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Expression, action and function of phosphoinositide 3-kinase p110delta

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Chapter 6

Dissociation between predisposition to Colitis and development of Colitis-Associated Cancer in $p110\delta^{D910A/D910A}\ PI3K\ mice$

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Abstract

The p110δ isoform of PI3K has attracted widespread attention for its role in allergy and inflammation and p110δ-specific inhibitors are currently being tested. Mice that express an inactivated p1108 PI3K isoform (p1108^{D910A/D910A}) develop mild caecal and rectal colitis. p1108^{D910A/D910A} mice were investigated for their propensity to inflammation and due to the causal link between inflammation and cancer also for their susceptibility to colorectal cancer in a colitis-associated cancer model. Here, we show that p110δ^{D910A/D910A} mice spontaneously develop colitis, but only in the presence of intestinal flora. Exposure to lipopolysaccharide leads to increased inflammatory cytokine secretion in p110 $\delta^{D910A/D910A}$ colons. We show that p110 $\delta^{D910A/D910A}$ mice have decreased numbers of peripheral CD4⁺CD25⁺ regulatory T cells and that these cells have a specific inability to migrate in response to the thymus exit chemokine sphingosine-1-phosphate, providing an additional explanation for the enhanced inflammatory phenotype. Finally, the absence of active p1108 PI3K results in aggravated inflammation but does not lead to more cancer in the colorectal cancer model. Also aged p1108^{D910A/D910A} mice do not develop colorectal or other types of cancer. Thus our results reveal important new insight into the relation between colonic inflammation and cancer as the lack of active p1108 predisposes to colitis but does not carry increased risk of colitis-associated colon cancer, the latter probably as a consequence of a negative role for p1108 in colon tumour surveillance.

Introduction

It is now generally accepted that inflammation and cancer are causally linked [1]. Many cancers arise from sites of infections and it is becoming clear that the inflammatory cells in the tumour environment can be participants in the neoplastic process [2]. The microenvironment in and around tumours contains cells of the innate immune system that secrete pro-inflammatory cytokines and chemokines, which can promote DNA damage and can also enhance cell proliferation, cell survival, cell migration and angiogenesis, thereby promoting tumour development. Thus the inflammatory cells and cytokines found around tumours can contribute to tumour growth, progression, and immunosuppression rather than to mount an effective host anti-tumour response [1,3]. It is usually envisioned that inflammation stimulates the formation of epithelium-derived tumours (carcinomas) through an indirect mechanism involving activation of surrounding inflammatory cells, such as in colorectal cancer associated with inflammatory bowel disease [4]. Colorectal cancer is the second most frequent carcinoma in the western world [5]. Although colorectal cancer related to inflammatory bowel disease (IBD) accounts only for 1-2% of all cases of colorectal carcinoma, the mortality rate in patients with a diagnosis of colorectal cancers in the setting of IBD is higher than for those afflicted with sporadic cases of colorectal cancer and accounts for more than 15% of all deaths in IBD patients [6]. Furthermore, during the last decades the incidence of IBD has continued to rise worldwide, reaching incidence rates of 16.6/100,000 in North America and 9.8/100,000 in Europe [7]. IBD forms, together with the hereditary syndromes of familial adenomatous polyposis and hereditary nonpolyposis colorectal cancer, the top three of high-risk conditions for colorectal cancer [8].

Phosphoinositide 3-kinases (PI3Ks) generate lipid second messengers that regulate a broad variety of cellular responses such as growth, proliferation, survival, differentiation, intracellular traffic and cell migration [9]. PI3K activity is critical in a wide variety of normal and pathological physiological responses, including immune regulation, metabolic control and cancer. p110δ is a class IA member of the PI3K family, that is preferentially expressed in leukocytes [10,11] and has generated a lot of interest because of its role in immunity [12] and allergy [13] and specific p110δ inhibitors are now being tested for their use in the treatment of allergy. Mice in which p110δ PI3K has been made catalytically inactive (p110δ^{D910A/D910A} or D910A) develop a mild form of colitis [12] and recently it has been suggested that this may be a consequence of attenuated regulatory T cell function [14]. That lack of active p110δ causes chronic inflammation raises the concern that systemic inhibition of p110δ would predispose to cancer development.

However, the relationship between increased activity of the immune system and the development of cancer is not clear-cut. The immune system also plays an important role in combating neoplastic lesions, also in the colon [15,16]. This raises very fundamental questions regarding the treatment of IBD. Colitis-associated colon cancer is an important clinical problem, thus combating colitis should decrease the incidence of cancer. On the other hand though, suppressing the immune system in order to diminish colitis may reduce the capacity of the body to deal with emerging (pre-) cancerous lesions. This problem is well-recognized in clinical practice, where screening of patients with otherwise quiescent IBD for the occurrence of cancer is considered an essential part of clinical care [6].

It is becoming increasingly clear that cancers can employ hyperactivation of the regulatory T cell compartment to suppress the action of the anti-tumour immune system [17,18], as is also evident from the clinical results obtained in –for instance- treatment of melanoma with therapy designed at reducing regulatory T cell activity [19,20]. Importantly though, such therapy is associated with the induction of colitis, showing that the main target for regulatory T cell activity is the colon [21].

These observations led us to investigate the role of p110 δ PI3K in colitis and the potential of colitis to induce malignant transformation in the absence of active p110 δ PI3K. Our results show that lack of active p110 δ predisposes to colitis but does not carry increased risk of colitis-associated colon cancer. Pro-inflammatory cytokine secretion is enhanced in p110 $\delta^{D910A/D910A}$ colons. Furthermore CD4⁺CD25⁺ regulatory T cells of p110 $\delta^{D910A/D910A}$ mice have impaired ability to migrate and are decreased in number in the periphery providing a further explanation for the inflammatory phenotype of these mice but also a possible link between the apparent uncoupling of colitis and colitis-associated cancer.

Results

$p110\delta^{D910A/D910A}$ mice develop colitis but only in the presence of colonic pathogens

Inflammatory bowel disease (IBD) is thought to be the consequence of an aberrant mucosal immune response to normal gut-dwelling bacteria, which damages tissues of the intestinal tract [22]. It has been shown that specifically the common gut pathogen *Helicobacter hepaticus* is associated with inflammatory large bowel disease in mice.

To assess whether the colitis observed in p110δ^{D910A/D910A} mice is an IBD-like colitis, we compared colon mucosa of mice bred and maintained under specific-pathogen-free (SPF) conditions with mice bred and maintained in conventional animal housing, where *H. hepaticus* is present. There was no enterocolitis seen in SPF-reared p110δ^{D910A/D910A} mice at any age (Figure 1A), while conventionally housed p110δ^{D910A/D910A} mice spontaneously develop mild colitis (Figure 1B), limited to the caecal and rectal sections of the large intestine, at advanced (> 15 weeks) of age. This suggests that components of the intestinal flora promote the deregulated inflammatory response encountered in conventionally maintained animals lacking active p110δ PI3K and equally that p110δ plays a role in controlling the innate immune response. Importantly, aged p110δ^{D910A/D910A} mice do not develop any type of cancer (representative histology of colon mucosa shown in Figure 1D and data not shown).

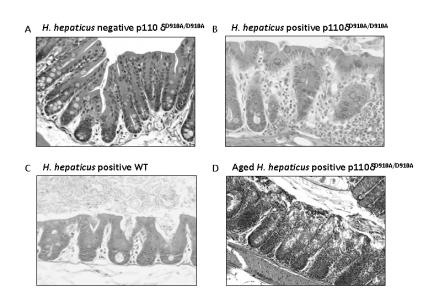


Figure 1. Histology of colon mucosa of wildtype and p110 $\delta^{D910A/D910A}$ mice, in the absence and presence of Helicobacter hepaticus. (A) Normal colon mucosa of p110 $\delta^{D910A/D910A}$ H. hepaticus-free mice. (B) Colon mucosa of p110 $\delta^{D910A/D910A}$ mice infected with H. hepaticus showing immune infiltrates in crypts and thickened mucosa. (C) Colon mucosa of WT mice infected with H. hepaticus showing normal mucosa and crypts. (D) Colons of aged p110 $\delta^{D910A/D910A}$ mice infected with H. hepaticus showing immune breakdown of crypts due to immune infiltrates and thickened mucosa but no malignant transformation.

Aggravated inflammation in response to dextran sulphate sodium salt in $p110\delta^{D910A/D910A}$ mice

To further study the role of p1108 in colonic inflammation we then compared the inflammatory response in p1108WT/WT (WT) and p1108D910A/D910A (D910A) mice in a dextran sulphate sodium (DSS) salt IBD-model. In total, 9 mice in each group were subjected to 1.5% DSS in the drinking water for 8 days. The severity of the inflammatory response was assessed by calculating bodyweight loss, calculating disease activity index (DAI, see Table 1) and by pathological scoring (Table 2 and 'Materials and Methods'). D910A mice suffered significantly (10%) more weight loss in response to the DSS treatment in comparison to WT mice (15% vs. 5% weight loss; Figure 2A; p<0.05) and had a substantial higher DAI (7.7/12 vs. 4.9/12 Figure 1B; p<0.05). Histological scoring revealed that monocyte influx, a characteristic of chronic inflammation is stronger in the mice without p110δ catalytic activity whereas granulocyte influx was equal, in agreement with a chronic Crohn's like response [23] in the D910A animals (p<0.05; Fig. 1C and D). Other histological parameters, (area involved, oedema, follicular involvement, crypt erosion, fibrosis, and ulceration) were not different between control and D910A animals (data not shown). We conclude that the lack of p110 δ enzymatic activity leads to an aggravated response to DSS.

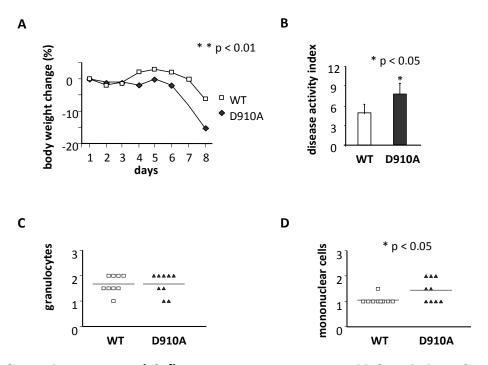


Figure 2. Aggravated inflammatory response to DSS in D910A mice. Mice were subjected to 8 days of DSS in the drinking water and subsequently sacrificed and analyzed for disease activity. (A) Weight loss per day as a percentage of the initial body weight. (B) Overall disease activity index [37]. (C) Granulocyte influx, histologically assessed (D) Monocyte influx, histologically assessed.

DISEASE ACTIVITY INDEX (DAI)				
Weight loss	0: <1%			
	1: 1-5%			
	2: 5-10%			
	3: 10-15%			
	4: >15%			
Stool consistency	0: normal			
	2: loose stools			
	4: diarrhoea			
Rectal bleeding	0: no rectal bleeding			
	2: rectal bleeding			
	4: gross rectal bleeding			

Table 1. Disease Activity index. Clinical assessment of disease activity was calculated using scoring of the disease parameters weight loss, stool consistency and rectal bleeding.

Enhanced cytokine production in colon explants of p110δ^{D910A/D910A} mice

To confirm the inflammatory phenotype of p1108^{D910A/D910A} colon, we next compared the amount of pro-inflammatory cytokines secreted in colon of WT and p1108^{D910A/D910A} mice in response to antigen stimulation. Colon explants were treated with the Gram negative bacterial cell wall component lipopolysaccharide (LPS, 100 ng/ml). Cytokine secretion production after 18 hours into the medium was compared using ELISA. Secretion of the pro-inflammatory cytokines IFN- γ , TNF- α , IL-6 and IL-12p70 was in colons of D910A mice (Figure 3A-D) while secretion of the anti-inflammatory cytokine interleukin 10 displayed lower levels in p1108 mutant mice (Figure 3E). Thus the inflammatory phenotype of the p1108^{D910A/D910A} colon is associated with the secretion of Th1 pro-inflammatory cytokine and concomitantly secretion of tolerogenic cytokines is decreased, the latter suggesting that a reduced regulatory component might be involved in the pathogenesis of this inflammatory phenotype.

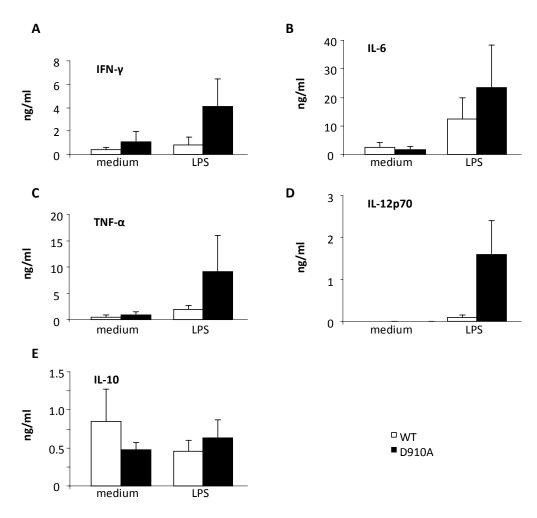


Figure 3. Loss of p110 δ enzymatic activity increases pro-inflammatory cytokine production and decreased interleukin 10 secretion in colonic explants. Cytokine secretion as measured in supernatant after 18 hours of stimulation of colonic explants with 100ng/ml LPS.

Defective migration of p110δ^{D910A/D910A} CD4+CD25+ regulatory T cells

Inflammatory bowel disease is ultimately a consequence of an exaggerated immune response to commensal flora and/or diminished immune suppression by regulatory T cells. It has been shown that spleen and lymph nodes of $p110\delta^{D910A/D910A}$ mice contain reduced proportions of $CD4^+CD25^+FoxP3^+$ Tregs, despite enhanced Treg selection in the thymus [14]. We confirmed the enhanced numbers of regulatory T cells in the thymus and decreased numbers peripherally (Figure 4A). We next investigated the ability of D910A regulatory T cells to migrate. We compared migratory responses in $p110\delta^{D910A/D910A}$ and WT mice to thymus exit chemokines and thymus entry cytokines (which increase and reduce the size of the peripheral T cell compartments, respectively), and performed such analysis for both regulatory and non-

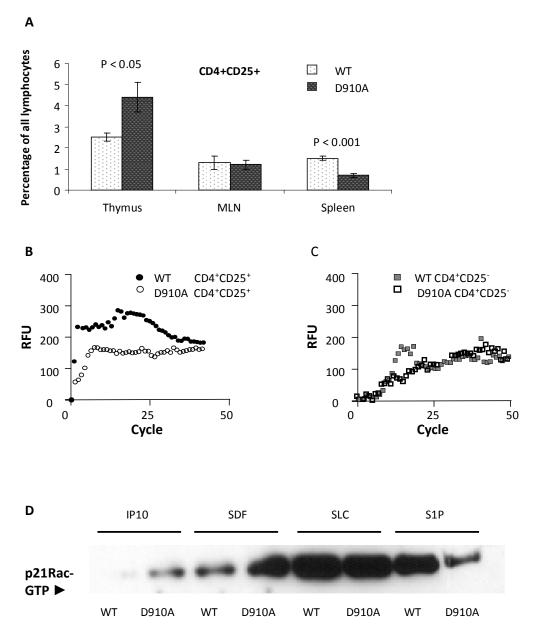


Figure 4. p110 δ enzymatic activity is required for migratory responses of regulatory T cells to S1P. (A) increased numbers of Tregs in thymus, decreased in spleen and mesenteric lymph nodes (MLN) (B,C) CD4 $^+$ CD25 $^+$ and CD4 $^+$ CD25 $^-$ T cells were isolated from thymi of WT and D910A mice, dyed with CellTracker Green and migration towards S1P assessed using 4000 CytoFluor Multi-Well Plate reader and 8 μ m pore size filters. (D) Activation of p21Rac in response to various stimuli in CD4 $^+$ CD25 $^+$ cells .

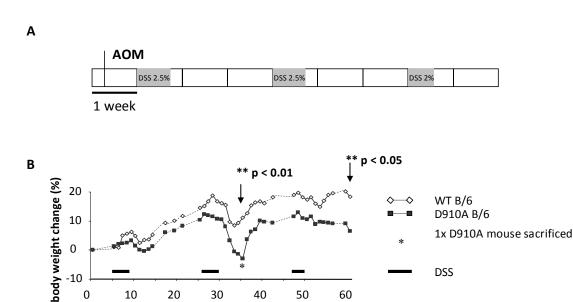
regulatory T cell subsets. CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were isolated from thymi of WT of D910A mice, dyed with CellTracker Green and migration towards the thymus exit chemokine Sphingosine-1-phosphate (S1P) [24] was assessed using 4000 CytoFluor Multi-Well Plate reader and 8 μm pore size filters. The CD4⁺CD25⁺ T cells displayed efficient migration towards S1P as evident by the appearance of fluorescence in the upper chamber. In contrast p110δ^{D910A/D910A} CD4⁺CD25⁺ cells were impaired in

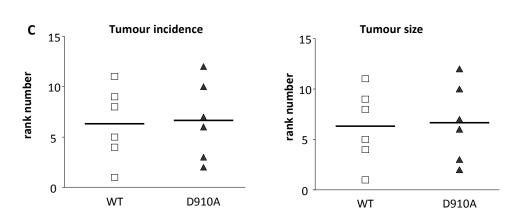
their migration towards S1P (Figure 4B). Thus lack of p110 δ enzymatic activity diminishes the capacity of regulatory T cells to respond to thymus exit cytokines. Importantly, such a difference was not apparent in D910A effector T cells (Figure 4C). In apparent agreement with the observations described above, were determinations of the activation of p21Rac, which is essential for cell migration [25]. In pull down assays, CD4⁺CD25⁺ T cells from wild type mice show efficient activation of p21 Rac following stimulation of S1P, but such stimulation was severely impaired in D910A regulatory CD4⁺CD25⁺ T cells. These effects were not seen when Rac activation was tested following stimulation of CD4⁺CD25⁺ cells with chemokines not involved in thymus exit (IP10, SDF, SLC)[26] (Figure 4D). Thus lack of p110 δ enzymatic activity results in incapacity in specifically the regulatory T cell compartment to respond to thymus egress signals.

Lack of catalytically active p1108 does not diminish anti-tumour surveillance

Azoxymethane (AOM) is a pro-carcinogen which upon metabolic activation causes formation of O⁶-methyl-guanine [27]. AOM induces tumours in the distal colon of rodents and is commonly used to elicit colorectal cancer in animal models [28]. To investigate the role of p110δ PI3K in colitis-associated cancer we used a model in which 6-10 week old mice were injected with a single dose of AOM (12.5 mg/kg) followed by three cycles of DSS administration (cycle 1: 2.5%, five days; cycle 2: 2.5%, 5 days; cycle 3: 2%, 4 days) in the drinking water [29] (Figure 5A). WT and D910A mice on a B/6 (6 each) or balb/c (12 each, data not shown) background and were subjected to this protocol. The severity of the colitis was investigated using body weight as indicator. DSS was effective in inducing colitis because control animals lost substantial body weight after each DSS cycle. Figure 5B shows weight loss as a percentage of weight on day 1. As shown previously, in D910A mice, the inflammatory response was more pronounced.

Mice treated with 3 cycles of Dextran Sulphate Sodium (DSS) over the course of 8 weeks were subsequently sacrificed and analyzed for polyp incidence and size. One p110δ^{D910A/D910A} mouse was sacrificed on day 35 (5 days post end second DSS cycle) due to severe weight loss (> 33%) and sepsis. All mice developed tumours. However, we observed no difference in tumour incidence or tumour size between the two groups. The tumours were in the distal and middle colon, the region of most severe changes during DSS colitis [30] suggesting that the inflammation correlates with the tumour incidence. The tumours were broad-based adenomas with high-grade dysplasia and varying degree of inflammatory cell infiltration. All tumours were non-invasive.





days (AOM injection on day 0)

Figure 5. Loss of p110 δ enzymatic activity leads to increased colitis but not to more cancer in a DSS colitis cancer model. (A) Experimental design of the colitis-induced cancer experiment. (B) Weight loss measure as % change in body weight from day 0 in WT and D910A mice in response to DSS added to drinking water. (C). Tumour size and tumour incidence in D910A and WT animals in the AOM-DSS colitis-associated cancer model.

Discussion

In the present study we compared p110 $\delta^{D910A/D910A}$ mice, lacking active p110 $\delta^{D910A/D910A}$ mice and we observed that the mice raised under conventional conditions developed colitis while the sterile group was unaffected. This phenotype was confirmed *ex vivo*, by increased inflammatory and reduced anti-inflammatory cytokines production in the colon of p110 $\delta^{D910A/D910A}$ mice. Thus the colitis seen in p110 $\delta^{D910A/D910A}$ mice is a consequence of reduced tolerance towards commensal flora.

Regulatory T cells are the most powerful regulators of immune responses in general and the intestinal mucosal immune system in particular. Transfer of regulatory T cells into rodent models of inflammatory bowel disease are capable of preventing the onset of disease and are even capable of reversing established disease. We observed accumulation of regulatory T cells in the thymus of p1108 mice, consistent with previously published data [14], possibly as a consequence of the impaired activation of sphingosine-1-phosphate-dependent signalling. The molecular mechanism by which genetic inhibition of p1108 activity retains regulatory T cells in the thymus remains only partly resolved, but seems to be critically dependent on the inhibition of sphingosine-1-phosphate signalling in regulatory T cells. A role for sphingosine-1phosphate as an exit factor for lymphocytes from lymphoid organs has now been described in various experimental conditions[31]. In the present study, the inhibition of sphingosine-1-phosphate-dependent migration showed a close correlation with the inhibition of the activity of p21Rac, a Ras-like GTPase well established to be a pivotal mediator of cell motility [25]. In this respect it is important to mention that sphingosine-1-phosphate plays an important role in exit of effector lymphocytes from secondary lymphoid organs as well. If in the migratory responses to sphingosine-1-phosphate these effector cells are attenuated in the $p110\delta^{D910A/D910A}$ mice as well, the relatively mild phenotype observed in these animals may contain a contribution from reduced egress of such effector cells from secondary lymphoid organs.

In a AOM/DSS colitis model, p110δ^{D910A/D910A} mice suffer more severe colitis. yet they do not developing more colitis-associated cancer as assessed by polyp size or polyp incidence, even though in the model used, the cancer develops as a direct consequence of colitis. We conclude that lack of active p1108 offers relative protection against colitis-associated cancer, at least in the model used. This relative protection too is possibly established through diminished peripheral presence of $\mathfrak{p}110\delta^{D910A/D910A}$ Tregs, thus increasing anti-tumour immunity. This principle is already used in a new treatment strategy for melanoma, that focuses on diminishing regulatory T cell activity to enhance anti-tumour immunity[19]. Importantly a side effect of this treatment is the induction of IBD. Thus suppression of inflammatory responses may aid malignant cells in suppression of anti-tumour immunity. These findings have important consequences for design of future therapy for IBD, as those that involve long-term increased regulatory T cell activity (e.g. chronic pre/probiotic therapy [32-34], L. lactis producing interleukin-10 [35], or mesenchymal stem cell therapy [36] may carry increased risk of colon cancer if a degree of inflammation remains present. Conversely, even as p1108 inhibitor therapy for e.g. allergic disease might be expected to increase chronic colonic inflammation to some extent, the data presented in the current study strongly suggest that such therapy will not be accompanied by an increased propensity of the colon of such patients to develop neoplastic lesion and might even in certain cases have a preventive effect on colonic cancer formation.

Materials and Methods

Dextran sulphate sodium salt colitis model

p110s^{D910A/D910A} mice [12] were backcrossed on a C57BL/6 or BALB/c background for more than 10 generations and kept in accordance with UK Home Office regulations. Age-matched, 6–10-week-old D910A and WT mice were used for all experiments. In dextran sulphate sodium (DSS) salt-induced colitis, $p110\delta^{D910A/D910A}$ mice and $p110\delta^{WT/WT}$ mice were fed 1.5 % (w/v) DSS (mol. wt. 40 kDa. MP Biomedical) in the drinking water for eight days. Body weight was assessed at baseline and every day for the duration of the experiment. A disease activity index (DAI)[37,38], based on weight loss, stool consistency and rectal bleeding was used to assess disease activity. Bodyweight loss was calculated as the percentage difference between the original bodyweight and the actual bodyweight on any particular day and animals were monitored clinically for stool consistency and rectal bleeding. The mice were sacrificed on day 8. Colons were removed, flushed with PBS, fixed as "Swiss-rolls" in 4% paraformaldehyde at 4°C overnight and paraffin-embedded. Sections (5 µm) were cut stepwise (200 µm) through the complete block and stained with haematoxylin and eosin. Histological scoring of inflammation was determined in a blind manner by a trained pathologist (Table 2). The sections were awarded scores from 0 to 3 for the following 8 parameters: amount of area involved and crypt loss: (0) 0%, (1) 1-10%, (2) 10-50%, (3) > 50%; follicles: (0) 0-1, (1) 2-3, (2) 4-5, (3) >6; oedema and fibrosis: (0) absent, (1) little, (2) moderate, (3) extensive; erosion/ulceration: (0) absent, (1) lamina propria, (2) submucosa, (3) transmural; granulocytes and mononuclear cells: (0) normal, (1) few, (2) moderate (3) extensive.

PA score	0	1	2	3
Area involved	0%	1-10%	10-50%	> 50%
Follicles	Normal (0-1)	Little (2-3)	Moderate (4-5)	Extensive
Oedema	Absent	Little	Moderate	Extensive
Fibrosis	Absent	Little	Moderate	Extensive
Erosion/ulceration	Absent	Lamina	Submucosa	Transmural
		propria		
Crypt loss	0%	1-10%	10-50%	> 50%
Granulocytes	Normal	Few	Moderate	Extensive
Mononuclear cells	Normal	Few	Moderate	Extensive

Table 2. Histological scoring of inflammation.

FACS, cell migration and Rac assays

Thymi were isolated and single-cell suspensions were made by cutting thymi with scissors. The resulting pieces were further digested at 37°C using Blenzyme 2 (Roche) and 100 U/ml DNase I (Roche) for 20 min while stirring continuously. Cell clumps were removed by pipetting the cells through a nylon mesh. The cells were washed and resuspended in PBS with 2% newborn calf serum. For isolation of regulatory T cells and effector T cells, cell were stained with anti-CD3 and anti-CD25 (clone PC61) (both from BD Pharmingen) and double positive cells were obtained with FACS, whereas CD4-positive but CD25-negative cells were considered effector T cells. Prior to experimentation cells were labelled for 1 h with 10 µM CellTracker Green in serum-free medium. The dye was fixed by 1 h incubation in medium with 10% serum, and subsequently cells were washed and resuspended in serum-free medium, pipetted through a 70 µM cell strainer (BD Falcon, Franklin Lakes, NJ), transferred to 8 µM pore size HTS FluoroBlok Cell Culture Inserts from BD Falcon and inserted in fitting 24-wells plates in which various attractant containing media were present. Promptly, fluorescence values representing the number of cells on the bottom side of the insert were read 4 times every 2 min on a Series 4000 CytoFluor Multi-Well Plate Reader (Perseptive Biosystems, Framingham, MA). The raw fluorescence data were corrected for background fluorescence and fading of the fluorophore. No-attractant controls were subtracted at each measured time point to correct for any effects not due to active migration to the chosen attractant. Migration start points of different conditions were set to a common zero to facilitate interpretation and curve fitting.

For Rac assays, S1P-treated CD4⁺CD25⁺ cells were incubated with MLB binding buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 10 mM MgCl₂, 10% glycerol, 25mM NaF, 150 mM NaCl, 1% Nonidet P-40, and 1:1000 dilution of protein inhibitors cocktail) in the presence of 10 µg of GST–Pak1 pzi-binding domain (PBD) agarose (Upstate) for 1 h at 4 C. The beads were washed three times with MLB buffer. The beads were suspended in sample buffer, separated by 15% SDS–PAGE, transferred to a nitrocellulose membrane, and blotted with a 1:1000 dilution of anti-Rac monoclonal antibody (Catalog # 05-389,Upstate), in 5% TBS-T skimmed milk.

Colitis-associated cancer model

Mice were injected intraperitoneally with 12.5 mg/kg azoxymethane (AOM) (Sigma, A2853). After five days, for mice on a C57BL/6 background, 2.5% DSS (MP Biomedical, 160110) was given in the drinking water for a period of five days, followed by 16 days of regular water. This cycle was repeated twice (five days of 2.5% DSS and four days of 2% DSS). For mice on a BALB/c background the cycles were four days 5% DSS, four days 2.5% DSS and four days 3.75% DSS. Mice were sacrificed ten days

after the last cycle and tumour counts were performed in a blind manner. Colons were given a rank score based on tumour size and tumour incidence by experienced pathologists (Johan Offerhaus and Fibo ten Kate). Cytokine determinations were performed using commercially available ELISA kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Cytokine release corresponded to the amount measured in supernatants. All values were adjusted for the weight of the colons.

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