $CD4^+$ cells.

We used T-cell depleted MLN from non-TG rats for APC. T cells were lysed using IgM anti-rat CD3 (clone 1F4, Pharmingen, San Diego, CA, USA), followed by incubation with rabbit complement (normal rabbit serum prepared in this laboratory). The cells remaining after complement-mediated lysis of $CD3^+$ cells were >95% surface Ig positive, <2% CD4 positive and <2% CD8 positive. The cells were pulsed overnight with caecal bacterial lysate from non-TG rats (100 μg/ml) or with unrelated protein antigen keyhole limpet haemocyanin (KLH; Pierce, Rockford, IL, USA) in complete medium. The pulsed APC were then washed to remove excess antigens and other bacterial products. Magnetic bead-enriched $CD4^+$ cells, 2×10^5 per well, were stimulated with antigen-pulsed APC, 3×10^5 per well. Supernatants were harvested after 3 days and stored at −20°C.

Flow cytometry

MLN cells before and after magnetic bead separation were evaluated by flow cytometry using the following fluorochrome labelled or unlabelled reagents. For detection of HLA-B27-expressing cells, we used culture supernatant from the murine hybridoma, designated ME-1, obtained from ATCC (Rockville, MD, USA), followed by FITC labelled goat anti-mouse IgG (γ chain specific) antibody (Southern Biotechnology, Birmingham, AL, USA). For surface immunoglobulin positive B cells, we used FITC labelled goat anti-rat IgG (H + L) antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). For $CD4^+$ and $CD8^+$ cells we used PE-anti-CD4 monoclonal antibody (clone W3/25) and FITC anti-CD8 monoclonal antibody (clone OX-8) (Caltag, Burlingame, CA, USA), respectively.

Cytokine and PGE_2 measurements

Cytokines in cell culture supernatants were measured by ELISA using unlabelled capture antibodies and biotin-labelled detection

antibodies, followed by horse-radish peroxidase labelled Streptavidin. The concentration of each cytokine was determined by comparison to a standard curve generated using recombinant proteins. For IFN-γ, we used unlabelled polyclonal anti-IFN-γ antibody and biotin-labelled monoclonal anti-IFN-γ antibody (clone DB-1) (Biosource International, Camarillo, CA, USA). For IL-10 we used unlabelled monoclonal anti-rat IL-10 antibody (clone A5-7) and biotin-labelled monoclonal anti-rat IL-10 antibody (clone A5-6) (BD Biosciences Pharmingen, San Diego, CA, USA). For IL-12 we used a rat IL-12 p40 CytoSet (Biosource International). For TNF we used a rat TNF BD OptEIA ELISA Set (BD Biosciences Pharmingen). TGF-β concentrations were measured after acidification and neutralization according to the manufacturer using a TGF-β1 specific ELISA (Promega, Madison, WI, USA). PGE_2 was measured using a competitive immunoassay Correlate-EIA (Assay Designs Inc., Ann Arbor MI, USA).

Cytokine mRNA expression in caecal tissues

Total RNA was extracted from caecal tissue using a standard technique as described previously [11]. One microgram of RNA isolated from each sample was reverse transcribed, and the cDNA (1-2 μg) was then amplified using primers specific for rat cytokines [11-16]. Negative controls without cDNA were included in each experiment. Aliquots of all samples were analysed by electrophoresis on 2% agarose gel containing GelStar® (BioWhittaker). The size of the PCR product was compared to the predicted size using a 100 bp DNA ladder (Gibco BRL, Grand Island, NY, USA). The DNA products were visualized by ultraviolet fluorescence and photographed (Polaroid 665, Polaroid Corp, Cambridge, MA). The cytokine mRNA was quantified by densitometry, and the ratio to β-actin mRNA was calculated.

Statistical analysis

Cytokine levels are expressed as mean ± standard deviation of triplicate measurements. A nonpaired Student *t*-test or alternate Welch *t*-test was used, in which a two-tailed *P*-value of $<0{\cdot}05$ was considered statistically significant.

RESULTS

Evaluation of colitis and of lymphoid cell subpopulations in MLN of SPF and of germ-free TG and non-TG rats

TG rats housed in SPF conditions showed significant gross and histological evidence of colitis of the entire colon and caecum, with over 95% of the TG rats demonstrating histology scores higher than 3·0 on a 0-4 scale. In contrast, their non-TG littermates exposed to the same microflora did not develop colitis, nor did germ-free TG or non-TG rats exhibit any disease (Fig. 1).

SPF TG rats with colitis had larger MLN, containing over 3-fold more cells, than did MLN of their non-TG littermates (Table 1). MLN from SPF TG rats contained a significantly higher percentage and total number of $CD4^+$ T cells than non-TG MLN (Table 1). The percentage of $CD8^+$ T cells and of B cells was lower in TG *versus* non-TG MLN. However, due to the higher number of total MLN cells, TG MLN contained significantly more $CD8^+$ cells and B cells than did MLN of non-TG littermates.

MLN of germ-free rats were small and contained much lower cell numbers than MLN of SPF rats. The total number of MLN cells was not significantly different between germ-free TG and germ-free non-TG rats. MLN from germ-free TG rats contained higher proportions and higher total numbers of $CD4^+$ cells but lower proportions and fewer total numbers of B cells compared to MLN of non-TG littermates, whereas the percentages and numbers of $CD8^+$ cells were similar (Table 1). All

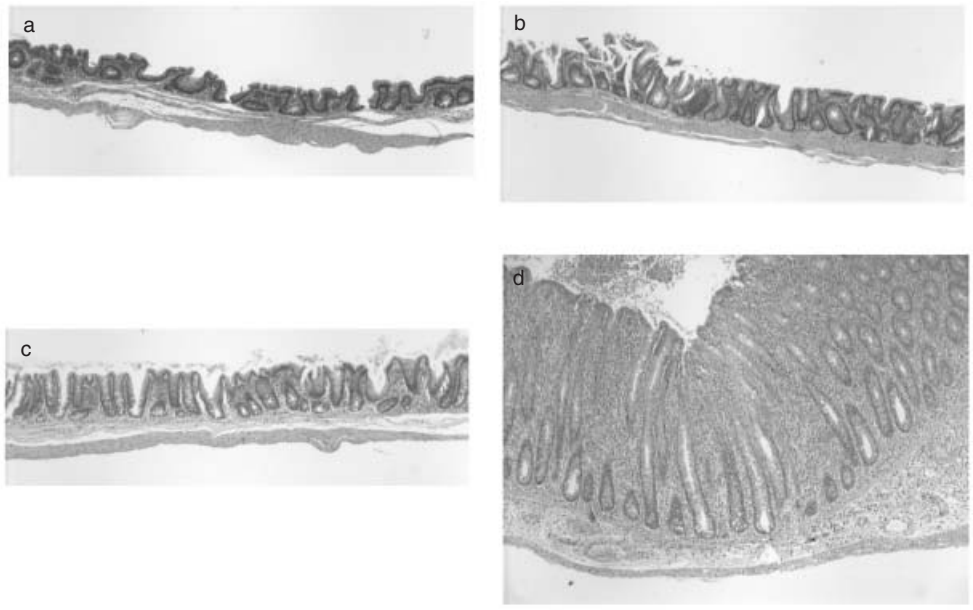

Fig. 1. Representative photomicrographs of tissue sections (×40) from ceca of 5-6 month old (a) germ-free non-TG; (b) germ-free TG; (c) SPF non-TG; (d) SPF TG rats. Note the extensive mucosal and submucosal inflammation as well as significant crypt hyperplasia in the caecum from a SPF TG rat (d), whereas no colitis was present in caecal tissue from germ-free rats or in the caecum from non-TG rats housed in an SPF environment (a–c).

Table 1. Cell numbers and cell subpopulations in mesenteric lymph nodes of specific pathogen free and germ-free HLA-B27 transgenic rats and their nontransgenic littermates

	Specific pathogen free		Germ-free	
	nontransgenic $n = 15$	transgenic $n = 15$	nontransgenic $n = 16$	transgenic $n = 12$
Total cells ($\times 10^6$)	87·6 ± 6·2	279·8 ± 22·7*	41·8 ± 4·8	41·7± 5·5
Cell subpopoulations (%)				
$CD4^+$	40·3 ± 0·9	56·2 ± 1·1*	28·1 ± 1·4	42·2± 1·9*
$CD8^+$	16·7 ± 0·6	13·4 ± 0·5†	11·9 ± 0·8	13·5± 0·6
Surface Ig	36·4 ± 1·8	24·9 ± 1·2*	51·3 ± 2·7	38·7± 2·1‡
Cell subpopoulations ($n \times 10^6$)				
$CD4^+$	35·5 ± 2·9	155·6 ± 11·9*	11·4 ± 1·1	17·7± 2·7‡
$CD8^+$	14·7 ± 1·2	37·6 ± 3·5*	5·0 ± 0·7	5·8± 1·0
Surface Ig	31·9 ± 2·7	70·3 ± 6·8*	22·2 ± 3·4	16·0± 2·1

Values represent mean ± SEM of cell numbers or of percentages of the different lymphoid cell subpopulations.*$P < 0{\cdot}001$, ‡$P < 0{\cdot}01$, †$P < 0{\cdot}05$ *versus* non-TG littermates.

animals identified as HLA-B27 TG by PCR analysis of tail DNA also expressed HLA-B27 on MLN cells as determined by flow cytometry.

Cytokine production in unseparated MLN cells from SPF TG versus *non-TG rats*

Unseparated TG MLN cells, stimulated *in vitro* with 100 μg/ml caecal bacterial lysate produced significantly more IFN-γ, IL-12, and TNF compared to MLN cells from non-TG rats (Figs 2a–c). In contrast, non-TG MLN cells stimulated with 10 μg/ml CBL produced significantly more IL-10 than TG MLN cells (Fig. 2d). TGF-β production by MLN cells followed the same trend as IL-10 after 10 μg/ml and also 100 μg/ml caecal bacterial lysate stimulation; non-TG unseparated MLN cells produced significantly more TGF-β than those from TG littermates (Fig. 2e). PGE_2 concentrations did not differ significantly in caecal bacterial lysate stimulated MLN cell cultures from TG *versus* non-TG rats (data not shown).

Stimulation with caecal bacterial lysate obtained from either TG rats or from non-TG rats showed the same pattern; both lysates induce more IFN-γ, IL-12, and TNF, and less IL-10 and TGF-β in MLN cell cultures of TG compared to non-TG rats (data not shown). In five separate experiments, we did not observe consistent differences in cytokine levels induced by bacterial lysates from either TG or from non-TG rats. Therefore, we chose to use caecal bacterial lysate from non-TG rats for the remainder of our experiments.

Caecal cytokine mRNA expression in SPF TG versus *non-TG rats*

The mRNA expression of pro-inflammatory cytokines was significantly higher in caecal tissues from TG compared to non-TG rats (Fig. 3). The cytokine/β-actin ratios for IL-1β, IFN-γ as well as for IL-12 were significantly higher in TG rats compared to non-TG littermates. Consistent with results from caecal bacterial lysate-stimulated unseparated MLN cultures, caecal IL-10 mRNA expression was significantly higher in non-TG *versus* TG rats. However, caecal mRNA expression for TGF-β did not differ between TG and non-TG rats.

Cytokine production in MLN cells from germ-free TG versus *non-TG rats*

Caecal bacterial lysate stimulation of MLN cells from germ-free rats did not induce IFN-γ, IL-12 or TNF production compared to unstimulated levels (data not shown), indicating that production of these cytokines in TG rats depends on *in vivo* exposure to commensal bacteria and their products. In contrast, MLN cells from both TG and non-TG germ-free rats produced IL-10 and TGF-β after stimulation with bacterial lysate (Fig. 4), indicating that these two cytokines can be produced by naive cells independent of *in vivo* exposure to commensal intestinal microorganisms. As in SPF rats, IL-10 detected in the supernatants of MLN cell cultures from germ-free non-TG rats was significantly more than that from germ-free TG rats after stimulation with 1 μg/ml and with 10 μg/ml caecal lysate (Fig. 4a). After stimulation with the optimal dose of 100 μg/ml, germ-free non-TG MLN cells produced almost twice as much TGF-β compared to germ-free TG MLN cells (Fig. 4b).

Cytokine production in MLN cells after T cell or B cell depletion or in cocultures of CD4 cells plus antigen-pulsed APC

To determine which cell type(s) are critical to the production of the cytokines that we evaluated, we carried out negative selection to deplete T cells or B cells from MLN cell preparations. After T cell depletion less than 2·5% of the total remaining cells were $CD4^+/CD8^+$, as determined by flow cytometry. Approximately 97% of the T cell-depleted MLN cells were positive for surface Ig, which is characteristic of B cells. In response to 100 μg/ml of caecal bacterial lysate, T cell-depleted MLN from TG rats did not produce IFN-γ (Fig. 5a). IFN-γ production was dramatically reduced but not absent in cultures of B cell depleted MLN. $CD4^+$ cell depletion of MLN cells, with less than 0·5% remaining $CD4^+$ cells, significantly reduced IFN-γ production to the same degree as T cell depletion (Fig. 5b). In contrast, $CD8^+$ cell depletion of MLN cells, with less than 0·5% $CD8^+$ cells, had no effect on IFN-γ production after bacterial lysate stimulation (Fig. 5b).

IL-12 production was significantly reduced after T cell depletion in TG MLN in response to 100 μg/ml caecal bacterial lysate (Fig. 5c). Supernatants of B cell-depleted MLN cell cultures

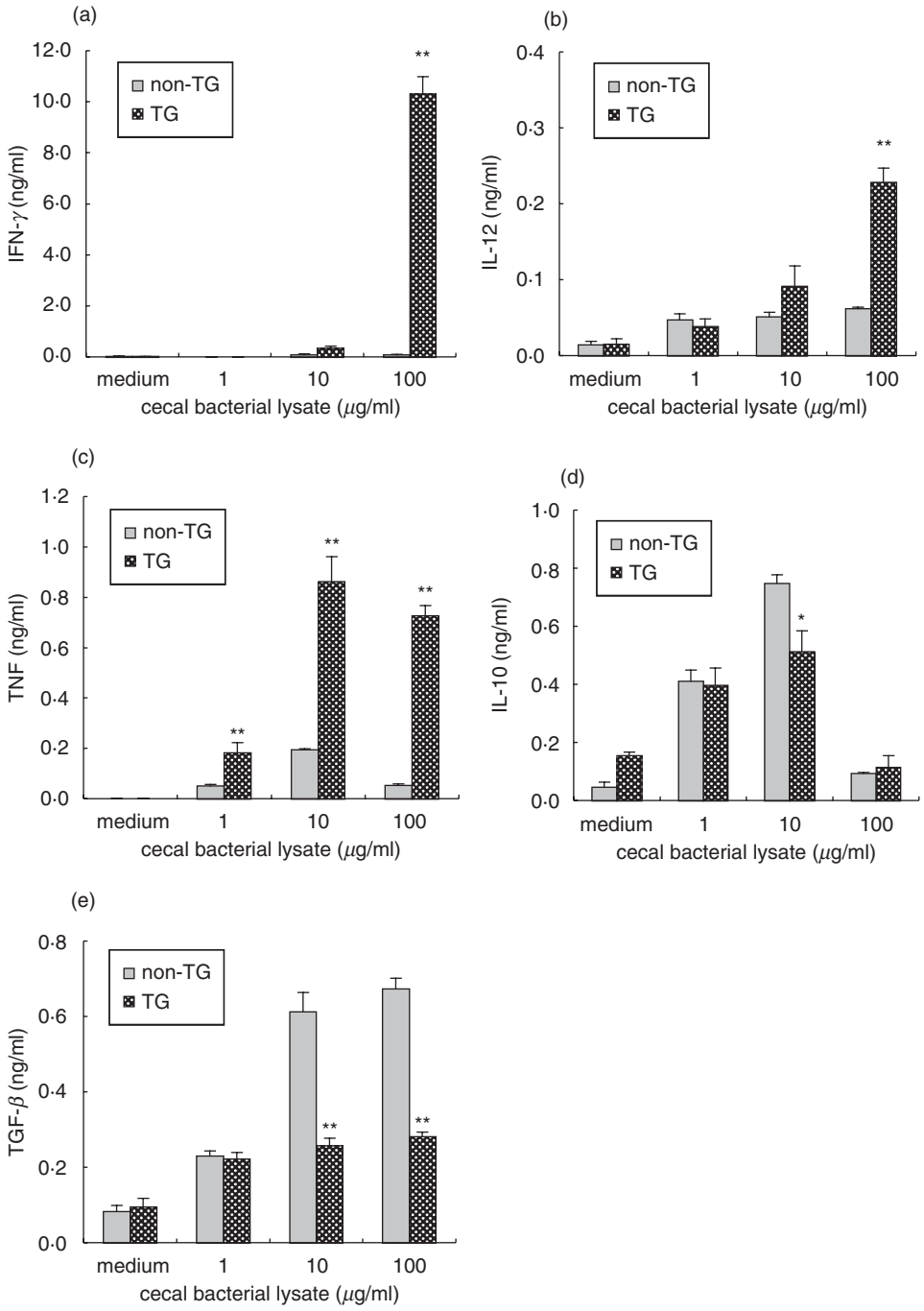

Fig. 2. Cytokine levels in supernatants of unseparated MLN cell cultures from 5 month old SPF non-TG (□) or TG (▩) rats after stimulation with various concentrations of caecal bacterial lysate (in μg/ml) from non-TG rats. (a) IFN-γ; (b) IL-12 (p40); (c) TNF; (d) IL-10; (e) TGF-β. Data shown are from a representative experiment out of two to seven separate experiments. Cytokines levels are expressed in ng/ml. Values represent the mean and standard deviations of each cytokine detected in triplicate supernatants. $^{**}P < 0{\cdot}01$ *versus* non-TG MLN cell supernatants, $^{*}P < 0{\cdot}05$ *versus* non-TG MLN cell supernatants.

contained slightly more IL-12 than did supernatants of unseparated MLN.

To more precisely evaluate $CD4^+$ T cell responses to caecal bacterial lysate, we cocultured CD4-enriched MLN cells with caecal bacterial lysate pulsed APC. As shown in Fig. 6, $CD4^+$ cells from TG but not from non-TG MLN produced high amounts of IFN-γ in coculture with caecal bacterial lysate pulsed APC. Interestingly, the *in vitro* response is not dependent on antigen presentation via the HLA-B27 molecule because $CD4^+$ cells from TG rats respond to antigen-pulsed non-TG APC. Further studies focusing on the potential differences between APC from TG and from non-TG rats to present bacterial antigens are in progress.

In contrast to IFN-γ and IL-12, supernatants of caecal bacterial lysate stimulated T cell-depleted MLN cells contained

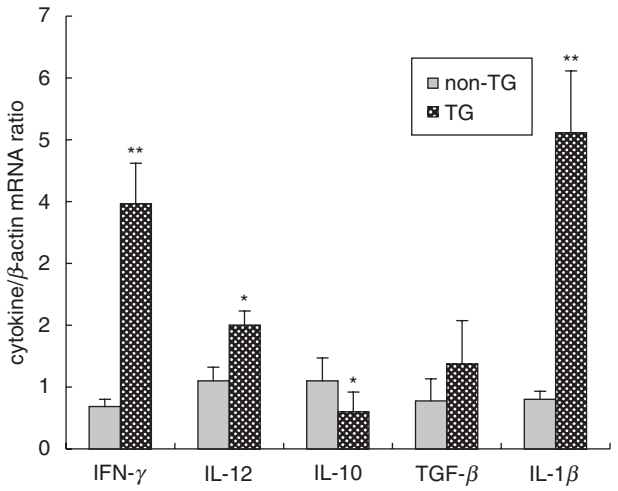

Fig. 3. Epression of IFN-γ, IL-12, IL-10, TGF-β, and IL-1β mRNA in caecal tissues from SPF TG rats (▩) and their non-TG littermates (□). Total RNA was reverse transcribed and amplified for various cycles by the polymerase chain reaction. Cytokine and β-actin mRNA expression was evaluated in 8 non-TG and 6 TG rats. Tissue from each individual animal was analysed separately. The results shown represent the averages of the ratios of specific cytokine *versus* β-actin mRNA obtained in two to three separate experiments. $^{**}P < 0{\cdot}01$ *versus* non-TG caecal tissue, $^{*}P < 0{\cdot}05$ *versus* non-TG caecal tissue.

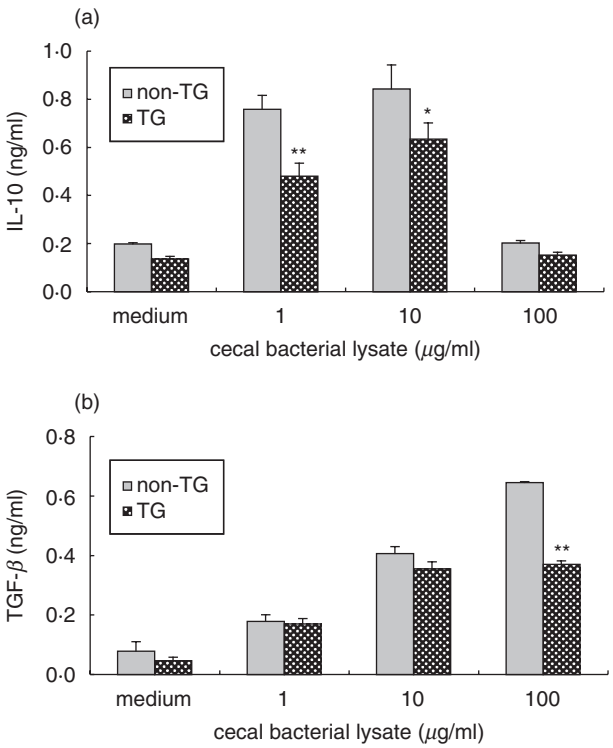

Fig. 4. Cytokine levels in supernatants of unseparated MLN cell cultures from 4 to 5 month old germ-free non-TG (□) or TG (▩) rats after stimulation with various concentrations of caecal bacterial lysates (in μg/ml) from non-TG rats. (a) IL-10; (b) TGF-β. Data shown are from a representative experiment out of five (IL-10) or two (TGF-β) separate experiments. Cytokines levels are expressed in ng/ml. Values represent the mean and standard deviations of each cytokine detected in triplicate supernatants. $^{**}P < 0{\cdot}01$ *versus* non-TG MLN cell supernatants, $^{*}P < 0{\cdot}05$ *versus* non-TG MLN cell supernatants.

equivalent or greater amounts of IL-10 and TGF-β compared to supernatants of unseparated MLN cells, indicating that these two cytokines are not produced by T cells (Fig. 7). Moreover, the levels of IL-10 and TGF-β in supernatants of B cell depleted MLN were even lower than amounts in supernatants of unstimulated cells, indicating that B cells either produce the majority of the IL-10 and TGF-β or that B cells are essential to their production (Fig. 7).

As also shown in Fig. 7, IL-10 and TGF-β levels were lower in supernatants of T cell-depleted TG compared to non-TG MLN cells after caecal bacterial lysate stimulation. In a total of seven separate experiments, there is a trend towards production of lower amounts of IL-10 by T cell-depleted TG MLN cells compared to non-TG cells (ratio IL-10 TG:IL-10 non-TG = $0{\cdot}78 \pm 0{\cdot}06$). These results suggest that there is only a marginal difference between the abilities of TG and non-TG MLN cells to produce IL-10 in response to commensal bacteria and their products.

DISCUSSION

We evaluated *in vitro* responses of MLN cells to physiologically relevant components of luminal contents, since MLN drain the diseased caecum and proximal colon in SPF HLA-B27 TG rats. For these studies, we prepared lysates of caecal contents as described by Cong *et al*. [10], rather than attempting to culture intestinal bacteria for the following reasons:

- the lysates contain bacterial products that are present at the site of maximal inflammation;
- not all enteric bacteria can be cultured;
- culturing can change bacterial antigen expression.

We showed that caecal bacterial lysate can stimulate significantly higher amounts of those cytokines generally associated with Th1 responses including IFN-γ, IL-12 and TNF from unseparated MLN cells collected from SPF TG rats than from non-TG littermates. These results correlated with caecal mRNA expression of the same cytokines in colitic SPF TG rats, which is in agreement with and extends a previous report by Rath *et al*. [3]. We therefore conclude that *in vitro* production of pro-inflammatory cytokines by draining MLN cells stimulated with commensal caecal bacterial lysates correlates with mucosal cytokine responses in the diseased caecum. These results, and our failure to detect IFN-γ, IL-12 or TNF in cultures of MLN cells from germ-free TG rats, indicate that caecal bacteria and their products can induce these pro-inflammatory cytokines in MLN and caecal tissues of disease-susceptible SPF HLA-B27 TG rats**.** T cell-depletion in our studies resulted in complete loss of IFN-γ responses by MLN from TG rats. Furthermore, CD4 T cell depletion of TG MLN cells abrogated IFN-γ production, whereas $CD8^+$ cell depletion had no effect. In addition, $CD4^+$-enriched MLN cells from TG but not from non-TG rats produced IFN-γ after stimulation with caecal bacterial lysate pulsed APC. Combined, these results indicate that caecal bacteria and their products stimulate MLN-derived CD4 T cells to produce IFN-γ in SPF TG rats. The disease-inducing capacity of these CD4 T cells was demonstrated by the ability of LN-derived $CD4^+$ cells from colitic SPF TG donor rats to transfer colitis into SPF nude TG recipients, which do not develop disease in the absence of T cells [17]. MHC class I molecules such as

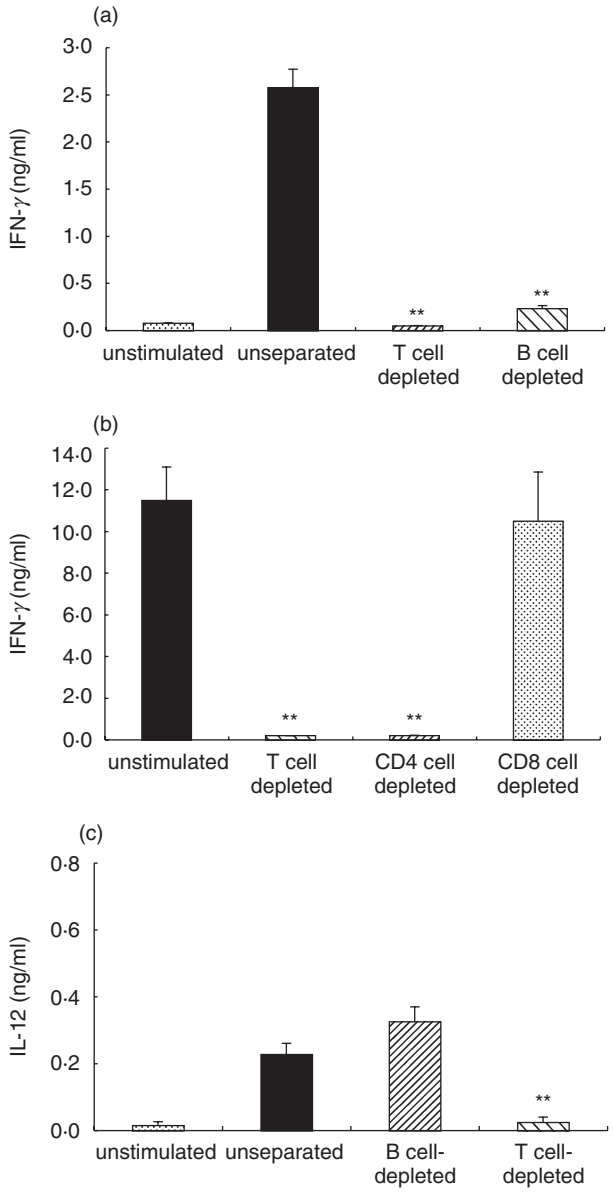

Fig. 5. Cytokine levels in MLN cell cultures from 4 to 5 month old SPF TG rats. (a, b) IFN-γ and (c) IL-12 production by TG MLN cells which were either unseparated, T cell depleted, $CD4^+$ cell depleted, $CD8^+$ cell depleted or B cell depleted, then stimulated with 100 μg/ml caecal bacterial lysate from non-TG rats. Cytokine levels are expressed in ng/ml. Values represent the mean and standard deviations of each cytokine detected in triplicate supernatants and are representative of three separate experiments. $^{**}P < 0{\cdot}01$ *versus* unseparated MLN cell supernatants.

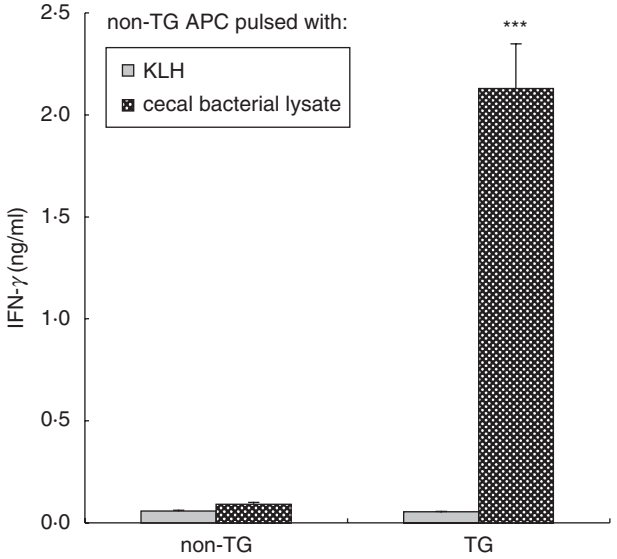

Fig. 6. IFN-γ production by $CD4^+$ MLN T cells in coculture with caecal bacterial lysate-pulsed APC. $CD4^+$ T cells were enriched from MLN of SPF TG or non-TG rats and stimulated for 3 days with either caecal bacterial lysate-pulsed (▩) or KLH-pulsed (▢) APC prepared from MLN of non-TG rats as described in Materials and Methods. Caecal bacterial lysate-pulsed APC alone do not produce detectable amounts of IFN-γ. Values represent the mean and standard deviation of IFN-γ in triplicate culture supernatants and are representative of six separate experiments. $^{***}P < 0{\cdot}005$ *versus* IFN-γ in supernatants of TG $CD4^+$ MLN cells in coculture with KLH-pulsed APC and *versus* IFN-γ in supernatants of non-TG $CD4^+$ MLN cells in coculture with caecal bacterial lysate-pulsed APC.

HLA-B27 are generally thought to activate $CD8^+$, not $CD4^+$ T cells. However, $CD8^+$ T cells were not essential to the pathogenesis of colitis in HLA-B27 TG rats [18]. While the role of the HLA-B27 molecule in development of inflammatory diseases has not been identified, a variety of plausible explanations have been proposed, based on the ability of HLA-B27 to activate $CD4^+$ T cells. Recent reports described $CD4^+$ T cells that recognized unusual forms of HLA-B27, such as heterodimers that lack an associated peptide, altered three dimensional structure of HLA-B27, or HLA-B27 homodimers [19]. HLA-B27 homodimers that are capable of binding peptide and thus taking on MHC class II-like conformation have been identified [20]. Moreover, MHC class I-restricted $CD4^+$ T cells derived from MHC class II-deficient mice induced colitis in congenic immunodeficient mice [21]. Any or all of the proposed mechanisms could explain the link between HLA-B27 expression and development of inflammatory disease in the transgenic rat model that we have employed.

Interestingly, in our studies, IFN-γ production by TG MLN cells was significantly reduced after B cell depletion, indicating either that B cells provide help for IFN-γ production by MLN-derived CD4 T cells through APC activity or, less likely, that these cells produce IFN-γ. In a comprehensive analysis reported by Harris *et al.* B cells, like T cells, could be divided into subsets (designated Be1 and Be2) [22]. Be1 cells, but not Be2 cells produce IFN-γ. In addition, different B cell subsets have the capacity to influence cytokine production by $CD4^+$ T cell subsets [22]. Furthermore, lower levels of IFN-γ were detected in spleen cell cultures of LCMV-infected B cell deficient mice compared to B cell replete mice after *in vitro* antigen stimulation [23].

IL-12 production in response to the optimal concentration of caecal bacterial lysate was reduced by 80% in T cell-depleted MLN cell cultures, indicating that IL-12 production is T cell-dependent. Although IL-12 is mainly produced by APC such as macrophages and dendritic cells (DC), activated T cells can stimulate IL-12 production by APC [24].

IFN-γ, IL-12 and TNF were produced by caecal bacterial lysate-stimulated TG MLN cells, but not by non-TG MLN cells. In addition, IFN-γ, IL-12 and TNF were not detected in supernatants of lysate-stimulated MLN cell cultures from germ-free rats. Thus,

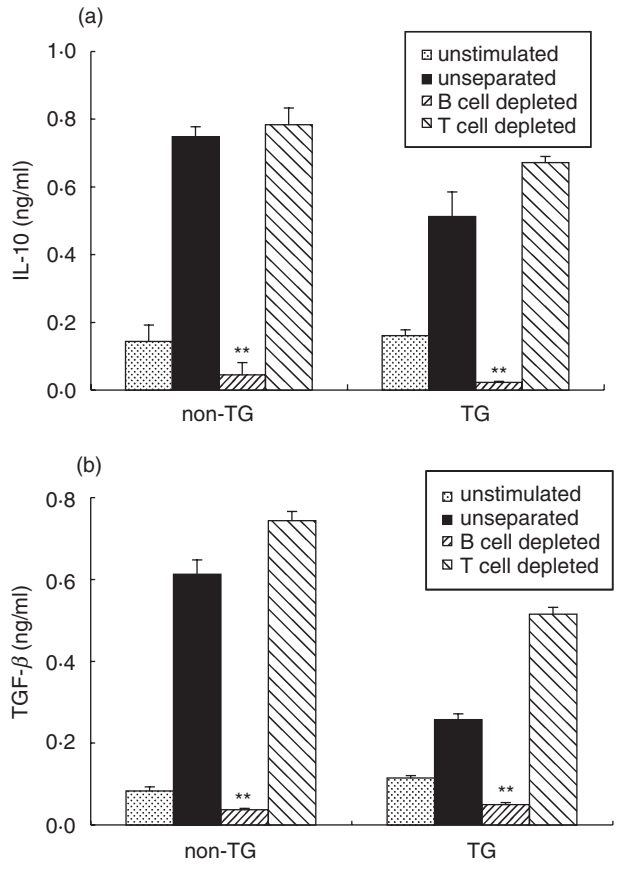

Fig. 7. Cytokine levels in MLN cell cultures from 4 to 5 month old SPF non-TG and TG rats. (a) IL-10 and (b) TGF-β production by MLN cells which were either unseparated, T cell depleted, or B cell depleted, then stimulated with 10 μg/ml of caecal bacterial lysate from non-TG rats. Cytokine levels are expressed in ng/ml. Values represent the mean and standard deviations of each cytokine detected in triplicate supernatants and are representative of seven (IL-10) or three (TGF-β) separate experiments. $^{**}P < 0{\cdot}01$ *versus* unseparated MLN cell supernatants.

the production of these three cytokines is dependent both on the genetic susceptibility of the host and on prior *in vivo* stimulation by commensal intestinal bacteria or the presence of colonic inflammation.

Of potential relevance to immunoregulation, we observed that caecal bacterial lysate-stimulated unseparated MLN cells from non-TG rats produced significantly more IL-10 and TGF-β relative to their TG littermates, whereas the production of another immunoregulatory molecule, PGE_2, was not significantly different in supernatants from lysate-stimulated TG and non-TG MLN cells. IL-10 is an immunoregulatory cytokine, produced by T regulatory cells (Tr-1) and also by B cells and DC, with the ability to prevent the development of colitis. IL-10 inhibits antigen-specific proliferation and cytokine production by Th1 lymphocytes and has down-regulatory effects on APC such as suppression of macrophage activation and IL-12 production [25-27]. The important anti-inflammatory effect of IL-10 is demonstrated by the observation that severe inflammatory disease develops in interleukin-10 deficient mice. The role of commensal bacteria in this model is underscored by the observation that germ-free IL-10 deficient mice do not develop colitis [28,29]. IL-12 and IFN-γ production in response to *in vitro* LPS stimulation is elevated in spleen cells from IL-10-deficient compared to wild type control mice [30]. Several *in vivo* studies have shown that parenteral administration of IL-10 or IL-10- producing T cells could prevent colitis in models of chronic intestinal inflammation [31-35]. However, parenterally administered recombinant IL-10 appeared to have limited capacity to reverse established experimental colitis [11] or human Crohn's disease [36]. A local mucosal delivery of IL-10 by genetically engineered *Lactococcus lactis* or by adenoviral vectors encoding IL-10 was able to reverse colitis in IL-10 deficient mice and dextran sodium sulphate-induced colitis [37,38].

TGF-β is another immunoregulatory molecule, which is produced by APC and by Th3/Tr1 lymphocytes, whereas TGF-β receptors are expressed on a variety of cells [39]. Of significant interest, we show here that caecal bacterial lysate stimulated B cells, but not T cells, produce IL-10 and TGF-β. The importance of TGF-β in immune homeostasis is demonstrated by the fact that TGF-β deficient mice die within 5 weeks of severe multiple organ inflammation [40]. $CD4^+$ $CD25^+$ T cells exert immunosuppression by a cell-cell interaction involving cell surface TGF-β [41]. TGF-β is important for the regulation of intestinal inflammation. Intranasal administration of a TGF-β-containing plasmid prevented TNBS induced colitis [42].

Our results show that the levels of IL-10 and TGF-β in supernatants of unseparated MLN cell cultures from either germ-free or SPF rats correlated with the percentage of B cells, and that neither IL-10 nor TGF-β were detected in B cell-depleted MLN cultures. B cells display several immune functions, such as production of immunoglobulins and cytokines, presentation of antigens, and potentiation of T cell responses [19,43]. B cells also have immunoregulatory functions [44-46], and murine B-1 B cells have been shown to produce IL-10 after LPS stimulation [47]. Mizoguchi *et al*. [48] demonstrated that IL-10-producing B cells are protective, since B cell/TCRα double deficient mice had more colitis than TCRα deficient mice with competent B cells. Also, transfer of B cells from IL-10/TCRα double deficient mice was unable to suppress chronic intestinal inflammation in B cell/TCRα double deficient mice [48]. Similarly, SPF Gαi 2 deficient mice that develop colitis lack IL-10 producing B cells [49]. IL-10-producing B cells can also prevent arthritis [50].

In our study MLN-derived B cells also produce TGF-β in both TG and non-TG rats, housed in either SPF or germ-free conditions. Earlier studies have shown that normal B cells can produce TGF-β, which limits their own clonal expansion and differentiation [51]. An immunoregulatory function of TGF-β-producing B cells was shown by Tian *et al*. [52] in a murine model of autoimmune diabetes in which activated B cells produced TGF-β. These B cells induced apoptosis of lymphocytes and prevented the development of autoimmune responses after cotransfer of B cells with disease-inducing T cells.

The bacterial components that stimulate cytokine production in our studies are not defined but are likely to be extremely heterogeneous, including LPS and peptidoglycan-polysaccharide complexes that stimulate polyclonal responses by T cells and B cells as well as bacterial antigens that induce antigen-specific responses. While we have not identified the nature of the components that induce the responses evaluated here, we predict that bacterial products activate B cells in a polyclonal fashion via Toll-like receptors as has been demonstrated [53]. In support of this view, we have found that LPS induces IL-10 and TGF-β, but not IFN-γ, in MLN cell cultures from TG and non-TG rats (data not

shown). In addition, we propose that bacterial antigens, presented by APC *in vitro*, restimulate T cells that have previously responded, *in vivo*, to the same bacterial antigens. A future direction of these studies is to further identify the effects of the different components of bacterial lysates.

In summary, our study indicates that MLN cells produce an array of cytokines in response to *in vitro* stimulation with normal, physiologically relevant caecal bacteria and their products. $CD4^+$ MLN cells from colitis-susceptible HLA-B27 TG rats, maintained in SPF conditions, produce cytokines that are found in association with Th1 immune responses. B cells, from both HLA-B27 TG rats and their non-TG littermates, maintained in SPF or germ-free conditions, produce IL-10 and TGF-β, cytokines that are often associated with protective immune response. Thus, our results reveal the diversity of responses of T cells and of B cells that can be activated by components of commensal bacteria in a rodent model of chronic intestinal inflammation.

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