Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Intestinal mononuclear cells primed by systemic interleukin-12 display long-term ability to aggravate colitis in mice

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doi:10.1111/imm.12685
Received 15 August 2016; revised 21 October 2016; accepted 23 October 2016.
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Summary

To address whether the burst of systemic interleukin-12 (IL-12) influences intestinal inflammation elicited by luminal stimuli, we induced IL-12 release by cDNA injection in C57BL/6 mice and simultaneously started dextran sulphate sodium administration. The sequence of the inflammatory response triggered by IL-12 release was characterized by assessing myeloperoxidase activity and histological damage in colon samples on days 1, 3, 5 and 7 after colitis induction. To evaluate the persistence of IL-12 priming, colitis was induced in mice 7 or 60 days after cDNA injection. Under IL-12 influence, the development of acute colitis presented a faster and selective infiltration of inflammatory mononuclear cells in the lamina propria. Recruitment was driven by systemic cytokines rather than luminal antigens. Interestingly, when colitis was triggered 7 or 60 days after the cytokine storm, cells maintained the ability to worsen clinical signs of intestinal inflammation. Together, a systemic IL-12 burst effectively primed intestinal cells that became more prone to develop inflammatory responses. Activation was long-lasting because intestinal cells maintained their inflammatory potential and their ability to aggravate colitis.

Keywords: colitis; interleukin-12; macrophage; priming; systemic T cell.

Introduction

Inappropriate recruitment and accumulation of leucocytes in the gut seem pivotal in inflammatory bowel disease.[1] Animal models are valuable tools to understand the sequence of inflammation and characterize mediators of intestinal injury.[2] The most widely used model of colitis in mice uses dextran sulphate sodium (DSS) to induce the disease. The DSS breaks the epithelial barrier, allowing the entry of luminal antigens and microorganisms into the mucosa resulting in overwhelming inflammation.[3] Models of acute, chronic and relapsing intestinal inflammation can be developed by changing DSS concentration and the administration schedule.[4] Acute colitis develops by continuous administration of 2–5% DSS for 4–9 days; clinical signs usually start after 1 day of treatment with increased intestinal permeability, diarrhoea, occult blood in stools, weight loss, anaemia and, depending on the experimental procedure, mortality.[4,5] Typically, an influx of neutrophils occurs into the lamina propria (LP) and the submucosa, which correlates with myeloperoxidase (MPO) activity in the colonic tissue. Apparently, the adaptive immune system does not play a major role in the acute model because T-cell-deficient and B-cell-deficient mice also develop severe intestinal inflammation.[6] Histological changes observed after DSS administration include mucin depletion, ulceration and submucosal inflammation, epithelial barrier disruption, neutrophil infiltration in the LP and submucosa, and abscesses.[4,5]

Interleukin-12 (IL-12) is a master regulator of innate and adaptive immune responses against pathogens and tumours[7] due to its ability to drive T helper type 1 (Th1) responses.[8] Hence, systemic IL-12 that is released during infections or in clinical applications could affect intestinal

Abbreviations: DAI, Disease Activity Index; DC, dendritic cell; DSS, dextran sulphate sodium; h.i., hydrodynamic injection; IFN-γ, interferon-γ; IL, interleukin; LP, lamina propria; MLN, mesenteric lymph nodes; MPO, myeloperoxidase; STAT, signal transducer and activator of transcription; Th1, T helper type 1; TNF-α, tumour necrosis factor-α

homeostasis. In fact, the gut can be the target of IL-12 biological activity[9,10] because mice lacking the IL-12p40 gene, a common subunit of IL-12 (a Th1 trigger) and IL-23 (a Th17 trigger) cytokines, develop attenuated colitis.[11] In the same way, mice treated with neutralizing antibodies against IL-12p40 develop milder colitis,[12,13] which emphasizes the key contribution of Th1/Th17 cell responses in intestinal inflammation.[14] Also, repeated doses of IL-12 alone or combined with other cytokines produce detrimental effects in BALB/c mice with mucosal damage, bloody diarrhoea and weight loss,[15] and they drive a chronic inflammation in DSS-induced colitis.[13] Despite these reports, the influence of systemic IL-12 in intestinal immunity has not yet been fully understood. After hydrodynamic injection (h.i.) of IL-12 cDNA in C57BL/6 mice, we found that T lymphocytes from Peyer's patches and mesenteric lymph nodes (MLN) become activated, exhibit a $CD44^{hi}$ $CD62L^{-}$ phenotype and up-regulate the $\alpha_4\beta_7$ integrin expression.[16] The increased $\alpha_4\beta_7$ expression depends on the axis formed by the IL-12 receptor and the signal transducer and activator of transcription 4 (STAT-4) and occurs independently of interferon-γ (IFN-γ), IL-4, IL-10 and tumour necrosis factor-α (TNF-α) signalling.[16] Mice injected with IL-12 cDNA do not develop intestinal inflammation; nonetheless, upon DSS administration, acute colitis shows higher severity.[16] To further characterize the effect of systemic IL-12, we evaluated the sequence of inflammatory response under IL-12 influence and the persistence of IL-12 priming away from the primary cytokine burst. This study shows that systemic IL-12 endowed intestinal mononuclear cells with inflammatory potential. This ability exacerbated the inflammatory response to luminal stimulation away from the initial burst of IL-12.

Materials and methods

Ethical considerations

All animal experiments were approved by and conducted in accordance with the guidelines of the Committee for Animal Care and Use of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba (Approval Number HCD 15-09-69596), and in strict conformity with the recommendation of the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (OLAW Assurance number A5802-01). Our animal facility obtained the NIH Animal Welfare Assurance (Assurance No. A5802-01, Office of Laboratory Animal Welfare, NIH, Bethesda, MD).

Mice

Six- to eight-week-old C57BL/6 (B6) mice from the Jackson Laboratory (Bar Harbor, ME) were used in these studies. The animals were maintained in specific pathogen-free conditions and housed in collective cages at $22 \pm 1°$ under a 12-hr light/dark cycle (lights on at 7:00 a.m.) with free access to laboratory chow and drinking water.

Reagents and antibodies

The IL-12 plasmid was kindly provided by Dr Howard Young (Frederick National Laboratory for Cancer Research, Frederick, MD). The DSS (MW 40 000) was a kind gift from George Usher, Dextran Products Limited (Scarborough, ON, Canada). Anti-CD3 (17A2), anti-CD28 (37.51), anti-CD4 (GK 1.5), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-MHC-II (25-9-17), CD11c (HL3) and anti-CCR5 (C34-3448) were provided by BD PharMingen (San Diego, CA); and CD103 (2E7) and anti Ly6C (HK.1.4) by BioLegend (San Diego, CA). Corresponding isotype-matched monoclonal antibodies were used as controls in flow cytometry experiments. Recombinant murine IL-12p70 (210-12) was from Peprotech (Colonia Narvarte, Mexico).

Hydrodynamic cDNA injection

The hydrodynamic gene transfer procedure was described previously.[16] Expression plasmids use the human elongation 1-α promoter to drive transcription. The animals were given an h.i. with 0·5 μg open reading frame empty vector control cDNA or pscIL-12 cDNA (p40-p35 fusion gene) in the tail vein in 1·6 ml of sterile 0·9% sodium chloride solution in 5–7 seconds. The marked splenomegaly observed on day 7 or at the end of each experiment confirmed the effectiveness of the treatment, as previously reported.[16–18] For transfer experiments, MLN cells from IL-12 or the control cDNA-injected mice were isolated 7 days after h.i., cultured with 50 ng/ml recombinant IL-12 (rIL-12) for 16 hr and injected ($2{\cdot}5 \times 10^6$ cells) intravenously in C57BL/6 mice. On the same day, administration of DSS was started for colitis induction, as detailed below.

Induction and assessment of DSS colitis

The mice were given a solution of filtered water containing 3% (weight/volume) DSS (MW 40 000) *ad libitum* over a 5-day period. Every other day, the DSS solution was replenished and at the end of this period it was replaced with normal drinking water for 2 days. The control mice received only normal drinking water. All the animals were examined once a day and the Disease Activity Index (DAI) that combines scores of weight loss, stool consistency and bleeding was assessed as previously described.[19] Briefly, for weight loss, a value of 0 was assigned if body weight increased or remained within 1%

of baseline, 1 for a 1–5% loss, 2 for a 5–10% loss, 3 for a 10–15% loss, and 4 for weight loss > 15%. For stool consistency, a value of 0 was for no diarrhoea, 2 for loose stools that did not stick to the anus and 4 for liquid stools that did stick to the anus. For faecal blood, 0 was for none, 2 for moderate and 4 for gross bleeding. When indicated, blood samples were taken from the tail vein and centrifuged immediately; sera were frozen for further analysis.[20] The animals were killed by cervical dislocation; their colons were removed and examined for weight, macroscopic appearance and length (which was measured from 1 cm above the anus to the top of the caecum).

Myeloperoxidase assay

Neutrophil infiltration into the colon was assessed indirectly by measuring MPO activity as previously explained.[19] The colon segments were homogenized in 5% EDTA/NaCl buffer (pH 4·7) and centrifuged at 10 000 ***g*** for 15 min at 4°. The pellets were resuspended in 0·5% hexadecyl trimethyl ammonium bromide buffer (pH 5·4), and the samples were frozen in liquid nitrogen and thawed three times. After the final thawing, they were centrifuged as above, and 25 µl of the supernatant was used for MPO assay. The MPO enzymatic reaction was assessed by adding 1·6 mmol/l tetramethylbenzidine, 80 mmol/l $NaPO_4$ and 0·3 mmol/l H_2O_2. The absorbance was measured spectrophotometrically at 690 nm and MPO was calculated with a standard curve generated for each experiment. The results were expressed as nanograms per milligram of tissue.

Histopathological analysis

The excised portions of the distal colon were immediately fixed in 4% (weight/volume) formaldehyde solution and embedded in paraffin. Next, 5-µm sections were mounted on glass slides, deparaffinized and stained using standard haematoxylin & eosin techniques. For histological analysis we used the histological score that represents the sum of histoarchitecture alterations, mononuclear and polymorphonuclear infiltrates in the epithelium, LP and submucosa, crypt alterations, pseudopolyp formation, oedema and ulcers, as described by Geboes *et al.*[21,22] Images were taken with a Nikon optical microscope (Nikon Eclipse TE2000-U, Melville, USA.).

Explant cultures and cell isolation

Extraintestinal fat tissue and blood vessels were carefully removed; large intestines were flushed with RPMI-1640 and opened along a longitudinal axis. Then, 5-mm^2 punch biopsies were obtained from the medial colon and incubated for 24 hr in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics (one punch biopsy per 1 ml medium). Supernatants were collected and kept at −20° until the production of cytokines was assessed as explained above.

Colonic LP cells were isolated as follows.[23] The colon was cut into small pieces and epithelial cells and mucus were removed by 45-min incubation with RPMI-1640 containing 5% fetal bovine serum, 2 mM EDTA and 1 mM dithiothreitol (Sigma, St Louis, MO) at 37° with 250 rpm shaking. The colon pieces were then digested in PBS containing 5% fetal bovine seurm and 1 mg/ml collagenase IV (Sigma) for 90 min at 37° and shaken at 250 rpm. The digested cell suspension was washed with PBS and passed through 40-µm cell strainers.

Cell culture

The MLN single-cell suspensions (1×10^6 cells/ml) were cultured with medium, 2 µg/ml anti-CD3/1 µg/ml anti-CD28 antibodies or 50 ng/ml rIL-12. When indicated, supernatants were harvested 24 hr later and assayed for cytokines as described below.

Measurement of cytokines and chemokines

Murine monocyte chemoattractant protein 1 (MCP-1) and IL-1β (BD Biosciences, San José, CA.) or IFN-γ, IL-6 and IL-10 (BD Pharmingen) were measured in sera or culture supernatants by ELISA kits, as specified by the manufacturers.

Flow cytometry

The cells were stained with the desired antibodies and, on the basis of forward and side light scatter, debris and dead cells were gated out; 10 000 events were analysed using a FACSAria flow cytometer (BD Bioscience). The flow cytometry analysis was performed with the FLOWJO software (Treestar, Ashland, OR, USA).

Statistical analysis

Data were expressed as mean ± SD. Statistical differences between groups were determined by a one-way analysis of variance followed by a Student–Newman–Keuls test. Statistical analyses were performed using GRAPHPAD PRISM 4 software (GraphPad Software, San Diego, CA). A P value of $< 0{\cdot}05$ was considered statistically significant.

Results

Systemic IL-12 modifies the development of acute colitis induced by DSS

We have recently shown that the systemic release of IL-12 exacerbates acute colitis in mice although, under

IL-12 influence, the typical increase of MPO activity on day 7 does not occur.[16] To address whether neutrophil infiltration could have occurred earlier in this experimental condition, mice were given h.i. with IL-12 cDNA and treated with DSS as described above.[16] The DAI was calculated daily, and colon length and MPO activity were assessed on days 1, 3, 5 and 7 of the treatment in representative mice from the control, IL-12, DSS and IL-12+DSS groups. As already stated, the combined treatment resulted in a higher DAI as early as day 4 (Fig. 1a) and in a significantly shortened colon length on day 5 (Fig. 1b). The progressive increase in MPO activity observed in acute DSS colitis from days 1 to 7 was not detected in the IL-12+DSS group (Fig. 1c), suggesting that IL-12 could be driving a different inflammatory response. To confirm the reduced neutrophil influx under IL-12 influence, we characterized the histopathological damage in the colon sections on days 1, 3, 5 and 7 after DSS administration. Representative microphotographs show that colon integrity was maintained on day 1 (Fig. 2a,e,i,m), although all groups had a moderate presence of inflammatory cells associated with the hydrodynamic process.[16–18] On day 3, the histological features of the control and IL-12 groups were normal (Fig. 2b,f), whereas colon damage started in the DSS group with oedema, inflammatory infiltrate and crypt alterations (Fig. 2j). On that day, IL-12+DSS mice presented more ulcers, a larger amount of mononuclear cells in the LP and epithelium, and severe crypt alterations (Fig. 2n). Changes observed in DSS-treated mice on day 5 were similar to those in the IL-12+DSS group as early as day 3 (Fig. 2k versus n), which reached a critical grade of colitis on day 7 with a marked infiltrate of polymorphonuclear cells (Fig. 2l). On that day, animals from the IL-12+DSS group showed mild to moderate oedema, ulcers and a considerable amount of mononuclear cells (Fig. 2p). The combined IL-12+DSS treatment induced injury and colon inflammation earlier on day 3, with a slight amelioration by day 7. In contrast, mice receiving only DSS reached the highest histopathological index by day 7. Table S1 (see Supplementary material) provides the detailed score that includes mononuclear and polymorphonuclear infiltrates (in the epithelium, LP and submucosa), crypt alterations, oedema and ulcers from each group calculated as described elsewhere[21,22]; the mean histological score is shown in Fig. S1 (see Supplementary material).

To correlate the histopathological analysis with the ongoing systemic inflammation, we measured serum cytokines in all experimental groups on days 1, 3, 5 and 7 (see Supplementary material, Fig. S2a). Mice from the IL-12 group produced more TNF-α, IFN-γ and IL-10 than the DSS group with an ongoing inflammatory response in the colon. Interestingly, the luminal stimuli were unable to expand serum cytokines over the values triggered by

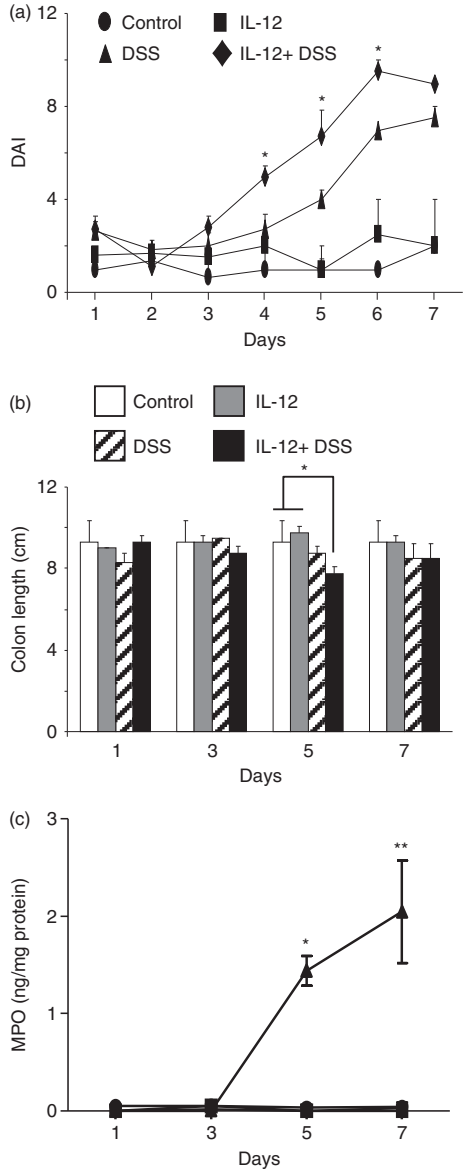

Figure 1. Evaluation of dextran sodium sulphate (DSS) -induced colitis under systemic interleukin-12 (IL-12) influence. The C57BL/6 mice (6–8 weeks old) were given a hydrodynamic injection (h.i.) with the control or IL-12 cDNAs; on the same day, they were administered DSS in drinking water. The mice were evaluated daily to calculate the Disease Activity Index (DAI) (a). Representative animals from each group were killed on days 1, 3, 5 or 7 and colon segments were removed to assess colon length (b) and myeloperoxidase (MPO) activity (c). Data are mean ± SD of two different experiments with three mice per group. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

IL-12 alone (i.e. IL-12 versus IL-12+DSS). The MLN cell suspensions stimulated with rIL-12 or aCD3/aCD28 antibodies from both groups injected with IL-12 cDNA produced higher levels of IFN-γ, which demonstrated that IL-12 was driving the Th1 response in mucosal lymphoid tissue during intestinal inflammation (see Supplementary material, Fig. S2b). On the other hand, the animals

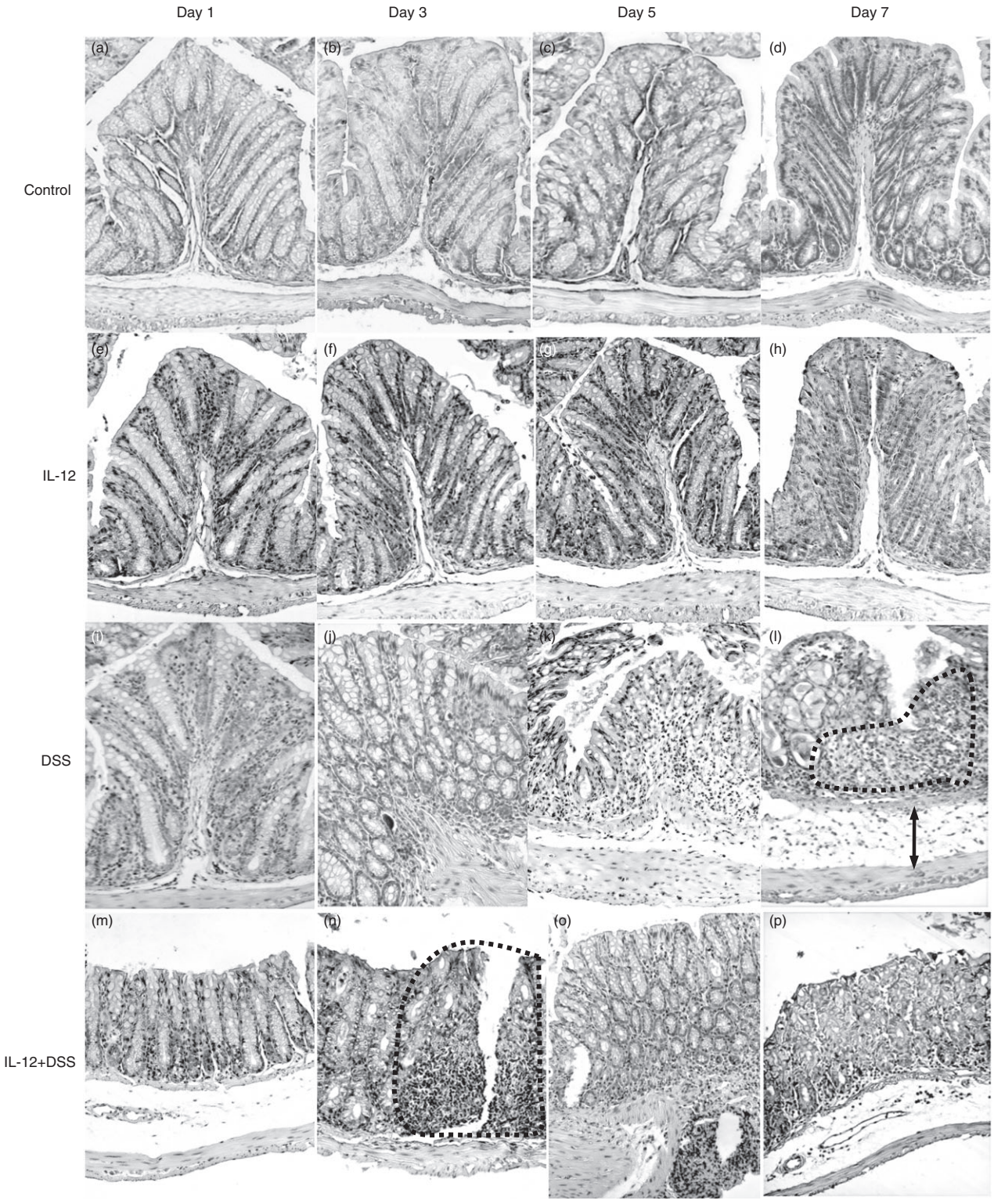

Figure 2. Daily histopathological evaluation of colon upon interleukin-12 (IL-12) and dextran sodium sulphate (DSS) administration. Histopathological alterations in representative distal colon sections stained with haematoxylin & eosin on days 1, 3, 5 and 7 in mice of the control (a–d), IL-12-treated (e–h), DSS (i–l) or IL-12+DSS (m–p) groups. Dashed lines (l and n) and arrows (l) highlight main alterations in the IL-12+DSS (day 3) and DSS (day 7) groups, respectively. Original magnification ×200.

exposed to both systemic and luminal stimuli exhibited the highest production of IL-10 upon T-cell receptor or IL-12 stimulation.

Cell subsets involved in acute colitis under systemic IL-12 influence

Considering that the presence of mononuclear and polymorphonuclear cells in colonic tissue was different in the DSS and IL-12+DSS groups, we evaluated by flow cytometry blood and LP mononuclear subsets that could be involved in the inflammatory response. Figure 3(a) shows that mice with the double treatment presented a 2- to 2·5-fold increment in $CD4^+$ and $CD8^+$ LP T cells, which was concurrent with the sharp reduction in blood (Fig. 3b). However, most of the remaining $CD4^+$ and $CD8^+$ lymphocytes expressed the CCR5 receptor (Fig. 3b), a marker of Th1 responses with an important role in localizing lymphocytes in the intestinal mucosa.[24] Changes in $CD4^+$ and $CD8^+$ T-cell subsets in the LP and blood were consistent with the increment of T cells bearing the $\alpha_4\beta_7$ integrin detected in MLN as early as day 5 (data not shown), as previously reported.[16] Considering that the $CD11c^+$ $CD103^+$ dendritic cell (DC) subset has the unique ability to induce gut-homing receptors on MLN T lymphocytes,[25] we evaluated the frequency of $CD11c^+$ MHC-II^+ $CD103^+$ DCs in MLN on day 5. Interestingly, on the same day, the IL-12 and IL-12+DSS groups showed a higher percentage of $CD11c^+$ MHC-II^+ $CD103^+$ DCs, in marked contrast with DSS mice (Fig. 4b). This increment could explain the expansion of activated $\alpha_4\beta_7^+$ T cells ready to migrate to the LP.

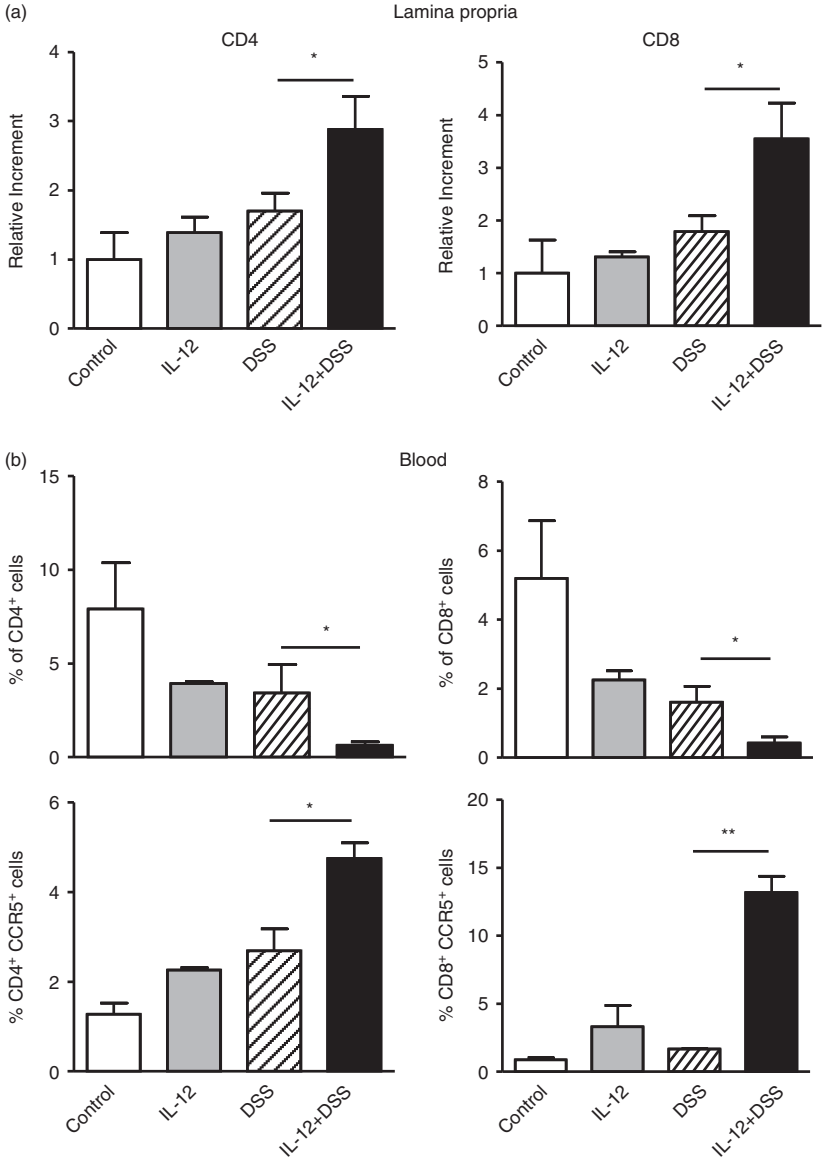

Figure 3. Lymphocyte traffic in interleukin-12 plus dextran sodium sulphate (IL-12+DSS) colitis. The C57BL/6 mice (6–8 weeks old) were given a hydrodynamic injection (h.i.) with the control or IL-12 cDNAs, and starting at the same time 3% DSS was administered. On day 5, blood and colon were collected; mononuclear cells were isolated as described in the Materials and methods and analysed by flow cytometry. Results are percentage of $CD4^+$ and $CD8^+$ lymphocytes in (a) LP or (b) blood and $CD4^+$ $CCR5^+$ and $CD8^+$ $CCR5^+$ blood lymphocytes in the control, IL-12, DSS and IL-12+DSS groups. Data are mean ± SD of two different experiments with $n = 4$ per group. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

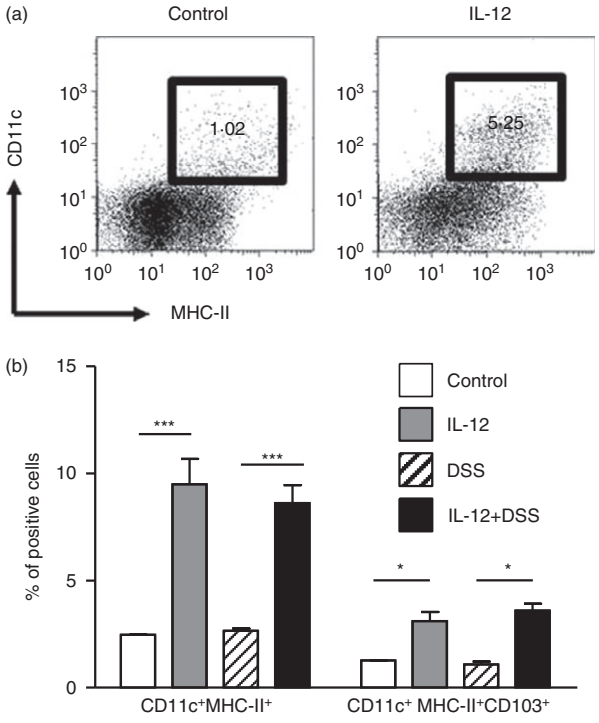

Figure 4. Enhanced dendritic cell (DC) migration to mesenteric lymph nodes (MLN) upon systemic interleukin-12 (IL-12) release. The C57BL/6 mice (6–8 weeks old) were given a hydrodynamic injection (h.i.) with the control or IL-12 cDNAs and at the same time administered 3% dextran sodium sulphate (DSS). Mice were killed on day 5. Single-cell suspensions prepared from MLN were stained with fluorochrome-labelled antibodies and assessed for CD11c, MHC-II and CD103 expression by flow cytometry. (a) Representative dot plots showing $CD11c^+$ $MHC\text{-}II^+$ DCs in the control and IL-12 injected groups. (b) Percentage of $CD11c^+$ $MHC\text{-}II^+$ or $CD11c^+$ $MHC\text{-}II^+$ $CD103^+$ DCs in MLN of the control, IL-12-injected, DSS and IL-12+DSS groups. Data are mean ± SD of two different experiments with $n = 5$ per group. $^*P < 0{\cdot}05$; $^{***}P < 0{\cdot}001$.

Taking into account that the strong Th1 milieu triggered by systemic IL-12 could be affecting the differentiation of monocytes arriving at the LP,[26] we evaluated $CD11b^+$ $MHC\text{-}II^+$ $Ly6C^+$ subsets in different groups. In agreement with the histopathological results, we found a significant increment of immature $CD11b^+$ $MHC\text{-}II^+$ $Ly6C^{hi}$ monocytes in the LP of IL-12+DSS mice (Fig. 5a), which was coincident with the blood reduction in the $CD11b^+$ $Ly6C^{hi}$ subset (Fig. 5c). On day 5, $CD11b^+$ $Ly6C^{hi}$ monocytes infiltrating the LP of DSS animals were mainly MHC-II negative (Fig. 5b). In supernatants of colon explants from both groups with colitis we found an increment in MCP-1, a chemokine involved in recruiting immature $Ly6C^{hi}$ monocytes,[26] indicating that the rise was not elicited by IL-12 (data not shown). On the other hand, IL-12+DSS-treated animals showed a 3·5-fold increase in IL-6 levels compared with the modest production of the DSS group (Fig. 5d). This strong inflammatory response may be elicited by a higher supply of luminal stimuli because intestinal permeability is impaired as early as 3 days after the IL-12 injection.[16]

To confirm that IL-12-primed cells instead of the cytokine storm were affecting inflammatory response, MLN cells from IL-12 cDNA-injected mice were isolated on day 7 and stimulated with rIL-12 before transfer into normal recipients that started DSS administration thereafter. As shown in the Supplementary material (Fig. S3a,b), recipients presented a more pronounced weight loss and a higher DAI compared with the controls, indicating that IL-12-primed cells did exacerbate intestinal inflammation. We tried several strategies to purify MLN cells from IL-12-injected mice for further characterization. Yet, the recovery of few viable cells due to the higher fragility associated with the activation status[16,18] precluded these experiments. The transference of MLN cells from normal donors stimulated with rIL-12 produced minor effects, demonstrating the relevance of the *in vivo* priming (data not shown).

Persistence of IL-12 priming

Finally, we wondered if cell priming could affect forthcoming intestinal inflammation. Hence, the mice were injected with IL-12 cDNA and again a week later, when cytokines regained baseline,[16] they were given DSS for 5 days. The IL-12+DSS mice developed a more severe colitis with early weight loss and higher DAI scores compared with the DSS group (Fig. 6a) To confirm the persistence of cell priming, a second group of mice started the DSS administration 60 days after the plasmid h.i. (Fig. 6b). Again, IL-12+DSS mice developed a more severe colitis with early weight loss and higher DAI scores than the DSS group. Supernatants of colon explants showed similar levels of MCP-1 and IL-1β; however, 60 days after the IL-12 burst, a higher production of IFN-γ was found in the IL-12+DSS group compared with the DSS mice (Fig. 6c). Together, primed cells instead of cytokines were mediating the distinctive intestinal inflammation in mice under the IL-12+DSS treatment. The IL-12 priming was long-lasting and similar to findings shown in Fig. 2(m–p) and Fig. S1 (see Supplementary material). Mice developing colitis 60 days after h.i. presented a marked mononuclear infiltration in tissue and vessels and a higher histological score (see Supplementary material; Fig. S4).

Discussion

Our results show that, after systemic release of IL-12, mice developed a severe intestinal inflammation when they were exposed to luminal stimuli. This inflammatory response involved the activity of immune effector cells

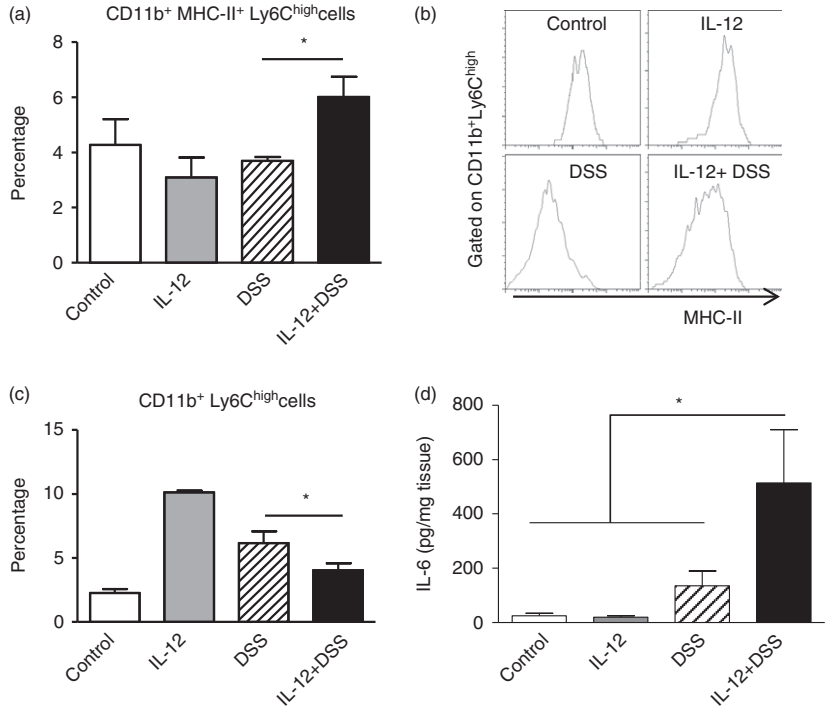

Figure 5. Phenotype and function of lamina propria (LP) subsets in interleukin-12 plus dextran sodium sulphate (IL-12+DSS) colitis. The C57BL/6 mice (6–8 weeks old) were given a hydrodynamic injection (h.i.) with the control or IL-12 cDNAs and at the same time administered 3% DSS. On day 5, mononuclear cells from LP (a,b) or blood (c) were analysed by flow cytometry. (a) Percentage of $CD11b^+$ MHC-II^+ $Ly6C^{hi}$ cells; (b) representative histograms of MHC-II expression in $CD11b^+$ $Ly6C^{hi}$ gated cells from different groups; (c) percentage of $CD11b^+$ $Ly6C^{hi}$ cells in blood; (d) IL-6 on supernatants of 24-hr colon explant cultures measured by ELISA. Data are mean ± SD of two different experiments with $n = 5$ per group. $^*P < 0{\cdot}05$.

and the production of mediators that are different from those typically involved in acute colitis. Furthermore, the robust and transient release of IL-12 endowed intestinal mononuclear cells with persistent inflammatory potential.

The combined IL-12+DSS treatment accelerated the clinical onset for 48 hr and the development of an inflammatory response with scarce polymorphonuclear infiltrate and reduced MPO activity, two features of acute DSS colitis.[4,5] Also, a decrease of MPO in colitis has been reported after sustained IL-12 administration.[13] This reduction may be related to the selective increment of IL-10 in serum, MLN and the LP, given that inhaled IL-10 weakens lung MPO activity, thereby attenuating the pulmonary but not the systemic inflammatory response in a haemorrhagic shock model.[27] Our findings highlight that systemic IL-12 promotes severe colon inflammation in acute DSS colitis with deficient recruitment of neutrophils. Previous reports have shown that inflammatory lesions in DSS colitis contain lymphocytes and macrophages, producing IL-12 and other mediators beyond neutrophils.[28] In this study, the simultaneous exposure to IL-12 and DSS enabled a predominant mononuclear infiltrate as early as day 3, and a significant entry of T-cell subsets and immature monocytes into the LP on day 5.

Up-regulation of pro-inflammatory cytokines in inflammatory bowel disease leads to cell traffic in an organ-specific manner in response to adhesion molecules, chemokines and chemokine receptors. Accordingly, $CD4^+$ T cells seem to take part in developing DSS-induced experimental colitis.[29] The localization within the intestinal mucosa is favoured by CCR5,[24] highly expressed in $CD4^+$ and $CD8^+$ subsets remaining in blood under our experimental conditions. Moreover, antibody depletion of $CD8^+$ T lymphocytes in acute DSS colitis in C57BL/6 mice lessens severity and reduces production of IFN-γ, TNF-α or IL-12 from LP cells.[30] In IFN-$\gamma^{-/-}$ mice, clinical as well as histological scores are attenuated in DSS colitis, which proves the indispensable role of Th1 cytokines in this model.[31] Yet, identical histological features observed in BALB/c and SCID (lacking T and B cells) mice led to the alternative aetiological hypothesis that the acute DSS model is a predominantly macrophage-driven colitis.[28,32] In fact, activated macrophages interacting with Th1 and Th2 cells seem to contribute to inflammation during the progression toward chronic forms of colitis.[32,33] Indeed, we believe that this could be the scenario recreated by systemic IL-12 in less than 4 days.

Circulating $Ly6C^{hi}$ monocytes can give rise to all subsets of macrophages present in healthy and inflamed colon. Upon entry into the LP, monocytes differentiate in a 'waterfall' sequence from macrophages that down-modulate (i.e. Ly6C) or up-regulate (i.e. MHC-II, CD64, F4/80) several markers.[33] The process is driven by the environment where the exceptional exposure of the epithelium to the commensal flora causes tonic sustained low-grade inflammation.[21,26,33] We found in this study that the systemic IL-12 burst affected LP replenishment in two ways: by speeding up the recruitment of $Ly6C^{hi}$ monocytes and stacking a particular pro-inflammatory subpopulation, as described in other experimental settings.[34] Indeed, on day 5 the $Ly6C^{hi}$ MHC-II^{hi} subset was most frequent in the LP of the IL-12+DSS group, possibly

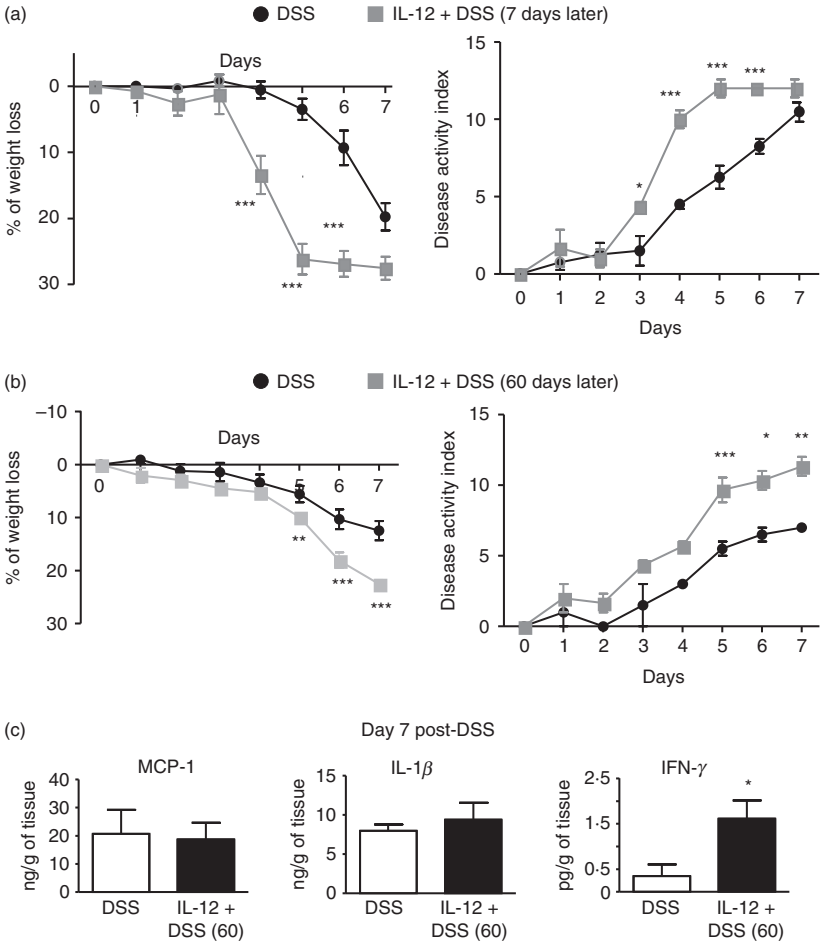

Figure 6. Persistent effect of systemic interleukin-12 (IL-12) priming in the development of dextran sodium sulphate (DSS) colitis. Mice were given a hydrodynamic injection (h.i.) with the control or IL-12 cDNAs; DSS administration started (a) 7 days or (b) 60 days after cDNA injection. Weight loss and the Disease Activity Index (DAI) were monitored daily; (c) monocyte chemoattractant protein 1 (MCP-1), IL-1β and interferon-γ (IFN-γ) were measured in supernatants of colon explant cultures after 7 days of DSS administration by ELISA. Data are mean $\pm$ SD of two different experiments with $n = 4$ or $n = 5$ mice per group. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$ versus DSS mice.

because the differentiation sequence started 24–48 hr earlier, according to previous reports.[35,36] In contrast, and at the same time, the LP of DSS mice displayed mostly Ly6C^{hi} MHC-II^{-} monocytes, i.e. newly arrived precursors. Selective accumulation of Ly6C^{hi} monocyte-like cells in both groups with colitis may be explained by the ongoing colon inflammation that disrupts differentiation from LP macrophages.[21,26,35] Consistently, an infiltrating pro-inflammatory Ly6C^{hi} MHC-II^{hi} subset producing IL-12, IL-23, IL-6, TNF-α and inducible nitric oxide synthase dramatically accumulates in the LP from colitic mice.[26,37] Still, early accumulation of Ly6C^{hi} MHC-II^{hi} equipped to interact with primed T cells could be a consequence of the sustained stimulation with IFN-γ in colonic LP.[34,38] Interestingly, when inflammation was limited to the DSS toxicity on the mucosal epithelium, the entry of this subset required at least 6 days. The systemic priming with IL-12, instead, dramatically accelerated the settlement of Ly6C^{hi} MHC-II^{hi} macrophage subpopulation by 3 days. The inflammatory characteristic of the predominant subset in IL-12+DSS mice could explain the robust IL-6 release, possibly elicited by luminal stimuli. In fact, as early as 3 days after IL-12 injection, paracellular permeability is altered, so favouring the supply of luminal stimuli that could support a premature and stronger inflammatory response.[16] Accordingly, Ly6C^{hi} monocytes display a robust inflammatory signature with the expression of inducible nitric oxide synthase and IL-6, and exhibit uncontrolled reactivity to bacterial products upon entering the inflamed intestinal tissue.[21,26] Considering that the decrease in colonic epithelial integrity even precedes histologically detectable colitis,[39] alteration in permeability in the IL-12+DSS-treated animals could even predate mononuclear recruitment.

Interestingly, these inflammatory macrophages cannot migrate to MLNs, thereby excluding their participation in the activation of naive T cells.[21] Due to their high MHC-II expression, however, they could potentially present antigens to resident or newly arrived T cells. In fact, incremented CD4^{+} and CD8^{+} T-cell subsets reaching the LP from IL-12+DSS mice could further enhance production of inflammatory mediators.[40] This subset could be intrinsically pro-inflammatory though, because Ly6C^{hi} MHC-II^{hi} cells sorted from the LP of wild-type mice induce a remarkable production of IFN-γ by OT II T cells compared with other macrophages and DCs.[21] If

this is the case, then the IL-12 or other IL-12-induced molecules in our model could be selectively expanding this subset. These findings illustrate the cooperative overlap of two inflammatory scenarios in which systemic IL-12 and local IL-6 orchestrate the setting of acute colitis with unique traits.

Interactions between DCs and lymphocytes condition the subsequent immune response because DCs not only expand antigen-specific T cells but also determine the localization of effector lymphocytes. In this study, the progressive increment of T cells bearing the $\alpha_4\beta_7$ integrin in MLN and LP could be linked to the higher frequency of $CD11c^+$ $CD103^+$ DCs in MLN, in agreement with the pivotal function of DCs in colonic inflammation. In fact, some authors claim that whereas neutrophils dominate the cellular infiltrate in the acute phase, monocytes/macrophages dominate the infiltrate in the resolution phase of colitis and that conventional DCs are pivotal for the cellular switch that regulates this type of colonic inflammation.[41] Our findings emphasize that the switch occurs faster under the influence of systemic IL-12.

In this work, after triggering a transient and huge release of cytokines, we demonstrate that IL-12 priming was long-lasting and favoured a worsened outcome of acute colitis even 8 weeks later. In agreement with this finding, T cells are susceptible to the stimulating effect of IL-12 in a very narrow temporal window and the priming for IFN-γ production can be maintained for several weeks.[37] Accordingly, explant cultures showed a significant release of IFN-γ 60 days after priming. Moreover, the role of IL-12 in maintaining colitis is more complex than in promoting IFN-γ production, acting also as a true growth stimulus for differentiated Th1 cells.[12] Colitogenic $CD4^+$ memory T cells, which are capable of reproducing colitis, continuously circulate through the body and are involved in the persistence of inflammatory bowel disease.[42] Although we have not addressed these possibilities, primed T cells in IL-12+DSS mice recruited in inductive and effector intestinal sites could be further expanded, so precipitating stronger Th1 responses upon re-stimulation.

In summary, we demonstrated that systemic IL-12 in normal hosts is able to switch the progress of colitis to a predominant mononuclear inflammation. Primed T cells bearing homing receptors, pro-inflammatory macrophages and luminal antigens assemble together in LP quickly and, in this environment, IL-12 and IL-6 become pivotal molecules. Cells instead of cytokines mediate a more severe colitis with a long-lasting effect. Our findings become increasingly relevant because IL-12 is considered to be the cytokine that can redirect developing Th17 or regulatory T lymphocytes into potentially harmful inflammatory Th1 effector cells.[43] Also, this model provides an opportunity to study the pathogenesis of this inflammatory disease and, more broadly, to understand the influence of systemic signals in mucosal surfaces.

Acknowledgements

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (FONCYT) [PICT 1216 2012 and PICT 1268 2013], Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET) and Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SECYT-UNC). The IL-12 plasmid was provided by Dr Howard Young (Frederick National Laboratory for Cancer Research, USA) and dextran sulphate sodium was a gift of George Usher, Dextran Products Limited (Canada). We would like to thank Paula Icely, Pilar Crespo, Paula Abadie, Luis Navarro and Fabricio Navarro for excellent technical assistance. We are indebted to Dr Howard Young (Frederick National Laboratory for Cancer Research, USA) for the IL-12 plasmid. MCRG and SGC belong to the CONICET research staff. L.P. Pedrotti, A.A. Sena and H. Cejas performed the experiments, A.A. Sena, M.C. Rodriguez Galan and S.G. Correa designed the study; A.A Sena and S.G. Correa wrote the paper.

Disclosures

The authors have no conflict of interest to declare.

References

1 Eksteen B, Liaskou E, Adams DH. Lymphocyte homing and its role in the pathogenesis of IBD. *Inflamm Bowel Dis* 2007; **14**:1298–312.
2 Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 2003; **3**:521–33.
3 Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* 2011; **474**:298–306.
4 Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr Protoc Immunol* 2014; **104**: Unit-15.25.
5 Perše M, Cerar A. Dextran sodium sulphate colitis mouse model: traps and tricks. *J Biomed Biotechnol* 2012; **2012**:718617.
6 Dieleman LA, Ridwan BU, Tennyson GS, Beagley KW, Bucy RP, Elson CO. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology* 1994; **107**:1643–52.
7 Jinushi M, Tahara H. Cytokine gene-mediated immunotherapy: current status and future perspectives. *Cancer Sci* 2009; **100**:1389–96.
8 Watford WT, Moriguchi M, Morinobu A, O'Shea JJ. The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Rev* 2003; **14**:361–8.
9 Car BD, Eng VM, Lipman JM, Anderson TD. The toxicology of interleukin-12: a review. *Toxicol Pathol* 1999; **27**:58–63.
10 Salem ML, Gillanders WE, Kadima AN, El-Naggar S, Rubinstein MP, Demcheva M, *et al.* Review: novel nonviral delivery approaches for interleukin-12 protein and gene systems: curbing toxicity and enhancing adjuvant activity. *J Interferon Cytokine Res* 2006; **26**:593–608.
11 Takagi H, Kanai T, Okazawa A, Kishi Y, Sato T, Takaishi H, *et al.* Contrasting action of IL-12 and IL-18 in the development of dextran sodium sulphate colitis in mice. *Scand J Gastroenterol* 2003; **38**:837–44.
12 Davidson NJ, Hudak SA, Lesley RE, Menon S, Leach MW, Rennick DM. IL-12, but not IFN-γ, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *J Immunol* 1998; **161**:3143–9.
13 Hans W, Schölmerich J, Gross V, Falk W. Interleukin-12 induced interferon-γ increases inflammation in acute dextran sulfate sodium induced colitis in mice. *Eur Cytokine Netw* 2000; **11**:67–74.
14 Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. *Annu Rev Immunol* 2010; **28**:573–621.

15 Chikano S, Sawada K, Shimoyama T, Kashiwamura SI, Sugihara A, Sekikawa K, *et al.* IL-18 and IL-12 induce intestinal inflammation and fatty liver in mice in an IFN-γ dependent manner. *Gut* 2000; **47**:779–86.
16 Pedrotti LP, Barrios BE, Maccio-Maretto L, Bento AF, Sena AA, Rodriguez-Galán MC, *et al.* Systemic IL-12 burst expands intestinal T-lymphocyte subsets bearing the α_4 β_7 integrin in mice. *Eur J Immunol* 2016; **46**:70–80.
17 Liu F, Song Y, Liu D. Hydrodynamic based transfection in animals by systemic administration of plasmid DNA. *Gene Ther* 1999; **6**:1258–66.
18 Rodriguez-Galan MC, Reynolds D, Correa SG, Iribarren P, Watanabe M, Young HA. Coexpression of IL-18 strongly attenuates IL-12-induced systemic toxicity through a rapid induction of IL-10 without affecting its antitumor capacity. *J Immunol* 2009; **183**:740–8.
19 Bento AF, Marcon R, Dutra RC, Claudino RF, Cola M, Leite DF, *et al.* β-Caryophyllene inhibits dextran sulfate sodium-induced colitis in mice through CB2 receptor activation and PPARγ pathway. *Am J Pathol* 2011; **178**:1153–66.
20 Hoff J. Methods of blood collection in the mouse. *Lab Anim* 2000; **29**:47–53.
21 Geboes K, Riddell R, Ost A, Jensfelt B, Persson T, Löfberg R. A reproducible grading scale for histological assessment of inflammation in ulcerative colitis. *Gut* 2000; **47**:404–9.
22 Sena AA, Pedrotti LP, Barrios BE, Cejas H, Balderramo D, Diller A, *et al.* Lack of TNFRI signaling enhances annexin A1 biological activity in intestinal inflammation. *Biochem Pharmacol* 2015; **98**:422–31.
23 Rivollier A, He J, Kole A, Valatas V, Kelsall BL. Inflammation switches the differentiation program of Ly6C^{hi} monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. *J Exp Med* 2012; **9**:139–55.
24 Agace WW, Roberts AI, Wu L, Greineder C, Ebert EC, Parker CM. Human intestinal LP and intraepithelial lymphocytes express receptors specific for chemokines induced by inflammation. *Eur J Immunol* 2000; **30**:819–26.
25 Annacker O, Coombes JL, Malmstrom V, Uhlig HH, Bourne T, Johansson-Lindbom B, *et al.* Essential role for CD103 in the T cell-mediated regulation of experimental colitis. *J Exp Med* 2005; **202**:1051–61.
26 Zigmond E, Varol C, Farache J, Elmaliah E, Satpathy AT, Friedlander G, *et al.* Ly6C hi monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen-presenting cells. *Immunity* 2012; **37**:1076–90.
27 Kobbe P, Lichte P, Schreiber H, Reiss LK, Uhlig S, Pape HC, *et al.* Inhalative IL-10 attenuates pulmonary inflammation following hemorrhagic shock without major alterations of the systemic inflammatory response. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**:G770–7.
28 Egger B, Bajaj-Elliott M, MacDonald TT, Inglin R, Eysselein VE, Büchler MW. Characterization of acute murine dextran sodium sulphate colitis: cytokine profile and dose dependency. *Digestion* 2000; **62**:240–8.
29 Shintani N, Nakajima T, Okamoto T, Kondo T, Nakamura N, Mayumi T. Involvement of CD4$^+$ T cells in the development of dextran sulfate sodium induced experimental colitis and suppressive effect of IgG on their action. *Gen Pharmacol* 1998; **31**:477–81.
30 Yoshihara K, Yajima T, Kubo C, Yoshikai Y. Role of interleukin 15 in colitis induced by dextran sulphate sodium in mice. *Gut* 2006; **55**:334–41.
31 Ito R, Shin-Ya M, Kishida T, Urano A, Takada R, Sakagami J, *et al.* Interferon-γ is causatively involved in experimental inflammatory bowel disease in mice. *Clin Exp Immunol* 2006; **146**:330–8.
32 Bai A, Yong M, Ma AG, Ma Y, Weiss CR, Guan Q, *et al.* Novel anti-inflammatory action of 5-aminoimidazole-4-carboxamide ribonucleoside with protective effect in dextran sulfate sodium-induced acute and chronic colitis. *J Pharmacol Exp Ther* 2010; **333**:717–25.
33 Dieleman LA, Palmen MJ, Akol H, Bloemena E, Peña AS, Meuwissen SG, *et al.* Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. *Clin Exp Immunol* 1998; **14**:385–91.
34 Sindrilaru A, Peters T, Wieschalka S, Baican C, Baican A, Peter HS, *et al.* An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. *J Clin Invest* 2011; **121**:985–97.
35 Tamoutounour S, Henri S, Lelouard H, de Bovis B, de Haar C, van der Woude CJ, *et al.* CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *Eur J Immunol* 2012; **42**:3150–66.
36 Bain CC, Scott CL, Uronen-Hansson H, Gudjonsson S, Jansson O, Gripp O *et al.* Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6C^{hi} monocyte precursors. *Mucosal Immunol* 2013; **6**:498–510.
37 Gerosa F, Paganin C, Peritt D, Paiola F, Scupoli MT, Aste-Amezaga M, *et al.* Interleukin-12 primes human CD4 and CD8 T cell clones for high production of both interferon-γ and interleukin-10. *J Exp Med* 1996; **183**:2559–69.
38 Sica A, Mantovani A. Macrophage plasticity and polarization: *in vivo veritas*. *J Clin Invest* 2012; **122**:787–95.
39 Garrett WS, Lord GM, Punit S, Lugo-Villarino G, Mazmanian SK, Ito S, *et al.* Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell* 2007; **131**:33–45.
40 Takeda K, Clausen BE, Kaisho T, Tsujimura T, Terada N, Förster I, *et al.* Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* 1999; **10**:39–49.
41 Abe K, Nguyen KP, Fine SD, Mo JH, Shen C, Shenouda S, *et al.* Conventional dendritic cells regulate the outcome of colonic inflammation independently of T cells. *Proc Natl Acad Sci USA* 2007; **104**:17022–7.
42 Nemoto Y, Kanai T, Kameyama K, Shinohara T, Sakamoto N, Totsuka T, *et al.* Long-lived colitogenic CD4$^+$ memory T cells residing outside the intestine participate in the perpetuation of chronic colitis. *J Immunol* 2009; **183**:5059–68.
43 Prochazkova J, Pokorna K, Holan V. IL-12 inhibits the TGF-β-dependent T cell developmental programs and skews the TGF-β-induced differentiation into a Th1-like direction. *Immunobiology* 2012; **217**:74–82.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Histopathological analyses.

Figure S2. Cytokines in ongoing acute colitis.

Figure S3. Primed cells mediate exacerbation of acute dextran sodium sulphate colitis after cytokine burst.

Figure S4. Histopathological damage in colon after persistent effect of systemic interleukin-12.

Table S1. Pedrotti *et al.*