 DOI: 10.1002/eji.201545585 Eur. J. Immunol. 2016. 46: 70–80

European Journal of Immunology

Systemic IL-12 burst expands intestinal T-lymphocyte subsets bearing the $\alpha_4\beta_7$ integrin in mice

Luciano P. Pedrotti[1], Bibiana E. Barrios[1], Lisa Maccio-Maretto[1], Allisson F. Bento[2], Angela A. Sena[1], María Cecilia Rodriguez-Galán[1], João B. Calixto[2] and Silvia G. Correa[1]

[1] Immunology, Department of Clinical Biochemistry, CIBICI (CONICET), Faculty of Chemical Sciences, National University of Córdoba, Córdoba, Argentina
[2] Department of Pharmacology, Centre of Biological Sciences, Universidade Federal de Santa Catarina, Florianopolis, Santa Catarina, Brasil

The intestinal immune system is complex and displays unique anatomic and functional characteristics. Numerous immune cell subsets are located beneath the epithelial barrier and their activity is highly regulated. Using hydrodynamic shear of IL-12 cDNA to achieve systemic expression of IL-12 in mice, we evaluated the effect of a transient burst of this cytokine on the activation status of T cells from Peyer's patches (PPs), mesenteric lymph nodes (MLNs), and colonic lamina propria (LP). Following systemic IL-12 release, intestinal T lymphocytes became activated, exhibiting a $CD44^{high}$ $CD62L^{-}$ phenotype. After 5 days of the cytokine burst, the frequency of $\alpha4\beta7^{+}$ $CD4^{+}$ and $CD8^{+}$ cells increased, and $CD8^{+}\alpha4\beta7^{+}$ cells mainly expressed T bet, a critical regulator of the Th1 differentiation program. The incremental increase in α4β7 expression involved the IL-12 receptor-signal transducer and activator of transcription (STAT)-4 axis, and occurred independently of IFN-γ, IL-4, IL-10, and TNF-α signaling. Moreover, IL-12 priming exacerbated the outcome of acute dextran sodium sulphate (DSS)-induced colitis with higher scores of weight loss, blood in stool, and diarrhea and lower hematocrit. Together, our findings demonstrate that systemic polarizing signals could effectively expand the number of effector cells able to home to the LP and contribute to local inflammation.

Keywords: $\alpha_4\beta_7$ · Colitis · Gut · Homing receptor · Systemic IL-12 · T cell

Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

The intestinal immune system is complex and displays unique anatomic and functional characteristics. The dynamic interplay between epithelium, microbes, and local immune cells is essential to maintain the intestinal homeostasis and build up protective immunity [1]. Heterogeneous subsets of immune cells reside beneath the single epithelial barrier and their activity is highly regulated [2–4]. Accordingly, the cytokine milieu [5] and the activity of gut epithelium [6] and intestinal DCs [7] contribute to regulate mucosal immune responses [8]. Lymphocytes activated in intestinal inductive mucosal sites such as MLNs upregulate homing receptors as the chemokine receptor CCR9 and the α4β7 integrin [9, 10], which direct the homing to gut effector sites [11]. Both extrinsic and intrinsic cell factors control levels of the α4β7 integrin suggesting that its expression involves the integration of a variety of signals [10].

IL-12 plays a central role in the induction of Th1 inflammation regardless of the initiating event or target organ [12]. This cytokine can skew an already ongoing T regulatory or

Correspondence: Dr. Silvia G. Correa
e-mail: scorrea@fcq.unc.edu.ar

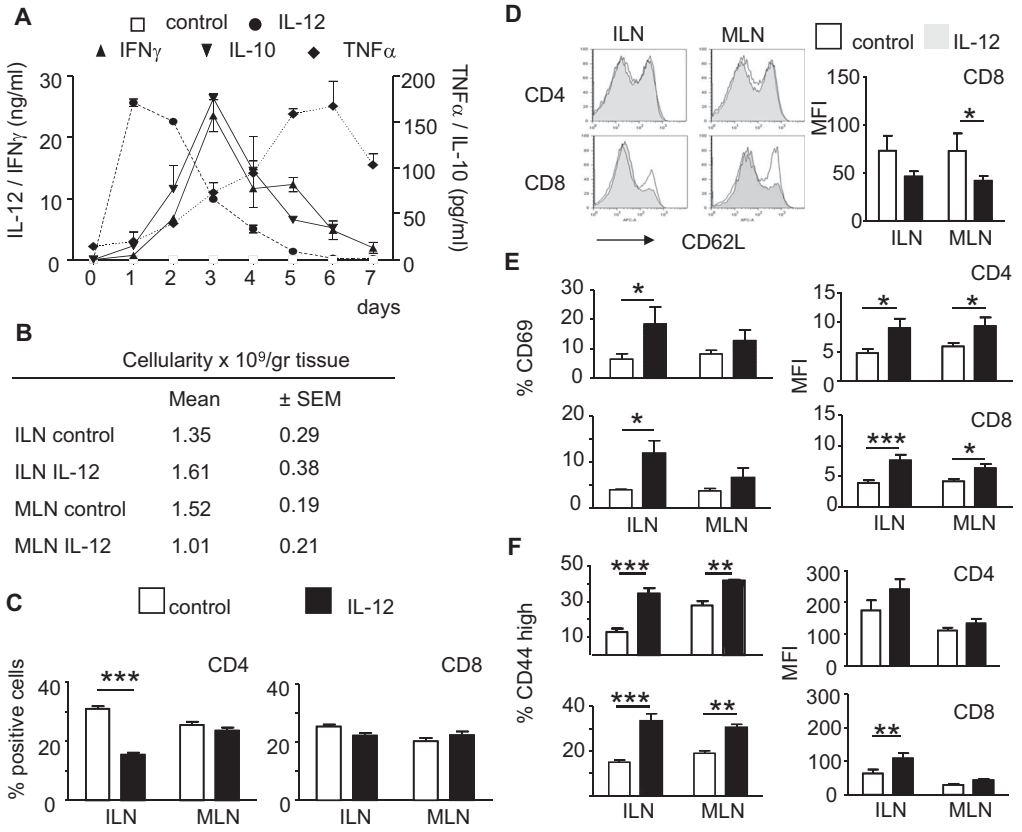

Figure 1. Systemic cytokines and phenotype of ILN and MLN lymphocytes after h.i. of IL-12 cDNA. C57BL/6 mice (6–8-week-old) were hydrodynamically injected with 0.5 μg of control or IL-12 cDNA. Animals were bled daily for 1 week postinjection. (A) Sera were assayed for IL-12, IFN-γ, IL-10, and TNF-α levels by ELISA; IL-12, IFN-γ, IL-10, and TNF-α serum levels in control mice. (B) Cellularity per gram of tissue in ILNs and MLNs from control or IL-12-injected groups. (C) On day 7, single-cell suspensions from ILNs and MLNs were prepared and stained with fluorochrome-labeled antibodies for flow cytometry analysis. The percentage of $CD4^+$ and $CD8^+$ lymphocytes in ILNs and MLNs are shown. (D–F) The expression of activation markers (D) CD62L, (E) CD69, and (F) CD44 was evaluated on gated $CD4^+$ and $CD8^+$ cells from ILNs and MLNs; control (open) or IL-12-injected (shaded);(D) Representative histograms of CD62L from mice and geometric mean fluorescence intensity (MFI) of CD62L expression in $CD8^+$ cells are shown. Results are shown as mean ± SEM of data pooled from three experiments with $n = 5$ per group in each experiment. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (One-way analysis of variance followed by a Student–Newman–Keuls post-test.)

Th17 developmental program into Th1-like direction [13]. Repeated and elevated doses of IL-12 alone or combined with other cytokines produce detrimental effects with prominent mucosal damage in Balb/c mice [14]. Recently, we triggered systemic expression of IL-12 by using hydrodynamic shear of IL-12 cDNA [15]. In this experimental model, IL-12 is expressed mainly in the liver and efficiently polarizes the spleen immune response toward a Th1 profile [15, 16]. As the effect of systemic release of IL-12 on intestinal immunity is not completely understood, we used the hydrodynamic injection (h.i.) to provoke a transient and robust IL-12 release before studying the activation status of intestinal lymphocytes. Following systemic IL-12 production, $CD8^+$ T lymphocytes from Peyer's patches (PPs) and MLNs became activated upregulating the α4β7 integrin. The cytokine burst increased the frequency of $\alpha4\beta7^+$ $CD4^+$ and $CD8^+$ cells expressing the transcription factor T bet, a critical regulator of the Th1 differentiation program. The increment of this homing molecule involved the IL-12 receptor-STAT-4 axis and was independent of IFN-γ, IL-4, IL-10, and TNF-α signals. Moreover, the IL-12 priming exacerbated the outcome of acute dextran sodium sulphate (DSS)-colitis modifying the effector response triggered by the luminal stimuli. Together, our findings demonstrate that systemic signals could expand effector cells able to recirculate to lamina propria (LP) and contribute to the outcome of a polarized inflammatory response.

Results

Systemic release of IL-12 primes local MLN T-cell subsets

We elicited the acute release of IL-12 by h.i. of IL-12 cDNA (working dose: 0.5 μg/mouse) in C57BL per 6 mice (Supporting Information Fig. 1) and we measured cytokine levels in serum samples every other day for a week. The treatment produced a sharp rise in IL-12 in the first 24 h (Fig. 1A) as well as a marked release of

other cytokines such as IFN-γ/ IL-10 and TNF-α that peaked on days 3 or 6 after injection, respectively. After 7 days, cytokines returned to control values; at that time the cellularity per gram of tissue of MLNs and inguinal lymph nodes (ILNs) included here for comparative purposes, was similar (Fig. 1B). In MLNs, the percentage of $CD4^+$ and $CD8^+$ T lymphocytes was unmodified by the systemic release of IL-12. Yet, the treatment diminished the proportion of $CD4^+$ lymphocytes in ILNs (Fig. 1C). Flow cytometry analysis showed reduced levels of L-selectin (CD62L) mainly in MLN $CD8^+$ lymphocytes (Fig. 1D). The percentage of $CD4^+$ and $CD8^+$ T cells expressing the activation marker CD69 was higher only in ILNs, although the treatment increased the fluorescence intensity in $CD4^+$ and $CD8^+$ lymphocytes from both ILNs and MLNs (Fig. 1E). The frequency of $CD4^+$ and $CD8^+$ T cells $CD44^{high}$ augmented both in ILNs and MLNs; still only ILN $CD4^+$ cells showed higher fluorescence intensity (Fig. 1F). MLN cell suspensions reacted promptly to TCR stimulation with a significant release of IFN-γ in 24-h cultures, compared with the negligible production of control group (data not shown). Together, the transient cytokine burst triggered by systemic IL-12 effectively activated ILN as well as MLN lymphocytes.

Systemic IL-12 release primes intestinal T-cell subsets to upregulate α4β7 integrin expression

Upon activation, mucosal lymphocytes acquire homing receptors to recirculate to the LP [9, 10]. Then, we evaluated the expression of α4β7 integrin in $CD4^+$ and $CD8^+$ T cells following the gating strategy shown in Supporting Information Fig. 2. Seven days after the h.i. of IL-12 cDNA the percentage of MLN $\alpha4\beta7^+$ lymphocytes increased significantly (Fig. 2A); yet, compared with the ~threefold rise of $CD8^+$ $integrin^+$ T cells, less $CD4^+$ lymphocytes became $\alpha4\beta7^+$. Nevertheless the integrin expression, evaluated as MFI, was higher both in $CD4^+$ and $CD8^+$ T lymphocytes (Fig. 2B). Interestingly, the frequency of $\alpha4\beta7^+$ T-cell subsets remained unmodified in ILNs even when $CD4^+$ and $CD8^+$ cells exhibited activation markers (Fig. 1D–F), suggesting a tissue-specific increment of the integrin. No changes were observed in B lymphocytes compared with control group (data not shown).

In other gut mucosal inductive site as PPs, the systemic IL-12 enlarged the percentage of CD4 and CD8 T subsets 7 days after h.i. (Fig. 2C) with a significantly higher frequency of $CD8^+$ $\alpha4\beta7^+$ lymphocytes. In additional experiments we confirmed that $CD8^+$ $\alpha4\beta7^+$ cells were $CD3^+$ lymphocytes (data not shown). Both $CD4^+$ (not shown) and $CD8^+$ $\alpha4\beta7^+$ MLN lymphocytes from IL-12-injected mice exhibited the activation phenotype $CD44^{high}$ $CD62L^{low}$ (Fig. 3A and B), with a low expression of CD69, possibly indicating that these cells were ready to exit the MLNs. As the IL-12 cDNA plasmid treatment triggers a Th1 bias in spleen or peripheral lymph nodes [15], we analyzed levels of the transcription factor T bet, a critical regulator of the Th1 differentiation program in gated $\alpha4\beta7^+$ $CD4^+$ and $CD8^+$ T cells. A significant rise in the frequency of $\alpha4\beta7^+$ $T\text{-}bet^+$ T lymphocytes occurred 7 days after IL-12 cDNA injection with a stronger effect in the $CD8^+$ compartment,

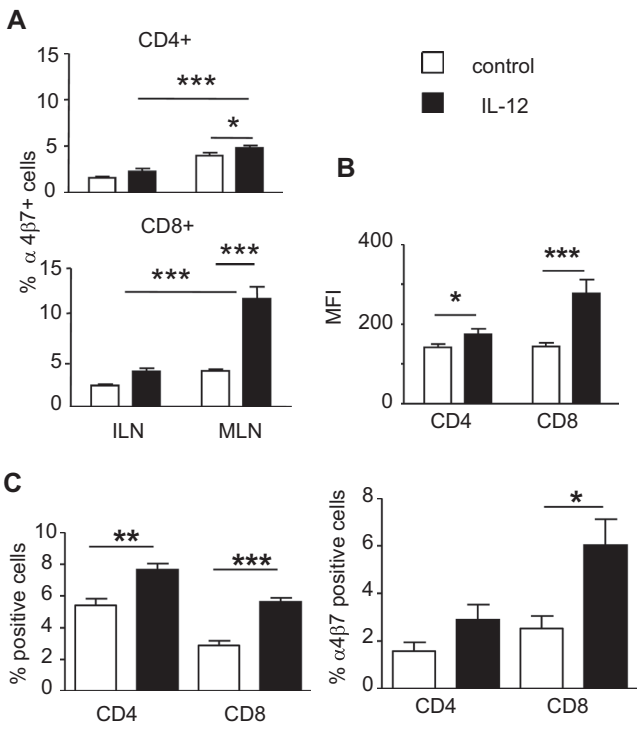

Figure 2. Expression of the α4β7 integrin in T-cell subsets upon systemic IL-12 release. Single-cell suspensions from ILNs, MLNs, and PPs were obtained on day 7 after IL-12 or control cDNA injection and stained with fluorochrome-labeled antibodies for flow cytometry analysis. (A) The percentage of $CD4^+$ and $CD8^+$ $\alpha4\beta7^+$ lymphocytes from ILNs and MLNs is shown. (B) The MFI of α4β7 expression in $CD4^+$ and $CD8^+$ MLN cells is shown. (C) The percentage of $CD4^+$ and $CD8^+$ subsets and $CD4^+$ and $CD8^+$ $\alpha4\beta7^+$ lymphocytes in PPs from control and IL-12-injected mice is shown. Results are shown as mean ± SEM of data pooled from three (A–C) or two (D) experiments with $n = 5$ per group in each experiment. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (One-way analysis of variance followed by a Student–Newman–Keuls post-test.)

suggesting the higher susceptibility of this subset to the IL-12 priming (Fig. 3C and D). Interestingly, the cytokine burst was also able to increase the frequency of $CD4^+$ cells expressing Foxp3, a master key gene for regulatory activity of Treg cells both in MLNs and PPs (Supporting Information Fig. 3). Yet, MLN and PP $CD4^+$ $Foxp3^+$ cells were α4β7 integrin negative (data not shown). Together, following the systemic cytokine burst, $CD8^+$ (from MLNs and PPs) and $CD4^+$ T cells (from MLNs) became activated and expressed differentiation markers and homing molecules that could favor the recirculation to LP.

We found that $CD4^+$ and $CD8^+$ lymphocytes isolated from colon LP exhibited the $CD44^+$ $\alpha4\beta7^+$ phenotype both in control and IL-12 groups (Supporting Information Fig. 4A), according to the fact that $CD4^+$ and $CD8^+$ T cells in human and rodent intestines display an effector memory phenotype [17]. Still, upon the cytokine storm the frequency of effector cells was higher, supporting the hypothesis that IL-12 primed cells could migrate to LP. To assess whether cells from LP could also exhibit the Th1 inflammatory profile observed in MLNs, 7 days after h.i. we isolated cells from colon LP and we evaluated the spontaneous release of several cytokines by a cytometric bead array. In IL-12-injected mice, the

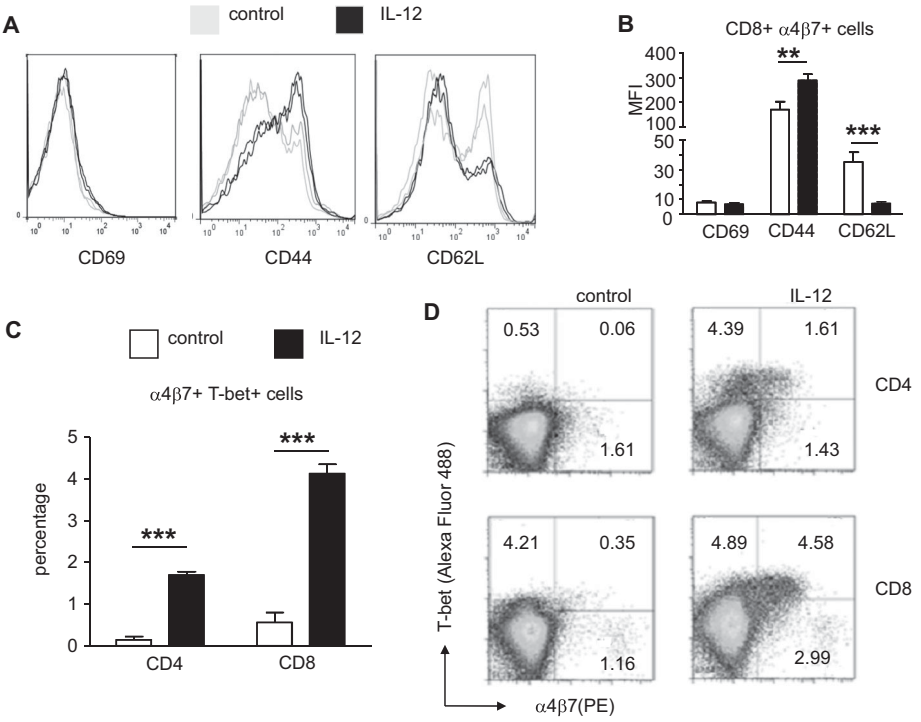

Figure 3. Expression of T bet in CD8$^+$ α4β7$^+$ cells from MLNs after systemic burst of IL-12. Single-cell suspensions from MLNs were obtained on day 7 after injection of 0.5 μg control cDNA or 0.5 μg IL-12 cDNA and stained with fluorochrome-labeled antibodies for flow cytometry analysis. (A) Representative histograms of CD69, CD44, and CD62L expression in CD8$^+$ α4β7$^+$ cells from two control (light gray) or two IL-12-injected (dark gray) mice. (B) MFI of activation markers CD69, CD44, and CD62L on gated CD8$^+$ α4β7$^+$ cells. (C) Percentage of CD4$^+$α4β7$^+$T-bet$^+$ and CD8$^+$α4β7$^+$T-bet$^+$ cells. (D) Representative dot plots of the expression of T bet in CD4$^+$ and CD8$^+$ α4β7$^+$ MLN T cells. Results are shown as mean ± SEM of data pooled from three experiments with $n = 5$ per group in each experiment. $^{**}p < 0.01$, $^{***}p < 0.001$. (One-way analysis of variance followed by a Student–Newman–Keuls post-test.)

release of IFN-γ and IL-10 was significant (Supporting Information Fig. 4B and C) while the production of IL-17A was similar to control group. This finding was confirmed with a standard ELISA, suggesting that the h.i. of IL-12 cDNA was not eliciting a Th17 profile in LP cells (Supporting Information Fig. 4D).

Signals involved in the upregulation of α4β7 on intestinal T cells

We hypothesized that the h.i. injection could be selectively expanding α4β7$^+$ lymphocytes specific for luminal antigens. To address this possibility, we injected the IL-12 cDNA plasmid in OT-I transgenic mice with CD8$^+$ T cells bearing a TCR ovalbumin-specific. As can be seen, h.i. mice also showed higher frequency of CD8$^+$ α4β7$^+$ cells in MLNs (Fig. 4A), suggesting that expanded α4β7$^+$ T subsets are not necessarily specific for luminal antigens. Still, an enhanced supply of ligands could sustain the activation status of the primed lymphocytes entering the LP. To check this possibility, we evaluated changes in intestinal permeability by feeding FITC-dextran. On day 7 after h.i. paracellular permeability was significantly higher in IL-12 mice and the alteration was evident already on day 3 postinjection (Supporting Information Fig. 5A), when the inflammatory wave is peaking (Fig. 1A).

As the cytokine storm lasted around a week, we evaluated the increment of integrin$^+$ CD8$^+$ cells in MLNs from wild type (WT) mice on days 1, 3, 5, and 7 after IL-12 cDNA injection. The CD8$^+$ α4β7$^+$ cells started to augment by day 5 reaching a three times higher frequency at the end of the week (Fig. 4B). Considering that large amounts of other cytokines had already been released at that time (Fig. 1A), we wondered if other signals beyond IL-12 could be also stimulating the expression of the homing molecule. To address this question we performed h.i. in WT or IL-4, IL-10, IFN-γ, and TNF-R1 knockout mice, and 7 days later we evaluated the percentage of α4β7$^+$ T lymphocytes in MLNs. In all mouse strains studied, the frequency of both CD4$^+$ (not shown) and CD8$^+$ α4β7$^+$ cells increased upon IL-12 release, independent of the cytokine deficiency (Fig. 4C). Interestingly, in basal condition, IL-10ko control mice had the higher percentage of α4β7$^+$ T cells and this strain experienced the greatest amplification of this subset upon IL-12 injection, suggesting that IL-10 could be tuning the levels of this homing receptor.

IL-12 receptor-STAT-4 axis mediates α4β7$^+$ induction in primed MLN T cells

To verify the involvement of IL-12 in the expansion of CD8$^+$ α4β7$^+$ T cells we used different strategies. First, we studied the expression of α4β7 integrin in MLN CD8$^+$ cells after 7 days of IL-12 cDNA h.i. in IL-12ko mice. Even when the frequency of CD8$^+$ α4β7$^+$ T cells in control IL-12ko was around 25% of untreated WT mice (see Figs. 2A and 5A), the single exposure to the cytokine expanded this subset three times, as observed with WT mice. Second, we stimulated isolated MLN cells from control and IL-12 cDNA injected WT mice for only 24 h with rIL-12 or anti-CD3/anti-CD28 to assess the frequency of CD8$^+$ α4β7$^+$ lymphocytes (Fig. 5B). Remarkably, the 24-h stimulation of unprimed control cells with rIL-12 was unable to upregulate the homing molecule, highlighting the

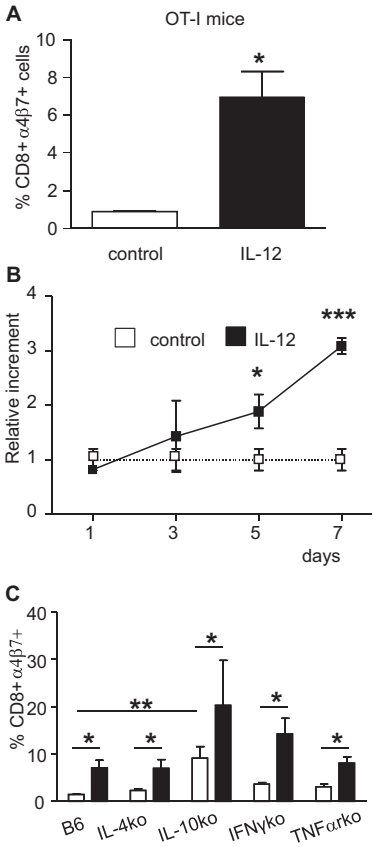

Figure 4. Kinetic and signals involved in α4β7 expression. (A) MLNs were isolated from transgenic OT-I mice bearing an ovalbumin-specific and MHC class I-restricted TCR and the percentage of $CD8^+$ $\alpha4\beta7^+$ cells was determined by flow cytometry. (B) α4β7 Expression in gated MLN $CD8^+$ cells from WT mice obtained on days 1, 3, 5, and 7 after control or IL-12 cDNA injection is shown. Data from IL-12-treated mice are expressed as an increment relative to control group. (C) The frequency of $CD8^+$ $\alpha4\beta7^+$ cells in MLNs of C57BL/6, IL-4ko, IL-10ko, IFN-γko, and TNF-R1ko mouse strains 7 days after control or IL-12 cDNA injection was determined by flow cytometry. Results are shown as mean ± SEM of data pooled from two experiments with $n = 5$ per group in each experiment. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (One-way analysis of variance followed by a Student–Newman–Keuls post-test.)

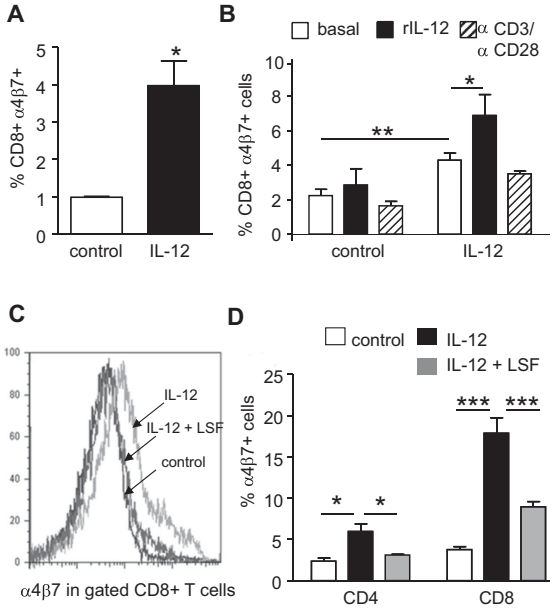

Figure 5. The IL-12R-STAT-4 axis is involved in α4β7 integrin induction on MLN T cells. (A) IL-12ko mice were injected with control or IL-12 cDNA and on day 7 the percentage of $CD8^+$ $\alpha4\beta7^+$ MLN cells was evaluated by flow cytometry. (B) C57BL/6 mice injected with control or IL-12 cDNA and on day 7 were restimulated for 24 h with medium (basal), 50 ng/mL rIL-12 or 2 μg/mL anti-CD3/1 μg/mL anti-CD28 antibodies. The percentage of $CD8^+$ $\alpha4\beta7^+$ MLN cells is shown. (C) C57BL/6 mice were injected with control cDNA, IL-12 cDNA, or IL-12 cDNA and LSF and 7 days later α4β7 expression in gated MLN $CD8^+$T cells was evaluated by flow cytometry. Representative histograms are shown. (D) The percentage of α4β7 expression in $CD4^+$ and $CD8^+$ MLN subsets in control or IL-12 cDNA-injected groups, with or without LSF treatment. Results are shown as mean ± SEM of data pooled from two experiments with $n =$ 4–5 mice per group in each experiment. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (One-way analysis of variance followed by a Student–Newman–Keuls post-test.)

need of previous in vivo IL-12 priming. However, IL-12-injected mice experienced an additional increment of $CD8^+$ $\alpha4\beta7^+$ cells upon rIL-12 stimulus, confirming that the observed effect is due to the ability of the cytokine to elicit the expression of the homing molecule. Interestingly, after TCR stimulation the percentage of $\alpha4\beta7^+$ cells remained unmodified, showing that this pathway is not implicated in the integrin increment (Fig. 5B). To confirm that the IL-12 signaling was involved in the integrin expression we used lisofylline (LSF), a synthetic modified methylxanthine able to block IL-12 signaling and STAT-4 activation in target cells and tissues [18]. Mice received six doses of LSF starting the same day of IL-12 cDNA injection and 7 days later, the expression of the integrin was evaluated in MLN T cells. As can be seen (Fig. 5C and D), LSF-treated animals had significantly reduced $CD4^+$ and $CD8^+$ $\alpha4\beta7^+$ cell percentage, showing the dependence of the IL-12-STAT-4 pathway in the higher frequency of integrin$^+$ subsets. Together, the signaling through the IL-12 receptor was the canonical pathway involved in the upregulation of this homing molecule in mucosal lymphoid tissue.

Effect of systemic IL-12 expression in acute colitis

As the activated status of intestinal lymphocytes could influence any inflammatory process at the intestinal mucosa, in additional experiments we started the oral administration of DSS the same day of h.i. Already on day 3 the combined treatment exacerbated weight loss (Fig. 6A) and produced higher disease activity index (DAI; Fig. 6B) and higher proportion of blood in feces (Fig. 6C). Compared with control mice, both DSS and IL-12 + DSS groups showed similar colon length reduction (Supporting Information Fig. 6A and B). The myeloperoxidase (MPO) activity, a marker of neutrophil infiltration, increased in colonic samples of DSS group on day 7 (Fig. 6D); remarkably, MPO activity in IL-12 + DSS group was similar to healthy controls. Histopathological damage in DSS and IL-12 + DSS groups included superficial and continuous inflammation, marked leukocyte infiltration and intense

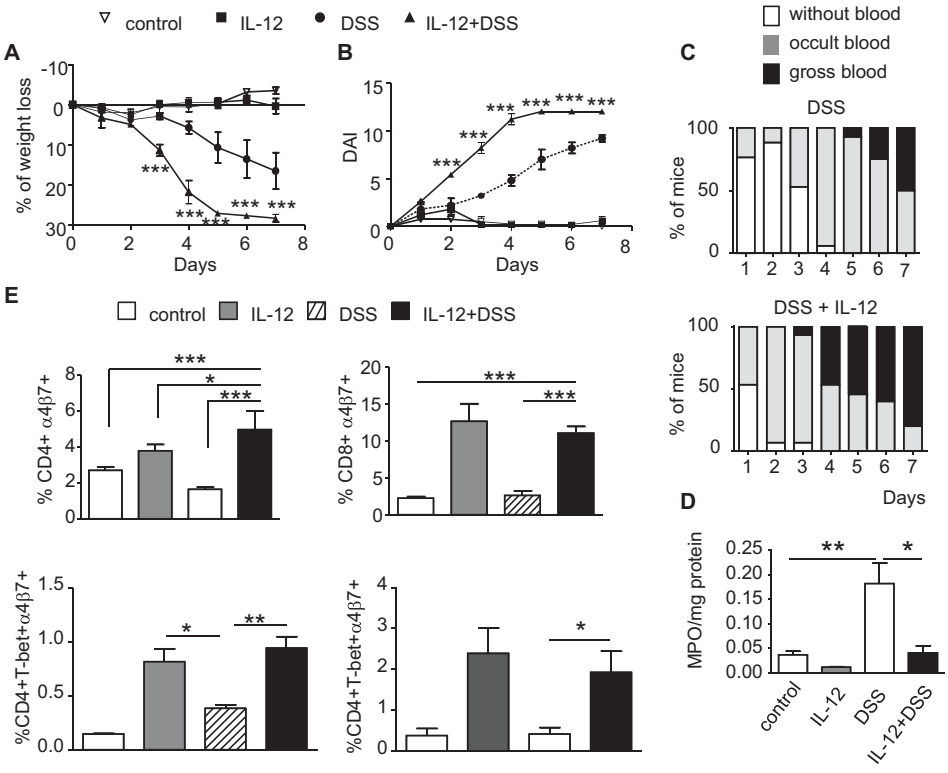

Figure 6. $\alpha4\beta7^+$ T-bet$^+$ T-cell subsets in DSS-induced colitis upon systemic IL-12 release. C57BL/6 mice (6–8-week-old) injected with control or IL-12 cDNAs received 3% DSS for 5 days and drinking water for the next 2 days. (A) Body weight loss over time was measured. (B) DAI is shown. (C) The percentage of mice without blood, with occult blood or gross blood in feces is shown. (D) MPO activity in colon homogenates was evaluated by colorimetric assay. (E) The percentage of $\alpha4\beta7^+$ CD4$^+$ or CD8$^+$ (top) and $\alpha4\beta7^+$T-bet$^+$ CD4$^+$ or CD8$^+$ (bottom) MLN cells was evaluated by flow cytometry. Results are shown as mean ± SEM of data pooled from two experiments with five mice per group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (One-way analysis of variance followed by a Student–Newman–Keuls post-test.)

submucosal edema while control and IL-12 cDNA injected groups showed intact architecture (Supporting Information Fig. 6C). Both groups exposed to systemic IL-12 had higher frequency of $\alpha4\beta7^+$ T cells in MLNs, in marked contrast with mice receiving only DSS (Fig. 6E). Moreover, integrin$^+$ T-bet$^+$ T subsets that could be relevant for the inflammatory outcome of colitis were significantly increased in MLN of mice exposed to both stimuli but absent in DSS group (Fig. 6D). Levels of the prototypical Th1 cytokine IFN-γ augmented in colon explant cultures after 5 days of treatment, while other inflammatory mediator such as MCP-1 was similar in DSS and IL-12 + DSS groups (Supporting Information Fig. 6D) and colon LP isolated cells from IL-12 + DSS mice exhibited a marked spontaneous release of IFN-γ and TNF-α cytokines evaluated by cytometric bead array (Supporting Information Fig. 6E) Together, the IL-12 priming exacerbated the clinical manifestations of acute colitis. The higher frequency of integrin$^+$ T-bet$^+$ T subsets and the Th1 cytokine detected in LP support the contribution of these subsets to the severity of the inflammatory response.

Discussion

We demonstrated here that the systemic and transient release of IL-12 increases the frequency of activated PP and MLN T cells equipped to migrate to LP. After 7 days of IL-12 plasmid injection CD4$^+$ and CD8$^+$ T lymphocytes exhibited phenotypic and functional traits of activated cells expressing high levels of $\alpha4\beta7$ and T bet as well as a bias to a Th1 cytokine profile. In agreement, using hydrodynamic shear of IL-12 cDNA to study antitumoral responses we found that the inflammatory cytokine provoked changes in the number of leukocyte subsets in spleen and the expression of activation markers in CD4$^+$ and CD8$^+$ lymphocytes [15]. The cytokine storm seems to produce an overall redistribution of lymphocytes that could lead to different outcomes, as the reduction in ILN CD4$^+$ subset observed here. Accordingly, after h.i. of IL-12 cDNA, among other experimental conditions tested, we found that peripheral B and T cells reenter the thymus. The phenomenon occurs in the absence of any antigenic stimulation and seems to be part of bystander activation of certain peripheral mature B and T cells [19].

Distinct secondary lymphoid organ microenvironments support the generation of effector cells with differential properties, including homing capacities [6, 20]. Differences between ILN and MLN cells in $\alpha4\beta7$ integrin expression suggest that systemic cytokine effect is tuned inside each particular lymphoid environment. In fact, upon activation the lymphoid compartment conditions the kinetic, level, and type of integrin expression [21]. Consistently, the majority of activated T cells present in MLNs express $\alpha4\beta7$ integrin [11, 20], and in vivo the net expression of gut homing receptors in primed T cells seems to depend on the route of antigen entry, the presence or absence of adjuvant, and the antigen dose [22]. As the microenvironment controls not only the proportion of cells that ultimately express the integrin but also its levels on individual cells [21], inflammatory signals in IL-12 cDNA injected mice could favor the higher expression of the integrin in CD4$^+$ and CD8$^+$ MLN cells. Importantly, systemic IL-12 displayed a stronger effect on the expression of $\alpha4\beta7$ and T bet

on the $CD8^+$ T subset. In agreement, the induction of gut homing receptors in vivo is more efficient on $CD8^+$- compared with $CD4^+$-primed T cells [22].

Changes observed in PP and MLN cells could mirror systemic activation triggered by IL-12, as the cocktail of cytokines present at the time of T-cell priming greatly influences the subsequent profile of the effector cells [23]. This observation is in keeping with the finding that increased levels of transcripts for T bet appear within 72 h after stimulation of T cells under Th1-inducing conditions [24]. Also, the expression of adhesion molecules can become clonally imprinted under certain conditions in the presence of IL-12, involving T-bet dependent and STAT-4-dependent signals [25]. Moreover, Th1 polarization is associated to the α4β7 expression on mouse $CD4^+$ T cells as primary activation in the presence of exogenous IL-12 plus anti-IL-4 antibody induced higher levels of α4β7 after 8–12 days of culture [26]. Our findings showing that IL-12 but not anti-CD3/anti-CD28 restimulation provoked increments in the integrin are in agreement with the previous report that OT-I $CD8^+$ lymphocytes remain CCR9 and α4β7-negative upon stimulation with anti-CD3 plus anti-CD28 antibodies [20]. At least two mechanisms could explain the higher frequency of $\alpha4\beta7^+$ and $\alpha4\beta7^+$ T-bet^+ T cells in MLNs upon IL-12 injection, including activation in situ or priming in different secondary lymphoid tissues, such as spleen, followed by migration. Accordingly, the α4β7 expression is transiently upregulated in early effector lymphocytic choriomeningitis virus-specific $CD8^+$ T splenocytes and then rapidly downregulated, suggesting a temporary ability of T cells to traffic from spleen to gut [27]. Although we have not addressed this issue, the results from in vitro assays support that bystander stimuli could enlarge $integrin^+$ Th1-primed subsets with ability to reenter the LP. Although in IL-12-injected group no signs of intestinal inflammation were observed, the early changes in paracellular permeability found in our experimental condition may favor the supply of luminal stimuli and therefore the expansion of $integrin^+$ T cells that are very reactive to restimulation with IL-12. Accordingly, mucosal inflammation may occur as a consequence of aberrant IL-12 response to constituents of the microbial flora that sets the pathological Th1 response [28].

To demonstrate the migration of activated $\alpha4\beta7^+$ lymphocytes to LP we tried the isolation of MLN cells from IL-12-injected mice. We failed to recover sufficient viable cells, possibly due to the higher fragility associated to the activation status of these cells [15], and the amount of mice required to isolate enough cells for in vivo tracking excluded this possibility. Other experiments using FTY720 were not very conclusive. Therefore, we took advantage of the T-bet polarization associated to the α4β7 expression and we used the Th1 cytokine production as an indirect way to assess the effect on these activated subsets in the outcome of intestinal inflammation. The acute DSS colitis model is particularly useful when studying the contribution of the innate immune system to the development of intestinal inflammation, and unlike the human disease does not required T and B cells for its development. We reasoned that both, higher frequency of activated $integrin^+$ cells in LP as well as spontaneous release of IFN-γ could be very conducive conditions to exacerbate inflammatory processes in the intestine. Indeed this seems to be the case because when mice started receiving DSS immediately after the delivery of the IL-12 plasmid, they developed a worse colitis with higher scores of weight loss, blood in stool, and diarrhea and lower hematocrit. Notably, the treatment anticipated the clinical onset and produced a marked reduction of MPO activity, in agreement with sustained IL-12 administration [29]. The reduction was possibly related to the selective increment of IL-10 detected in MLNs and LP, as inhaled IL-10 reduced lung MPO activity, attenuating the pulmonary but not the systemic inflammatory response in hemorrhagic shock model [30].

Interestingly, our findings highlight that systemic IL-12 prompted a severe gut inflammation but also that IL-12 + DSS group developed not only worse but also different acute DSS colitis with a deficient recruitment of neutrophils, the typical effector cells of this experimental model [31]. Comparatively, in the group with conventional DSS colitis the increment of $\alpha4\beta7^+$ T cells in MLNs did not occur. Finally, on day 5 under the IL-12 influence, the cytokine profile in colon was Th1, in agreement with the increment of $\alpha4\beta7^+$ T-bet^+ cells found in MLNs. Additional evidence supports the link between systemic activation and mucosal inflammation, as mice with a targeted mutation in the gene for the G protein subunit Gai2 exhibit several alterations in the systemic immune system such as substantially increased production of IL-2, IFN-γ, and TNF-α and develop intestinal inflammation [32]. In agreement, colitis-susceptible SLJ/J mice develop higher IL-12p70 responses following systemic administration of LPS [33]. Similar to our model, LP and MLN $CD4^+$ cells from these mice show decreased expression of CD62L and increased expression of the mucosal α4β7 integrin that most likely influences homing of lymphocytes to mucosal tissues [27].

Interestingly, in spite of the robust systemic inflammatory response elicited in our model, some regulatory mechanisms might be operating. Herein, systemic as well as mucosal release of IL-10 overlapped in timing and magnitude the production of IFN-γ. The superimposed increment could represent the production of IL-10 by differentiated Th1 cells after full activation, in order to moderate or inhibit the inflammatory response, as previously suggested [34]. Moreover, the cytokine storm provoked the increment in the frequency of $CD4^+$ $Foxp3^+$ T cells in PPs and MLNs. The biological relevance of the increment of $CD4^+Foxp3^+$ cells in PPs and MLNs is unclear at this time, and could actually be part of a general regulatory mechanism as stimuli able to increase the production of IL-12, IL-10, and IFN-γ such as gut microbiota or LPS-rich diet expand $CD4^+$ Foxp3-expressing T cells [35]. The Treg function could also be influenced by Th1 cytokines, as activated Treg express IFN-γR and IL-12p70R [36] and the development of Tregs upon activation may rely on IL-12 responsiveness [37]. Moreover, in vitro rIL-12p70 induces proliferation of rIL-2-activated Treg (named Ts1) that express receptors for Th1 cytokines [36].

Our findings suggest that systemic IL-12 could represent a translator of the peripheral immune activation into the intestinal lymphoid tissue, allowing the recruitment of more effector lymphocytes to carry out gut immune surveillance. In summary,

our data support a model by which the transient expression of the IL-12 cDNA was able to expand $\alpha4\beta7^+$ T-bet$^+$ CD4$^+$ and CD8$^+$ T ready to migrate to mucosal effector sites. In this microenvironment, concomitant exposure to luminal stimuli could further expand differentiated T cells, favoring the outcome of the gut inflammation.

Materials and methods

Mice

Six- to 8-week-old C57BL/6 (B6), IL-10$^{-/-}$, IFN-γ, Foxp3-GFP, and OT-I (from Jackson Laboratory); TNF-R1$^{-/-}$, IL-4$^{-/-}$, and IL-12$^{-/-}$ mice (kindly provided by Dr. Silvia DiGenaro, National University of San Luis, Argentina) were used in these studies. These animals were maintained in specific pathogen-free conditions. Animals were housed in collective cages at 22 ± 1°C under a 12-h light/dark cycle (lights on at 7:00 a.m.) with free access to laboratory chow and drinking water. All animal experiments were approved by and conducted in accordance with guidelines of the Committee for Animal Care and Use of the Chemical Science Faculty, National University of Córdoba (Approval No. HCD 15-09-69596) in strict accordance with the recommendation of the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (OLAW Assurance No. A5802-01). Our animal facility obtained NIH animal welfare assurance (Assurance No. A5802-01, Office of Laboratory Animal Welfare, NIH, Bethesda, MD, USA).

Reagents and antibodies

The IL-12 plasmid was kindly provided by Dr. Howard Young (Frederick National Laboratory for Cancer Research, USA). Anti-CD3 (17A2), anti-CD28 (37.51), anti-CD4 (GK 1.5), anti-CD8 (53-6.7), anti-CD19 (SJ25C1), anti-CD62L (Dreg56), anti-CD44 (IM7), anti-CD69 (H1.2F3), anti-T bet (O4-46), anti-α4β7 integrin (DATK32) were from BD PharMingen (San Diego, CA, USA). Corresponding isotype-matched mAbs were used as controls in flow cytometry experiments. Recombinant murine IL-12p70 (210-12) was from Preprotech (Colonia Narvarte, Mexico). LSF was purchased from Santa Cruz Biotechnology. Fluorescein isothiocyanate–dextran average molecular weight 4000 (FITC-Dextran; Sigma–Aldrich).

Hydrodynamic cDNA and LSF injections

The hydrodynamic gene transfer procedure (h.i.) was described previously [15, 16, 38]. As previously shown, with this technique, the large volume and high injection rate used forces the flow of DNA solution into tissues directly linked to the inferior vena cava. A large portion of DNA solution is forced into the liver and plasmid DNA molecules are likely transferred inside the liver cells by the hydrodynamic process during DNA administration [38]. In the first step, animals were injected in the tail vein with 0.5, 1, and 3 μg of IL-12 cDNA (pscIL-12, p40-p35 fusion gene) or 3 μg of control cDNA (ORF empty vector control cDNA) in 1.6 mL of sterile 0.9% sodium chloride solution in 5–7 s. Taking into account that 0.5 and 3 μg elicited comparable levels of IL-12 (Supporting Information Fig. 1), we selected 0.5 μg as the working dose to minimize undesirable side effects [15, 16] in our study. Then, animals were injected in the tail vein with 0.5 μg of IL-12 cDNA (pscIL-12, p40-p35 fusion gene) in 1.6 mL of sterile 0.9% sodium chloride solution in 5–7 s. Control animals were h.i. with control (0.5 μg of ORF empty vector control cDNA) or 12 (0.5 μg of IL-12 cDNA). The expression plasmids utilize the human elongation 1-α promoter to drive transcription. Blood samples were collected every day during a week to measure IL-12, IFN-γ, IL-10, and TNF-α levels. LSF was administered i.p. (0.5 mg per mouse) starting the same day of the IL-12 cDNA h.i., every 12 h during 3 days.

Isolation of ILN, PP, MLN, and LP cells

On day 7 we removed ILNs, PPs, MLNs, and colon. We prepared single-cell suspensions by mechanical dispersion (ILNs, PPs, and MLNs) in RPMI medium supplemented with gentamicin, heparin, and 5% fetal calf serum (FCS) [39]. We isolated colonic LP cells as previously with slight modifications [40]. Briefly, extraintestinal fat tissue and blood vessels were carefully removed and colons were flushed with cold PBS, opened longitudinally, and cut into small pieces. Epithelial cells and mucus were removed by 45-min incubation in RPMI containing 5% FCS and 2 mM EDTA and 1 mM dithiothreitol (DTT; Sigma–Aldrich) at 37°C with 250 rpm shaking. Colon pieces were then digested in PBS containing 5% FBS, 1 mg/mL collagenase IV (Sigma–Aldrich) for 90 min and 37°C shaking at 250 rpm. The digested cell suspension was washed with PBS and passed through 40 μm cell strainers.

Cell culture

Single-cell suspensions (1×10^6 cells/mL) of MLNs were cultured 24 h with medium, 2 μg/mL anti-CD3/1 μg/mL anti-CD28 antibodies or 50 ng/mL rIL-12. Isolated 1×10^6 LP cell aliquots were cultured for 16 h in gentamicin and 10% FCS supplemented RPMI medium.

Flow cytometry

Flow cytometric analysis was performed to evaluate cell subsets, activation markers, homing receptor, T bet, or Foxp3 expression. Cells from ILNs, MLNs, PPs, and LP were washed and resuspended in FACS buffer (PBS, 5% FBS, 0.02% NaN_2) and incubated with mAbs to cell-surface markers for 30 min at 4°C. To perform

intracellular staining, cells were washed twice with staining buffer, fixed and permeabilized using Cytofix/Cytoperm solution (BD Pharmingen) for 1 h at 4°C. Cells were stained for intracellular T bet with mAb diluted in PermWash solution for 30 min at 4°C. Live cell events of 80 000–100 000 were acquired on an FACSCanto (BD Pharmingen) and analyzed using FlowJo software (Tree Star, Inc.).

Cytokines

Murine IL-12 and TNF-α (BD Biosciences), IFN-γ, and IL-10 (BD Pharmingen) were measured in sera and IL-17A in cell-free supernatants by using ELISA kits, as specified by the manufacturers. IL-2, IL-4, IL-6, IL-17A, IL-10, IFN-γ, and TNF-α were quantified in cell-free supernatants using the cytometric bead array CBA assay from BD Biosciences following manufacturer's instruction (Supporting Information Fig. 4A).

Explant cultures and LP cell isolation

Colons were flushed with RPMI and open along a longitudinal axis. Then, 5 mm two punch biopsies were obtained from the medial colon and incubated for 24 h in RPMI supplemented with 10% FCS and antibiotics (one punch biopsy per 1 mL medium). Supernatants were collected and kept in −20°C. Cytokines and chemokines were assessed as described above.

FITC-dextran permeability assay

Intestinal permeability was assessed by luminal enteral administration of FITC-dextran 4000 (Sigma), a nonmetabolizable macromolecule that is used as a permeability probe as previously [41]. Mice were gavaged with FITC-dextran (40 mg/100 g body weight) 2–4 h before killing. Whole blood was obtained by tail vein (2 h) or cardiac puncture (4 h) and FITC-dextran measurements were performed in a fluorometer at 488 nm. Fluorescence intensity was expressed in arbitrary units.

Induction and assessment of DSS-induced colitis

Mice received a solution of 3% w/v DSS (MW 40 000 kDa) in filtered water ad libitum over a 5-day period. Every other day, the DSS solution was replenished and at the end of this period, DSS was replaced by normal drinking water for 2 days. Control mice received only normal drinking water. All animals were examined once a day; the DAI was assessed during 7 days as described previously [42]. Briefly, DAI was the combined score of weight loss, stool consistency, and bleeding. 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 diarrhea, 2 for loose stool that did not stick to the anus, and 4 for liquid stool that did stick to the anus. For fecal blood, 0 was for none, 2 for moderate, and 4 for gross bleeding.

MPO assay

The MPO assay was performed as previously [42]. When indicated animals were killed and colon tissue segments homogenized in 5% EDTA/NaCl buffer (pH 4.7) and centrifuged at 10 000 × *g* for 15 min at 4°C. Pellets were resuspended in 0.5% hexadecyl trimethyl ammonium bromide buffer (pH 5.4), and samples were frozen in liquid nitrogen and thawed three times. On final thawing, the samples were similarly centrifuged, and 25 μL of the supernatant was used for MPO assay. The MPO enzymatic reaction was assessed by the addition of 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. Results were expressed as nanogram per milligram tissue protein.

Histopathological analysis

Excised portions of the distal colon were fixed immediately in a 4% w/v formaldehyde solution and embedded in paraffin. Next, 5 μm sections were mounted on glass slides, and deparaffinized. For histological analysis, slices were stained using standard H&E techniques. Images were taken with a Nikon optical microscope (Nikon eclipse TE2000-U, USA).

Statistical analysis

Data were expressed as means ± SEM. Statistical differences between groups were determined by 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, USA). A value of $p < 0.05$ was considered statistically significant.

Acknowledgments: We would like to thank Paula Icely, Luis Navarro, and Fabricio Navarro for excellent technical assistance. We thank Howard Young (Frederick National Laboratory for Cancer Research, USA) for the IL-12 plasmid. This work was supported by grants of Agencia Nacional de Promoción Científica y Tecnológica (FONCYT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), and Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SECyT-UNC). Fellows were funded by CONICET (LPP and BEB), FONCYT

(LMM), and CAPES (ASP). M.C.R.-G. and S.G.C. belong to the research staff of CONICET.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Maloy, K. J. and Powrie, F.**, Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* 2011. **474**: 298–306.

2 **Brandtzaeg, P., Baekkevold, E. S., Farstad, I. N., Jahnsen, F. L., Johansen, F. E., Nilsen, E. M. and Yamanaka, T. L.**, Regional specialization in the mucosal immune system: what happens in the microcompartments? *Immunol. Today* 1999. **20**: 141–151.

3 **Chen, K. and Cerutti, A.**, Vaccination strategies to promote mucosal antibody responses. *Immunity* 2010. **33**: 479–491.

4 **Strauch, U. G., Grunwald, N., Obermeier, F., Gurster, S. and Rath, H. C.** Loss of CD103$^+$ intestinal dendritic cells during colonic inflammation. *World J. Gastroenterol.* 2010. **16**: 21–29.

5 **Barnes, M. J. and Powrie, F.** Regulatory T cells reinforce intestinal homeostasis. *Immunity* 2009. **31**: 401–411.

6 **Mora, J. R., Cheng, G., Picarella, D., Briskin, M., Buchanan, N. and von Andrian, U. H.** Reciprocal and dynamic control of CD8 T cell homing by dendritic cells from skin- and gut-associated lymphoid tissues. *J. Exp. Med.* 2005. **201**: 303–316.

7 **Matteoli, G., Mazzini, E., Iliev, I. D., Mileti, E., Fallarino, F., Puccetti, P., Chieppa, M. et al.**, Gut CD103$^+$ dendritic cells express indoleamine 2,3-dioxygenase which influences T regulatory/T effector cell balance and oral tolerance induction. *Gut* 2010. **59**: 595–604.

8 **Watford, W. T., Moriguchi, M., Morinobu, A. and O'Shea, J. J.**, The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Rev.* 2003. **14**: 361–368.

9 **Cassani, B., Villablanca, E. J., Quintana, F. J., Love, P. E., Lacy-Hulbert, A., Blaner, W. S., Sparwasser, T. et al.**, Gut-tropic T cells that express integrin alpha4beta7 and CCR9 are required for induction of oral immune tolerance in mice. *Gastroenterology* 2011. **141**: 2109–2118.

10 **DeNucci, C. C., Pagan, A. J., Mitchell, J. S. and Shimizu, Y.**, Control of alpha4beta7 integrin expression and CD4 T cell homing by the beta1 integrin subunit. *J. Immunol.* 2010. **184**: 2458–2467.

11 **Campbell, D. J. and Butcher, E. C.** Rapid acquisition of tissue-specific homing phenotypes by CD4$^+$ T cells activated in cutaneous or mucosal lymphoid tissues. *J. Exp. Med.* 2002. **195**: 135–141.

12 **Davidson, N. J., Hudak, S. A., Lesley, R. E., Menon, S., Leach, M. W. and Rennick, D. M.**, IL-12, but not IFN-gamma, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *J. Immunol.* 1998. **161**: 3143–3149.

13 **Prochazkova, J., Pokorna, K. and 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.

14 **Chikano, S., Sawada, K., Shimoyama, T., Kashiwamura, S. I., Sugihara, A., Sekikawa, K., Terada, N. et al.**, IL-18 and IL-12 induce intestinal inflammation and fatty liver in mice in an IFN-gamma dependent manner. *Gut* 2000. **47**: 779–786.

15 **Rodriguez-Galan, M. C., Reynolds, D., Correa, S. G., Iribarren, P., Watanabe, M. and Young, H. A.**, 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**: 740–748.

16 **Schultz, J., Heinzerling, L., Pavlovic, J. and Moelling, K.**, Induction of long-lasting cytokine effect by injection of IL-12 encoding plasmid DNA. *Cancer Gene Ther.* 2000. **7**: 1557–1565.

17 **Mowat, A. M. and Agace, W. W.**, Regional specialization within the intestinal immune system. *Nat. Rev. Immunol.* 2014. **14**: 667–685.

18 **Coon, M. E., Diegel, M., Leshinsky, N. and Klaus, S. J.**, Selective pharmacologic inhibition of murine and human IL-12-dependent Th1 differentiation and IL-12 signaling. *J. Immunol.* 1999. **163**: 6567–6574.

19 **Hodge, D. L., Reynolds, D., Cerbán, F. M., Correa, S. G., Baez, N. S., Young, H. A. and Rodriguez-Galan, M. C.**, MCP-1/CCR2 interactions direct migration of peripheral B and T lymphocytes to the thymus during acute infectious/inflammatory processes. *Eur. J. Immunol.* 2012. **42**: 2644–2654.

20 **Johansson-Lindbom, B., Svensson, M., Wurbel, M. A., Malissen, B., Márquez, G. and Agace, W.**, Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): requirement for GALT dendritic cells and adjuvant. *J. Exp. Med.* 2003. **198**: 963–969.

21 **Sheasley-O'Neill, S. L., Brinkman, C. C., Ferguson, A. R., Dispenza, M. C. and Engelhard, V. H.** Dendritic cell immunization route determines integrin expression and lymphoid and nonlymphoid tissue distribution of CD8 T cells. *J. Immunol.* 2007. **178**: 1512–1522.

22 **Agace, W. W.**, T-cell recruitment to the intestinal mucosa. *Trends Immunol.* 2008. **29**: 514–522.

23 **Kemper, C., Chan, A. C., Green, J. M., Brett, K. A., Murphy, K. M. and Atkinson, J. P.**, Activation of human CD4$^+$ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature* 2003. **421**: 388–392.

24 **Grogan, J. L., Mohrs, M., Harmon, B., Lacy, D. A., Sedat, J. W. and Locksley, R. M.**, Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* 2001. **14**: 205–215.

25 **Underhill, G. H., Zisoulis, D. G., Kolli, K. P., Ellies, L. G., Marth, J. D. and Kansas, G. S.**, A crucial role for T-bet in selectin ligand expression in T helper 1 (Th1) cells. *Blood* 2005. **106**: 3867–3873.

26 **Abramson, O., Qiu, S. and Erle, D. J.**, Preferential production of interferon-gamma by CD4$^+$ T cells expressing the homing receptor integrin alpha4/beta7. *Immunology* 2001. **103**: 155–163.

27 **Masopust, D., Choo, D., Vezys, V., Wherry, E. J., Duraiswamy, J., Akondy, R., Wang, J. et al.**, Dynamic T cell migration program provides resident memory within intestinal epithelium. *J. Exp. Med.* 2010. **207**: 553–564.

28 **Bouma, G. and Strober, W.**, The immunological and genetic basis of inflammatory bowel disease. *Nat. Rev. Immunol.* 2003. **3**: 521–533.

29 **Hans, W., Schölmerich, J., Gross, V. and Falk, W.**, Interleukin-12 induced interferon-g increases inflammation in acute dextran sulfate sodium induced colitis in mice. *Eur. Cytokine Netw.* 2000. **11**: 67–74.

30 **Perše, M. and Cerar, A.**, Dextran sodium sulphate colitis mouse model: traps and tricks. *J. Biomed. Biotechnol.* 2012. **2012**: 718617.

31 **Kobbe, P., Lichte, P., Schreiber, H., Reiss, L. K., Uhlig, S., Pape, H. C. and Pfeifer, R.**, Inhalative IL-10 attenuates pulmonary inflammation following hemorrhagic shock without major alterations of the systemic inflammatory response. *Mediators Inflamm.* 2012. **2012**: 512974.

32 **Hörnquist, C. E., Lu, X., Rogers-Fani, P. M., Rudolph, U., Shappell, S., Birnbaumer, L. and Harriman, G. R.**, G(alpha)i2-deficient mice with colitis exhibit a local increase in memory CD4+ T cells and proinflammatory Th1-type cytokines. *J. Immunol.* 1997. **158**: 1068–1077.

33 **O' Garra, A. and Vieira, P.**, T(H)1 cells control themselves by producing interleukin-10. *Nat. Rev. Immunol.* 2007. **7**: 425–428.

34 **Bouma, G., Kaushiva, A. and Strober, W.**, Experimental murine colitis is regulated by two genetic loci, including one on chromosome 11 that regulates IL-12 responses. *Gastroenterology* 2002. **123**: 554–565.

35 **Hrncir, T., Stepankova, R., Kozakova, H., Hudcovic, T. and Tlaskalova Hogenova, H.**, Gut microbiota and lipopolysaccharide content of the diet influence development of regulatory T cells: studies in germ-free mice. *BMC Immunology* 2008. **9**: 65.

36 **Verma, N. D., Hall, B. M., Plain, K. M., Robinson, C. M., Boyd, R., Tran, G. T., Wang C. et al.**, Interleukin-12 (IL-12p70) promotes induction of highly potent Th1-like CD4+CD25+ T regulatory cells that inhibit allograft rejection in unmodified recipients. *Front. Immunol.* 2014. **5**: 190.

37 **Zhao, Z., Yu, S., Fitzgerald, D. C., Elbehi, M., Ciric, B., Rostami, A. M. and Zhang, G. X.**, IL-12R beta 2 promotes the development of $CD4^+CD25^+$ regulatory T cells. *J. Immunol.* 2008. **181**: 3870–3876.

38 **Liu, F., Song, Y. and Liu, D.**, Hydrodynamics based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* 1999. **6**: 1258–1266.

39 **Porporatto, C., Bianco, I. D. and Correa, S. G.**, Local and systemic activity of the polysaccharide chitosan at lymphoid tissues after oral administration. *J. Leukoc. Biol.* 2005. **78**: 62–69.

40 **Davies, M. D. and Parrott, D. M.**, Preparation and purification of lymphocytes from the epithelium and lamina propria of murine small intestine. *Gut* 1981. **22**: 481–488.

41 **Wang, Q., Fang, C. H. and Hasselgren, P. O.**, Intestinal permeability is reduced and IL-10 levels are increased in septic IL-6 knockout mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2001. **281**: R1013–R1023.

42 **Bento, A. F., Marcon, R., Dutra, R. C., Claudino, R. F., Cola, M., Leite, D. F. and Calixto, J. B.**, β-Caryophyllene inhibits dextran sulfate sodium-induced colitis in mice through CB2 receptor activation and PPARγ pathway. *Am. J. Pathol.* 2011. **178**: 1153–1166.

Abbreviations: **DAI**: disease activity index · **DSS**: dextran sodium sulphate · **h.i.**: hydrodynamic injection · **ILNs**: inguinal lymph nodes · **LP**: lamina propria · **LSF**: lisofylline · **MLNs**: mesenteric lymph nodes · **MPO**: myeloperoxidase · **PPs**: Peyer's patches

Full correspondence: Dr. Silvia G. Correa, Inmunología, Departamento de Bioquímica Clínica, CIBICI (CONICET), Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, 5000 Córdoba, Argentina
Fax: 54-351-4333048
e-mail: scorrea@fcq.unc.edu.ar

Received: 14/2/2015
Revised: 11/9/2015
Accepted: 6/10/2015
Accepted article online: 14/10/2015

