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T helper type 1 memory cells disseminate postoperative ileus over the entire intestinal tract

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Localized abdominal surgery can lead to disruption of motility in the entire gastrointestinal tract (postoperative ileus). Intestinal macrophages produce mediators that paralyze myocytes, but it is unclear how the macrophages are activated, especially those in unmanipulated intestinal areas. Here we show that intestinal surgery activates intestinal CD103$^+$CD11b$^+$ dendritic cells (DCs) to produce interleukin-12 (IL-12). This promotes interferon-γ (IFN-γ) secretion by CCR9$^+$ memory T helper type 1 (T_H1) cells which activates the macrophages. IL-12 also caused some T_H1 cells to migrate from surgically manipulated sites through the bloodstream to unmanipulated intestinal areas where they induced ileus. Preventing T cell migration with the drug FTY720 or inhibition of IL-12, T-bet (T_H1-specific T box transcription factor) or IFN-γ prevented postoperative ileus. CCR9$^+$ T_H1 memory cells were detected in the venous blood of subjects 1 h after abdominal surgery. These findings indicate that postoperative ileus is a T_H1 immune-mediated disease and identify potential targets for disease monitoring and therapy.

Postoperative ileus is a frequent and severe complication after intestinal surgery[1–5]. The bowel dysmotility associated with this condition can last for days, and it necessitates parenteral nutrition and leads to risk of aspiration and infectious complications. The resulting costs in the US exceed $1 billion per year[6,7]. The clinically most relevant problem is the dissemination of postoperative ileus over the entire intestinal tract, the so-called 'gastrointestinal field effect', which halts peristalsis in areas that had not been surgically handled[2,3,8,9]. Inhibitory neuronal signals in the intestine have traditionally been considered responsible[5,8,10], but the underlying mechanisms are unclear. Neither prophylaxis nor effective treatments are available.

Intestinal dysmotility is typically accompanied by increased expression of proinflammatory cytokines such as IL-1, IL-6 and tumor necrosis factor-α (TNF-α), followed by infiltration of polymorphonuclear neutrophilic granulocytes (PMNs) and macrophages[3,10]. Activated macrophages in the intestinal muscularis express inducible nitric oxide synthase (iNOS), whose product nitric oxide directly inhibits smooth muscle cell function[11]. Macrophage depletion or inhibition prevents ileus and inflammation[4,12], demonstrating their essential role in postoperative ileus. Macrophages can be activated by CD4$^+$ T helper cells[13], however, the role of T helper cells in postoperative ileus is unclear.

DCs activate T helper cells and promote differentiation into T_H1, T_H2 or T_H17 effector cells by the cytokines they secrete. T_H1 cells are induced by IL-12, a heterodimer consisting of a p40 and a p35 chain, and express the transcription factor T-bet[14]. They promote immunity against bacteria and viruses and can precipitate Crohn's disease, a type of chronic inflammatory bowel disease (IBD)[15–18]. By contrast, T_H2 cells are involved in containing parasite infections, promote allergy and ulcerative colitis and secrete IL-4 and IL-5 (ref. 17). T_H17 cells have been implicated in autoimmune diseases[19–21] including Crohn's disease[13,22–24]. They are regulated by distinct transcription factors such as signal transducer and activator of transcription-3 (STAT3), are maintained by IL-23, a heterodimer of p40 and p19, produce IL-17 and TNF-α and recruit PMNs[20]. Differentiated T effector cells circulate through the body, a proportion of which develop into memory cells for more rapid recall responses to re-infection[25]. There is evidence that some memory T cells reside in nonlymphoid tissues for even faster local recall responses[26] that are induced by tissue-resident DCs[27,28]. In the intestine, distinct DC subsets have been linked to the induction of regulatory T cells for maintenance of intestinal homeostasis or to the stimulation of T_H1 or T_H17 cells[29–31]. Although postoperative ileus is more prevalent than IBD, the role of DCs in postoperative ileus has not been studied yet.

RESULTS

CD4$^+$ T memory cells are essential for postoperative ileus

We studied the role of T cells in a mouse model of postoperative ileus induced by standardized surgical manipulation of the small intestine[32]. We measured transit of orally administered fluorescent dextran through the intestinal tract by fluorometry. In sham-operated control mice, most dextran had progressed to the colon after 1.5 h, whereas intestinal manipulation retarded its progression such that the dextran reached only the upper jejunal segments (**Fig. 1a**,**b**). This delay did not occur in CD4-knockout mice (**Fig. 1a**,**b**), indicating that T helper

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cells were essential. The geometric center of dextran progression confirmed these findings and will be used here as quantitative parameter for intestinal dysmotility and postoperative ileus (**Fig. 1b**).

To investigate whether the general lack of T helper cells in these mice was responsible for protection from postoperative ileus, we used OT-II mice, which have normal T helper cell numbers, but all of them are specific for ovalbumin. These mice were also protected from postoperative ileus (**Fig. 1b**), indicating that the T helper cell specificity was crucial. These results also suggested that T helper cells need to be activated, as the OT-II cells were naive in our model, owing to the lack of their cognate antigen in our experimental system. We tested this interpretation by depleting T cells expressing the activation marker CD25. Indeed, this prevented postoperative ileus (**Fig. 1b**), indicating that either memory T cells or T regulatory cells (T_{reg} cells) were involved. We found that T_{reg} cells were not responsible, as intestinal manipulation did not lead to postoperative ileus in DEREG mice[33] that had been depleted of T_{reg} cells by injecting diphteria toxin (**Fig. 1b**), implying that memory T helper cells mediated postoperative ileus.

Postoperative ileus is accompanied by inflammation of all intestinal segments, which is marked by increased proinflammatory cytokine and iNOS expression and PMN infiltration (**Supplementary Fig. 1**)[3,32]. We determined whether these parameters depend on memory T helper cells by using CD4-knockout mice. Although TNF-α, IL-1, IL-6 and iNOS expression was still increased in the small intestine of these mice, this increase was much lower than in CD4-competent control mice (**Fig. 1c** and **Supplementary Fig. 1c**). PMN infiltration was unaffected. Thus, although a local inflammatory response was generated after intestinal manipulation in CD4-knockout mice, it was less intense, suggesting that T helper cells locally enhanced inflammation. In the large intestine, all parameters of inflammation were decreased in the CD4-knockout mice (**Fig. 1d**), indicating a requirement for T helper cells for colon inflammation.

T_H1-responses are essential for postoperative ileus

Of the inflammatory mediators studied above, only iNOS was regulated in a T helper cell–dependent manner in both the small and large intestine after intestinal manipulation (**Fig. 1c**,**d**). Because iNOS is essential for postoperative ileus[11], we speculated that T helper cells stimulated iNOS production in intestinal macrophages. To identify the T helper effector cell type stimulating iNOS production, we measured mRNA levels of the cytokines that induce T_H1, Th2 and T_H17 cells or are produced by them. We found that mRNA for IL-12 p35 and T-bet were upregulated in the muscularis of the manipulated small intestine as compared with sham-operated controls (**Fig. 2a**), suggesting that a T_H1 response was generated. IFN-γ and T-bet mRNA were increased in both the small and large intestine over sham-operated controls (**Fig. 2a**,**b**), consistent with the presence of T_H1 cells. mRNA encoding IL-12 and IL-23 p40, IL-23 p19, IL-4, IL-5, IL-17A and STAT3 were not upregulated (**Fig. 2a**,**b**), suggesting neither T_H2 nor T_H17 responses were generated. Flow cytometry confirmed the presence of IFN-γ–producing memory T_H1 cells in the manipulated, but not the sham-operated small intestine (**Fig. 2c**,**d**). Neither IL-17–producing nor IL-4–producing T helper cells were detected (**Fig. 2d**), verifying that intestinal manipulation induced a local T_H1, but not a T_H2 or T_H17, response.

To determine whether this T_H1 response is required for postoperative ileus, we used IFN-γ–knockout, IL-12 p35–knockout mice and, as a control, IL-23 p19–knockout mice. Manipulated IFN-γ–knockout and IL-12 p35 knockout mice showed near-normal FITC-dextran transit, as opposed to IL-23 p19–knockout mice (**Fig. 2e**), indicating that a T_H1, but not T_H17, response was needed. We confirmed this finding by measuring the excretion time of a glass ball after rectal insertion (hereafter referred to as colon transit time), which selectively probes for dysmotility of the colon as a measure of the field effect. Colon transit time was also delayed in C57BL/6 and IL-23p19–knockout mice but partially normalized in IFN-γ–knockout mice and completely normalized in IL-12 p35–knockout mice (**Fig. 2f**). We also used mice deficient for the T_H1 transcription factor T-bet on the less T_H1-prone BALB/c background. BALB/c mice also showed impaired dextran transit and colon motility after intestinal manipulation, and these symptoms were completely attenuated in T-bet–knockout mice (**Fig. 2e**,**f**). These findings indicated that IL-12 p35, T-bet and IFN-γ are necessary for postoperative ileus.

IFN-γ was not produced by $CD8^+$ T cells, and only small amounts were released by natural killer T cells in response to intestinal manipulation (**Supplementary Fig. 2a**,**b**). However, depletion of natural killer cells did not alter colon transit time (**Supplementary Fig. 2c**), confirming that T_H1 cells were the source of functionally relevant IFN-γ in the context of postoperative ileus.

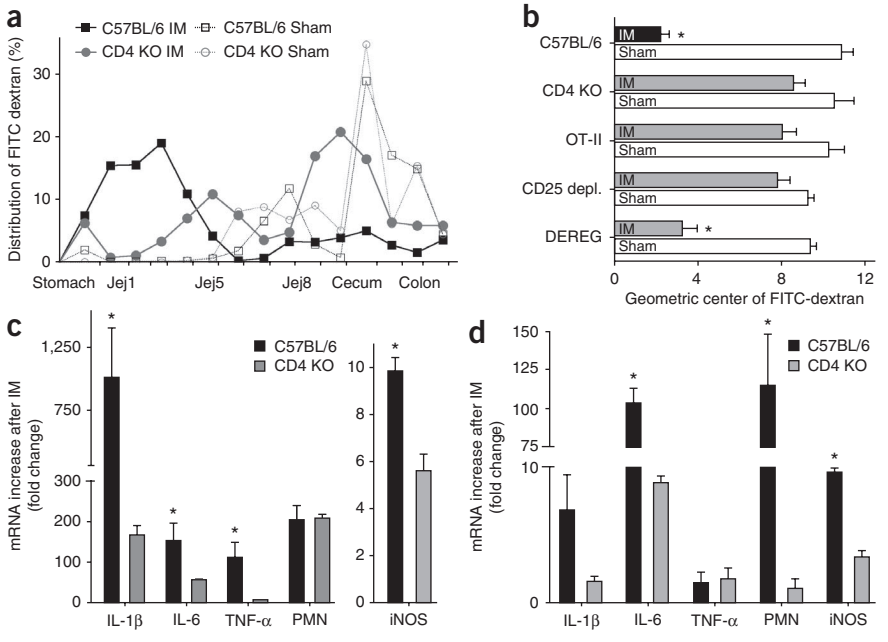

Figure 1 T helper memory cells are essential for ileus. (**a**) Dextran transit through the intestinal segments in C57BL/6 and CD4-knockout (KO) mice who were sham-operated or intestinally manipulated (IM). Jej, jejunal segments. (**b**) Mean geometrical center of dextran transit in C57BL/6, CD4-knockout, OT-II, CD25 cell–depleted and DEREG mice after intestinal manipulation or sham operation (Sham). (**c**,**d**) Ratio of mRNA levels for IL-1, IL-6, TNF-α, iNOS and numbers of PMNs in the muscularis of the small intestine (**c**) and colon (**d**) of manipulated over sham-operated C57BL/6 or CD4-knockout mice. Results are representative of five (**a** and **b**) and three (**c** and **d**) independent experiments in groups of four to five mice, and data are means ± s.e.m. Statistical analysis was done by Kruskal-Wallis with Dunn's *post hoc test*; $^*P < 0.05$.

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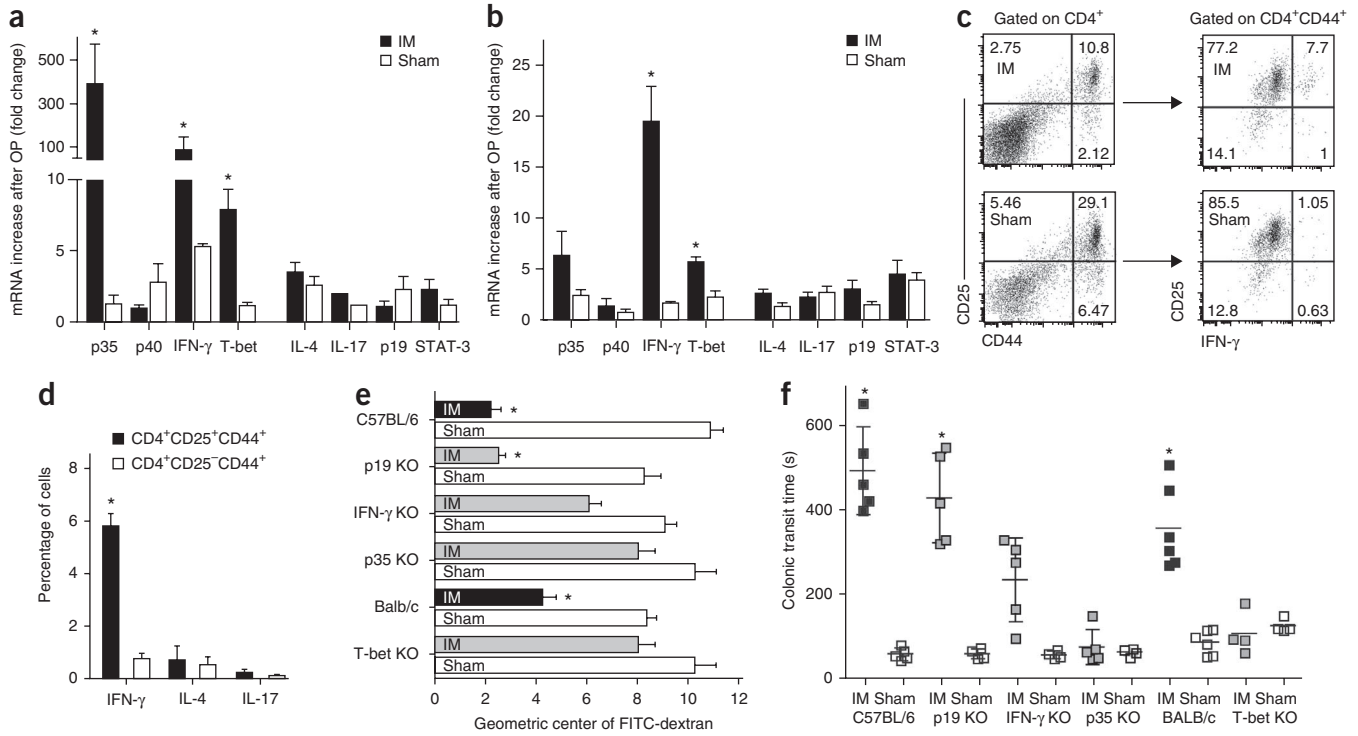

Figure 2 Intestinal manipulation induces T_H1 responses that are essential for postoperative ileus. (**a**,**b**) Ratio of mRNA levels of IL-12 p35, IL-12 and IL-23 p40, IFN-γ, T-bet, IL-4, IL-17, IL-23p19 and STAT3 in the muscularis of the small intestine (**a**) or colon (**b**) of C57BL/6 mice after operation (OP), either intestinal manipulation or sham operation, over nonoperated controls, as determined by RT-PCR. (**c**) FACS analysis of $CD4^+$ T helper cells from the small intestinal muscularis re-stimulated and stained for CD25 and CD44 and intracellularly for IFN-γ. (**d**) $CD25^+$ and $CD25^-$ $CD4^+CD44^+$ memory T helper cells from the small intestinal muscularis were re-stimulated and intracellularly stained for IFN-γ, IL-4 and IL17A. Numbers indicate proportions of cells expressing the respective cytokine, as measured by flow cytometry. (**e**,**f**) Mean geometrical center of orally administered FITC-dextran (**e**) or colonic transit time of a 2-mm glass ball (**f**) after intestinal manipulation or sham operation. C57BL/6 mice served as controls for p19-knockout, IFN-γ–knockout and p35-knockout mice, and BALB/c mice for T-bet–knockout mice. Results are representative of four (**a** and **b**), three (**c** and **d**) and six (**e** and **f**) independent experiments in groups of six mice, and data are means ± s.e.m. Statistical analysis was done by Mann-Whitney test; $^*P < 0.05$.

Next we determined mRNA expression of IL-12 p35, T-bet, IFN-γ and iNOS in mice deficient in these molecules (**Supplementary Fig. 3**) to clarify their sequence of action. We calculated how much these mRNAs were upregulated in manipulated knockout mice over sham-operated mice as a percentage of the upregulation in wild-type mice (**Table 1**). The results were consistent with the sequence of IL-12 inducing T-bet, resulting in upregulation of IFN-γ, which then induced iNOS expression, and thus supported our hypothesis of T_H1 cell–induced macrophage activation. Some results deviated from the classical T_H1 cascade as IL-12 p35 induction was partially reduced in IFN-γ–knockout mice, and IFN-γ induction was only partially reduced in T-bet–knockout mice (**Table 1** and **Supplementary Fig. 3**). This might be explained by positive feedback loops between IL-12, T-bet and IFN-γ, through which these mediators affect each other's expression[34]. Collectively, these findings indicate that the T_H1 memory response induced by intestinal manipulation is required for postoperative ileus and the field effect.

T_H1 memory cells migrate between intestinal segments

The major problem in postoperative ileus is paralysis of the entire intestinal tract. To test whether migration of T_H1 memory cells to unmanipulated areas might be responsible, we quantified the number of $CD25^+$ T helper cells in the manipulated small intestine and the unmanipulated colon. T helper cell numbers declined in the manipulated small intestinal muscularis but increased in the colon (**Fig. 3a**), supporting cell migration, although the increase in the colon was not significant. To unequivocally show migration, we painted the small intestine with fluorescent carboxyfluorescein succinimidyl ester (CFSE) to label resident cells and performed intestinal manipulation. One day later, we found CFSE-labeled $CD25^+$ T helper cells in the colonic muscularis of manipulated, but not sham-operated, mice (**Fig. 3b**,**c**). Flow cytometry and immunofluorescence microscopy confirmed the presence of $CD4^+IFN\text{-}\gamma^+$ cells in the colon of manipulated mice (**Supplementary Fig. 4**).

$CFSE^+$ T helper cell numbers were increased in the portal vein and peaked 45 min after intestinal manipulation (**Fig. 3d**), suggesting that they left the small intestine through this route. These $CFSE^+$ cells expressed the T cell memory markers CD25 and CD44, as expected, and also the chemokine receptor CCR9 (**Fig. 3e**), which facilitates T cell homing to the intestine[35]. Emigration of T_H1 memory cells through the portal vein was slightly increased also in sham-operated mice (**Fig. 3d**). We speculated this might be due to CFSE-induced local inflammation and therefore manipulated mice without CFSE labeling and determined the numbers of $CCR9^+CD25^+CD44^+$ T helper cells that produce IFN-γ in the portal vein blood. This analysis confirmed that T_H1 memory cells exited the small intestine through the portal vein (**Fig. 3f**) but did not exclude that these cells also used other routes, such as lymphatic vessels. Approximately 75% of the exiting $CCR9^+$ $CD25^+$ $CD44^+$ T helper cells produced IFN-γ, partially explaining why the absolute cell numbers were lower than in the CFSE analysis (**Fig. 3d**).

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Table 1 Expression of T_H1 mediators following intestinal manipulation in knockout mice

	p35			T-bet				IFN-γ				iNOS			
Small intestine	Mean		s.d.	Mean		s.d.		Mean		s.d.		Mean		s.d.	
Wild type	100.0	±	21.0	100.0	±	17.7		100.0	±	12.9		100.0	±	40.7	
IFN-γ knockout	63.7	±	21.1	110.7	±	40.9			ND			**40.6**	±	**13.7**	**
T-bet knockout	87.9	±	18.8		ND			**40.7**	±	**10.3**	*	**43.8**	±	**14.8**	**
p35 knockout		ND		**23.3**	±	**9.0**	**	**7.9**	±	**0.9**	***	**51.0**	±	**6.6**	*
	p35			T-bet				IFN-γ				iNOS			
Colon	Mean		s.d.	Mean		s.d.		Mean		s.d.		Mean		s.d.	
Wild type	100.0	±	39.0	100.0	±	23.9		100.0	±	29.8		100.0	±	18.9	
IFN-γ knockout	150.8	±	33.9	88.6	±	9.7			ND			**39.3**	±	**29.0**	*
T-bet knockout	92.3	±	24.4		ND			**32.9**	±	**10.5**	*	**47.7**	±	**17.8**	*
p35 knockout		ND		**9.7**	±	**5.7**	***	**27.5**	±	**6.7**	**	**32.4**	±	**13.7**	*

The table indicates how much the respective gene was upregulated in manipulated knockout mice as compared to sham-operated mice as percentage of the upregulation in wild-type (WT) mice, which had been defined as 100% (intestinal $manipulation_{knockout}$ / $sham_{knockout}$)/(intestinal $manipulation_{WT}$ / $sham_{WT}$). Values are based on the ratios given in **Supplementary Figure 3**. Results are representative of three independent experiments in groups of five mice. Data are means ± s.e.m., and statistical analysis was done by Kruskal-Wallis with Dunn's *post hoc* test; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. ND, not determined.

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The presence of migrating $CCR9^+$ memory T_H1 cells in the venous blood of mice (**Fig. 3f**) suggested that analogous cells may be increased in the blood of humans undergoing abdominal surgery. We therefore studied five individuals undergoing hemicolectomy, gastrectomy or resection of jejunal segments and compared them with subjects after exploratory laparotomy, thoracotomy or vascular surgery (details in **Supplementary Table 1**). One hour after abdominal surgery, numbers of $CCR9^+CD45RO^+$ (memory) IFN-γ–producing T helper cells were nearly tenfold elevated in the blood compared to those in subjects after nonabdominal surgery (**Fig. 3g**), supporting a role of the T_H1 cascade in humans.

IL-12-induced T_H1 cell migration causes postoperative ileus

To determine whether T_H1 cell migration from the manipulated intestine contributed to postoperative ileus, we treated mice with FTY720, an immunosuppressive drug that sequesters lymphocytes in lymphatic[36] and mucosal[37] tissues, and thereby prevents their migration between organs, including the intestine[38]. First we confirmed that this drug prevented the exit of T_H1 memory cells through the portal vein of mice after intestinal manipulation (**Fig. 4a**). FTY720-treated mice were protected from both postoperative ileus and the field effect (**Fig. 4b**,**c**), confirming the necessity of T cell migration for the field effect. IFN-γ expression in the manipulated small intestine was substantially reduced after treatment (**Fig. 4d**), explaining why FTY720 improved peristalsis also locally within the manipulated small intestine.

We next asked how the intestinal T_H1 cascade was initiated. Expression of IFN-γ and IL-12 p70 (the bioactive form of IL-12) was elevated in the muscularis of the manipulated small intestine 30 min after intestinal manipulation (**Fig. 4e**). We focused on IL-12 because it was the most upstream T_H1 component in our analysis (**Table 1**). However, IL-12 p35–knockout mice could not be used to clarify the role of intestinal IL-12 because these mice possess very few T_H1 cells as a result of the necessity of this cytokine for differentiation of naive T cells into T_H1 cells[14]. Therefore, we treated IL-12–competent mice before intestinal manipulation with antibodies that block IL-12 p40. This was possible despite the presence of p40, a subunit shared with IL-23, because IL-23 was neither induced nor relevant in postoperative ileus (**Figs. 2** and **3**). Blocking IL-12

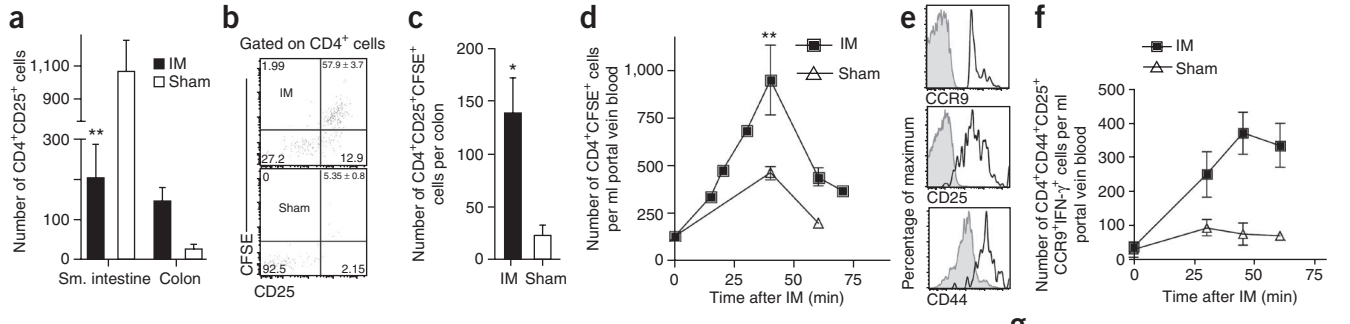

Figure 3 T_H1 memory cells migrate from manipulated to unmanipulated intestinal segments. (**a**) Absolute numbers of $CD4^+$ $CD25^+$ T helper cells in the small intestine or colon 1 d after intestinal manipulation or sham operation. (**b**–**e**) The small intestine was painted with CFSE 10 min before intestinal manipulation or sham operation. After 1 d, the colonic muscularis (**b**,**c**) or the portal vein blood (**d**,**e**) was analyzed for the presence of $CD4^+CD25^+CFSE^+$ cells. The dot plots in **b** were gated for $CD4^+$ cells; the bars in **c** show the numbers of T_H1 memory cells in the colon that originated in the small intestine. The histograms in **e** show expression of CCR9, CD44 and CD25 by $CD4^+CFSE^+$ cells in the portal vein. Shaded areas indicate staining with an isotype control. (**f**) Numbers of $CD4^+CCR9^+CD44^+CD25^+IFN\text{-}\gamma^+$ T_H1 cells per ml portal vein blood after intestinal manipulation or sham operation. (**g**) Numbers of $CD4^+CCR9^+CD45RO^+IFN\text{-}\gamma^+$ T_H1 cells in peripheral blood of five subjects after abdominal surgery (IM) and five subjects after thoracic or vascular surgery (Ctrl). Results are representative of three (**e** and **f**), four (**b**–**d**) or five (**a**) independent experiments in groups of four or five mice, and data are means ± s.e.m. Statistical analysis was done by Mann-Whitney test; $*P < 0.05$; $**P < 0.01$.

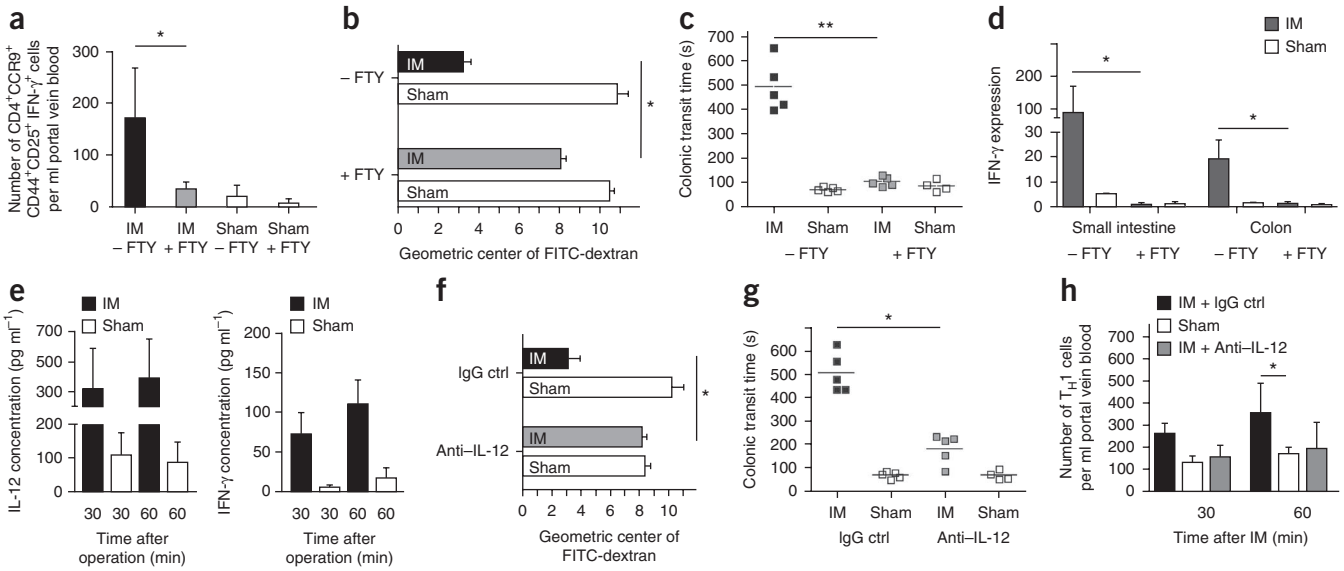

Figure 4 Pharmacological inhibition of memory T_H1 cell activation and migration protects mice from postoperative ileus. (**a**) Numbers of $CD4^+CCR9^+CD44^+CD25^+IFN\text{-}\gamma^+$ T_H1 cells in portal vein blood of FTY720- or PBS-treated C57BL/6 mice 45 min after intestinal manipulation or sham operation. (**b**,**c**) C57BL/6 mice were injected with 0.7 mg per kg body weight FTY720 or PBS 24 h and 0 h before intestinal manipulation or sham operation. Dextran transit (**b**) and colonic transit time (**c**) were determined 1 d later. (**d**) IFN-γ mRNA levels in the muscularis of the small intestine and colon of C57BL/6 mice 40h after FTY720 or PBS treatment and 24 h after intestinal manipulation or sham operation. (**e**) IL-12 p70 and IFN-γ concentrations in small intestinal muscularis homogenates 30 and 60 min after intestinal manipulation or sham operation. (**f**–**h**) C57BL/6 mice were injected with antibodies against IL-12 p40 (anti–IL-12) or control immunoglobulin 24 h and 0 h before intestinal manipulation or sham operation. Gastrointestinal dextran transit (**f**), colonic transit (**g**) and exit of T_H1 cells through the portal vein (**h**) were determined. Results are representative of two (**a**, **e** and **h**), three (**b**, **c**, **d**, **f** and **g**) independent experiments in groups of four or five mice, and data are means ± s.e.m. Statistical analysis was done by Mann-Whitney test; $^*P < 0.05$; $^{**}P < 0.01$.

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p40 indeed suppressed postoperative ileus (**Fig. 4f**) and prevented the field effect (**Fig. 4g**), indicating that IL-12 produced after intestinal manipulation is required for postoperative ileus. To clarify the mechanism whereby IL-12 acted in postoperative ileus, we examined the exit of T_H1 memory cells after blocking IL-12. Inhibition of IL-12 completely prevented egress of cells via the portal vein (**Fig. 4h**), indicating that local production of IL-12 initiated T_H1 memory cell migration and the field effect.

DCs produce pathogenic IL-12 after intestinal manipulation

Next, we identified the source of IL-12 in the manipulated intestine. Only $CD11c^+$ cells expressing class II major histocompatibility complex expressed this cytokine (**Fig. 5a** and **Supplementary Fig. 5**), suggesting DCs are the main source of IL-12. Nearly all muscularis DCs, including the IL-12–producing ones, expressed the subtype markers CD11b and CD103 (**Fig. 5a**). Few DCs were present in the muscularis of sham-operated mice, but the numbers increased by 30-fold after intestinal manipulation (**Fig. 5b**). This recruitment was probably a consequence of surgical trauma, the resulting local inflammation or both, because DCs were not recruited to unmanipulated colons (**Fig. 5b**). DCs in the manipulated small intestine expressed high amounts of the co-stimulatory molecules CD40, CD80 and CD86 (**Fig. 5c** and **Supplementary Fig. 6a**) and induced proliferation of naive T helper cells in co-culture (**Supplementary Fig. 6b–d**). These T helper cells secreted IFN-γ, but little IL-17 and IL-4 (**Fig. 5d**), indicating that DCs from the manipulated intestine induced T_H1 but not T_H17 or T_H2 differentiation. These findings support the scenario that small intestinal DCs induce a T_H1 response via IL-12.

We postulated that if this were the case, depletion of DCs should prevent postoperative ileus. We used CD11c-DTR mice, which allow for conditional ablation of $CD11c^+$ cells[39], and depleted DCs before intestinal manipulation. Depletion efficiency in the small intestinal muscularis was typically about 95% (**Supplementary Fig. 7**). This prevented postoperative ileus (**Fig. 5e**) and the field effect (**Fig. 5f**). IL-12 p35 mRNA was only eightfold increased in the small intestine (**Fig. 5g**) of DC-depleted mice as compared to a more than 400-fold elevation in undepleted controls. Colonic IL-12 p35 mRNA was marginally increased (**Fig. 5h**). mRNA levels of several T_H1 mediators were reduced in both the small and the large intestines following DC depletion (**Fig. 5g**,**h**). These findings confirm that DCs in the small intestine initiate a T_H1 response leading to postoperative ileus.

DISCUSSION

Ileus is a serious complication after abdominal surgery that traditionally was thought to result from neuronal dysfunction. The discovery that macrophages are essential[1,3–5] indicated that immune processes are important, but failed to explain why unmanipulated intestinal segments were subsequently paralyzed. Pharmacological or electrical stimulation of the vagus nerve can reduce macrophage activation and attenuate postoperative ileus[40]. However, it remains to be seen how neuronal dysfunction can lead to dysregulation of macrophages during postoperative ileus, particularly given, postoperative ileus still occurs after small bowel transplantation, where the intestine is denervated[41].

Here we have shown that local inflammation after intestinal manipulation causes ileus only when a T_H1 response is generated. T_H1 memory cells had at least two roles: they amplified local inflammation, which paralyzed the small intestine, and they migrated via the blood to the colon, which caused the field effect. TNF-α expression and PMN infiltration did not coincide with the onset of symptoms of ileus and were not dependent on T helper cells. Instead, T_H1 cells and mediators were essential in initiating postoperative ileus. These findings do not rule out the contribution of neuronal signals

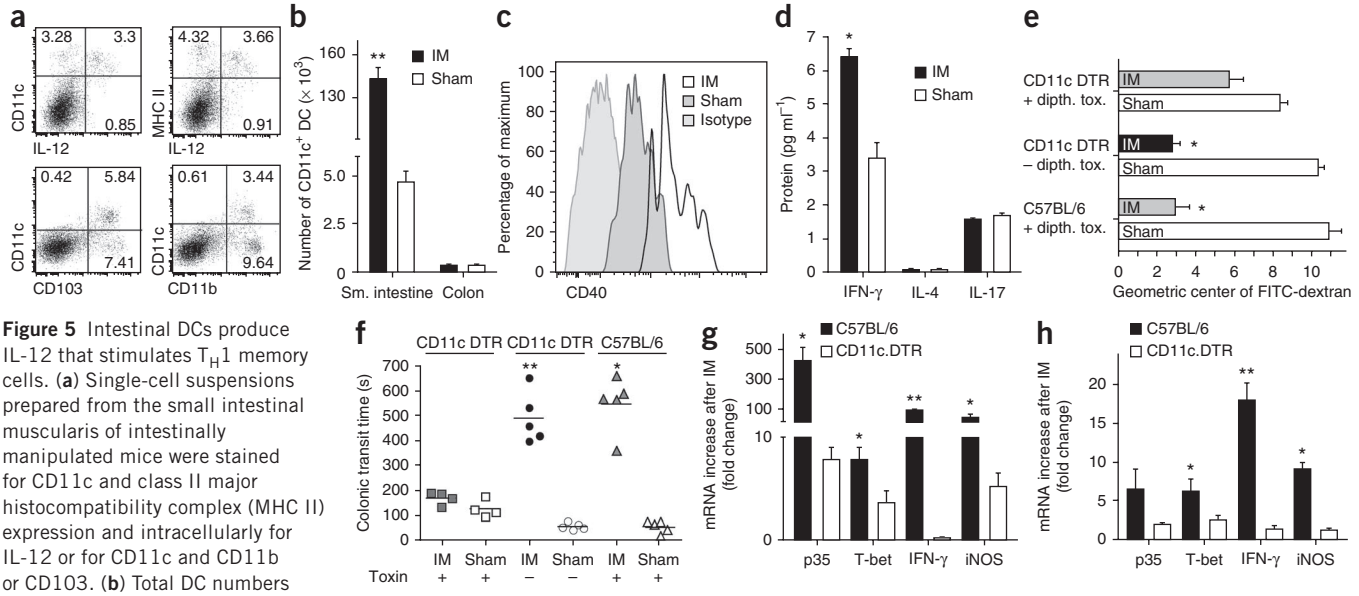

Figure 5 Intestinal DCs produce IL-12 that stimulates T_H1 memory cells. (**a**) Single-cell suspensions prepared from the small intestinal muscularis of intestinally manipulated mice were stained for CD11c and class II major histocompatibility complex (MHC II) expression and intracellularly for IL-12 or for CD11c and CD11b or CD103. (**b**) Total DC numbers in the muscularis and colon after intestinal manipulation or sham operation. (**c**) CD40 expression on $CD11c^+$ DCs from the muscularis of the small intestine 1 d after intestinal manipulation or sham operation. (**d**) $CD11c^+$ cells from the small intestinal muscularis 1 d after intestinal manipulation or sham operation were loaded with $ovalbumin_{323–339}$ peptide and cultured with OT-II cells. After 1 d, cytokine concentrations were determined in the supernatant by ELISA. (**e**,**f**) Mean geometrical center of dextran transit (**e**) or colon transit time (**f**) in CD11c-DTR or C57BL/6 mice injected with diphtheria toxin after intestinal manipulation or sham operation. (**g**,**h**) IL-12 p35, T-bet, IFN-γ and iNOS mRNA in the muscularis of the small intestine (**g**) or colon (**h**) of diphtheria toxin-injected C57BL/6 or DC-depleted CD11c-DTR mice after intestinal manipulation. Results are representative of (**a**), three (**d**–**h**) or six (**b** and **c**) independent experiments in groups of five mice, and data are means ± s.e.m. Statistical analysis was done by Mann-Whitney test (**b** and **d**) and Kruskal-Wallis with Dunn's *post hoc* test (**e**–**h**); $*P < 0.05$; $**P < 0.01$.

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to the development of postoperative ileus. Indeed, there is cross-talk between such the nervous and immune system, both in postoperative ileus and IBD[10,40,42], and further examination of this cross-talk and its implications for postoperative ileus is warranted.

T_H1 cells were thought to be responsible for most immune cell–mediated inflammatory diseases, including IBD[15–18]. Later, IL-23 and T_H17 cells were implicated in some IBD models[22,24,43–46]. We found that IL-23 was dispensable for postoperative ileus, excluding a role for IL-23–dependent T_H17 cells. This cytokine not only maintains T_H17 cell responses but also regulates T_{reg} cells[43], which did not contribute to the development of postoperative ileus. The dispensability of T_H17 cells may be explained by the time needed for T_H17 differentiation (1 week[47]), which exceeds the time frame of hours during which postoperative ileus takes place. This short time frame alone was strong evidence that naive T helper cells cannot be involved, because their differentiation into T_H1 effector cells requires at least 1 d. In contrast, memory T_H1 cells contain preformed T-bet and can secrete high amounts of IFN-γ immediately after IL-12 stimulation[48], and therefore they are theoretically capable of migrating to the colon and activating macrophages in that time span. A role for antigen-specific memory T_H1 cells was verified by the lack of postoperative ileus in OT-II mice. These mice cannot mount T cell responses with specificity for the antigens relevant in postoperative ileus. It is likely that numerous intestinal antigens are relevant, and these need to be identified in future studies.

The T_H1 memory response leading to postoperative ileus was initiated by IL-12, indicating that this cytokine governs not only differentiation of T_H1 cells but also their migration in the effector phase. A role of T_H1 memory cell migration was supported by the effectiveness of FTY720, which prevents T cell egress from lymphoid and mucosal tissues[36–38]. FTY720 also prevented IFN-γ production by intestinal T_H1 cells, which may explain why it prevented postoperative ileus also locally. These findings suggest that FTY720-related drugs might be useful in postoperative ileus.

The IL-12–producing DCs were of the $CD11b^+CD103^+$ subset, which can imprint CCR9 expression in T helper cells[49,50]. This is consistent with our detection of circulating $CCR9^+$ T_H1 memory cells. Such DCs have been reported to induce T_{reg} cell differentiation[50,51] or to prime T_H17 cells under inflammatory conditions[52,53]. A role for $CD11b^+CD103^+$DCs in pathogenic T_H1 responses has not been previously reported[31].

Developing new therapies for postoperative ileus requires identification of mediators that are essential for disease development. We identified DCs, IL-12 p35, T-bet and IFN-γas essential mediators and excluded the T_H17 pathway. Furthermore, antibodies against IL-12 prevented postoperative ileus and the field effect. This effect on T_H1 cell migration may also be relevant in Crohn's disease, where IL-12 blockade was effective in clinical trials[54]. Depletion of the DCs that produce IL-12, or inhibiting T_H1 cell migration by treatment with FTY720, was also effective, suggesting new prophylactic or therapeutic opportunities. Finally, the detection of $CCR9^+$ memory T_H1 cells in the bloodstream may allow monitoring postoperative ileus in subjects after abdominal surgery.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

D.R.E., A.K., M.S., L.F. and J.M. performed the experiments. D.R.E., A.K., J.C.K. and C.K. designed the study and wrote the manuscript. J.C.K. supervised the surgical part and C.K. supervised the immunological part of the study. S.W., A.H. and P.A.K. provided crucial ideas. T.S., B.S. and A.L. provided crucial reagents. All authors discussed and interpreted results.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Kalff, J.C., Schraut, W.H., Billiar, T.R., Simmons, R.L. & Bauer, A.J. Role of inducible nitric oxide synthase in postoperative intestinal smooth muscle dysfunction in rodents. *Gastroenterology* **118**, 316–327 (2000).
2. Bauer, A.J., Schwarz, N.T., Moore, B.A., Turler, A. & Kalff, J.C. Ileus in critical illness: mechanisms and management. *Curr. Opin. Crit. Care* **8**, 152–157 (2002).
3. Schwarz, N.T. *et al.* Selective jejunal manipulation causes postoperative pan-enteric inflammation and dysmotility. *Gastroenterology* **126**, 159–169 (2004).
4. Wehner, S. *et al.* Inhibition of macrophage function prevents intestinal inflammation and postoperative ileus in rodents. *Gut* **56**, 176–185 (2007).
5. Bauer, A.J. Mentation on the immunological modulation of gastrointestinal motility. *Neurogastroenterol. Motil.* **20**(Suppl 1), 81–90 (2008).
6. Goldstein, J.L. *et al.* Inpatient economic burden of postoperative ileus associated with abdominal surgery in the United States. *P&T Journal* **32**, 82–90 (2007).
7. Iyer, S., Saunders, W.B. & Stemkowski, S. Economic burden of postoperative ileus associated with colectomy in the United States. *J. Manag. Care Pharm.* **15**, 485–494 (2009).
8. Barquist, E. *et al.* Neuronal pathways involved in abdominal surgery–induced gastric ileus in rats. *Am. J. Physiol.* **270**, R888–R894 (1996).
9. Barada, K.A. *et al.* Localized colonic inflammation increases cytokine levels in distant small intestinal segments in the rat. *Life Sci.* **79**, 2032–2042 (2006).
10. de Jonge, W.J. *et al.* Postoperative ileus is maintained by intestinal immune infiltrates that activate inhibitory neural pathways in mice. *Gastroenterology* **125**, 1137–1147 (2003).
11. Türler, A. *et al.* Leukocyte-derived inducible nitric oxide synthase mediates murine postoperative ileus. *Ann. Surg.* **244**, 220–229 (2006).
12. Wehner, S. *et al.* Inhibition of p38 mitogen–activated protein kinase pathway as prophylaxis of postoperative ileus in mice. *Gastroenterology* **136**, 619–629 (2009).
13. Dardalhon, V., Korn, T., Kuchroo, V.K. & Anderson, A.C. Role of T_H1 and T_H17 cells in organ-specific autoimmunity. *J. Autoimmun.* **31**, 252–256 (2008).
14. Szabo, S.J. *et al.* Distinct effects of T-bet in T_H1 lineage commitment and IFN-γ production in CD4 and CD8 T cells. *Science* **295**, 338–342 (2002).
15. Neurath, M.F., Finotto, S. & Glimcher, L.H. The role of T_H1/T_H2 polarization in mucosal immunity. *Nat. Med.* **8**, 567–573 (2002).
16. Bouma, G. & Strober, W. The immunological and genetic basis of inflammatory bowel disease. *Nat. Rev. Immunol.* **3**, 521–533 (2003).
17. Strober, W., Fuss, I. & Mannon, P. The fundamental basis of inflammatory bowel disease. *J. Clin. Invest.* **117**, 514–521 (2007).
18. Gutcher, I. & Becher, B. APC-derived cytokines and T cell polarization in autoimmune inflammation. *J. Clin. Invest.* **117**, 1119–1127 (2007).
19. Cua, D.J. *et al.* Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* **421**, 744–748 (2003).
20. Bettelli, E., Oukka, M. & Kuchroo, V.K. T_H-17 cells in the circle of immunity and autoimmunity. *Nat. Immunol.* **8**, 345–350 (2007).
21. Paust, H.J. *et al.* The IL-23/T_H17 axis contributes to renal injury in experimental glomerulonephritis. *J. Am. Soc. Nephrol.* **20**, 969–979 (2009).
22. Yen, D. *et al.* IL-23 is essential for T cell–mediated colitis and promotes inflammation via IL-17 and IL-6. *J. Clin. Invest.* **116**, 1310–1316 (2006).
23. Mucida, D. *et al.* Reciprocal T_H17 and regulatory T cell differentiation mediated by retinoic acid. *Science* **317**, 256–260 (2007).
24. Leppkes, M. *et al.* RORγ-expressing T_H17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. *Gastroenterology* **136**, 257–267 (2009).
25. Sallusto, F., Lenig, D., Forster, R., Lipp, M. & Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**, 708–712 (1999).
26. Gebhardt, T. *et al.* Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat. Immunol.* **10**, 524–530 (2009).
27. Wakim, L.M., Waithman, J., van Rooijen, N., Heath, W.R. & Carbone, F.R. Dendritic cell-induced memory T cell activation in nonlymphoid tissues. *Science* **319**, 198–202 (2008).
28. Heymann, F. *et al.* Kidney dendritic cell activation is required for progression of renal disease in a mouse model of glomerular injury. *J. Clin. Invest.* **119**, 1286–1297 (2009).
29. Coombes, J.L. & Powrie, F. Dendritic cells in intestinal immune regulation. *Nat. Rev. Immunol.* **8**, 435–446 (2008).
30. Rescigno, M. & Di Sabatino, A. Dendritic cells in intestinal homeostasis and disease. *J. Clin. Invest.* **119**, 2441–2450 (2009).
31. Varol, C., Zigmond, E. & Jung, S. Securing the immune tightrope: mononuclear phagocytes in the intestinal lamina propria. *Nat. Rev. Immunol.* **10**, 415–426 (2010).
32. Kalff, J.C., Schraut, W.H., Simmons, R.L. & Bauer, A.J. Surgical manipulation of the gut elicits an intestinal muscularis inflammatory response resulting in postsurgical ileus. *Ann. Surg.* **228**, 652–663 (1998).
33. Lahl, K. *et al.* Selective depletion of Foxp3$^+$ regulatory T cells induces a scurfy-like disease. *J. Exp. Med.* **204**, 57–63 (2007).
34. Schulz, E.G., Mariani, L., Radbruch, A. & Hofer, T. Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-γ and interleukin-12. *Immunity* **30**, 673–683 (2009).
35. Mora, J.R. *et al.* Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* **424**, 88–93 (2003).
36. Cyster, J.G. Chemokines, sphingosine-1-phosphate and cell migration in secondary lymphoid organs. *Annu. Rev. Immunol.* **23**, 127–159 (2005).
37. Tomita, T. *et al.* Colitogenic CD4$^+$ effector-memory T cells actively recirculate in chronic colitic mice. *Inflamm. Bowel Dis.* **14**, 1630–1640 (2008).
38. Kunisawa, J. *et al.* Sphingosine 1-phosphate dependence in the regulation of lymphocyte trafficking to the gut epithelium. *J. Exp. Med.* **204**, 2335–2348 (2007).
39. Jung, S. *et al. In vivo* depletion of CD11c$^+$ dendritic cells abrogates priming of CD8$^+$ T cells by exogenous cell-associated antigens. *Immunity* **17**, 211–220 (2002).
40. de Jonge, W.J. *et al.* Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat. Immunol.* **6**, 844–851 (2005).
41. Schaefer, N. *et al.* Role of resident macrophages in the immunologic response and smooth muscle dysfunction during acute allograft rejection after intestinal transplantation. *Transpl. Int.* **21**, 778–791 (2008).
42. Boeckxstaens, G.E. & de Jonge, W.J. Neuroimmune mechanisms in postoperative ileus. *Gut* **58**, 1300–1311 (2009).
43. Ahern, P.P., Izcue, A., Maloy, K.J. & Powrie, F. The interleukin-23 axis in intestinal inflammation. *Immunol. Rev.* **226**, 147–159 (2008).
44. Weigmann, B. *et al.* The transcription factor NFATc2 controls IL-6–dependent T cell activation in experimental colitis. *J. Exp. Med.* **205**, 2099–2110 (2008).
45. Ogawa, A., Andoh, A., Araki, Y., Bamba, T. & Fujiyama, Y. Neutralization of interleukin-17 aggravates dextran sulfate sodium-induced colitis in mice. *Clin. Immunol.* **110**, 55–62 (2004).
46. Haak, S. *et al.* IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation in mice. *J. Clin. Invest.* **119**, 61–69 (2009).
47. McGeachy, M.J. *et al.* The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17–producing effector T helper cells *in vivo*. *Nat. Immunol.* **10**, 314–324 (2009).
48. Hu, J. & August, A. Naive and innate memory phenotype CD4$^+$ T cells have different requirements for active Itk for their development. *J. Immunol.* **180**, 6544–6552 (2008).
49. Johansson-Lindbom, B. *et al.* Functional specialization of gut CD103$^+$ dendritic cells in the regulation of tissue-selective T cell homing. *J. Exp. Med.* **202**, 1063–1073 (2005).
50. Sun, C.M. *et al.* Small intestine lamina propria dendritic cells promote *de novo* generation of Foxp3 T_{reg} cells via retinoic acid. *J. Exp. Med.* **204**, 1775–1785 (2007).
51. Coombes, J.L. *et al.* A functionally specialized population of mucosal CD103$^+$ DCs induces Foxp3$^+$ regulatory T cells via a TGF-β and retinoic acid–dependent mechanism. *J. Exp. Med.* **204**, 1757–1764 (2007).
52. Denning, T.L., Wang, Y.C., Patel, S.R., Williams, I.R. & Pulendran, B. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17–producing T cell responses. *Nat. Immunol.* **8**, 1086–1094 (2007).
53. Uematsu, S. *et al.* Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nat. Immunol.* **9**, 769–776 (2008).
54. Mannon, P.J. *et al.* Anti–interleukin-12 antibody for active Crohn's disease. *N. Engl. J. Med.* **351**, 2069–2079 (2004).

ONLINE METHODS

Mice and reagents. Mice had been backcrossed for more than ten generations to C57BL/6 and were used at 8–12 weeks of age. Governmental review boards (Bezirksregierung Köln, from 2008 Landesamt für Natur, Umwelt und Verbraucherschutz NRW in Recklinghausen, Germany) approved all mouse experiments. We depleted $CD25^+$ cells with 300 μg per mouse PC61 antibody, which removed 85–90% of $CD25^+$ cells[55], and natural killer cells with 300 μg per mouse NK1.1-specific antibody. We blocked IL-12 p40 with 500 μg per mouse clone C17.8 antibody. We used FTY720 at 0.7 mg per kg body weight. All reagents, if not otherwise specified, were from Sigma (Taufkirchen).

Standardized intestinal manipulation in mice. We performed standardized surgical intestinal manipulation of the small intestine as previously described[56–58]. Briefly, after isoflurane narcosis, the peritoneal cavity was opened by a midline incision and the small intestine was placed onto sterile moist gauze and manipulated by moist cotton applicators once from the oral to aboral direction, strictly avoiding contact with stomach or colon. Then, two layers of running suture with 5.0-size silk closed the laparotomy. Sham-operated mice underwent laparotomy without manipulation.

For CFSE labeling of the small intestine, we placed the small intestine into PBS containing 10 μM CFSE and incubated for 10 min (see also **Supplementary Methods**).

Functional studies. We measured gastrointestinal FITC-dextran transit as previously described[56–58]. Briefly, 24 h after intestinal manipulation, we anesthetized the mice and applied fluorescent-labeled 70-kDa FITC-dextran via gavage into the stomach. After 90 min, we divided the entire gastrointestinal tract into 15 pieces (stomach, ten equal parts from the small bowel, cecum and three equal parts of the colon) and measured the fluorescence intensity in each intestinal segment by fluorometry. We calculated the geometric center of FITC-dextran as (Σ (% FITC per segment × segment number)) / 100 and displayed as a horizontal bar graph.

We performed colonic transit measurement 24 h after intestinal manipulation as previously described[56–58]. Briefly, we inserted a 2-mm glass ball retrogradely 3 cm deep into the colon of slightly isoflurane-anesthetized mice and measured the time until excretion after recovery from anesthesia.

Isolation of intestinal immune cells. After removal of the Peyer's patches, we separated the muscularis from the mucosa by slipping 5-cm-length sections from the intestine or colon over a glass rod, stripped the muscularis from the mucosa and isolated immune cells as previously described[59]. We cut the muscularis into 5-mm pieces and digested these for 45 min at 37 °C with 100 U ml^{-1} collagenase type VIII and 50 U ml^{-1} DNase I in HBSS containing 10% FCS and 10 mM HEPES. Then samples were shaken vigorously for 10 s, and supernatants were passed through 40-μm nylon mesh.

Analysis of human blood samples by flow cytometry. Analysis had been approved by the Ethics Board of the University Clinic of Bonn, and the subjects gave informed consent for the analysis. We collected 5 ml of human blood and stained the leukocytes with antibodies against CCR9 (clone 3C7), CD45RO (UCHL1) and CD4 (SK3), followed by intracellular staining for IFN-γ (B27) after a 4-h incubation in the presence of brefeldin A and CD3/CD28-specific beads (BD).

Statistical analyses. We compared two non-normally distributed groups by Mann-Whitney test with Prism software (GraphPad) and more groups by Kruskal-Wallis with Dunn's *post hoc* test. Results are given as means ± s.e.m.; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Additional methods. We performed cell culture experiments, immunofluorescence histology, RT-PCR, ELISA and flow cytometry by standard methods as previously described[60]. Detailed methodology (for example, primer sequences and antibodies used) is described in the **Supplementary Methods.**

55. Ludwig-Portugall, I., Hamilton-Williams, E.E., Gottschalk, C. & Kurts, C. Cutting edge: CD25+ regulatory T cells prevent expansion and induce apoptosis of B cells specific for tissue autoantigens. *J. Immunol.* **181**, 4447–4451 (2008).
56. Kalff, J.C., Schwarz, N.T., Walgenbach, K.J., Schraut, W.H. & Bauer, A.J. Leukocytes of the intestinal muscularis: their phenotype and isolation. *J. Leukoc. Biol.* **63**, 683–691 (1998).
57. Schaefer, N. *et al.* Resident macrophages are involved in intestinal transplantation-associated inflammation and motoric dysfunction of the graft muscularis. *Am. J. Transplant.* **7**, 1062–1070 (2007).
58. Wehner, S. *et al.* Measurement of gastrointestinal and colonic transit in mice. *Nat. Protoc.* published online, doi:10.1038/protex.2010.1158 (28 November 2010).
59. Chirdo, F.G., Millington, O.R., Beacock-Sharp, H. & Mowat, A.M. Immunomodulatory dendritic cells in intestinal lamina propria. *Eur. J. Immunol.* **35**, 1831–1840 (2005).
60. Semmling, V. *et al.* Alternative cross-priming through CCL17-CCR4–mediated attraction of CTLs toward NKT cell-licensed DCs. *Nat. Immunol.* **11**, 313–320 (2010).

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