Keratin-8-deficient mice develop chronic spontaneous Th2 colitis amenable to antibiotic treatment

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Summary

Keratin 8 (K8) is the major intermediate filament protein present in intestinal epithelia. Depending on the mouse genetic background, absence of K8 causes embryonic lethality or colonic hyperplasia and colitis. We studied disease progression, the inflammatory responses, and role of luminal bacteria in K8-null mice in order to characterize the intestinal pathology of K8-associated colitis. Colon lymphocytes were isolated for analysis of their phenotype and cytokine production, and vascular and lymphocyte adhesion molecule expression in K8–/– mice of varying ages. K8–/– mice had a marked increase in TCR$_{\beta}$-positive/CD4-positive T cells infiltrating the colon lamina propria, in association with enhanced Th2 cytokine (IL-4, IL-5 and IL-13) production. K8–/– mice show early signs of inflammation even prior to weaning, that increases with age, and their epithelial cells overexpress MHC class II antigens. The chronic colitis is related to increased CD4-positive infiltrating T cells displaying memory and naive phenotypes, and an altered vascular endothelium with aberrant expression of peripheral node addressin. Analysis of normal gut-specific homing molecules, reveals an increased number of $\alpha_4\beta_7$-positive cells and vascular mucosal addressin cell adhesion molecule-1 in K8-null colons. Antibiotic treatment markedly decreased colon inflammation and ion transporter AE1/2 mistargeting, indicating that luminal bacteria play an important role in the observed phenotype. Therefore, K8-null mice develop chronic spontaneous Th2-type colitis due to a primary epithelial rather than immune cell defect, which is amenable to antibiotic therapy. These mice provide a model to investigate epithelial-leukocyte and epithelial-microbial cross-talk.

Key words: Colitis, inflammatory bowel disease, intermediate filaments, Th2 cytokine

Introduction

Keratins (Ks) make up the intermediate filament cytoskeleton of epithelial cells, and exist as obligate non-covalent heteropolymers of type I (K9-K20) and type II (K1-K8) keratins (Coulombe and Omary, 2002). In the intestine, the intermediate filament cytoskeletal network consists of the simple epithelial keratins K7, K8, K18, K19 and K20 (Moll et al., 1982; Zhou et al., 2003). Of these, K8 and K19 are major keratins of enterocytes (Zhou et al., 2003). Although the function of keratins in intestinal epithelial cells is poorly understood, keratins play a role in protecting various tissues from mechanical and non-mechanical stresses (Fuchs and Cleveland, 1998; Coulombe and Omary, 2002; Lane and McLean, 2004). In humans, K8 and K18 mutations appear to pose a risk factor for subsequent development of liver disease (Ku et al., 2003; Omary et al., 2004). The phenotypes resulting from K8 deletion not only support the importance of keratins in liver disease but also suggest an essential role for keratins in the intestine. For example, whereas 95% of C57BL/6 mice lacking K8 die in utero probably because of trophoblast layer dysfunction (Jaquemar et al., 2004), 50% of FVB/n K8–/– mice have a normal life span, but develop colonic hyperplasia, rectal prolapse and colitis (Baribault et al., 1994). The colonic inflammation in K8-null mice represents a unique model for inflammatory bowel disease (IBD) resulting from a primary epithelial rather than an immune cell defect. Moreover, K8 miss-sense mutations were recently shown in a subset of patients with IBD (Owens et al., 2004).

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory bowel diseases of unknown etiology. Several animal models of IBD have been established mostly via genetic targeting of the immune system or via application of acute chemical injuries (Bouma and Strober, 2003; Strober et al., 2002). Despite different mechanisms for the cause of experimental IBD, pathogenic CD4-positve (CD4+) T cells infiltrate the lamina propria (LP) and the inflammation appears to be mediated via an excessive T helper 1 (Th1) (resembling CD) or via Th2 (resembling UC) cell response (Podolsky, 2002). These two responses have different cytokine profiles, with an increased secretion of IL-12, IFNγ and/or TNF$_{\alpha}$ in the Th1 response, and IL-4, IL-5 and/or IL-13 in the Th2 response. Regardless of their phenotypic responses or primary defect/insult that ultimately causes colonic inflammation, intestinal microflora play a major role in the pathogenesis of IBD. In support of this, IBD models raised in a germ-free environment do not develop colitis, and treatment with broad-spectrum antibiotics reduces or prevents the colonic inflammation (Dianda et al., 1997; Madsen et al., 2000).

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An important aspect of colonic inflammation is the mechanism by which pathogenic CD4+ T cells are able to home to the colon. Lymphocyte recruitment into tissues is specific and requires a multistep process involving differential expression and activation of lymphocyte homing receptors and their interaction with counter-receptors on tissue vasculature or high endothelial venules (HEVs) (Butcher and Picker, 1996). In intestinal tissues, $\alpha_4\beta_7$ (integrin on gut homing lymphocytes) interacts with its ligand mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed on gut associated HEVs. MAdCAM-1 expression is increased in colitis, and blocking antibodies against MAdCAM-1 and/or its ligand α_4 reduce inflammation in animal models (Picarella et al., 1997; Podolsky et al., 1993) and human patients (Ghosh et al., 2003) with IBD.

Leukocyte recruitment involves dynamic multiple steps, and differences in trafficking exists between acute and chronic inflammation. Naive T cells express high levels of L-selectin and are able to home into peripheral lymph nodes (PLN) via their interaction with peripheral node addressin (PNAd), a vascular ligand for L-selectin (Butcher and Picker, 1996; Michie et al., 1993). PNAd initiates rolling of naive but not memory lymphocytes and is normally expressed by HEV in PLNs and to a lesser extent by HEVs in Peyer's Patches. Although naive T cells are generally excluded from non-lymphoid compartments, in chronic, but not acute, inflammation significant numbers of naive T cells accumulate in chronically inflamed tissues, often forming lymphoid aggregates reminiscent of lymph node architecture (Girard and Springer, 1995). The mechanism by which naive T cells accumulate within chronically inflamed tissues is not fully understood but may involve aberrant PNAd expression. Such expression occurs in various tissues of patients with chronic autoimmune diseases including IBD (Renkonen et al., 2002), and T cells isolated from involved intestines of IBD patients (but not from healthy colons) interact with PNAd in vitro (Salmi et al., 1994; Salmi and Jalkanen, 2001). Whether similar events occur in animal models of IBD is not known. In this study we show that K8–/– mice develop chronic colitis and describe the nature of the colonic inflammation and infiltrating T cells, and examine the effect of antibiotic therapy in the early phase of colitis.

Materials and Methods

Mice

K8–/– mice were kindly provided by Robert Oshima (The Burnham Institute, La Jolla, CA, USA) and Helene Baribault (Amgen, South San Francisco, CA, USA). K8-null mice and their wild-type littermates, in an FVB/n background, were generated by interbreeding of K8+/– mice under specific pathogen-free environment, except that the mouse colony tested positive for *Helicobacter hepaticus* and *Helicobacter bilis* by stool PCR analysis (not shown). Mice were genotyped using tail DNA and PCR (Baribault et al., 1994), and age- and sex-matched mice (2 weeks to 6 months old) were studied. The animals were treated according to NIH guidelines and approved animal study protocols.

Cell isolation

Intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were isolated as described previously (Lefrancois and Lycke, 1996) with some modifications. Briefly, colons were removed and rinsed with Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS) containing 1 mM EDTA and 15 mM Hepes (buffer A). The intestines were opened longitudinally, cut into 5 mm pieces, rewashed, then placed in buffer A for two sequential 20-minute incubations with constant stirring at 37°C to remove epithelial cells and IELs. The remaining tissues were incubated with constant stirring (10 minutes, 37°C) in RPMI 1640 medium supplemented with 10% bovine calf serum (BCS) and 15 mM Hepes (buffer B) to remove residual EDTA. The tissues were then digested with 300 U/ml type VIII collagenase (Sigma-Aldrich, St Louis, MO, USA) in buffer B for three sequential 30-minute incubations (37°C) with constant stirring to release the LPLs. Mesenteric lymph node (MLN) lymphocytes were isolated by mechanical dispersion through a wire mesh followed by a wash and resuspension in HBSS with 2% BCS for analysis.

Flow cytometry

The primary antibodies (PharMingen, San Diego, CA, USA) used for flow cytometric analysis included: TCR_β-APC (allophycocyanin), $TCR_{\gamma\delta}$-PE, CD4-FITC or CD4-PerCP, $CD8_\alpha$-PerCP, CD44-FITC, CD45RB-PE, CD62L (L-selectin)-APC and CD69-PE. Isolated cells were stained with the primary antibodies then analyzed with a FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA, USA).

Intracellular cytokine analysis

Intracellular cytokine production by isolated colon LPLs was detected as described previously (Campbell and Butcher, 2002). In brief, cells were stimulated for 4 hours with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) and ionomycin (1 μg/ml; both from Sigma-Aldrich) in buffer B. Monensen (10 μg/ml; Sigma-Aldrich) was added in the last 2 hours to prevent extracellular cytokine secretion and the cells were stained for CD4. The cells were fixed and permeabilized using the cytofix-cytoperm kit (PharMingen) and subsequently stained with anti-cytokine antibodies according to the manufacturer's instructions and analyzed by FACS. To confirm specificity, cells were pre-incubated with excess (fivefold) unlabeled recombinant (r) IL-4 and IL-13 cytokines (R&D Systems, Minneapolis, MN, USA) prior to staining with fluorophore-labeled anti-cytokine antibodies. All samples were pre-incubated with anti-Fc_γRII/III (PharMingen) and purified rat IgG (Sigma-Aldrich).

Culture of LP cells for cytokine production assay

Isolated LP cells were cultured in RPMI 1640 medium supplemented with 3 mM L-glutamine, 10 mM Hepes, 100 U/ml of penicillin and streptomycin, and 10% BCS. Cells were cultured (10^6 cells/ml, 48 hours) over coated (murine anti-CD3ε antibody; PharMingen) or uncoated 24-well culture plates (Costar Corp., Cambridge, MA, USA). Anti-CD28 (2 μg/ml; PharMingen) was added to cells cultured in the anti-CD3-coated plates. The culture supernatants were harvested and stored at –20°C until further analysis of cytokine concentrations by ELISA (Hornquist et al., 1997).

Immunofluorescence staining

Tissues from K8+/+ and K8–/– mice were embedded in optimum cutting temperature (OCT) compound and frozen at –80°C. Frozen 6 μm tissue sections were fixed in acetone (–20°C, 10 minutes) and blocked with PBS containing 2% BSA and 2% goat serum (10 minutes). The tissue sections were incubated (22°C) with primary antibodies: anti-EPCAM (G8.8) (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), anti-PNAd (MECA 79) and anti-MAdCAM-1 (MECA 367), or an isotype control antibody followed by secondary antibodies (40 minutes, 22°C) PE goat anti-rat $F(ab')_2$ IgM or IgG (Jackson ImmunoResearch Laboratories Inc., West Grove,

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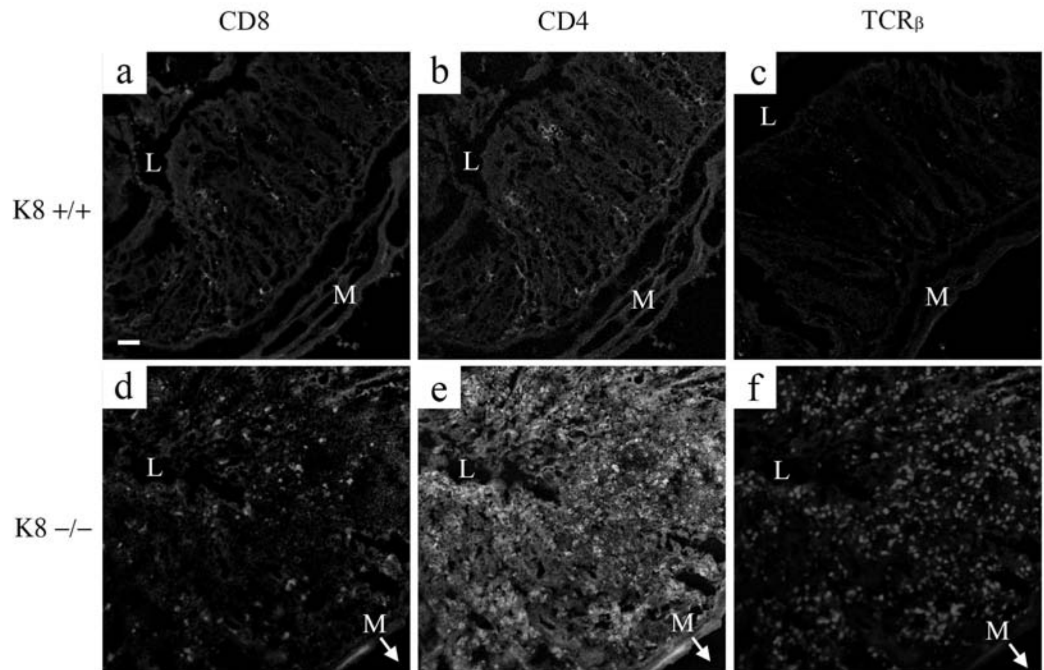

Fig. 1. Immunohistochemical staining of K8+/+ and K8–/– colons with fluorescent antibodies to T cell surface markers; the original color staining is depicted in black and white. Frozen sections from 6-month-old K8+/+ and K8–/– colons were triple-stained with fluorophore-conjugated antibodies directed to: CD8 (PE, red), CD4 (FITC, green), and TCR_β (APC, blue). A dramatic increase in the number of TCR_β and CD4+ cells are present within the LP and mucosa of the K8–/– (d-f) as compared to the K8+/+ (a-c) colons. L, lumen; M, muscle layer. Scale bar: 50 μm.

PA, USA). Alternatively, directly conjugated antibodies [CD4-FITC, CD8-PE, TCR_β-APC, $\alpha_4\beta_7$-APC, I-A/I-E (MHC class II)-FITC, PECAM (CD31)-FITC; PharMingen] were used. Nuclei were stained using Toto-3 (Molecular Probes, Eugene, OR, USA) as described (Toivola et al., 2004). The slides were washed with PBS containing 2% BSA before and after the incubation steps, and were examined with a confocal microscope equipped with Lasersharp software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Antibiotic treatment and histology scoring

K8+/+ and K8–/– mice were treated with vancomycin and imipenem administered in drinking water at 50 mg/kg body weight/day for 8 weeks starting at 18-19 days after birth. Following completion of antibiotic treatments, mice were sacrificed, colonic tissues were fixed in 10% formalin, embedded in paraffin, and sections were stained with Hematoxylin and Eosin. Proximal colon sections were assessed using a previously validated scoring system (Hoentjen et al., 2003; Sellon et al., 1998): 0 (no inflammation) to 4 (severe inflammation). In addition, colon sections were stained with rabbit antibody to AE1/2 (5288; provided by R. Kopito, Stanford University, USA) as described (Toivola et al., 2004). Unless specified, all values are expressed as mean±s.e.m. Student's *t*-test was used for analysis of significance. Differences were considered significant if P was <0.05.

Results

TCR_β+CD4+ T cells are increased in the colon LP of K8-null mice

In order to determine the phenotype of infiltrating lymphocytes within the inflamed K8–/– colons, immunohistochemical and flow cytometric analysis were performed. A significant increase in TCR_β+ CD4+ T cells was seen in K8–/– colon LP by immunohistochemical staining (Fig. 1d-f) compared with K8+/+ mice (Fig. 1a-c). Most of the infiltrating CD4+ T cells were seen within the LP, while in areas of severe inflammation aggregates of CD4+ T cells were also noted in the mucosa. Flow cytometric analysis revealed similar findings, with expansion of LP-derived T cells positive for CD4 and TCR_β in association with increasing age in K8–/– mice (Fig. 2). A smaller but significant increase in $CD8_\alpha$ and $TCR_{\gamma\delta}$ was seen in older K8–/– mice (Fig. 2). Using the same surface markers (CD4, $CD8_\alpha$, TCR_β and $TCR_{\gamma\delta}$) no difference in IEL numbers or phenotypes was observed between K8+/+ and K8–/– colons (not shown). Because significant chronic inflammation is present in the K8–/– colon by the age of 3 months, we studied mice at various ages following birth. K8–/– mice showed early signs of inflammation with an increase in CD4+ T cells at 2 weeks (Fig. 3Ad,e, arrows). By one month, early signs of chronic inflammation were noted in a few areas with fibrous tissue deposition and mononuclear infiltration (Fig. 3Af, arrows) in association with gross colon thickening (not shown). However, despite the chronic colon inflammation, K8–/– mice continued to gain weight (25.9±2.4 g for K8–/– vs. 26.9±4.1 g

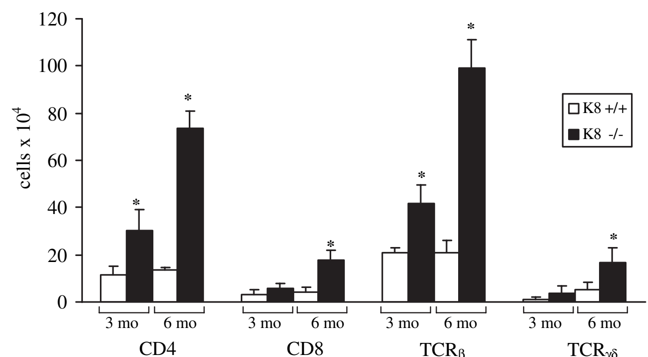

Fig. 2. Colon lamina propria lymphocytes stained with surface markers and analyzed by flow cytometry. LPL were isolated from K8+/+ and K8–/– colons, stained with the indicated markers then analyzed as described in Materials and Methods. Markedly higher numbers of TCR_β and CD4+ T cells are recovered from the K8–/– compared to K8+/+ mouse colons in an age-dependent manner. The data is presented as mean cell number±s.e.m. (n=4). *P<0.05 when comparing K8–/– with K8+/+.

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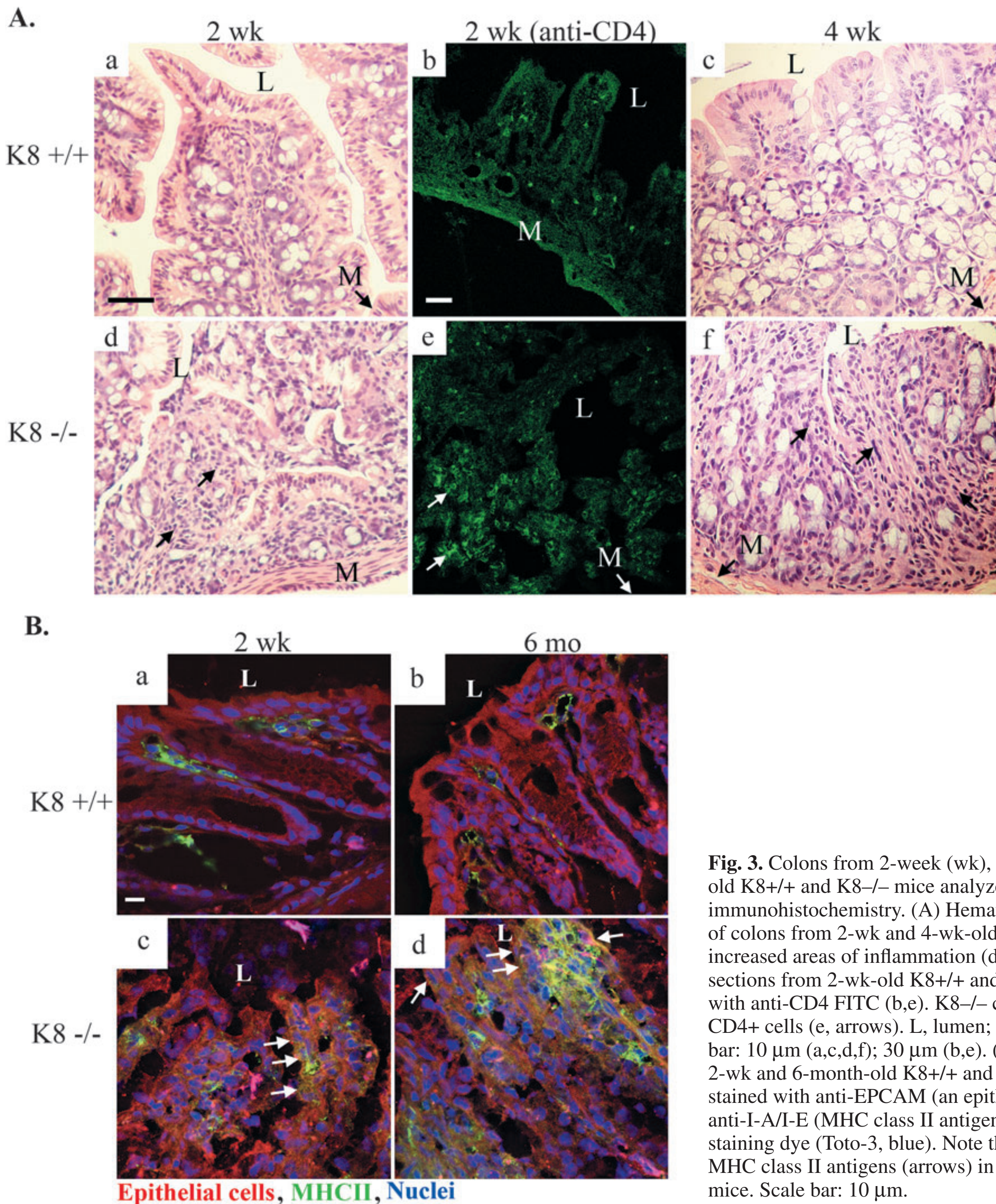


Fig. 3. Colons from 2-week (wk), 4-wk and 6-month (mo)-old K8+/+ and K8–/– mice analyzed by histology and immunohistochemistry. (A) Hematoxylin and Eosin staining of colons from 2-wk and 4-wk-old K8–/– mice show increased areas of inflammation (d,f, arrows). Frozen colon sections from 2-wk-old K8+/+ and K8–/– mice were stained with anti-CD4 FITC (b,e). K8–/– colon LP shows increased CD4+ cells (e, arrows). L, lumen; M, muscle layer. Scale bar: 10 μm (a,c,d,f); 30 μm (b,e). (B) Frozen sections from 2-wk and 6-month-old K8+/+ and K8–/– colons were stained with anti-EPCAM (an epithelial cell marker, red), anti-I-A/I-E (MHC class II antigens, green), and a nucleus-staining dye (Toto-3, blue). Note the overexpression of MHC class II antigens (arrows) in colonocytes of K8–/– mice. Scale bar: 10 μm.

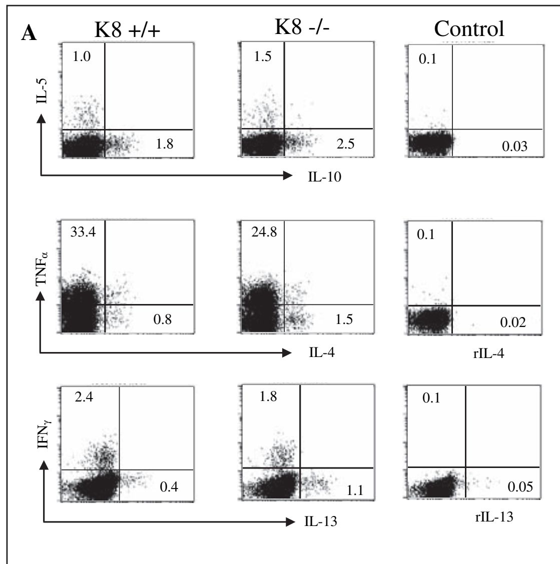
for K8+/+, at 4-5 months, *n*=5) and no lethality was observed up to >12 months after birth.

Colon epithelium from K8-null mice express MHC class II antigens

Intestinal epithelial cells over-express MHC class II antigens during intestinal inflammation (Hornquist et al., 1997; Mayer et al., 1991). Since K8–/– mice have a primary epithelial cell defect with early signs of colonic inflammation, we stained frozen colon sections from wild-type and K8–/– mice with anti-I-A/I-E antibody. In contrast to K8+/+ mice where MHC class II antigen expression was limited to the LP (Fig. 3Ba,b), K8–/– colon epithelial cells expressed MHC class II antigens (Fig. 3Bc,d). In less inflamed areas and in younger mice (2 weeks old), fewer colonocytes expressed MHC class II antigens (Fig. 3Bc).

Colonic inflammation in K8-null mice is associated with increased Th2 cytokines

We used an intracellular cytokine assay to examine the Th1 (CD-like) and Th2 (UC-like) cytokine profiles associated with colonic inflammation in K8–/– colons isolated from mice aged 3-4 months. Colon LP CD4+ T cells from K8–/– mice



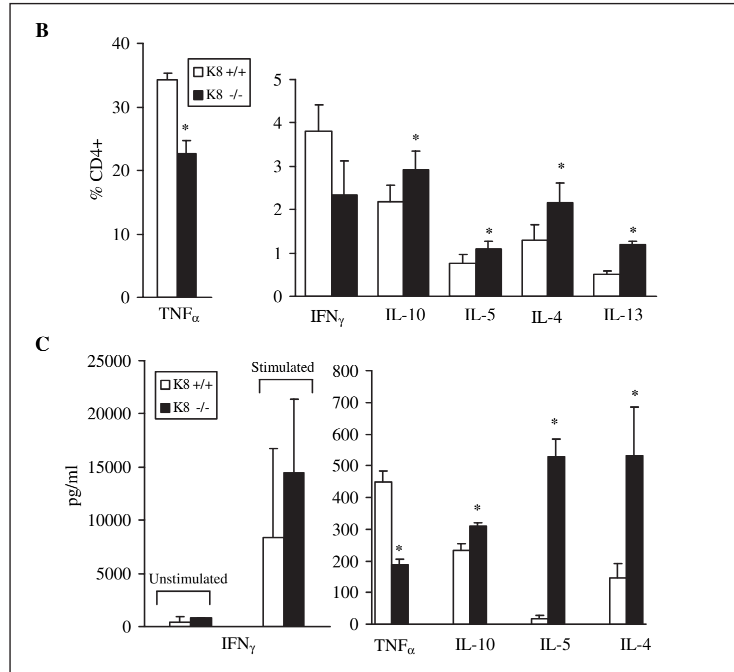


Fig. 4. Cytokine production by K8+/+ and K8–/– colon LP cells. (A) Cells were stimulated with PMA/ionomycin and stained for surface CD4 and intracellular cytokines (IL-5, IL-10, TNFα, IL-4, IFNγ or IL-13) or isotype control antibodies. Samples were analyzed by FACS with gating on CD4+ T cells. To confirm specificity of the IL-4 and IL-13 staining, stimulated cells were pre-incubated with excess unlabeled recombinant (r) cytokines (rIL-4 or rIL-13). The frequency of cytokine-producing CD4+ T cells is indicated in the quadrants as percentages (e.g. 1% for IL-5 and 1.8% for IL-10 for K8+/+ CD4+ T cells). (B) Summary of the result shown in A. CD4+ T cells from K8–/– colons produced higher Th2 cytokines (IL-5, IL-4, and IL-13), and IL-10. Data is presented as mean±s.e.m. (n=4). (C) Cytokine production by K8+/+ and K8–/– colon LP cell culture. Cells were cultured in plates coated with anti-CD3ε and soluble anti-CD28 (Stimulated) or in the presence of medium alone (Unstimulated) for 48 hours. Culture supernatants from the stimulated cells were analyzed by ELISA for IFN_γ, TNF_α, IL-10, IL-5 and IL-4 production. Note that K8–/– cultures produced higher levels of Th2 cytokines. Data are presented as mean±s.e.m. (n=3). *P<0.05 when comparing K8–/– with K8+/+.

produced higher Th2 cytokines (IL-4, IL-5 and IL-13) and lower levels of TNF_α (Fig. 4). In addition to using the appropriate isotype controls, specific increases in IL-4 and IL-13 were verified by blocking with recombinant IL-4 and IL-13 antibodies (Fig. 4A; middle and bottom rows, right-sided plots). Furthermore, ELISA analysis of total K8–/– colon LP cell cultures stimulated with anti-CD3ε and anti-CD28 gave similar results, of higher Th2 cytokines than cultures from their K8+/+ littermates (Fig. 4C). Limited spontaneous IFNγ (but not IL-4, IL-5, IL-10, or TNF_α) production in unstimulated cells was similarly detected in cultures of K8+/+ and K8–/– colon LP cells (Fig. 4C). Hence, Th2 cytokine profile (i.e. increased IL-4, IL-5 and IL-13) is seen in K8-null colons.

Memory and naive CD4-positive T cells are increased in colon LP of K8-null mice

Further phenotypic analysis of the colon LP and MLN CD4+ infiltrating T cells in 3-4-month-old mice was performed using memory and naive cell surface markers. The MLNs from K8–/– mice were larger because of an increase in the number of cells recovered from K8–/– MLN than from their K8+/+ littermates (mean of 51±8 $\times 10^6$ vs. 24±5 $\times 10^6$, respectively, n=5). However, no significant difference in the percentage of CD4+ T cells displaying memory ($CD44^{hi}/CD45RB^{lo}$ or $CD62L^{lo}/CD45RB^{lo}$) and naive ($CD44^{lo}/CD45RB^{hi}$ or $CD62L^{hi}/CD45RB^{hi}$) phenotypes were observed between K8+/+ and K8–/– colon LP cells (not shown). Similarly, no difference in the proportion of CD4+ T cells with memory or naive phenotypes was seen between K8+/+ and K8–/– MLNs (not shown).

K8–/– colon LP have increased $\alpha_4\beta_7$+ cells, enhanced vascular MAdCAM-1 and aberrant PNAd expression

Since colonic inflammation in mice and patients with IBD is associated with increased expression of gut homing molecules, we studied the expression of adhesion molecules in colons of K8–/– mice. An increase in $\alpha_4\beta_7$+ cells was seen within the inflamed colons of K8–/– mice (Fig. 5Ab). In K8+/+ colon LP a higher percentage of $\alpha_4\beta_7$+ cells were CD4+ than in K8-null mice (not shown). Moreover, enhanced expression of MAdCAM-1 was noted within K8–/– colons (Fig. 5Ad and f as compared with c and e). Since high numbers of naive CD4+ T cells (L-selectin+ or CD62L+) were recovered from the inflamed colons of K8-null mice, and aberrant expression of PNAd in chronically inflamed tissues in human patients has been observed (Renkonen et al., 2002), we assessed the presence of PNAd+ venules in the colon. Unlike sections from K8+/+ mice where PNAd staining is absent, several PNAd+ venules were visible in K8–/– colons (Fig. 5Bc,d). Higher PNAd expression was noted in areas of increased inflammation and with increased age, since only 1 of 3 younger mice (3-4-months) but all older mice (6 months, n=4) had aberrant PNAd

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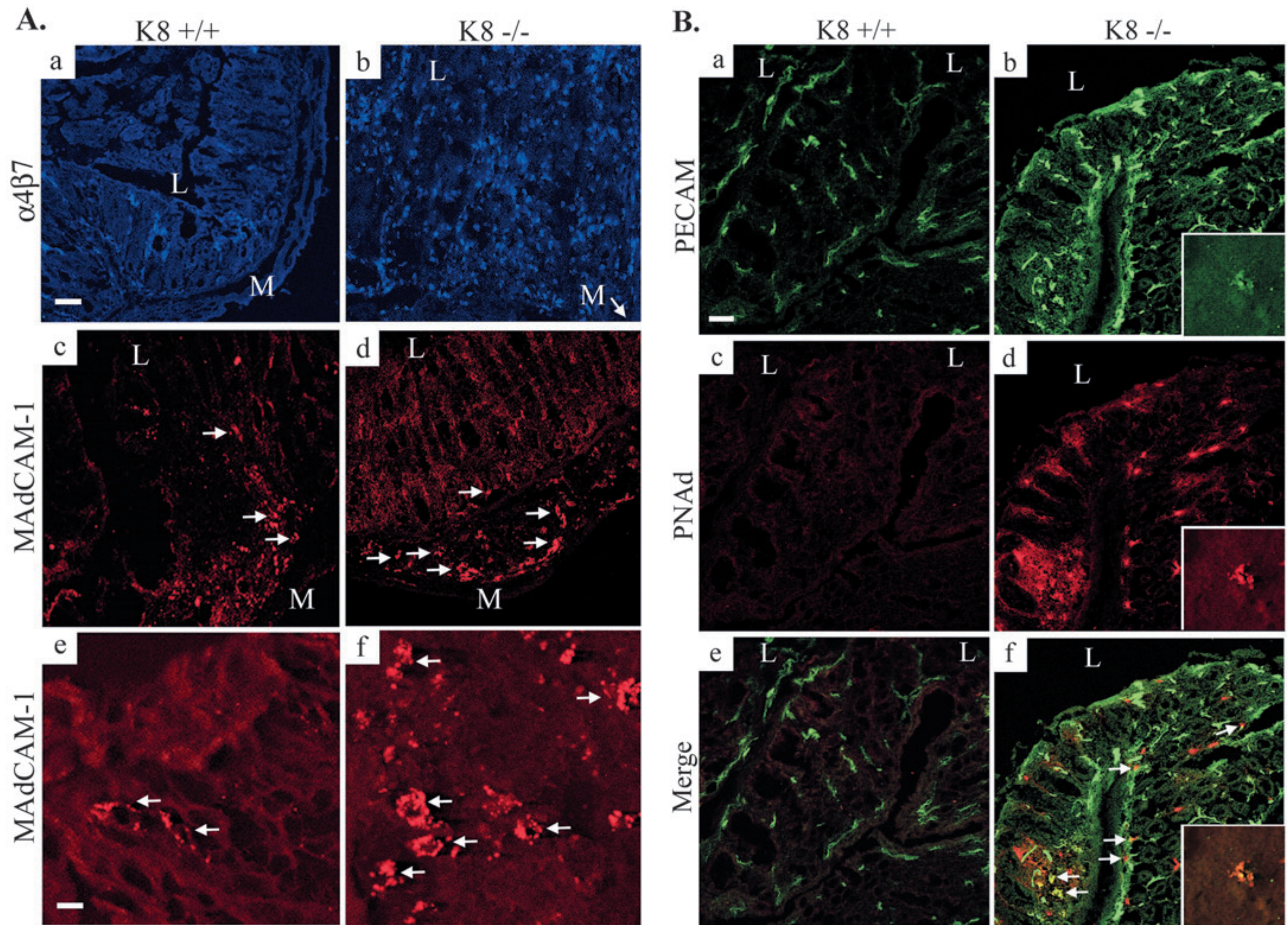

Fig. 5. Expression of $\alpha_4\beta_7$ and endothelial markers by K8+/+ and K8–/– colons. (A) Frozen sections from 3-month-old K8+/+ (a) and K8–/– (b) colons were stained with anti-$\alpha_4\beta_7$. Sections from 6-month-old K8+/+ (c,e) and K8–/– (d,f) colons were stained with anti-MAdCAM-1. Increased $\alpha_4\beta_7$+ cells (b) and MAdCAM-1+ venules (d,f, arrows) were seen in K8–/– colons. L, lumen; M, muscle layer. Scale bars: (a-d) 50 µm; (e,f) 30 µm. (B) Colons from K8+/+ (a,c) and K8–/– (b,d) mice were double-stained for the endothelial markers anti-PECAM/CD31 (green) and the vascular adhesion molecule PNAd (red). Merged images are shown in e and f. Inserts show a magnified view of a positive double-stained venule. Note the aberrant expression of PNAd in K8-null colon (compare c and d). Arrows in f indicate areas of co-localization. Scale bar: 50 µm.

expression. In contrast, the non-affected small intestine from both K8+/+ and K8–/– mice did not express PNAd (not shown). Areas of PNAd expression include regions of inflammation with increased vascularization, as confirmed by double staining with anti-PECAM antibody (Fig. 5B).

Broad-spectrum antibiotic treatment reverses the colitis and protein mistargeting in K8–/– mice

Because K8-null mice have a primary epithelial cell defect, we sought to investigate the dependence of colitis in these mice on luminal bacteria. Such approaches have been utilized in other animal models of IBD to examine and determine the essential role of bacteria in colitis (Hoentjen et al., 2003; Madsen et al., 2000). Treatment of K8–/– mice 18-19 days after birth, using a combination of vancomycin and imipenem in their drinking water for 8 weeks, prevented colonic inflammation and thickening (Fig. 6A). There were no difference in colon histology scores between antibiotic-treated K8–/– mice and antibiotic or non-antibiotic treated K8+/+ mice (Fig. 6B). Furthermore, antibiotic treatment reversed the AE1/2 ion transporter mistargeting (Fig. 7), which was previously shown to be mislocalized in K8–/– mice colons (Toivola et al., 2004).

Discussion

We investigated the inflammatory response associated with K8-null mice. Relative to the larger number of Th1 colitis models, few models are associated with Th2 cytokine production. Unlike TCRα–/– (Mizoguchi et al., 1996) and WASP–/– (Snapper et al., 1998) spontaneous Th2 colitis mouse models, K8-null mice have a primary epithelial cell defect. Moreover, unlike the other two Th2 models, trinitrobenzene sulfonic acid (TNBS)- (Dohi et al., 1999) and oxazolone-induced colitis (Boirivant et al., 1998), K8-null mice develop spontaneous chronic colitis without lethality. Mice with barrier or epithelial

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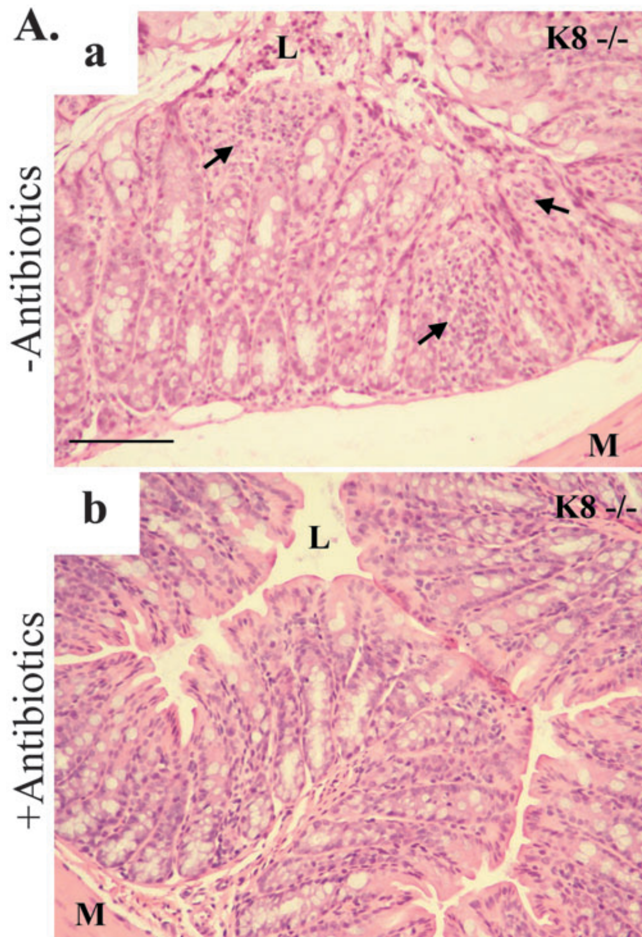

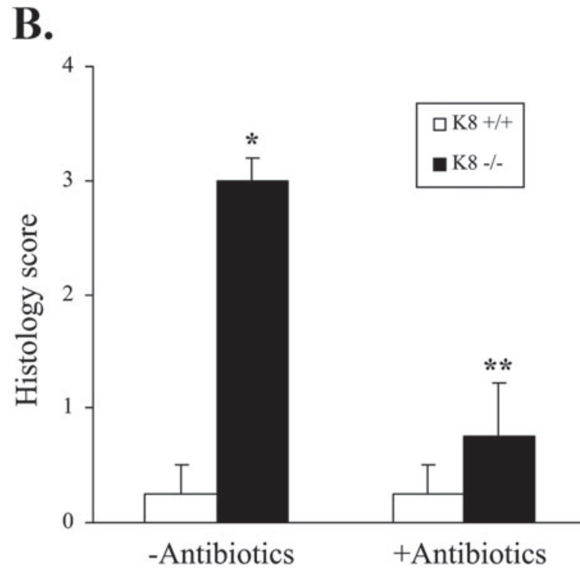

Fig. 6. K8–/– colon histology following treatment with broad-spectrum oral antibiotics. (A) Hematoxylin and Eosin staining of proximal colons from K8–/– mice that were given normal drinking water for 8 weeks (a) or water containing vancomycin and imipenem at 50 mg/kg body weight (b). Significant inflammation (arrows) is present in the non-treated K8–/– colon. L, lumen; M, muscle layer. Scale bar: 10 μm, (B) Summary of the colon inflammation histology score (as described in Materials and Methods) is shown for the non-treated (–Antibiotics) and treated (+Antibiotics) groups. Data is presented as mean±s.e.m. (n=4). *P<0.005 when comparing untreated K8–/– with K8+/+, **P<0.01 when comparing untreated with treated K8–/–.

cell dysfunction such as an N-cadherin mutation (Hermiston and Gordon, 1995), intestinal trefoil factor (Mashimo et al., 1996) or multiple drug resistant (mdr1a) ablation (Panwala et al., 1998) also develop colitis. However, mdr1a deficiency is not limited to epithelial cells, since the *mdr1a* gene is also expressed in T cells. In contrast to K8–/–, trefoil factor-deficient mice do not develop spontaneous colitis unless treated with dextran sodium sulfate. This is not to downplay the important pathogenic mechanisms provided by the IBD models mentioned above, but rather to highlight the differences and the uniqueness of K8-null mice and their attractive use in addressing a novel potential association with IBD.

Overexpression of MHC class II antigens occurs in animal models (Hornquist et al., 1997) and patients (Mayer et al., 1991) with IBD, and intestinal epithelial cells from IBD patients abnormally activate CD4+ T cells (Toy et al., 1997).

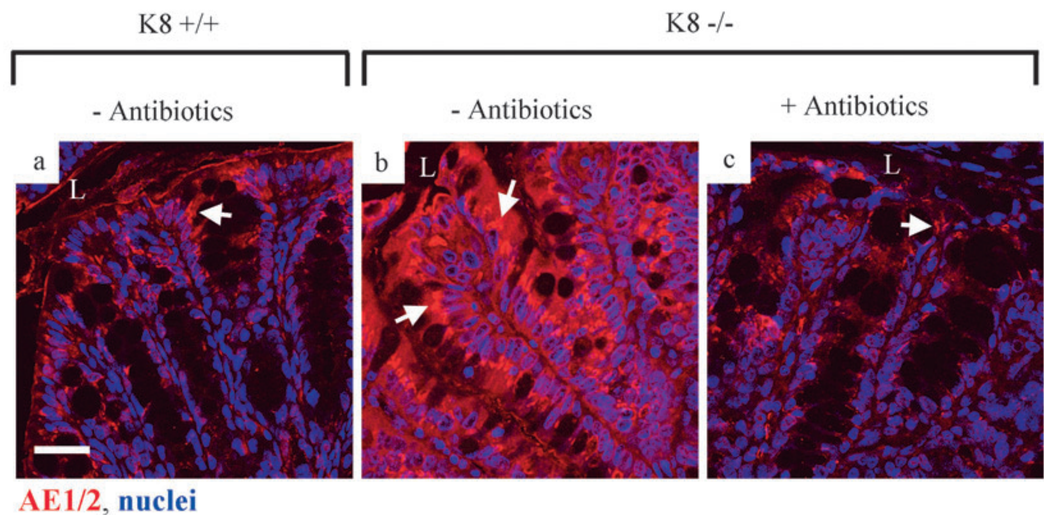

Fig. 7. Effect of oral antibiotic treatment on AE1/2 ion transporter localization. Proximal colon from non-antibiotic-treated K8+/+ (a), K8–/– (b), and antibiotic-treated K8–/– (c) mice were fixed in formalin, paraffin embedded and sectioned, then double-stained with anti-AE1/2 (red) and nuclear dye (blue). Note the reversal and normalization of the brighter and supranuclear AE1/2 staining in K8–/– colon (b, arrows) following antibiotic treatment (c), to resemble the staining of non-antibiotic treated K8+/+ colon (a). Scale bar: 50 μm.

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Table 1. Summary of features associated with K8–/– colonic inflammation

Colon phenotype	K8–/– colitis
Primary cell defect	Epithelial
Onset of inflammation	Early (≤2 weeks)
T cell phenotype	TCR_{β}+/CD4+
Naive	↑
Memory	↑
Cytokine profile	Th2
MHC II colonocyte expression	↑ (≤2 weeks)
Lymphocyte/vascular adhesion molecules	
$\alpha_4\beta_7^+$ lymphocytes	↑
MAdCAM-1$^+$ venules	↑
PNAd$^+$ venules	Aberrant new expression

MHC II, major histocompatibility complex; MAdCAM-1, mucosal addressin cell adhesion molecule-1; PNAd, peripheral node addressin; TCR, T cell receptor; Th2, T helper 2.

Moderate MHC class II antigen induction is present in K8–/– mice, and is noted in mice as young as 2 weeks. It is possible that MHC II induction in K8–/– colonocytes allows the presentation of luminal antigens and activating CD4+ T cells, as demonstrated with murine enterocytes (Kaiserlian et al., 1989) and epithelial cells from IBD patients (Toy et al., 1997). Assessment of K8–/– enterocyte interaction with CD4+ T cells in vitro and raising K8–/– mice in a germ-free environment may provide information as to whether keratin deficiency alters enterocyte antigen processing or presentation.

Similar to other animal IBD models, K8–/– colons have increased accumulation of CD4+ TCR_{β}+ T cells within their LP. This suggests a multifaceted immune interaction between leukocytes, enterocytes and the luminal environment, whereby disruption leads to a phenotypic outcome of inflammation with recruitment of CD4+ T cells. However, variations in inflammatory mediators generated by infiltrating CD4+ T and other immune cells exist, which may depend on genetic differences (Bouma and Strober, 2003). For example, induction of colitis using TNBS in BALB/c and SJL/J mice results in a Th2 and Th1 response, respectively (Neurath et al., 1995). K8–/– mice in an FVB/n background develop colitis and as shown in this study, the inflammation is associated with a Th2 cytokine profile (summarized in Table 1).

The differences in cytokine production, as estimated by the percentage of CD4+ T cells in K8–/– and K8+/+ mice, are modest but significant (Fig. 4A). This is probably related to the less dramatic increase in the percentage of memory CD4+ T cells isolated from the inflamed colons of K8–/– mice. The K8–/– inflamed colon is infiltrated by a larger absolute number of activated T cells as reflected by the increased production of Th2 cytokines in anti-CD3ε- and anti-CD28-stimulated colon LP cell cultures. In contrast to other models of IBD, such as Gαi2–/– mice (Hornquist et al., 1997), the increase in the percentage of LP memory CD4+ T cells infiltrating K8–/– colons is not associated with a decrease in percent naive CD4+ T cells.

Naive, but not memory T cells home to PLNs by interacting with PNAd in PLN venules (Butcher and Picker, 1996; Michie et al., 1993). Abnormal expression of PNAd in inflamed tissues occurs in several chronic autoimmune diseases including IBD (Renkonen et al., 2002), as noted in the K8–/– inflamed colons. PNAd induction is probably dependent on chronicity of the colitis since it becomes more prominent in older mice. The mechanism of PNAd induction, and whether the induced PNAd functions normally, remain to be determined. Alternatively, the lymphocytes that interact with the induced PNAd may not behave normally. For example, immunoblasts isolated from IBD lesions of patients are able to interact with PLN HEVs in vitro, while cells isolated from non-IBD control patients do not (Salmi et al., 1994).

Gut homing molecules, such as MAdCAM-1 are highly expressed in enteritis, and a role in recruitment of pathogenic immune cells has been demonstrated, since blocking with anti-MAdCAM-1 (or its ligand $\alpha_4\beta_7$ integrin) antibodies blunts inflammation (Ghosh et al., 2003; Picarella et al., 1997; Podolsky et al., 1993). Consistent with previous findings in animal models and patients with IBD, enhanced vascular MAdCAM-1 expression is observed in chronically inflamed K8–/– colons. Moreover, higher number of $\alpha_4\beta_7$+ cells are present within the colon LP of K8–/– mice. Thus K8–/– mice provide an ideal model to test the role of PNAd and MAdCAM-1 (with or without $\alpha_4\beta_7$) in chronically inflamed colons.

It is well known that intestinal microflora plays a key role in inducing or perpetuating colitis in all tested IBD animal models (Bouma and Strober, 2003; Sadlack et al., 1993; Strober et

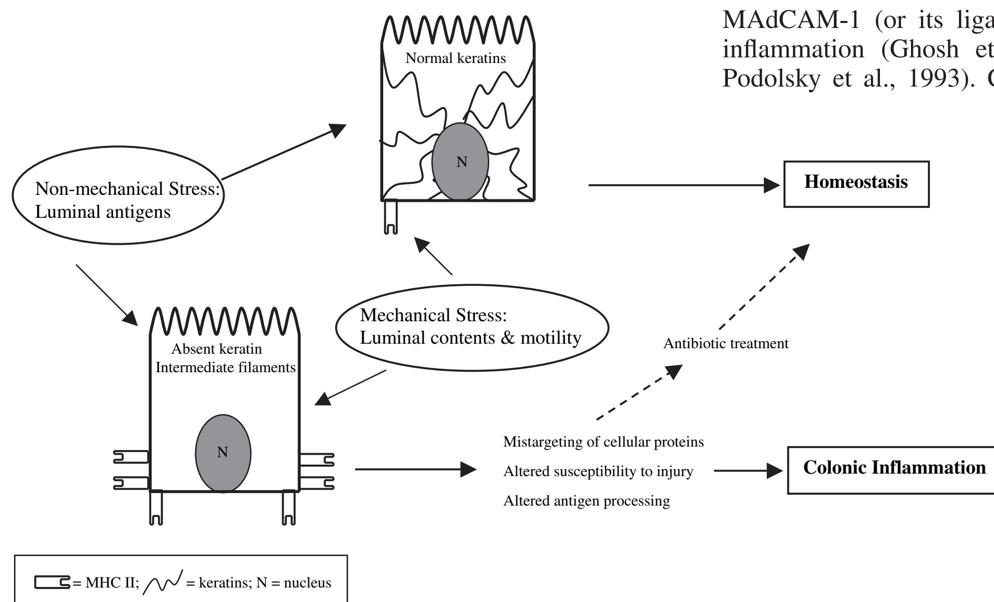

Fig. 8. A model depicting the effects of mechanical and non-mechanical stresses on intestinal epithelial cells, depending on the presence or absence of keratin intermediate filaments.

Journal of Cell Science

al., 2002), which led us to investigate the role of bacteria in K8–/– mouse colitis. Our findings show that K8–/– colitis is amenable to antibiotic treatment, which indicates that luminal bacteria are likely to play an important role in triggering the colitis in K8–/– mice. Our mouse colony (K8+/+ and K8–/–) tested positive for *H. bilis* and *H. hepaticus*, and *Helicobacter* species have been associated with enterocolitis in immune-deficient animal model of IBD (Cahill et al., 1997). We cannot exclude a role for *H. bilis* and *H. hepaticus* alone relative to other bacterial species.

The exact mechanism by which K8–/– mice develop colitis and the functional role of keratins in the colon are poorly understood (Fig. 8). However, K8–/– colons have normal tight junction permeability and paracellular transport but are defective in their ion transport in association with mistargeting of ion transport proteins observed as early as 1-2 days after birth (Toivola et al., 2004). The normalization of AE1/2 mistargeting in K8–/– colons after antibiotic treatment (Fig. 7) suggests that luminal bacteria and/or their consequent inflammatory response promote the observed protein mistargeting. Alternatively, mistargeted ion transporters may create an attractive environment for pathogenic bacteria that in turn stimulate the colitis and maintenance of the mislocalized transport proteins.

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