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Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Treatment with a Novel Chemokine-Binding Protein or Eosinophil Lineage-Ablation Protects Mice from Experimental Colitis

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Eosinophils are multifunctional leukocytes implicated in numerous inflammatory diseases. The present study was conducted to clarify the precise role of eosinophils in the development of colitis by using eosinophil-depleted mice and a novel chemokine-binding protein that neutralizes CCL11 action. Colitis was induced by administration of dextran sodium sulfate (DSS) to wild-type and eosinophil-deficient *ΔdblGATA-1* mice. Accumulation of eosinophils in the gut of mice given DSS paralleled worsening of clinical score and weight loss. In response to DSS, *ΔdblGATA-1* mice showed virtual absence of eosinophil recruitment, amelioration of clinical score, weight loss, and tissue destruction, and no lethality. There was a decrease in CXCL1 and CCL3 production and decreased neutrophil influx in the intestine of *ΔdblGATA-1* mice. Transfer of bone marrow cells from wild-type mice reconstituted disease manifestation in DSS-treated *ΔdblGATA-1* mice, and levels of CCL11 were increased after DSS treatment and localized to inflammatory cells. Treatment with the chemokine-binding protein evasin-4 at a dose that prevented the function of CCL11 greatly ameliorated clinical score, weight loss, overall tissue destruction, and death rates. In conclusion, the influx of eosinophils is critical for the induction of colitis by DSS. Treatment with a novel chemokine-binding protein decreased eosinophil influx and greatly ameliorated colitis, suggesting that strategies that interfere with the recruitment of eosinophils may be useful as therapy for colitis. ***(Am J Pathol 2009, 175:2382–2391; DOI: 10.2353/ajpath.2009.090093)***

Inflammatory bowel diseases (IBD), which include ulcerative colitis (UC) and Crohn's disease, are chronic, relapsing, and remitting inflammatory conditions of unknown origin that affect individuals of both sexes throughout life.[1–3] The etiology of IBD remains unclear but presumably involves down-regulation of immunomodulatory mechanism and an uncontrolled activation of proinflammatory mediators. UC is a condition that primarily affects the superficial layer of the colon mucosa and presents clinical manifestation of disease, which include weight loss, diarrhea accompanied by blood and/or mucus, fever, and shortening of the colon. The colon of patients with UC is characterized by an increase in inflammatory infiltrate, which is composed predominantly by neutrophils, lymphocytes, mast cells, and eosinophils.[1–3]

Studies of the gut mucosa have emphasized the involvement of neutrophil granulocytes in the pathogenesis of IBD, but little attention has been given to the role of eosinophils. Recently, there has been increasing interest in the understanding of the involvement of these cells in the pathogenesis of IBD.[4] Eosinophils are present in the healthy lamina propria of the gastrointestinal tract, where

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they are believed to play an important role in host defense against helminth infection.[5,6] However, eosinophils are powerful proinflammatory cells possessing the capacity to initiate or potentiate inflammatory reactions through the release of a range of inflammatory cytokines (interleukin (IL)-2, IL-4, IL-5, and IL-10), and eosinophilic granular proteins, such as major basic protein and eosinophil peroxidase (EPO).[4–6] Clinical investigations of bowel biopsy specimens from UC patients have demonstrated a correlation between eosinophil numbers in the mucosa, the levels of theses proteins in perfusion fluid samples, and disease severity.[7–10] Furthermore, eosinophil-active cytokines and chemokines are expressed and could be relevant for the control of colonic eosinophilia in UC.[11–13] However, the precise role of eosinophils and their mediators in UC remains unclear.

The present study was designed to investigate the role of eosinophils in the pathogenesis of UC by using a previously described model of UC induced by dextran sodium sulfate (DSS).[14] We explored the potential contribution of eosinophils to colitis pathogenesis by using mice (*Δdbl*GATA) in which the eosinophil lineage had been completely ablated.[15] *Δdbl*GATA mice contain an engineered deletion of a palindromic double-enhancer binding site for GATA proteins in the 5′ region to the 1E exon of the gene encoding GATA-1 and are reported to be devoid of eosinophils both at baseline and in response to cytokine challenge, without reported effects on other hematopoietic lineages.[15] Finally, we also tested the effects of treatment evasin-4, a novel chemokine-binding protein cloned from tick saliva that binds to eosinophil-active chemokines CCL11 and CCL5.[16,17]

Materials and Methods

Animals

*Δdbl*GATA mice (BALB/c genetic background) were donated by Dr. A. Humbles[15] (Harvard, Boston, MA) and were bred in the animal facility of Universidade Federal de Minas Gerais (UFMG). BALB/c mice (wild-type) were purchased from the Bioscience unit of UFMG. Animals were housed under standard conditions and had free access to commercial chow and water. Only females weighing 20 to 24 g were used in our experiments. All procedures described here had prior approval from the local animal ethics committee.

Induction of DSS Colitis

Mice received 4% (w/v) DSS (36,000 to 50,000 kDA; MP Biomedicals) in their drinking water *ad libitum* for 7 days, then switched to autoclaved drinking water. Mice were sacrificed in the seventh day, and the colon was excised and cut into three equal segments—proximal, middle and distal colon—for the determination of histological score, cytokines, myeloperoxidase (MPO) activity, and EPO activity. For survival studies, mice were followed for 25 days after start of DSS treatment. Animals were euthanized after presenting hunched posture, weight loss >20% of initial weight, and severe hemorrhage, and this was used as a surrogate marker of death. Mice were weighed every other day for the determination of percent weight change. This was calculated as: percent weight change (weight at day × day 0/weight at day 0) × 100. For kinetics studies, a separate series of animals were sacrificed at days 1, 3, 5, and 7 after the start of DSS administration. In these animals, weight and clinical changes were also assessed. For the enzymatic determination of cells content and cytokines and chemokines measurement, the entire colon was divided in three equal portions in length. From distal to proximal, portions were used respectively for EPO, MPO, and enzyme-linked immunosorbent assays, as described below. Another experiment was conducted for histological analysis.

Assessment of Colitis Activity (Clinical Score Means)

Mice were monitored clinically every other day. They were left alone in a box for 10 minutes to determine the consistency of feces and to obtain samples for further evaluation. Fecal blood was tested using Hemaccult cards (INLAB-Diagnostica, São Paulo, Brazil). Graded score was conducted as follows: 0 = normal feces and no blood, 1 = normal feces and trace of blood in the fecal blood test, 2 = feces with smooth consistency and positive fecal blood test, 3 = feces with pasty consistency and positive fecal blood test, 4 = liquid feces and positive fecal blood test, 5 = liquid feces and moderate rectal bleeding, 6 = liquid feces and severe rectal bleeding, 7 = diarrhea and hemorrhage, and 8 = diarrhea, hemorrhage, and general signs of morbidity, including hunched posture and failure to groom.

Differential Blood Cell Count

Animals were sacrificed by cervical displacement, and 20 μl of blood was collected from the brachial plexus. The blood was diluted in Turk's solution, and total cell counts were performed in a modified Neubawer chamber. A blood smear was prepared and stained with Giemsa and May-Grumwald stains. Percentage of lymphocyte, monocytes, neutrophils, and eosinophils were determined using standard morphological criteria by counting at least 300 cells.

Determination of the EPO Activity and MPO Activity

The extent of tissue eosinophil infiltration was assessed by measuring EPO as described previously.[18] Briefly, for each 100 mg of colon tissue of the animals were weighted and homogenized with 1.9 ml of PBS and centrifuged at 12,000 × *g* for 10 minutes. The supernatant was discarded, and the erythrocytes were lysed. The samples were then centrifuged, the supernatant was discarded, and the pellet was suspended in 1.9 ml of 0.5% hexadecyltrimethyl ammonium bromide in PBS, frozen three times in liquid nitrogen, and centrifuged at 4°C at

12,000 × *g* for 10 minutes. The supernatant was used in the enzymatic assay by the addition of an equal amount substrate (1.5 mmol/L *o*-phenylenediamine and 6.6 mmol/L H_2O_2 in 0.075 mmol/L Tris-HCl (pH 8)). The reaction was stopped with 50 μl of 1 M H_2SO_4, and the absorbance was read at 492 nm.

The extent of neutrophil accumulation in the colon tissue was measured by assaying MPO activity, as described previously.[19] Briefly, a portion of colon of animals that had undergone colitis were removed and snap frozen in liquid nitrogen. On thawing and processing, the tissue was assayed for MPO activity by measuring the change in OD at 450 nm using tetramethylbenzidine. Results were expressed as the relative unit that denotes activity of MPO related with casein-elicited murine peritoneal neutrophils processed in the same way.

Histological and Immunohistochemistry Analysis of Colon

The proximal section of the colon was excised and fixed in 10% buffered formalin, paraffin-embedded, sectioned, and stained with H&E. Paraffin-embedded tissues were sectioned (3 μm) and collected in serial sections on glass slides coated with 2% 3-aminopropyltriethylsilane (Sigma-Aldrich, St. Louis, MO). The specimens were routinely processed and analyzed, as described previously.[20] The slides were then incubated with the following primary antibodies: rabbit anti-mouse CCL11 polyclonal antibody (a gift from Dr. N. Lukacs University of Michigan Medical School) at 1/1000, at 4°C overnight in a humidified chamber stained with chromogen solution (K3468; Dako, Carpinteria, CA). Negative controls were obtained from sections of DSS-treated mice on day 7 by the omission of primary antibodies, which were substituted by 1% PBS-bovine serum albumin and by nonimmune rabbit serum (X0902; Dako).

For eosinophil counts in tissue, 20 fields from H&E-stained sections were randomly chosen at ×1000 (0.01 mm^2/field) measurement. Ten fields were chosen randomly at ×400 magnification (0.07 mm^2/field) from immunohistochemistry sections to count the number of positive inflammatory cells, and the data are reported as number of positive cells/field.

All colon sections, H&E-stained and at ×100 magnification (11 mm^2/field), were evaluated in a blinded manner using a previously validated scoring system.[21] Three independent parameters were measured: severity of inflammation (0 to 3: none, slight, moderate, severe), depth of injury (0 to 3: none, mucosal, mucosal and submucosal, transmural), and crypt damage (0 to 4: none, basal one-third damaged, basal two-thirds damaged, only surface epithelium intact, entire crypt and epithelium lost of globlets cells). The score of each parameter was multiplied by a factor reflecting the percentage of tissue involvement (1, 0 to 25%; 2, 26 to 50%; 3, 51 to 75%; and 4, 76 to 100%) and added to a sum. The maximum possible score is 40.

Measurement of Cytokine/Chemokine Concentrations in the Colon

The concentration of IL-4, IL-5, CXCL1 (KC), CCL-3 (MIP-1α), and CCL11 (eotaxin-1) was measured in colon of animals using commercially available kits and according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN).

Transfer of Bone Marrow Cells

Bone marrow cells were collected by flushing the two femurs of mice with RPMI 1640 medium containing 10% FCS, washed, and counted. Cells were collected, centrifuged, and resuspended in 1 ml of saline 0.9%. For transfer, 1 to 2 × 10^6 cells were injected in the tail of mice after DSS-induced colitis. Bone marrow cells were injected every other day in both Δ*dbl*GATA- and wild-type naive mice (without endogenous cell ablation by irradiation).

Treatment with Evasin-4 in Allergic Pleurisy, CCL11 Pleurisy Challenge, and Colitis Model

Initial experiments examined the dose-dependent (0.001 to 10 μg/mouse) effects of evasin-4 in models of eosinophil recruitment in the pleural cavity.[22] In CCL11-induced pleural eosinophil recruitment, mice received an injection of human eotaxin/CCL11 (300 ng per cavity) and cell influx evaluated 24 hours later. Evasin-4 (0.01 to 10 μg/animal) was administrated 45 minutes before administration of CCL11. In the allergic pleurisy model,[22] antigen (ovalbumin; 1.0 μg per pleural cavity) or vehicle was injected i.p. in immunized mice, and animals were sacrificed 48 hours later. The cells present in the cavity were harvest using 2 ml of PBS, and total cell counts were performed in a modified Neubauer chamber using Turk's stain. Differential cell counts were performed on cytospin preparations (Shandon III) stained with May-Grumwald-Giemsa using standard morphological criteria to identify cell types. The results are presented as the number of cells per cavity.

In the DSS model, evasin-4 (0.1 $\mu g \cdot kg^{-1}$, s.c.) or vehicle (saline; 2 ml/kg) was given 8 hours before induction of colitis and then every at 8 hours.

Statistical Analysis

Results are shown as the mean ± SEM. Differences were evaluated by using analysis of variance (analysis of variance), followed by Student-Newman-Keuls *post hoc* analysis. Results with $P < 0.05$ were considered significant. For survival curves, differences between groups at different time points were compared using Fisher's exact test and considered significant when $P < 0.05$.

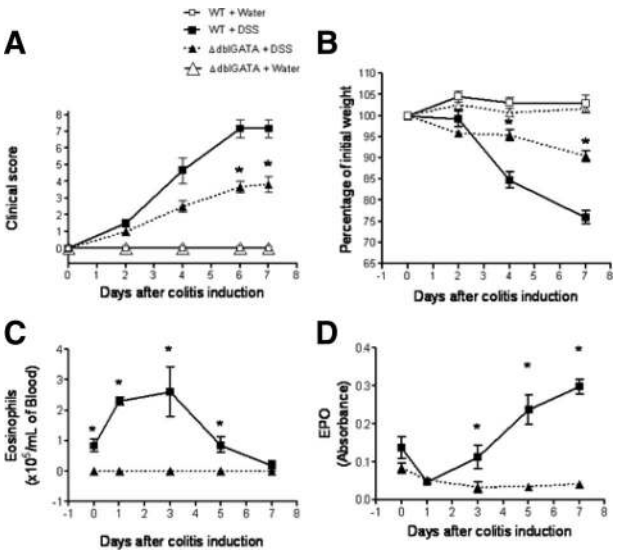

Figure 1. Decreased clinical score, weight loss, eosinophilia, and eosinophil accumulation in Δ*dbl*GATA mice given DSS. Wild-type (WT) and Δ*dbl*GATA mice were given water or water with 4% DSS for 7 days and the following parameters were assessed: clinical score (**A**); change in body weight (**B**); eosinophil counts in blood (**C**); and EPO activity in colon tissue (**D**). Each data point represents the mean ± SEM of $n = 6–8$. For kinetics studies, the animals were sacrificed on days 0, 1, 3, 5, and 7. In all experiments, clinical score and weight were determined but the kinetic graphs (**A** and **B**) represent the results of a single experiment done with the sole purpose of measuring these parameters. Each experiment was repeated twice. $^{*}P < 0.05$ when comparing wild-type and Δ*dbl*GATA mice given DSS.

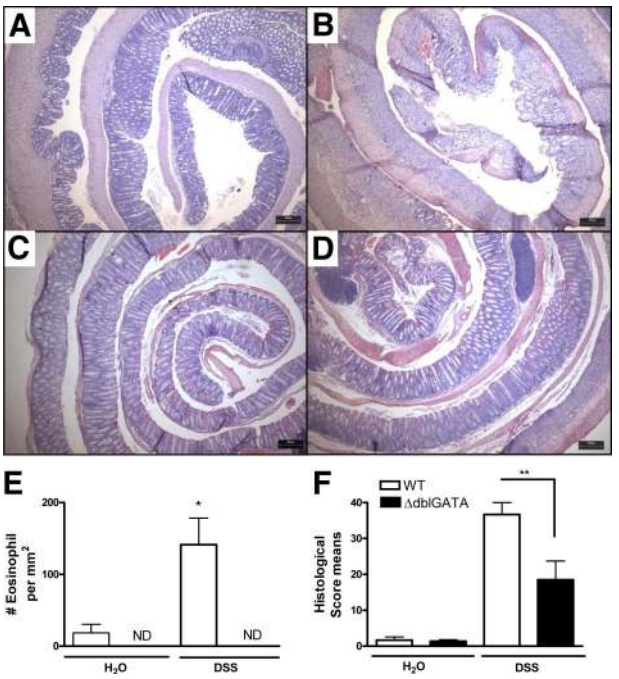

Figure 2. Decreased eosinophil accumulation and pathological score in ΔdblGATA mice given DSS. Wild-type (WT) and Δ*dbl*GATA mice were given water or water with 4% DSS for 7 days, and colon samples were obtained at day 7. Representative pictures of WT and Δ*dbl*GATA mice given water are shown in **A** and **C**, respectively. WT and Δ*dbl*GATA mice given 4% DSS are shown in **B** and **D**, respectively. Scale bar = 200 μm. Blind counting of eosinophils in tissue (**E**) and histological assessment of colitis (**F**) was performed on $n = 6$ animals in each group, as described in *Materials and Methods*. Results are mean ± SEM $^{*}P < 0.05$ and $^{**}P < 0.01$ when comparing WT and Δ*dbl*GATA mice given DSS. Scale bar = 200 μm.

Results

Clinical Course of DSS-Induced Colonic Inflammation and Eosinophil Infiltration in Wild-Type and ΔdblGATA-1 Mice

Administration of DSS to BALB/c mice induced an acute inflammation of the colon with diarrhea and rectal bleeding, as demonstrated in the clinical score, and pronounced weight loss (Figure 1, A and B). Clinical symptoms were first apparent 2 days after DSS exposure and reached a peak at day 6 (Figure 1*A*). Weight loss was significant in the fourth day after exposure (Figure 1*B*). The number of peripheral blood eosinophils in wild-type mice peaked in the first day after DSS treatment was elevated until day 3 and decreased thereafter (Figure 1C). The reduction of the number of eosinophils in blood of DSS-treated wild-type mice was accompanied by an increase in the number of eosinophils in the colon, as measured by EPO (Figure 1D).

To better explore the role of eosinophils in the pathogenesis of colitis, we evaluated the course of DSS-induced colitis in eosinophil lineage-ablated Δ*dbl*GATA-1 mice. Δ*dbl*GATA-1 mice given DSS in drinking water showed lower clinical score and lower weight loss as compared with wild-type controls (Figure 1, A and B). No eosinophils were detected in the blood count, and there was no EPO activity above baseline in the colon of Δ*dbl*GATA-1 mice (Figure 1, C and D). Thus, complete eosinophil lineage-ablated mice are less susceptible to DSS-induced disease than wild-type mice.

Reduction of Colonic Damage in ΔdblGATA-1 Mice

Histological injury was analyzed in the distal colon of wild-type and Δ*dbl*GATA-1 mice 7 days after treatment with 4% DSS. There were no eosinophils in the gut of naive Δ*dbl*GATA-1 mice (data not shown), but there were no further differences in the intestine of naive wild-type and Δ*dbl*GATA-1 mice (Figure 2, A and C). In wild-type mice, histological examination showed that DSS-treated wild-type mice developed extensive ulceration of the epithelial layer, crypt damage, and dense infiltration of the superficial layers of the mucosa with mononuclear and granulocytes cells (Figure 2B). These parameters were confirmed by the histological score as demonstrated in Figure 2F. In agreement with the decreased EPO levels (Figure 1D), there was also a significant decrease in number of infiltrating eosinophils in colonic tissues of Δ*dbl*GATA-1 mice subjected to DSS treatment (Figure 2E). Δ*dbl*GATA-1 mice consistently showed an attenuated injury response to DSS, with reduction of all alterations previously described for wild-type mice (Figure 2D). In the latter experiments, there was also protection from clinical disease and weight loss in Δ*dbl*GATA-1 mice subjected to DSS treatment (Figure 1, A and B).

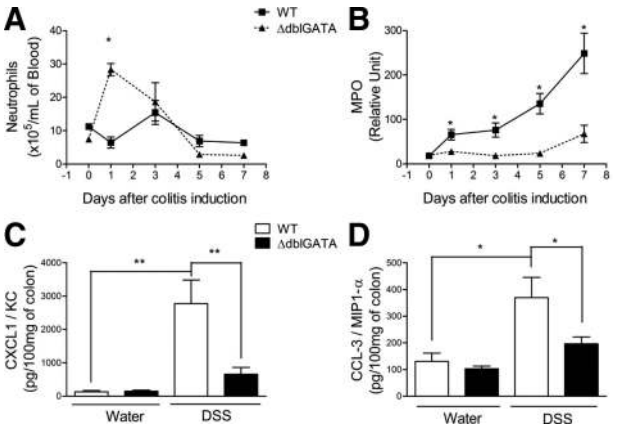

Figure 3. Decreased neutrophil influx and neutrophil-active chemokines in Δ*dbl*GATA mice given DSS. Wild-type (WT) and Δ*dbl*GATA mice were given water or water with 4% DSS for 7 days and the following parameters assessed daily: blood neutrophils (**A**) and MPO levels in colon tissue (**B**). Levels of CXCL1 (**C**) and CCL3 (**D**) were evaluated at day 7 after DSS. Results are mean ± SEM of $n = 6$ animals in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ when comparing WT and Δ*dbl*GATA mice given DSS.

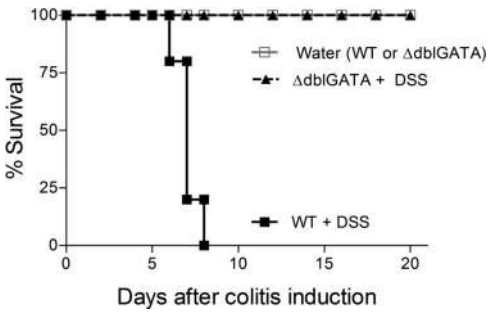

Figure 4. Enhanced survival in Δ*dbl*GATA mice given DSS. Wild-type (WT) and Δ*dbl*GATA mice were given water or water with 4% DSS for 7 days and survival monitored until day 20 after the start of DSS treatment. Results are shown as percent survival ($n = 6$ for water groups and $n = 8$ for DSS-treated groups) and were repeated twice. There was significant protection ($P < 0.01$) in Δ*dbl*GATA when compared with WT mice given DSS.

Reduced Neutrophil Influx and Neutrophil-Active Chemokines in ΔdblGATA-1 Mice

Neutrophils have been suggested to play a role in the development of colitis.[23] In our experiments, there were no significant changes in the number of neutrophils in blood of wild-type mice subjected to DSS colitis (Figure 3A). However, there was a significant increase in the number of neutrophils infiltrating the tissue, as assessed by the measurement of tissue MPO (Figure 3B), and confirmed qualitatively by histology (data not shown). Neutrophil influx in the gut was detected as early as 1 day after DSS administration and increased thereafter. There was an increase in the number of neutrophils in blood of Δ*dbl*GATA-1 mice at day 1 after DSS administration, but neutrophilia dropped thereafter to baseline levels (Figure 3*A*). There was no detectable MPO activity in the colon of Δ*dbl*GATA-1 mice treated with DSS throughout the 7 days of DSS administration (Figure 3*B*).

In mice, the chemokines CCL3 and CXCL1 are known to play a role in the recruitment of neutrophils to sites of inflammation.[16] Moreover, these chemokines are expressed in human colitis[24] and mediate neutrophil recruitment in experimental colitis.[25,26] There was a significant increase in the colonic production of both CXCL1 and CCL3 at day 7 after DSS administration (Figure 3, C and D). However, colonic levels of both chemokines in Δ*dbl*GATA-1 mice subjected to DSS colitis was significantly lower than those of wild-type mice (Figure 3). Neutrophils from Δ*dbl*GATA-1 mice responded normally to chemoattractants as seen by a similar recruitment of neutrophils in response to the injection of CXCL1 in wild-type and Δ*dbl*GATA-1 mice (data not shown). These results suggest that the lower levels of CXCL1 and CCL3 in the colon could explain the lower levels of neutrophils observed in Δ*dbl*GATA-1 given DSS.

Reduced Lethality Rates in ΔdblGATA-1 Mice

Administration of DSS to wild-type mice induced significant clinical disease (Figure 1) and pathology (Figure 2), culminating in 100% lethality at day 8 (Figure 4). In contrast to the latter findings, none of the Δ*dbl*GATA-1 mice were dead, even at day 21 after the beginning of the administration of DSS (Figure 4). The protection from lethality is consistent with the better clinical score and overall tissue pathology observed in Δ*dbl*GATA-1 mice.

Adoptive Transfer of Bone Marrow Cells from Wild-Type Mice to Eosinophil Lineage-Ablated Mice Re-Established DSS-Induced Tissue Injury

To re-establish the eosinophil lineage in Δ*dbl*GATA-1 mice, we transferred bone marrow cells from wild-type mice to Δ*dbl*GATA-1 mice and examined the clinical score and eosinophil numbers in the colon. As seen in Figure 5, the transfer of wild-type bone marrow to Δ*dbl*GATA-1 re-established the phenotype in these mice, as seen by the increased weight loss, reduction of colon length, and greater histological injury (Figure 5, A–C). Wild-type bone marrow transfer restored to wild-type levels the influx of eosinophils, as assessed by EPO assay and eosinophil counts, to the colon of Δ*dbl*GATA-1 mice given DSS (Figure 5, D and E). There was also a restoration of the neutrophil influx, as assessed by counting neutrophils, to the colon of Δ*dbl*GATA-1 mice given DSS (Figure 5F). The enhanced eosinophil influx observed in the Δ*dbl*GATA-1 mice given wild-type bone marrow was accompanied by worsening of the clinical score, which was similar to that observed in wild-type mice given DSS (eg, clinical score at day 7: wild-type→Δ*dbl*GATA-1 + DSS 5.0 ± 0.7 and wild-type + DSS 6.4 ± 0.7).

Enhanced Production and Role of CCL11-Induced Eosinophil Influx in DSS-Induced Colitis

The chemokine CCL11, also known as eotaxin, was initially discovered as an eosinophil chemoattractant obtained from the lungs of guinea pigs immunized and challenged with ovalbumin.[27] CCL11 appears to play an

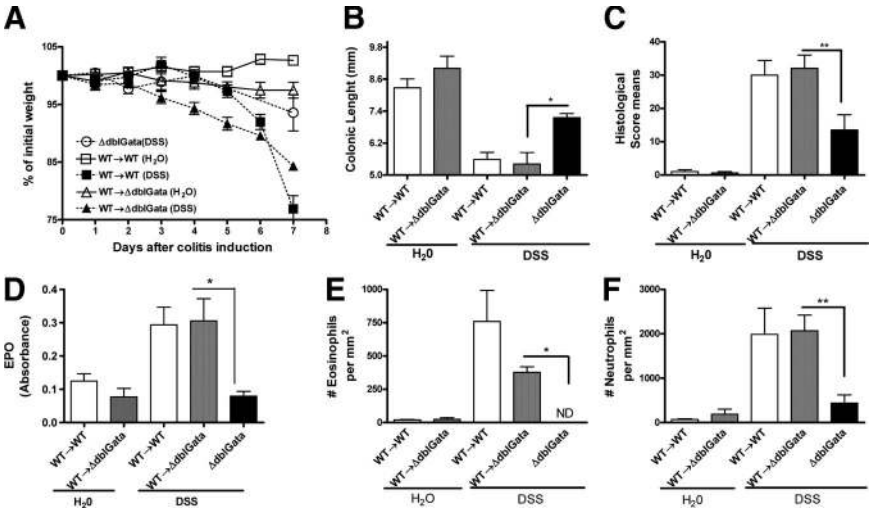

Figure 5. Adoptive transfer of bone marrow leukocytes reconstitutes colitis in *Δdbl*GATA mice given DSS. Wild-type (WT) and *Δdbl*GATA mice were given water or water with 4% DSS for 7 days. Animals received adoptive transfer of bone marrow leukocytes from WT and *Δdbl*GATA mice, as indicated in the figure, every other day from the day of colitis induction. Donor and recipient mice are indicated by the direction of arrows. **A:** Weight loss was monitored daily. At day 7, mice were sacrificed and the following parameters assessed in the colon, as shown in *Materials and Methods:* length (**B**); histological score (**C**); EPO levels (**D**); and numbers of eosinophils (**E**) and neutrophils (**F**). Results are mean ± SEM of $n = 6–8$ animals in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ as indicated in the figure. Experiments were repeated twice.

important role in mediating eosinophil influx into mucosal surfaces.[4] In wild-type mice, administration of DSS induced an increase of CCL11 expression, as measured by immunohistochemistry. CCL11 immunoreactivity was detectable at days 0, 3, 5, and 7 after the start of DSS administration (Figure 6). Expression of CCL11 was low under basal conditions (day 0) but expression increased with time and colocalized with infiltrating leukocytes (days 3, 5, and 7). A semiquantitative score showed that CCL11 expression was significant from day 3 after DSS administration (Figure 6). We also evaluated CCL11 production by enzyme-linked immunosorbent assay in the intestinal homogenates. Colonic levels of CCL11 were significantly increased in wild-type mice from day 5 of DSS administration (basal, 1257 ± 172 pg CCL11/100 mg of tissue; day 3, 715 ± 250; day 5, 2916 ± 840; and day 7, 7769 ± 1374; $n = 4$). Levels of CCL11 were decreased in *Δdbl*GATA-1 mice subjected to DSS, as assessed by enzyme-linked immunosorbent assay (basal, 674 ± 256 pg CCL11/100 mg of tissue; day 3, 662 ± 198; day 5, 318 ± 131; and day 7, 1439 ± 552; $n = 4$) and immunohistochemistry (data not shown).

The Novel Chemokine-Binding Protein Evasin-4 Inhibits Eosinophil Recruitment and Ameliorates Inflammation in DSS-Induced Colitis

Evasin-4 is a novel chemokine binding protein obtained from cloning of proteins expressed in tick saliva.[17] This molecule binds to CCL11 and CCL5 *in vitro* (Ref. [28] and our unpublished observation). Our *in vivo* studies confirmed that evasin-4 (0.1 or 1.0 μg/mouse, s.c. 45 minutes before stimulation) treatment inhibited the recruitment of eosinophils induced by CCL11, and this inhibition did not affect the influx of other inflammatory cells (Figure 7, A, C, E). Furthermore, treatment with evasin-4 prevented the influx of eosinophils induced by antigen challenge of immunized mice (Figure 7, B, D, F), an event shown to be dependent on the endogenous production of CCL11.[22]

Treatment of mice with evasin-4 significantly improved clinical symptoms and weight loss in mice subjected to DSS-induced colitis (Figure 8, A and B). This better outcome was associated with markedly reduced eosinophil recruitment in the colon, as assessed by measurement of

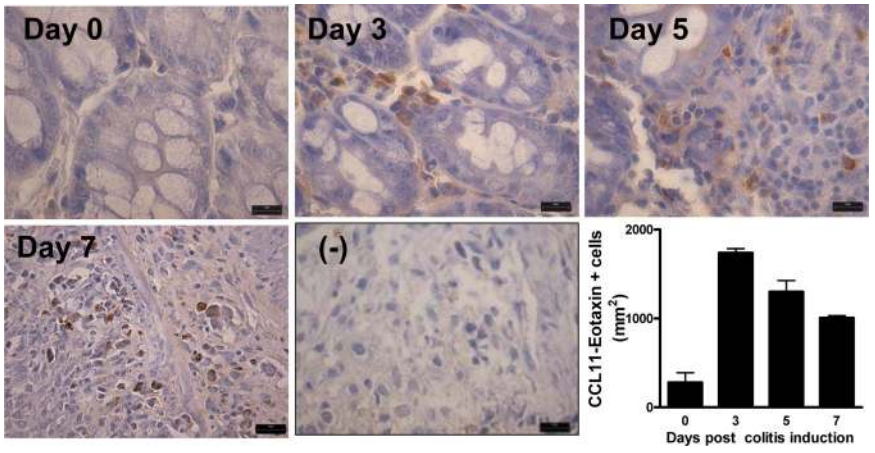

Figure 6. Production of CCL11/eotaxin in the colon of wild-type (WT) mice given DSS. The panels show CCL11-stained sections of DSS-treated wild-type mice at days 0, 3, 5, and 7 after the start of DSS. **Panel** (−) shows a section from an animal given DSS at day 7 in which anti-CCL11 was substituted for preimmune rabbit control serum. The quantification of CCL11 expression is shown in the graph as number of CCL11-positive cells per mm^2. Results are mean ± SEM of $n = 4$ animals in each group. Scale bar = 10 μm.

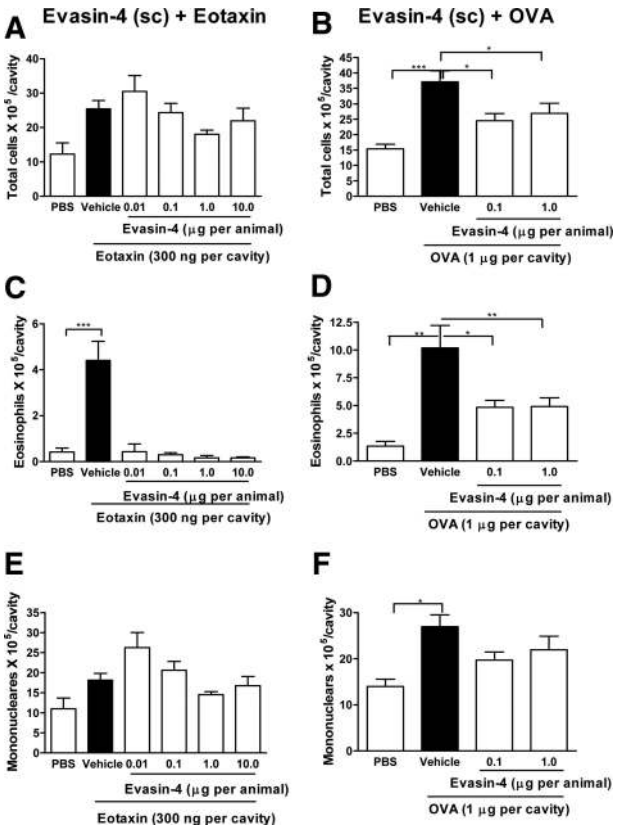

Figure 7. Treatment with evasin-4 decreases eosinophil influx induced by CCL11 or antigen challenge of immunized mice. Mice received an intrapleural injection of CCL11(300 ng per cavity) and total number of leukocytes (**A**), eosinophils (**C**), and monocytes (**E**) evaluated 24 hours later. Evasin-4 (0.001 to 10 μg per animal) was given s.c. 45 minutes before CCL11. Immunized animals were challenged with OVA or PBS and total number of leukocytes (**B**), eosinophils (**D**), and monocytes (**F**) evaluated 48 hours later. Evasin-4 (0.1 or 1.0 μg per animal) was s.c. 45 minutes before and 6 hours after OVA challenge. Values are mean ± SEM of $n = 6$ mice in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ as indicated in the graph.

EPO levels (Figure 8*C*) and counting eosinophils in the colon (water, 18 ± 10 eosinophils/mm^2; DSS, 446 ± 109; DSS + evasin-4, 42 ± 10). Akin to the results observed in eosinophil lineage-ablated mice, treatment with evasin-4 also decreased the MPO contents in tissues (Figure 8D). The latter findings were mirrored in the evaluation of tissue pathology in which there was decreased leukocyte influx and overall amelioration of tissue damage (Figure 8F). More importantly, treatment with evasin-4 reduced the mortality of mice with DSS-induced colitis (Figure 8E).

Discussion

The major findings of our study can be summarized as follows: i) administration of DSS to mice induces the accumulation of eosinophils in the colon, which correlates temporarily with the clinical score, weight loss, and tissue injury; and ii) prevention of eosinophil influx, as observed in eosinophil lineage-ablated *Δdbl*GATA-1 mice, prevents DSS-induced clinical disease, pathological changes, and lethality. The latter findings are in agreement with those of Ahrens et al[29] in another strain of eosinophil-deficient mice. In addition we provide the novel findings that: iii) there is a decrease in colonic expression of neutrophil-active chemokines and neutrophil influx in *Δdbl*GATA-1 mice given DSS; and iv) eosinophil influx is associated with local expression of CCL11 by inflammatory cells and blockade of CCL11 with a novel chemokine-binding protein, evasin-4, decreased eosinophil and neutrophil influx, clinical disease, tissue injury, and death.

Eosinophil recruitment to the colon of animals given DSS correlated with disease activity, which is in agreement with studies showing an increase of eosinophils in colon of UC patients.[11–13] More importantly, deficiency in the eosinophilic lineage resulted in attenuation of pathology, clinical disease, and lethality caused by DSS administration. *Δdbl*GATA mice reconstituted with wild-type hematopoietic cells presented eosinophil recruitment and developed colitis similarly to their wild-type counterparts. The latter results suggest that hematopoietic-derived cells, most likely eosinophils, were indeed responsible for triggering injury. Taken together with results in another line of eosinophil-deficient mice[29] and results showing a role for EPO in colitis development,[30] these data suggest that eosinophils and their degranulation products are pivotal in the pathogenesis of DSS colitis.

Stevceva et al[31] showed that IL-5 deficiency results in reduced eosinophilia after DSS treatment. However, the disease severity was not affected by the reduction in eosinophil mobilization. In their work, eosinophil recruitment after DSS treatment was not totally reduced. The remaining amount of active eosinophils could be sufficient to unleash the inflammatory response, leading to tissue injury and disease. This is in accordance with our hypothesis, in which eosinophils are of great importance to initiate disease and by promoting recruitment and activation of other inflammatory cells such as neutrophils. It is important to mention that not only IL-5 but also CCL11 is able to induce eosinophil mobilization, recruitment, and activation. CCL11 production was not analyzed by Stevceva et al[31] in their IL-5-deficient mice. The production of CCL11 could overcome IL-5 deficiency and lead to the remaining eosinophils to become active and to maintain inflammatory activation, tissue injury, and disease onset.

Eosinophils have been suggested to have a catapult-like antibacterial effect in a model of cecal ligation and puncture.[32] In contrast to the latter model in which bacterial translocation plays a major role, we could not observe consistent bacteremia in our model of DSS-induced colitis (our unpublished data). Therefore, any potential antibacterial effects of eosinophils may have not been disclosed under our experimental conditions.

In humans, the accumulation of neutrophils and the local production of IL-8 have also been noticed in rectal biopsies of UC patients.[24,25,33–35] UC models in mice, including that induced by DSS, are also characterized by significant neutrophil migration to the colon.[11,14,26] Neutrophil recruitment after administration of DSS is associated with the production of CXC chemokines and activation of CXCR2[25] and also on the production and action of CCL3.[26] In our experiments, there was local production of the chemokines CXCL1 and CCL3 and significant neu-

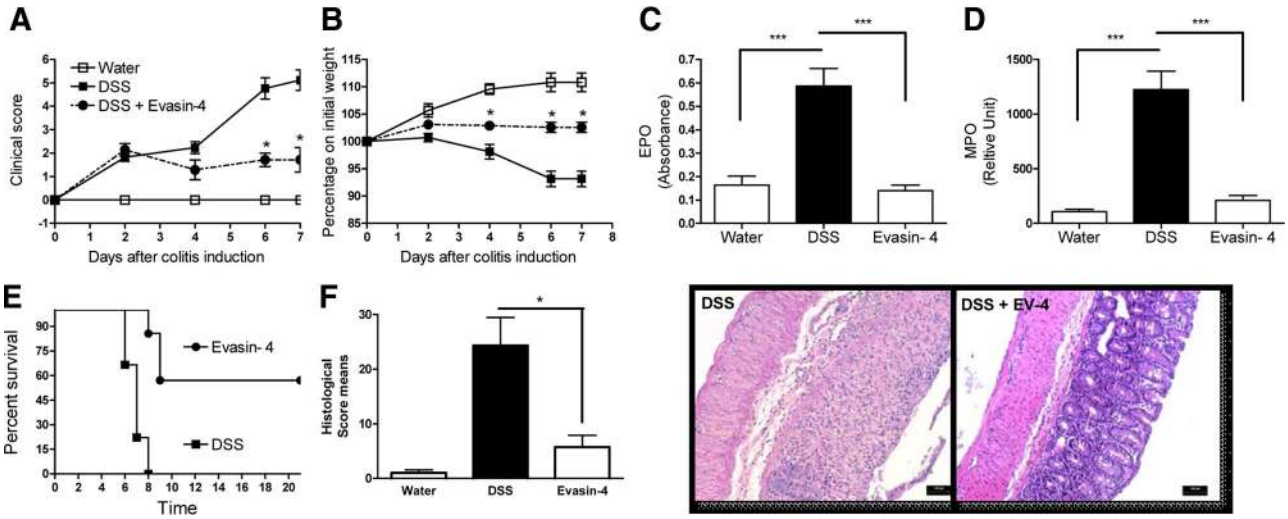

Figure 8. Treatment with evasin-4 protects mice from DSS-induced colitis. Mice were given water or water with 4% DSS for 7 days. Vehicle (saline) or evasin-4 was given s.c. at the dose of 0.1 μg/kg, 8 hours before colitis induction and every 8 hours thereafter. The following parameters were assessed: clinical score (**A**); change in body weight (**B**); EPO activity in colon tissue (**C**); MPO activity in colon tissue (**D**); lethality rate (**E**); and histopathological score (**F**). Each data point represent the mean ± SEM of $n = 6–8$ animals in each group, with the exception of histopathological score ($n = 4$). $^{*}P < 0.05$ when comparing vehicle and evasin-4-treated mice and $^{***}P < 0.001$ as indicated in the figure.

trophil influx after DSS administration. Experiments in *Δdbl*GATA animals showed that there was decreased local expression of CXCL1 and CCL3, which correlated with a decreased local influx of neutrophils. Eosinophils themselves have been shown to be relevant sources of CCL3 and CXC chemokines.[36,37] In addition, eosinophils may release granule contents and free radicals, which may activate resident cells to release mediators of inflammation, including chemokines.[38] Finally, neutrophils are potential sources of both CXCL1 and CCL3,[39,40] amplifying their own recruitment to inflammatory sites. The major sources of neutrophil-active chemokines in our system were not investigated here. Altogether, however, our data do demonstrate that eosinophil influx was relevant directly or indirectly for production of neutrophil-active chemokines and accumulation of neutrophils after DSS-induced colitis. The inhibition of neutrophil influx may contribute to the protective effects afforded by the eosinophil-deficient phenotype.

High levels of CCL11 have been found in serum and in biopsies of UC patients, which is consistent with the eosinophil accumulation in the gastrointestinal tract during this pathology.[4,12,28] Therefore, it seems that the eosinophil migration to the intestinal colon of animals with colitis is dependent on the local release of chemokines, particularly CCL11. The results presented here reinforce such hypothesis. The concentration of this CCL11 was increased after DSS administration and correlated with the influx of eosinophils. It is interesting to observe that there was some basal expression of CCL11 in epithelial cells, suggesting these cells may be initial source of the chemokine. Subsequently and coinciding with a greater influx of eosinophils, CCL11 expression was mostly found on leukocytes, especially macrophages and eosinophils. Ahrens et al[29] have suggested that macrophages are major source of CCL11 in DSS colitis. Our results concur with the latter finding and show that eosinophils themselves can be a source of CCL11, as demonstrated by immunohistochemistry and the decrease of CCL11 expression in eosinophil-deficient mice.

We have recently described a family of chemokine-binding proteins derived from tick saliva that we termed "evasions."[16,17] One of these molecules, evasin-4, was shown to bind to CCL11 and CCL5. The present study shows that evasin-4 was able to reduce the recruitment of eosinophils to the pleural cavity induced by CCL11 and antigen challenge of immunized mice. The latter model has been shown to depend on the endogenous production and action of CCL11.[22] Supporting the role of eosinophils and CCL11 in DSS-induced colits, treatment with evasin-4 greatly inhibited the eosinophil recruitment to the intestinal colon induced by DSS administration. This was accompanied by improvement of the characteristic clinical signs of colitis, weight loss, and lethality rates. Similarly to experiments in *Δdbl*GATA mice, the administration of evasin-4 also resulted in decrease of neutrophil content in the colon of animals with colitis, emphasizing the role of eosinophils in driving the migration of neutrophils in the model. The decreased neutrophil recruitment could contribute to the beneficial effects of evasin-4 in the model.

Although evasin-4 significantly decreased clinical score/weight loss and inhibited eosinophil accumulation to levels similar to those found in *Δdbl*GATA mice, there was still a 40% lethality rate in treated mice given DSS. It is possible that the few eosinophils, which are present after evasin-4 treatment but not in *Δdbl*GATA mice, were sufficient to produce a degree of damage to the gut, leading to death. Therefore, the greater protection afforded by *Δdbl*GATA mice could be due to the greater effect on eosinophil accumulation in the gut.

In addition to blocking CCL11, evasin-4 also binds to CCL5.[17] Hence, the possibility that blockade of CCL5 by evasin-4 plays a role in the model cannot be excluded. It has been shown that CCL5 does not induce eosinophil recruitment directly in mice.[41] However, there is still the

possibility that CCL5 may be acting on other cell types to induce eosinophil influx and tissue injury after DSS. Indeed, CCL5 may orchestrate recruitment and activation of activated T cells and NK cells to inflammatory sites[42,43] and correlation between CCL5 production, effector T cell activation, and recruitment during IBD has been reported by several groups.[44–46] It is suggested that CCL5 could be responsible for $CCR5^+$ T cell recruitment to inflammatory sites during IBD, favoring a shift to a Th1 response. We could not detect any increase in CCL5 or in IFN-γ production in our DSS-induced colitis model (data not shown). Furthermore, T cells do not seem to be key effector population during DSS-induced colitis.[47] Thus, it seems that CCL5 modulation by evasin-4 may not be the major mechanism of protection induced by treatment with the drug.

In conclusion, our study shows that eosinophils play a pivotal role in the pathogenesis of DSS-induced colitis. Moreover, it is shown that blockade of eosinophil recruitment with a CCL11-active chemokine-binding protein prevents clinical disease, tissue destruction, and death. Eosinophils are, therefore, potential target cells to the development of new therapies, and mechanisms that interfere with the recruitment and/or the activation of these cells may represent important therapeutic strategies for the treatment of colitis. Finally, a CCL11-binding protein effectively prevented eosinophil influx, clinical symptoms, tissue pathology, and lethality in DSS-induced colitis, suggesting that evasin-4 may represent a potential novel anti-inflammatory agent for the treatment of IBD.

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