The CCL3/Macrophage Inflammatory Protein-1 α–Binding Protein Evasin-1 Protects from Graft-versus-Host Disease but Does Not Modify Graft-versus-Leukemia in Mice


*J Immunol* 2010; 184:2646-2654; Prepublished online 25 January 2010; doi: 10.4049/jimmunol.0902614

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The CCL3/Macrophage Inflammatory Protein-1α–Binding Protein Evasin-1 Protects from Graft-versus-Host Disease but Does Not Modify Graft-versus-Leukemia in Mice


CCL3 is a protein of the CC chemokine family known to be important for T cell recruitment in inflammatory diseases. The aim of the current study was to evaluate the effects and putative mechanism of action of evasin-1, a novel CCL3-binding protein, in the pathogenesis of acute graft-versus-host disease (GVHD). GVHD was induced by the transplantation of splenocytes from C57BL/6J to B6D2F1 mice. Treatment of recipient mice with evasin-1 prevented mortality associated with GVHD. This was correlated with reduced weight loss and clinical disease severity. Analysis of the small intestine showed that evasin-1 treatment reduced the histopathological score and decreased levels of IFN-γ and CCL5. Mechanistically, evasin-1 treatment reduced the number of CD4+ and CD8+ T cells infiltrating the small intestine, as assessed by immunohistochemistry, and the adhesion of leukocytes to intestinal venules of recipient mice, as assessed by intravitral microscopy. Evasin-1 was also able to decrease liver damage, as seen by reduction of inflammatory infiltrate and IFN-γ levels. Treatment with evasin-1 did not interfere with graft-versus-leukemia. Altogether, our studies demonstrate that CCL3 plays a major role in mediating GVHD, but not graft-versus-leukemia in mice and suggest that blockade of CCL3 with evasin-1 has potential therapeutic application in patients undergoing bone marrow transplantation. The Journal of Immunology, 2010, 184: 2646–2654.

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Graft-versus-host disease (GVHD) is a major complication of allogeneic bone marrow transplantation (BMT), leading to significant morbidity and mortality in humans (1). Allogeneic BMT is a transplantation between two genetically nonidentical individuals (2). This therapy is currently indicated for treatment of a number of malignant and nonmalignant diseases, including acute and chronic leukemia, myelomas, lymphomas, aplastic anemia, solid tumors, and severe immunodeficiency (1, 2). Acute and chronic GVHD occurs when transplanted donor-derived T cells recognize and react to histo-incompatible recipient Ags and cells (3). Acute GVHD occurs within 100 d and is a rapidly progressive syndrome characterized by profound wasting, immunosuppression, and tissue injury in a number of organs, including the intestines, spleen, skin, liver, and lung (1, 4–6).

Mononuclear phagocytes and other leukocytes are thought to be responsible for both initiation of graft-versus-host reaction and for the subsequent injury to host tissues after complex interactions with cytokines and chemokines (1, 2, 7–9). Three phases have been described in experimental GVHD. During the first phase, the conditioning regimen (irradiation and/or chemotherapy) leads to damage, activation of host tissues, including intestinal mucosa, and induces the secretion of inflammatory cytokines (8–11). The second phase consists of donor T cell activation, proliferation and differentiation into effector T cells (3, 8, 9). Activated donor T cells recognize allogeneic Ags presented by host APC and are further activated by costimulation expressed by APC (3, 8, 9). At the final phase, reactive effector T cells are recruited by chemokines, including CCL3/MIP-1α, into GVHD target organs, resulting in host tissue injury (2, 3, 8–13). CCL3 is a member of the CC chemokine subfamily that has been shown to be expressed in GVHD and to affect T cell recruitment and proliferation in GVHD (8, 9, 12, 13).

A number of distinct chemokine-binding proteins (CBP) have been isolated from virus and other parasites, including ticks, that inhibit chemokine activity both in vitro and in vivo and play an important role in immune evasion (14–20). CBP derived from ticks, named evasins, appear to have unusually stringent chemokine selectivity, differentiating them from broader spectrum viral CBPs. In vitro, evasin-1 binds and inactivates CCL3 (17) and, in vivo, treatment with evasin-1 decreased rolling, adhesion, and transmigration of leukocytes induced by CCL3 and reduced bleomycin-induced lung fibrosis and death (18). The present study examined the effect of the treatment with evasin-1 in the pathogenesis of acute GVHD in mice and compared findings with results obtained in CCL3-deficient (CCL3−/−) mice or mice treated with dexamethasone. We also evaluated whether CCL3

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0902614
would affect the ability of the engrafted murine host to deal with challenge from a leukemic cell line.

Materials and Methods

Mice

Eight- to twelve-week-old mice were housed under standard conditions in a temperature-controlled room (23 ± 1°C) on an automatic 12-h light/dark cycle and had free access to commercial chow and water. All procedures described in this study were approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (number: 077/08). Male C57BL/6J and (C57BL/6J × DBA/2) F1 were obtained from the Bioscience unit of Universidade Federal de Minas Gerais., CCL3-deficient mice (21) were purchased from The Jackson Laboratory (Bar Harbor, ME), and they were maintained in the animal facilities of the Universidade Federal de Minas. Transgenic mice expressing enhanced GFP (22) were kindly donated by Professor Okabe (Osaka University, Osaka, Japan) and maintained in the animal facilities of on Animal the Universidade Federal de Minas.

Induction of GVHD and treatment protocols

For GVHD induction, 3 × 10^7 splenocytes from either syngeneic (B6D2F1) or semiallogeneic (C57BL/6J) donors were injected into recipient B6D2F1 mice that had been irradiated with 4 Gy total body irradiation (source: 60Co). C57BL/6J or C57BL/6J-CCL3-/- mice were considered the control group. The GVHD group received splenocytes from C57BL/6J mice (C57BL/6J → B6D2F1) and developed classic disease. Animals in this group were treated with PBS (the vehicle for drugs used) s.c. in 200 μl twice per day. The evasin-1 group received splenocytes from C57BL/6J (C57BL/6J → B6D2F1) and 10 μg evasin-1 s.c. in 200 μl PBS, 30 min before transplantation and then twice per day during the entire duration of the experiments. The dexamethasone group received splenocytes from C57BL/6J (C57BL/6J → B6D2F1) and 25 μg dexamethasone (Hipo-labor Pharmaceuticals, Sao Paulo, Brazil) s.c. in 200 μl PBS once a day. A group of B6D2F1 mice (CCL3-/- group) received C57BL/6J splenocytes from CCL3-deficient mice (C57BL/6J CCL3-/- → B6D2F1). Evasin-1 was produced in HEK293 cells and purified by Merck Serono (Geneva, Switzerland) according to a previously published study (17).

Mortality rate and assessment of GVHD clinical score

A group of mice was monitored daily for survival posttransplantation and assessed clinically by a standard scoring system that generates a composite GVHD score comprising individual scores for weight loss, posture (hunching), activity, fur texture, skin integrity, diarrhea (11, 23), and occult blood in feces. Each parameter was evaluated every 2 d and graded 0–2 (maximum index = 14).

Histopathology

A set of experiments were conducted for quantification of histopathological parameters in the intestine and liver, important target organs in GVHD. The intestinal rolls and liver were fixed in buffered formaldehyde (10% in PBS) for 24 h and routinely processed for paraffin embedding. Sections 5 μm-thick were obtained and stained with H&E. The entire extension of the jejunum and ileum wall and liver was examined under an optical microscope (Olympus BX51, Tokyo, Japan) using a 10× objective. The images were captured under the

![FIGURE 1. Kinetics of the expression of CCL3 after GVHD induction. GVHD was induced by the transfer of splenocytes from allogeneic (WT C57BL/6J or C57BL/6J-CCL3-/- mice) donors to B6D2F1 mice. Mice that received splenocytes from syngeneic (B6D2F1) mice did not develop disease and were considered the control group. Intestinal samples were collected at the indicated time points after GVD induction, and CCL3 was detected by ELISA. Results are presented as the mean ± SEM (n = 6). *p < 0.01 compared with control; #p < 0.01 compared with GVHD.

![FIGURE 2. Evasin-1 treatment prevented mortality and clinical GVHD. GVHD was induced by the transfer of splenocytes from allogeneic (WT C57BL/6J or C57BL/6J-CCL3-/- mice) donors to B6D2F1 mice. Mice that received splenocytes from syngeneic (B6D2F1) mice did not develop disease and were considered the control group. Evasin-1 (10 μg in 200 μl of PBS, s.c.) was given to WT mice 30 min before transplantation and twice per day during the entire duration of the experiments. After induction of GVHD, mice were monitored every 2 d for survival (A), body weight (B), and GVHD clinical scores (C). Control group (●), n = six mice/group; GVHD group (▲), n = eight mice/group; evasin-1 group (×), n = nine mice/group; and CCL3-/- group (▼), n = seven mice/group. #p < 0.05 when compared with the control group; ##p < 0.01 when compared with the GVHD group.

According to occurrence and degree of intensity. A clinical index was subsequently generated by summation of scores of the seven criteria (maximum index = 14).
microscope with a resolution of 720 × 480 pixels (Cool SNAP-Proof Color, Media Cybernetics, Bethesda, MD) and evaluated using the program Image ProExpress version 4.0 for Windows (Media Cybernetics).

Histopathological scores were determined according to the criteria previously established (11, 24, 25). Samples were obtained from mice at days 3, 10, and 20 posttransplantation, which correspond to the following phases of clinical disease in mice: latency, onset of clinical disease, and mortality, respectively. Some mice had intestines removed for histopathological analysis 24 h postirradiation and before the transplantation to control for the consequences of irradiation to the intestine.

Cytokine and chemokine quantification
Cytokines and chemokines were measured according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN). Briefly, 100 mg duodenum or liver was homogenized in 1 ml PBS (0.4 mol/l NaCl and 10 mmol/l NaPO₄) containing antiproteases (0.1 mmol/l phenylmethyl sulfonfl fluoride, 0.1 mmol/l benzothenium chloride, 10 mmol/l EDTA, and 20 KI aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 min at 3000 × g. and the supernatants immediately used for ELISA assays at a 1:4 dilution in PBS.

Intravital microscopy
GVHD was induced in B6D2F1 mice, using GFP⁺C57BL/6j mice splenocytes. At day 20 posttransplantation, mice were anesthetized, and the jejunum-ileum was exposed in a perfusion system containing warm bicarbonate-buffered saline (pH 7.4). An intravital microscope (Olympus BX50/F4) with a 10× objective lens was used to examine the mesenteric microcirculation. A video camera (5100 HS; Panasonic, Osaka, Japan) was used to project the images onto a monitor, and images were recorded for playback analysis, using a conventional videocassette recorder.

Intestinal venules (20–40 µm) were selected and the number of rolling and adherent GFP⁺leukocytes determined offline during video playback analysis. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. The flux of rolling cells was measured as the number of rolling cells passing by a given point in the venule per minute. A leukocyte was considered adherent if it remained stationary for at least 30 s, and total leukocyte adhesion was quantified as the number of cells in the intravascular space within an area of 100 µm.

Evasin-1 was given on two separate treatment schedules. In the first group of experiments, evasin-1 (10 µg/mouse) was administered just before GVHD induction and then every 12 h until the intravital microscopy procedure. In the second group, mice were treated with evasin-1 (10 µg/mouse) 30 min before the intravital procedure; that is, in the latter group, animals only received the compound just before evaluation of leukocyte and endothelial cell interactions.

Immunohistochemistry of intestine
Paraffin-embedded jejunum-ileum obtained from mice at day 20 post-transplantation were sectioned (3 µm), placed on glass slides coated with 2% 3-amino propyltriethoxysilane (Sigma-Aldrich, St. Louis, MO), deparaffinized, and incubated with 3% hydrogen peroxide diluted in methanol (1:1) for 20 min. Sections were then immersed in citrate buffer (pH 6.0) for 20 min at 95°C for Ag retrieval. The material was blocked by incubation with 3% normal goat serum diluted, at room temperature, for 20 min. The slides were then incubated with anti-mouse CD8 or CD4 monoclonal Abs diluted at 1:1000, at 4°C overnight in a humidified chamber. After washing in TBS, the sections were revealed using the LSAB+ System-HRP (DakoCytomation, Carpinteria, CA) in accordance with instructions given by the supplier. Sections were counterstained with Mayer’s hematoxylin. Negative controls were obtained by the omission of primary Abs, which were substituted with 1% PBS-BSA and with mouse serum (X501-1, Dako).

The number of positive cells was counted in 10 alternate high-power microscopic fields (×400) under an optical microscope, Olympus BX50 Q (Melville, NY), using an integration reticule (4740680000000–Netzkirkrometer 12.5 ×, Carl Zeiss, Göttlingen, Germany) with the aid of Q-Capture Pro software (QImaging, Surrey, British Columbia, Canada).

Quantification of macrophage infiltration
The relative number of macrophages infiltrating the intestine and liver were quantified by measuring N-acetyl glucosaminidase (NAG) activity at day 20 posttransplantation. A portion of 100 mg of the small intestine was resuspended in saline 0.9% (4°C) containing 0.15 v/v Triton X-100 (Merck Sharp & Dohme, Sao Paulo, Brazil), homogenized, and centrifuged at 4°C for 10 min at 1500 rpm. The supernatants were collected and immediately assayed for NAG using a 1:10 dilution, as described previously (26).

Graft-versus-leukemia induction
P815, a mouse mastocytoma cell line (H-2d, American Type Culture Collection, Rockville, MD) transduced with a lentiviral vector (EF1αGFP), was kindly provided by A.C. Leal and M. Bonamino from Divisão de Medicina Experimental, Instituto Nacional de Cancer (Rio de Janeiro, Brazil). These cells were used for graft-versus-leukemia (GVL) experiments and were maintained in RPMI 1640/10% FCS at 37°C, 5% CO₂. The same protocols for irradiation and GVHD induction were used in these experiments. A total of 6000 GFP⁺ P815 cells were injected i.v. in B6D2F1 mice 30 min before the intravital procedure; that is, in the latter group, animals only received the compound just before evaluation of leukocyte and endothelial cell interactions.

FIGURE 3. Evasin-1 treatment reduced intestinal damage in mice subjected to GVHD. GVHD was induced by the transfer of splenocytes from allo-genic (C57BL/6j) donors to B6D2F1 mice. Mice that received splenocytes from syngeneic (B6D2F1) mice did not develop disease and were considered the control group. Evasin-1 (10 µg in 200 µl of PBS s.c.) was given 30 min before transplantation and twice per day during the entire duration of the experiments. After induction of GVHD, mice were killed and the jejunum-ileum sampled for histopathological analysis and scored at 3 (A), 10 (B), and 20 (C) days posttransplantation. Results are presented as the mean ± SEM (n = 6). ∗p < 0.05 compared with control; #p < 0.05 compared with GVHD. D–F, Histological aspects of small intestine (jejenum-ileum) of control, GVHD, and evasin-1-treated mice, respectively, at day 20 posttransplantation. Scale bar, 50 µm for all panels.
recipients on day 0 just after the splenocyte transplantation. At day 10 posttransplantation, mice were sacrificed and lymph nodes prepared for FACS analysis. Erythrocytes were lysed and cells centrifuged for 5 min at 350 g. Cells (1 × 10⁷) were resuspended in buffer containing 5% BSA and 0.01% sodium azide and fixed in a solution containing 2% formaldehyde in PBS. Flow cytometry was performed using a FACScan (BD Biosciences, San Jose, CA), and 20,000 events per sample were evaluated. The presence of tumor cells was determined by assessing the frequency of GFP+ cells, using the program FlowJo (Tree Star, Ashland, OR). The frequency of GFP+ P815 cells was analyzed in tumor-bearing mice that received or did not receive evasin-1 treatment (10 µg/mouse, s.c., twice per day).

Statistical analysis
Data in text are given as mean ± SEM. Comparison between groups was carried out by performing ANOVA followed by Student-Newman-Keuls test. A p < 0.05 was considered significant.

Results
Initial experiments were performed to evaluate the kinetics of CCL3 production in our model (Fig. 1). The transplantation of splenocytes from wild-type (WT) C57BL/6J mice to WT B6D2F1 mice (GVHD group) induced an increase in levels of CCL3 in intestine when compared with B6D2F1 mice that received transplants of isogenic cells (control group). When WT B6D2F1 mice received parental splenocytes of C57BL/6J CCL3−/− (CCL3−/− group), the levels of CCL3 in intestine were reduced at 10 and 20 d, but not at day 3, posttransplantation (Fig. 1). The latter results indicate that resident cells (which are CCL3+) contribute to the initial production of CCL3.

All B6D2F1 mice that received splenocytes from C57BL/6J mice (GVHD group) died before day 45, as shown in Fig. 2A. In contrast, the control group did not develop GVHD and were all alive by the end of the experiment. The transplantation of splenocytes from C57BL/6J CCL3−/− mice to WT B6D2F1 mice (CCL3−/− group) resulted in marked protection (88% survival rate), confirming the role of CCL3 in GVHD (Fig. 2A). The evaluation of weight (Fig. 2B) and clinical score (Fig. 2C) of mice concurred with the mortality data. Indeed, whereas there was a marked weight loss in mice subjected to GVHD, weight loss and clinical score were greatly reduced in CCL3−/− mice (Fig. 2B, 2C), indicating the role of CCL3 in GVHD development.

Reduced clinical disease and mortality in evasin-1–treated mice subjected to GVHD
Once the role of CCL3−/− was determined in our model, we tested whether treatment with evasin-1 was efficient to ameliorate GVHD. Evasin-1 treatment, 30 min before transplantation and every 12 h thereafter, prevented mortality and provided partial alleviation of the GVHD clinical presentation (Fig. 2). Overall, the protection afforded by evasin-1 was similar to that observed in CCL3−/− mice (Fig. 2). For comparison, experiments conducted with the glucocorticosteroid dexamethasone showed that 75% of mice were alive at day 45 after GVHD induction and there was decrease of clinical disease similar to that observed with evasin-1 treatment (data not shown). Evasin-1 treatment was able to decrease CCL3 levels in the gut at 10 d after GVHD induction (pg/100 mg of tissues: control mice, 63 ± 9; vehicle-treated GVHD mice, 2988 ± 165; evasin-1–treated mice, 2281 ± 233; n = 6 and p < 0.05) and 20 d after GVHD induction (pg/100 mg of tissues: vehicle-treated GVHD mice. 1518 [±123]; evasin-1–treated mice: 756 [±49]; n = 6 and p < 0.05).

Reduced intestinal damage in evasin-1–treated mice subjected to GVHD
Intestinal sections were evaluated at 3, 10, and 20 d posttransplantation. The dose of irradiation given to the animals appeared not to cause significant changes in the intestine of mice when they were evaluated 24 h postirradiation (data not shown). In contrast, the grafted transplant caused significant changes in the intestine that were already apparent at day 3 and evolved to much more significant disease at day 20 posttransplantation (Fig. 3). At day 3, mild edema and congestion of the lamina propria were present, and this condition was associated with focal areas of villous enlargement (data not shown). These initial changes were not modified significantly by treatment with evasin-1 or in experiments in CCL3−/− mice (Fig. 3A).

At day 10 after disease induction, there was increased cellularity in the lamina propria and the inflammatory process reached the muscular layer, interposing between degenerated muscle cells. Peyer patches were increased in volume and exhibited signs of reactivity; phagocytosis events were frequent, and swelling and increased vascularization were observed in the surrounding mucosa (data not shown). Treatment with evasin-1 induced a significant preservation of the muscular and serous layer, resulting in a reduction in overall score (Fig. 3B). The effects of the treatment were qualitatively and quantitatively similar to results obtained in CCL3−/− mice (Fig. 3B).

At day 20 posttransplantation, epithelial changes were significant, including crypt cell hyperplasia, increased cellularity of the lamina propria, edema, and congestion. There were also degenerative changes and necrosis of the muscular and serous layers in the GVHD group (Fig. 3C–E). Treatment with evasin-1 caused a significant amelioration of the overall pathological score. Protection of the mucosa was observed; it showed only superficial and rare erosions, with regenerative cell changes restricted to the top

**FIGURE 4.** Evasin-1 treatment reduced concentration of CCL5 and IFN-γ in the small intestine of mice subjected to GVHD. GVHD was induced by the transfer of splenocytes from allogeneic (C57BL/6J) donors to B6D2F1 mice. Mice that received splenocytes from syngeneic (B6D2F1) mice did not develop disease and were considered control group. Evasin-1 (10 µg in 200 µl of PBS s.c.) was given 30 min before transplantation and twice per day during the entire duration of the experiments. At day 20 after induction of GVHD, mice were killed and the concentrations of CCL5 (A) and IFN-γ (B) evaluated by ELISA. Results are presented as the mean ± SEM (n = 6). *p < 0.05 compared with control; #p < 0.05 compared with GVHD.
reduced production of CCL5 and IFN-γ and recruitment of CD4+ and CD8+ cells in the intestine of evasin-1–treated mice subjected to GVHD. As seen in Fig. 5, there was a marked infiltration of CD8+ and CD4+ cells in the lamina propria of the small intestine of GVHD mice, and treatment with evasin-1 greatly decreased CD8+ and CD4+ cell recruitment.

Histological analysis of samples showed an overall decrease in inflammatory cell influx in the intestine of evasin-1–treated mice when compared with vehicle-treated mice (Fig. 3). Moreover, a significant decrease in NAG activity (a surrogate marker for macrophage influx) (26) was noted in the intestine of evasin-1–treated mice (Relative number of macrophages/mg of tissue: control mice, 5.6 ± 10^7 [±0.2]; vehicle-treated GVHD mice, 8.4 × 10^7 [±0.2]; evasin-1–treated mice, 6.0 × 10^7 [±0.1]; n = 6 and p < 0.05).

The experiments above showed inhibition of the total recruitment of CD8+ and CD4+ cells and macrophages in GVHD mice given evasin-1. To examine whether inhibition of chemokine-induced leukocyte adhesion and consequent migration was the mechanism by which evasin-1 functioned in the system, we

Thus, we evaluated whether the treatment with evasin-1 would modify the recruitment of CD8+ and CD4+ cells into the intestine of mice subjected to GVHD. As seen in Fig. 5, there was a marked infiltration of CD8+ and CD4+ cells in the intestine of mice subjected to GVHD when compared with vehicle-treated mice (Fig. 3). Moreover, a significant decrease in NAG activity (a surrogate marker for macrophage influx) (26) was noted in the intestine of evasin-1–treated mice (Relative number of macrophages/mg of tissue: control mice, 5.6 × 10^7 [±0.2]; vehicle-treated GVHD mice, 8.4 × 10^7 [±0.2]; evasin-1–treated mice, 6.0 × 10^7 [±0.1]; n = 6 and p < 0.05).

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performed intravital microscopy of an intestinal venule, postcapillary. Irradiated B6D2F1 or C57BL/6J mice received leukocytes from GFP$^+$ C57BL/6J mice. Mice were treated with vehicle or evasin-1 (10 μg/mouse) using two different protocols, and the interaction of GFP$^+$ cells with endothelium was evaluated on day 20 after GVHD induction. As seen in Fig. 6, there was a marked increase in the number of leukocytes adhering to the intestinal venules of mice undergoing GVHD. In the first protocol, mice were treated daily with evasin-1 from the onset of GVHD (Fig. 6, top panel). Consistent with its ability to decrease lethality and tissue injury, daily evasin-1 treatment greatly reduced leukocyte adhesion (Fig. 6). Of more interest, administration of evasin-1 just before intravital microscopy on day 20 after GVHD induction greatly decreased the adhesion of leukocytes to the mesenteric venules (Fig. 6). These results suggest that blockade of chemokine-induced leukocyte adhesion is a major mechanism for the protective action of evasin-1 in the system.

Evasin-1–treated mice subjected to GVHD exhibited reduction of IFN-γ production and inflammatory infiltrate in liver

In addition to the effects on the intestine, hepatic damage is an important feature of GVHD. Liver damage was scored at 3, 10, and 20 d posttransplantation. There was no significant liver damage at days 3 and 10 after induction of GVHD (data not shown). At 20 d, a marked inflammatory infiltrate was noted in periportal and intralobular areas in mice subjected to GVHD. Treatment with evasin-1 decreased leukocyte infiltration in animals subjected to GVHD (Fig. 7A, 7D–F). Consistent with the overall leukocyte influx observed on histological sections, evasin-1–treated mice subjected to GVHD showed a significant decrease in NAG activity 2m the liver at day 20 posttransplantation (Fig. 7B). Levels of IFN-γ were enhanced by GVHD at day 20, and this increase was prevented by treatment with evasin-1 (Fig. 7C). Hepatic levels of CCL5 and IL-10 did not increase in mice subjected to GVHD at day 20 (data not shown).

Evasin-1 treatment maintains GVL effect after GVHD induction

GFP$^+$ P815 cells were injected at day 0 of splenocyte transplantation, and the frequency of these cells in the lymph nodes was determined by FACS at day 10 posttransplantation. A group of mice that received splenocyte transplant and did not receive tumor cells served as negative controls to set the gates for GFP$^+$ cells. There was a marked increase in the frequency of GFP$^+$ P815 cells in the lymph nodes of mice that were not subjected to GVHD (i.e., did not receive the splenocyte transplant) (Fig. 8). In contrast, transfusion of splenocytes into mice that had the tumor implanted resulted in a significant decrease in the frequency of GFP$^+$ P815 tumor cells, suggesting a significant GVL effect (Fig. 8). Treatment with evasin-1 had no major effect on the ability of the graft to decrease the frequency of GFP$^+$ P815 tumor cells in the lymph nodes (Fig. 8). Entrapment of effector T cells could have accounted for the antitumor effect in evasin-1–treated animals. However, treatment with evasin-1 did not interfere with accumulation of activated donor (GFP$^+$) in spleen or lymph nodes (number of GFP$^+$CD$^+$CD69$^+$ cells/spleen: vehicle-treated GVHD mice, 56.5 × 10$^3$ [±13.4]; evasin-1–treated mice, 66.3 × 10$^3$ [±11.8]; number of GFP$^+$CD$^+$CD69$^+$ cells/lymph node: vehicle-treated GVHD mice, 9.2 × 10$^3$ [±4.2]; evasin-1–treated mice, 6.1 × 10$^3$ [±2.4]; n = 5 and p > 0.05).

Discussion

CCL3 is expressed during experimental GVHD and may be important for the inflammatory response caused by acute GVHD (12, 13). The results reported hereewith confirm the relevance of CCL3 in mediating GVHD in mice. Moreover, we report for the first time that blockade of CCL3 with a CBP, evasin-1, which prevents CCL3 function (17), ameliorated GVHD and prevented death. Treatment with evasin-1 prevented the influx of leukocytes, especially CD$^+$, CD4$^+$ cells and macrophages, to the small intestine and decreased tissue damage in the liver. Mechanistically, inhibition of leukocyte influx to the intestine was due to inhibition by evasin-1 of the ability of leukocytes to adhere to endothelial cells in affected tissues. The latter results suggest that mediation of leukocyte adherence and subsequent migration is the major mechanism by which CCL3 participates in murine GVHD. Finally, the protective effects of evasin-1 against GVHD did not interfere with the beneficial effect of the graft against a leukemic cell line, hence showing that GVL was still operative in evasin-1–treated mice.

The production of CCL3 peaked at day 10 after GVHD but was already noticeable at day 3 and remained elevated at day 20, suggesting that the chemokine was generated continuously throughout the course of the disease. Studies in which donor cells were obtained from C57BL/6/JCCL3$^{-/-}$ mice showed that initial levels of CCL3 were mostly derived from cells of recipient mice, whereas a considerable proportion of the production of CCL3 derived from donor cells at days 10 (>80%) and 20 (>95%) postinduction. Hence, the cells transferred during grafting become major contributors of CCL3 production as the disease progresses. In agreement with previous studies (12, 13), transfer of CCL3-deficient cells was accompanied by decreased GVHD-associated clinical score, weight loss, and lethality. Treatment with evasin-1 resulted in protective effects that were qualitatively and quantitatively similar to those observed in GVHD experiments using cells from CCL3$^{-/-}$ mice. Indeed, the ability of evasin-1 to

![FIGURE 6. Effects of treatment with evasin-1 on the number of cells adherent to the mesenteric venules of mice subjected to GVHD, as assessed by intravital microscopy. GVHD was induced by the transfer of splenocytes from allogeneic (C57BL/6) donors to B6D2F1 mice. Mice that received splenocytes from syngeneic (B6D2F1) mice did not develop disease and were considered the control group. In one group (evasin-1 daily), evasin-1 (10 μg in 200 μl of PBS s.c.) was given 30 min before transplantation and twice per day during the entire duration of the experiments. In another experiment (evasin-1 30 min), evasin-1 was given at a single dose 30 min before the intravital microscopy. At day 20 after induction of GVHD, mice were anesthetized, and intestinal venules (20–40 μm) were selected for counting the number of adherent leukocytes, using intravital microscopy. Results are presented as the mean ± SEM. *p < 0.01 compared with control; #p < 0.01 compared with GVHD; n = 6.](image-url)
FIGURE 7. Evasin-1-treatment reduces inflammation and levels of IFN-γ in the liver of mice subjected to GVHD. GVHD was induced by the transfer of splenocytes from allogeneic (C57BL/6J) donors to B6D2F1 mice. Mice that received splenocytes from syngeneic (B6D2F1) mice did not develop disease and were considered the control group. Evasin-1 (10 µg in 200 µl of PBS s.c.) was given 30 min before transplantation and twice per day during the entire duration of the experiments. After induction of GVHD, mice were killed and the liver sampled for histopathological analysis and scored at 20 d post-transplantation (A). The relative numbers of macrophages (B) and IFN-γ (C) also were verified. Results are presented as the mean ± SEM (n = 6). *p < 0.05 compared with GVHD.

FIGURE 8. Evasin-1 treatment does not interfere with GVL in mice subjected to GVHD. GVHD was induced by the transfer of splenocytes from allogenic (C57BL/6J) donors to B6D2F1 mice. Mice that received splenocytes from syngeneic (B6D2F1) mice did not develop disease and were considered the control group. Evasin-1 (10 µg in 200 µl of PBS s.c.) was given 30 min before transplantation and twice per day during the entire duration of the experiments. GFP+P815 cells were injected i.v. into B6D2F1 recipients on day 0 of transplantation. At day 10 of transplantation, mice were killed and the frequency of GFP+P815 cells evaluated in inguinal lymph nodes. Results are presented as the mean ± SEM (n = 6). *p < 0.01 compared with mice that received no tumor cells; #p < 0.01 compared with mice that received tumor but were not subjected to GVHD.
protection and decreased death rates, is in line with the ability of evasin-1 to decrease, but not abolish, disease. Whether these maintained levels of TNF-α contribute to effective GVT (see below) remains to be determined.

BMT has become standard treatment for a number of malignant and nonmalignant diseases, including acute and chronic leukemia, myelomas, lymphomas, aplastic anemia, solid tumors, and severe immunodeficiency (1, 2). In these diseases, it is thought that BMT may help in the elimination or decrease in growth of the tumor (41). The ability of grafted leukocytes to reduce tumor growth is referred to as the GVL effect (41–46). Unfortunately, allografted cells become activated against the host and cause GVHD (41–46). Therefore, it is generally accepted that, in the context of hematopoietic stem cell transplantation, a therapy should decrease or prevent GVHD but ideally should not modify the associated GVL (42–46). In our study, there was significant GVL after the transplantation of splenocytes, as assessed by the inhibition of the growth of P815-GFP+; a mastocytoma cell line that becomes leukemic. Importantly, treatment with evasin-1 had no effect on GVL against P815-GFP+ cells, demonstrating that it prevented disease without disrupting the beneficial effect of the host against the leukemic cell line.

CBPs have arisen as new proteins capable of modulating inflammatory responses and might be explored as potential therapeutic strategies for inflammatory diseases. In this study, we observed decidedly beneficial effects of the CCL3-binding protein evasin-1 in inflammatory responses and might be explored as potential therapeutic strategies for inflammatory diseases. In this study, we observed decidely beneficial effects of the CCL3-binding protein evasin-1 in inflammatory responses and might be explored as potential therapeutic strategies for inflammatory diseases.


