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Ig-Free Light Chains Play a Crucial Role in Murine Mast Cell-Dependent Colitis and Are Associated with Human Inflammatory Bowel Diseases

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Traditionally, mast cells were regarded as key cells orchestrating type I hypersensitivity responses. However, it is now recognized that mast cells are widely involved in nonallergic (non-IgE) chronic diseases. Also, in inflammatory bowel disease (IBD), a disease not associated with increased IgE concentrations, clear signs of activation of mast cells have been found. In this study, we investigated if Ig-free L chain-induced hypersensitivity-like responses through activation of mast cells could contribute to the pathophysiology of IBD. As a mast cell-dependent model for IBD, mice were skin-sensitized with dinitrofluorobenzene followed by intrarectal application of the hapten. In this murine IBD model, F991 prevented mast cell activation and also abrogated the development of diarrhea, cellular infiltration, and colonic lymphoid follicle hyperplasia. Furthermore, passive immunization with Ag-specific Ig-free L chains (IgLCs) and subsequent rectal hapten challenge elicited local mast cell activation and increased vascular permeability in the colon of mice. Clinical support is provided by the observation that serum concentrations of IgLCs of patients suffering from Crohn's disease are greatly increased. Moreover, increased presence of IgLCs was evident in tissue specimens from colon and ileum tissue of patients with IBD. Our data suggest that IgLCs may play a role in the pathogenesis of IBD, which provides novel therapeutic means to prevent or ameliorate the adverse gastrointestinal manifestations of IBD. *The Journal of Immunology*, 2010, 185: 000–000.

Inflammatory bowel disease (IBD) comprises chronic, spontaneously relapsing inflammatory conditions of the gastrointestinal tract, with Crohn's disease (CD) and ulcerative colitis (UC) as major representatives (1). Patients diagnosed with this disorder are suffering from severe abdominal pain and cramps, diarrhea, rectal bleeding, and a substantial personal burden. Although the exact etiology remains unclear, it is thought to be a complex interaction of genetic, environmental, and immunological factors (2).

Several inflammatory cells are involved in the pathology of IBD, including the mast cell (3). An increased number of mast cells and mast cell activation are found in the mucosa of ileum and colon of patients with IBD (3–5). In IBD, not only can increased numbers of mast cells be found, but also the cellular content of these cells is greatly changed. For example, granules contained a higher expression of IL-16 (6), TNF- α (7), and substance P (8). Moreover, abundant evidence for mast cell degranulation is provided by the

observation of increased concentrations of mast cell mediators in the gastrointestinal tract of patients with IBD (9–11). Mast cell mediators, like proteases, cytokines, PGs, and chemokines have been shown to induce microvascular leakage and recruit inflammatory cells to the site of inflammation and, therefore, enhance the inflammatory response (12, 13).

Ag-specific mast cell activation occurs predominantly via cross-linking of two IgE Abs bound to the high-affinity IgE receptor Fc ϵ RI. However, Ag-specific mast cell activation also takes place in the absence of IgE (14, 15). Because IBD is a disorder not associated with elevated IgE levels (16), mast cell activation is likely to be elicited by other mechanisms. In our laboratory, an alternative route for Ag-specific mast cell activation mediated by Ig-free L chain (IgLC) was demonstrated. IgLCs are produced by B lymphocytes in excess over H chains during the production of Abs and subsequently secreted into the circulation (17). Ag-specific IgLC was demonstrated to bind to mast cells, and succeeding local stimulation with the corresponding Ag resulted in mast cell-dependent plasma extravasation and infiltration of inflammatory cells. In the skin, this is accompanied with tissue swelling (18), whereas acute bronchoconstriction and airway hyperresponsiveness is observed in the airways (19).

In the study presented in this paper, we suggest a role for IgLC in a murine colonic hypersensitivity model for IBD (20). This model was shown to be mast cell-dependent by the observation that the colonic hypersensitivity response could not be elicited in mast cell-deficient mice and could be restored upon reconstitution with in vitro-cultured bone marrow-derived mast cells (20). To explore the role of IgLC in this model, we treated the mice with the IgLC antagonist F991 previously described to prevent and inhibit IgLC-mediated hypersensitivity responses in skin and airways (18, 19, 21). This treatment with F991 prevented mast cell activation,

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Abbreviations used in this paper: 5-ASA, 5-aminosalicylic acid; CD, Crohn's disease; DNFB, 2,4-dinitrofluorobenzene; DNS, dinitrobenzene-sulfonic acid; IBD, inflammatory bowel disease; IgLC, Ig-free L chain; mMCP-1, mouse mast cell protease-1; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNP, trinitrophenol; UC, ulcerative colitis.

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ameliorated diarrhea, and prevented infiltration of inflammatory cells in the colon. Passive transfer of Ag-specific IgLCs led to mast cell activation and plasma extravasation in the colonic wall after intrarectal challenge with the corresponding Ag. Clinical relevance for IgLC was demonstrated by the observation that serum κ and λ IgLC levels were significantly increased in patients with CD compared with healthy controls. Moreover, it is shown that IgLC can be found in colonic lesions as well as the inflamed and normal mucosa of both the ileum and colon of patients with IBD. The results obtained in this study suggest IgLC as a potential therapeutic target in IBD.

Materials and Methods

Animals

Male BALB/c mice were obtained from Charles River Laboratories (Maastricht, The Netherlands). The mice were 6–8 wk of age and weighed 20–25 g by the time of use. The animals were housed in groups not exceeding eight mice per cage. Tap water and chow food were allowed ad libitum; there was a 12-h day-night cycle. All experiments were conducted in accordance with The Animal Care Committee of Utrecht University (Utrecht, The Netherlands).

Induction of colonic hypersensitivity

Mice were sensitized on day 0 by application of either 2,4-dinitrofluorobenzene (DNFB) (Sigma-Aldrich, St. Louis, MO) (0.6% in acetone/olive oil 4:1) or vehicle (acetone/olive oil 4:1) epicutaneously on the shaved abdomen (50 μ l) and all four paws (50 μ l). On day 1, the mice received a boost of DNFB or vehicle on the abdomen only (50 μ l). All of the animals were challenged intrarectally with dinitrobenzenesulfonic acid (DNS) (Sigma-Aldrich) (0.6%) dissolved in 10% ethanol on day 5. The sensitization, boost, and challenge took place under light inhalation anesthesia (isoflurane 3%). The mice were macroscopically scored on days 6, 7, and 8 for stool consistency. Thereafter, the mice were sacrificed on day 8, 72 h postchallenge, with an overdose of pentobarbital to determine in vivo mast cell activation, mast cell infiltration, colonic vascular permeability, cytokine production in the colon, and presence and number of colonic patches.

Passive immunization with IgLC

Male BALB/c mice were injected i.p. with κ IgLC specific for trinitrophenol (TNP) (5 μ g/mouse) or PBS. To determine vascular permeability changes, 1.25% Evans blue dye was injected simultaneously. Thirty minutes after passive sensitization, the mice were challenged intrarectally with 0.6% 2,4,6-trinitrobenzene sulfonic acid (TNBS) in 10% ethanol under light inhalation anesthesia (isoflurane 3%). The mice were sacrificed with an overdose of pentobarbital 30 min postchallenge to determine in vivo mast cell activation, mast cell infiltration, colonic vascular permeability, and number and presence of colonic patches. Optimal concentrations for κ IgLC sensitization have been determined in previous studies (18, 19).

F991 treatment

F991 is a 9-mer peptide analogous to the specific IgLC-binding domain of THP (amino acid sequence: AHWSGHCCCL; produced by Bachem, Bubendorf, Switzerland). F991 was administered i.p. 50 μ g in 100 μ l sterile saline 24 h prechallenge, at time of challenge, and 24 h and 48 h postchallenge. Previous studies have demonstrated a dose-dependent inhibition of DNFB-induced cutaneous hypersensitivity responses (18).

Clinical scoring of the disease

Stool consistency was monitored daily 24–72 h postchallenge by placing the mice separately in cages without bedding. They were left in the cages until they discharged enough feces to establish the consistency. The feces were taken out immediately by a spatula and smeared on a piece of carton. The scoring was as follows: 0 for well-formed solid pellets, 1 for easy to smear and loose stool, and 2 for diarrhea and watery stool.

Macroscopical scoring of the disease

After sacrificing the animals 72 h postchallenge, the colon was carefully dissected from anus to cecum and placed in saline. The colon was opened longitudinally along the mesenteric border and washed gently in saline. The number of colonic patches, which appear like bulges in the tissue, was counted with the naked eye at the mucosal side.

Histology and immunohistochemistry

The colon was routinely fixed as a Swiss roll and embedded in paraffin as described previously (20). For every mouse, three different longitudinal sections (5 μ m) of the colon tissue were stained with H&E and microscopically analyzed. One observer evaluated all sections and scored according to Hartmann et al. (22) and as described previously (20): for cellular infiltration, rare inflammatory cells in the lamina propria were counted as 0; increased numbers of inflammatory cells in the lamina propria as 1; confluence of inflammatory cells extending into the submucosa as 2; and a score of 3 was given to transmural extension of the infiltrate. For tissue damage, no mucosal damage was counted as 0; discrete lymphoepithelial lesions were counted as 1; surface mucosal erosion as 2; and a score of 3 was given to extensive mucosal damage and extension through deeper structures of the bowel wall. The combined histological score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage). Per mouse, three different longitudinal sections of the colon tissue were observed, and a score was given to the total of all sections. Pictures shown are representatives of the different treatment groups.

To detect mast cells, 5- μ m sections were immunohistochemically stained for mouse mast cell protease-1 (mMCP-1) as described previously (20). For every mouse, three different longitudinal sections of the colon tissue were stained and analyzed. The number of mMCP-1-positive cells was quantified by microscopical visualization and manually counted. Results are expressed as median average number of cells (minimum–maximum) per colon section.

Preparations of tissue homogenates

To determine mast cell infiltration into the tissue, whole colon homogenates were made as described previously (20) and stored frozen until further use to assess mast cell infiltration by means of measuring mMCP-1 and TNF- α levels.

Mast cell activation and infiltration in vivo

To monitor mast cell activation in time, blood samples of DNFB- and vehicle-sensitized mice were taken 30 min after intrarectal DNS challenge. Blood samples were collected via heart puncture, and 4% EDTA was added (10% v/v) to obtain plasma. Postcentrifugation, the plasma was stored at -70°C until use. Levels of mMCP-1 in plasma were measured as described previously using a commercially available ELISA assay (Moredu Scientific, Midlothian, U.K.) (23). Results are expressed as nanograms of mMCP-1 per milliliter of plasma. To determine mast cell infiltration in the colon, mMCP-1 levels were measured in the supernatant of colon homogenates with the commercially available ELISA kit. Total protein content of the supernatant of colon homogenates was quantified with a commercial available detection kit (BCA protein assay, Pierce, Rockford, IL). Results are expressed as nanograms of mMCP-1 per milligram of total protein.

TNF- α in colon tissue

To determine TNF- α levels in vivo, TNF- α was measured in the supernatant of colon homogenates 72 h postchallenge with a commercially available TNF- α ELISA kit (BioSource International, Camarillo, CA). Total protein content of the supernatant of colon homogenates was quantified with a commercial available detection kit (BCA protein assay, Pierce). Results are expressed as picograms of TNF- α per milligram of total protein.

Western blotting

Spleen was dissected from vehicle and DNFB-sensitized mice 72 h after DNS challenge, and single-cell splenocytes were cultured in RPMI 1640 without FCS for 24 h. Supernatant was harvested, filtered, and frozen until use.

Equal amounts of proteins of boiled nonreduced samples were separated electrophoretically (SDS-PAGE 12%) and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Veenendaal, The Netherlands). The membrane was blocked with Tween-PBS containing 2% milk proteins. HRP-labeled goat anti-mouse κ IgLC (1:5000) was applied for 1 h at room temperature as primary Ab. Blots were washed in Tween-PBS three times for 10 min, incubated in commercial ECL reagents (Amersham Biosciences, Roosendaal, The Netherlands), and exposed to photographic film. Films were scanned on a GS710 Calibrated Imaging Densitometer (Bio-Rad).

Isolation of Ag-specific IgLC

Ag-specific IgLC was isolated from TNP-specific IgG (1B7-11, American Type Culture Collection, Manassas, Virginia) and purified as described previously (18).

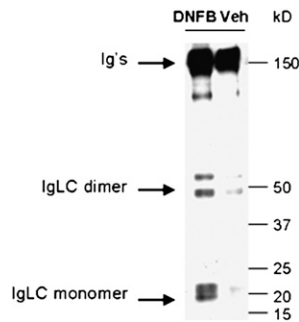


FIGURE 1. IgLC levels in mouse spleen. Spleen κ IgLC levels are increased after DNFB-sensitization and DNS challenge. Immunoblotting with Ab specific for mouse κ IgLC of spleen supernatants from DNFB- (lane 1) and vehicle-sensitized (lane 2) mice 72 h postchallenge. Immunoreactive proteins were visualized with ECL and quantified by densitometry. DNFB-sensitized group: IgLC (dimer)/Ig ratio: 0.18 and IgLC (monomer)/Ig ratio: 0.21; vehicle group: IgLC (dimer)/Ig ratio: 0.060 and IgLC(monomer)/Ig ratio: 0.041.

Determination of colonic vascular permeability

Vascular permeability changes were determined as described previously with minor modifications (20). In addition to the earlier described method, Evans blue (1.25% in sterile saline, 50 μ l) was injected i.v. in the tail vein 1 h prior to the end of an experiment and simultaneously with passive immunization with Ag-specific IgLC. The vascular permeability was expressed as microliters of exudated plasma per milligram dry weight of the colon.

Human serum κ and λ IgLC assays

The concentration of κ and λ IgLC in serum samples obtained from patients with CD [male, 36; female, 55; age 35.7 ± 11.4 y ($\pm$ SD)] and healthy controls [male, 6; female, 5; age 25 ± 5.4 y ($\pm$ SD)] was measured by ELISA with mAbs (kindly provided by A. Solomon, University of Tennessee, Knoxville, TN) specific for human IgLC as described previously (24). Serum specimens were appropriately diluted to obtain at least three data points within the linear portion of the standard curve and measured in a double-blind way. Serum samples from patients with CD were obtained at different time points, and concentrations of λ and κ IgLC were determined at all of these time points. Subsequently, the concentrations were averaged per patient with CD. All patients with CD received treatment with infliximab either or not comedicated with immunosuppressive compounds like azathioprine ($n = 29$), methotrexate ($n = 23$), 5-aminosalicylic acid (5-ASA) and methotrexate ($n = 6$), or thioguanide ($n = 8$).

Human tissue specimens

Intestinal tissue specimens of ileum and colon were obtained during endoscopy from patients suffering from CD ($n = 8$) or UC ($n = 8$) who underwent internal inspection. Specimens were fixed in formalin and

routinely embedded in paraffin. Sequential sections of 5 μ m were subjected to immunohistochemical staining described below. Control tissue specimens ($n = 5$) were obtained from patients enduring endoscopy because of suspected IBD and were defined as controls postinspection by microscopical observation by a pathologist based on morphology, cellular infiltration, and damage of the tissue.

Immunohistochemical staining for IgLC

Paraffin sections of intestinal biopsies of 5 μ m were routinely deparaffinized and blocked for endogenous peroxidase for 30 min. Primary mouse Abs directed against human λ IgLC were incubated for 60 min (concentration 1:500). Postincubation with the primary Ab, the sections were incubated with anti-mouse HRP-labeled polymer (DakoCytomation, Heverlee, Belgium) for 30 min. Color was developed with AEC substrate chromogen. Between incubation steps, the sections were intensively rinsed with 0.05 M TBS containing 0.05% Tween. Within each test, negative controls were included, and they were all found not to contain any specific staining. Immunohistochemistry for κ IgLC was also performed, but no positive staining could be detected, probably due to nonoptimal staining conditions and Ab used.

Statistics

Stool consistency data, total tissue damage score, and mast cell and colonic patch numbers were analyzed from raw scoring data using a distribution free Kruskal-Wallis ANOVA followed by a Dunn's multiple comparison test. The following data were analyzed by two-way ANOVA and a Bonferroni multiple comparison test: mMCP-1 content in plasma and colon tissue, TNF- α concentrations in the colon, and λ and κ IgLC concentrations in serum of patients with CD. Vascular leakage was tested with a Student t test. The analysis of infliximab treatment comedicated or not with azathioprine, methotrexate, 5-ASA, and methotrexate or thioguanide was performed using a one-way ANOVA followed by a Bonferroni multiple comparison test. In the figures, group means $\pm$ SEM are given. $p < 0.05$ was considered to be statistically significant. All data manipulations and statistical analysis were conducted with GraphPad Prism (version 3.0, GraphPad, San Diego, CA).

Results

Non-IgE-mediated hypersensitivity responses in the colon are associated with increases in IgLC production

In previous studies, we have shown that contact sensitization of BALB/c mice with DNFB followed by an intrarectal challenge with the Ag results in colonic hypersensitivity (20). This Ag-specific hypersensitivity reaction is associated with the development of diarrhea, lymphoid structure hypertrophy, and cellular infiltration 72 h postchallenge (20). In this study, we show that increased levels of κ IgLC were present in spleen of DNFB-sensitized mice 72 h postchallenge compared with vehicle-sensitized mice (Fig. 1), indicating production of κ IgLC. In the next set of experiments, we investigated if IgLC played a functional role in this model for IBD.

Table I. Disease symptoms in colonic tissue of vehicle- or DNFB-sensitized mice 72 h postchallenge treated with either PBS or F991

Treatment	DNFB	Stool Consistency ^a	Colonic Patch ^b	Total Damage Score ^c	Mast Cells ^d
PBS	—	0 (0–1)	2.5 (1–4)	0 (0–1)	29 (16–35)
	+	1 (0–2)*	6 (2–10)*	2 (1–5)*	74 (52–90)*
F991	—	0 (0–0)	3 (2–4)	0 (0–1)	14 (4–29)
	+	0 (0–1)**	5.5 (3–8)*.e	1 (0–1)**	53 (30–72)*.e

^aResults are expressed as median stool score (minimum–maximum); $n = 8$ mice.

^bResults are expressed as median number of colonic patches per colon (minimum–maximum); $n = 8$ mice.

^cColon tissue was scored for cellular infiltration and tissue damage after HE staining. For every mouse, three sections of colon tissue were examined at three different longitudinal depths, leaving at least 100 μ m in between. A score was given to the total appearance of the colon. Results are expressed as median tissue damage score (minimum–maximum); $n = 3$ mice.

^dMast cells were counted after mMCP-1 staining. For every mouse, three different longitudinal sections were counted and averaged. Results are expressed as median average number of mMCP-1 positive cells per colon section (minimum–maximum); $n = 3$ mice.

^eNS compared with PBS-treated DNFB-sensitized mice (distribution-free Kruskal-Wallis ANOVA followed by a Dunn's multiple comparison test).

* $p < 0.05$ compared with vehicle-sensitized mice subjected to the same treatment; ** $p < 0.05$ compared with DNFB-sensitized mice subjected to PBS treatment.

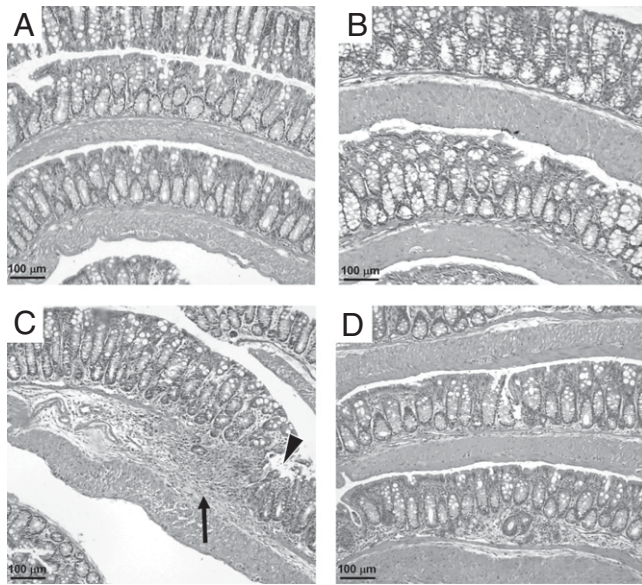


FIGURE 2. F991 prevents cellular infiltration and tissue damage in mouse colon. The IgLC antagonist F991 inhibits Ag-induced cellular infiltration and tissue damage 72 h postchallenge of vehicle- and DNFB-sensitized mice. H&E staining of 5- μ m sections of paraffin-embedded colon tissue shows a normal mucosal lining in vehicle-sensitized mice treated with PBS (A) or F991 (B) (original magnification $\times 200$). Colon tissue of DNFB-sensitized mice 72 h postchallenge treated with PBS shows cellular infiltration in the submucosa (arrow) and disruption of the mucosal lining (arrowhead) (C), which is not seen posttreatment with F991 (D). See also Table I.

The IgLC antagonist F991 reduces Ag-induced inflammation and disease symptoms in the colon

The presence of diarrhea and loose stool is indicative for a damaged and inflamed colon. As shown in Table I, treatment with F991 led to complete prevention of the development of diarrhea in DNFB-sensitized mice 72 h postchallenge. Administration of F991 had no influence on stool consistency in vehicle-sensitized mice.

Colonic patches are small lymphoid follicles that appear at the mucosal side of the colon. They consist predominantly of B lymphocytes, but also clusters of T lymphocytes are found (observations obtained with FACS analysis and immunohistochemistry as previously reported) (20). Colonic patches are spread irregularly and differ in size and shape between animals. The number of colonic patches increases significantly in PBS-treated DNFB-sensitized mice 72 h postchallenge compared with vehicle-sensitized mice (Table I). Treatment of DNFB-sensitized mice with F991 had no effect on the number of colonic patches present in the colon (Table I).

In PBS-treated DNFB-sensitized mice, cellular infiltration was observed 72 h after DNS challenge (Fig. 2C). This was accompanied

with loss of structure of the colonic lining and mucosal swelling. Treatment with F991 significantly reduced this inflammation in DNFB-sensitized mice 72 h postchallenge (Fig. 2D, cellular infiltration; Table I, damage score). F991 pretreatment did not influence the colonic structure of vehicle-sensitized mice (Fig. 2A, 2B).

F991 reduces mMCP-1 release in plasma but not tissue mMCP-1

mMCP-1 is a protease specific for mouse mucosal mast cells and appears in the bloodstream postactivation of mast cells (25). To assess mast cell activation, mMCP-1 levels were determined 30 min postchallenge in plasma. A significant rise in mMCP-1 was observed in DNFB-sensitized PBS-treated mice compared with vehicle-sensitized PBS-treated mice (Fig. 3A). This increase in mMCP-1 could be significantly attenuated by pretreatment with F991 (Fig. 3A). Infiltration of mast cells was determined by total mMCP-1 levels in supernatant of colon homogenates. Fig. 3B shows significantly increased mMCP-1 levels in both PBS- and F991-treated DNFB-sensitized mice at 72 h postchallenge. Immunohistochemical staining for mMCP-1 confirmed that F991 treatment did not influence this increase in the presence of mast cell numbers in the colon in DNFB-sensitized and challenged animals at 72 h postchallenge (Table I).

Total TNF- α levels in colon tissue increase posttreatment with F991

TNF- α measured in supernatant of colon homogenates was measured as an estimate of the total amount of TNF- α present in the tissue. DNFB sensitization leads to a significant increase in TNF- α in the colon 72 h postchallenge compared with vehicle-sensitized mice (Fig. 4). Due to F991 treatment, total TNF- α levels in the colon of both vehicle- and DNFB-sensitized mice 72 h postchallenge are significantly enhanced compared with PBS-treated mice subjected to the same sensitization protocol (Fig. 4).

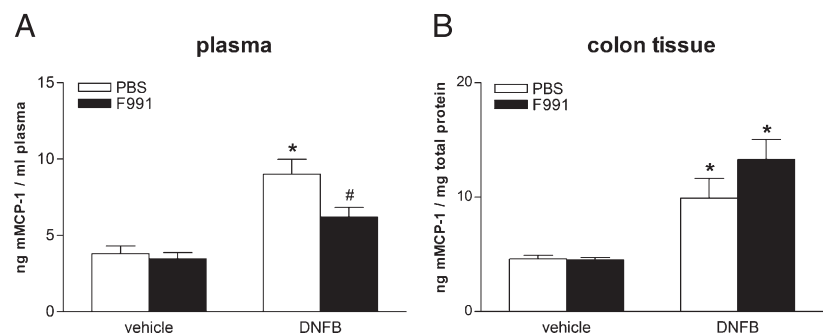
Passive immunization with TNP-specific IgLC induces mast cell activation and increases vascular permeability after intrarectal challenge with the hapten

To assess if IgLC could elicit early symptoms of colonic hypersensitivity, mice were passively immunized i.v. with TNP-specific κ IgLC or PBS as a control 30 min prior to intrarectal challenge. Mast cell activation determined by means of mMCP-1 levels in plasma 30 min postchallenge was significantly increased in κ IgLC-immunized mice compared with PBS-immunized mice (Fig. 5A). This was associated with a rapid increase in vascular permeability in κ IgLC-immunized mice quantified by the accumulation of Evans blue dye, which is one of the early signs of inflammation (Fig. 5B).

Patients with CD show increased levels of circulating λ and κ IgLC

Serum samples from patients suffering from CD ($n = 91$) and healthy controls ($n = 11$) were randomly selected from a large

FIGURE 3. F991 treatment prevents mast cell activation but not mast cell infiltration into the colon. A, mMCP-1 levels in plasma were assessed 30 min after DNS challenge. Results are expressed as mean $\pm$ SEM; $n = 8$ mice/group. B, Increased numbers of mast cells in the colon are indicated by changes in mMCP-1 levels in supernatant of colon homogenates 72 h after DNS challenge. Results are expressed as mean $\pm$ SEM; $n = 5$ –6 mice/group (two-way ANOVA and a Bonferroni multiple comparison test). * $p < 0.05$ compared with vehicle-sensitized mice subjected to the same treatment; # $p < 0.05$ compared with PBS-treated mice subjected to the same sensitization protocol.



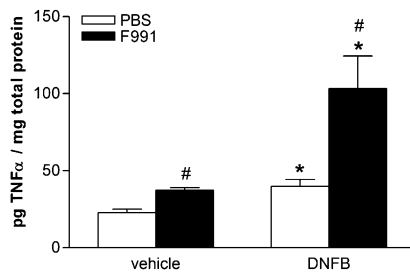


FIGURE 4. F991 does not affect the increased levels of TNF- α in the colon of DNFB-sensitized mice. TNF- α is determined in supernatant of colon homogenates 72 h postchallenge and expressed as mean TNF- α (picograms)/total protein (milligrams) $\pm$ SEM; $n = 6$ –12 mice (two-way ANOVA and a Bonferroni multiple comparison test). * $p < 0.05$ compared vehicle-sensitized mice subjected to the same treatment; # $p < 0.05$ compared with PBS-treated mice subjected to the same sensitization protocol.

specimen bank for quantitative λ and κ IgLC assays. We found that the serum concentrations of λ and κ IgLC were increased 8.0 ± 0.5 -fold and 2.8 ± 0.2 -fold, respectively, in patients with CD compared with healthy controls (Fig. 6A). Absolute levels (in milligrams per liter) were: for controls: 39.0 ± 3.7 (λ IgLC) and 68.2 ± 8.5 (κ IgLC); and for patients with CD: 311 ± 19 (λ IgLC) and 188 ± 12 (κ IgLC).

When the above-analyzed serum samples were categorized according to the received medication of the patients, it became evident that treatment with immunosuppressive drugs reduced IgLC serum concentrations in CD. In this analysis, patients with CD received either infliximab (murine/human chimeric anti-TNF- α mAb) alone ($n = 25$) or comedicated with an immunosuppressive drug like azathioprine ($n = 29$), methotrexate ($n = 23$), thioguanide ($n = 8$), or the combination of 5-ASA and methotrexate ($n = 6$). Fig. 6B demonstrates that patients receiving treatment with infliximab alone showed significantly higher concentrations of serum λ and κ IgLC compared with patients comedicated with azathioprine, methotrexate, thioguanide, or 5-ASA and methotrexate.

IgLCs are located in the colon of patients with IBD

To analyze the localization of IgLC, immunohistochemical staining with primary Abs specific for λ IgLC was performed on intestinal tissue sections of patients with CD and patients with UC. Fig. 7 demonstrates representatives of positive λ IgLC staining in ileum and colon biopsies of patients with IBD. Positive IgLC staining was observed in inflamed lesions as well as in inflamed mucosa of

the colon (Fig. 7A, 7B) and ileum (Fig. 7C, 7D) of patients with CD. Moreover, in pathological noninflamed mucosa from IBD patients, increased IgLC staining compared with mucosa from healthy controls was observed (pictures not shown). Colon biopsies of patients with UC also demonstrated positive λ IgLC staining in the lesions and the mucosa (Fig. 7E, 7F).

Discussion

In this study, we show that IBD is associated with increased expression of IgLC. Serum concentrations of IgLC in patients with CD are significantly increased compared with healthy controls. Use of immunosuppressive medication could significantly reduce serum levels of IgLC. Increased expression of IgLC was found to be associated with the presence of inflamed regions in intestinal specimen from patients with CD and patients with UC. Moreover, we provide evidence that development of clinical symptoms of disease can be prevented by treatment with F991, an antagonist for IgLC in a murine model for colitis.

mMCP-1 levels in supernatant of colon homogenates are an indication of total amount of mast cells present in the tissue. Both PBS- and F991-treated DNFB-sensitized mice showed a significant increase in mMCP-1 levels in colon tissue compared with vehicle-sensitized mice. These data are in correspondence with immunohistochemical mMCP-1 staining of the colon. However, mMCP-1 levels in plasma were significantly decreased in F991-treated mice. Overall, it can be concluded that mast cell infiltration and/or proliferation is unaffected by F991 treatment but that mast cell degranulation was inhibited by F991 treatment.

The increased presence of TNF- α in colon tissue of DNFB-sensitized animals after F991 administration could be explained by the increased number of nondegranulated mast cells in the tissue (Fig. 3A). We have previously shown that TNF- α present in colon tissue in this colonic hypersensitivity model is mainly derived from mast cells (20). Measuring TNF- α in supernatant of colon homogenates determines total TNF- α levels of intracellular prestored TNF- α . Because TNF- α is abundantly prestored in mast cell granules, the significantly higher TNF- α levels in both F991-treated vehicle- and DNFB-sensitized mice 72 h postchallenge compared with the corresponding PBS-treated group (Fig. 4) can be explained by the prevention of mast cell degranulation by the IgLC antagonist. A similar trend is also found in mMCP-1 tissue levels. This significant increase in both PBS- and F991-treated DNFB-sensitized mice compared with the corresponding vehicle-sensitized mice further indicates that mast cell activation is

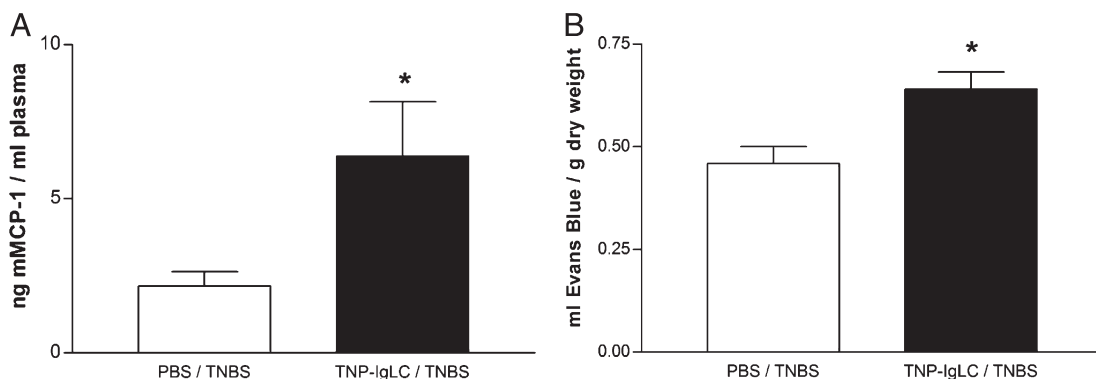


FIGURE 5. Ag-specific IgLCs mediate acute mast cell-associated plasma extravasation. Open bars represent PBS-treated mice and closed bars TNP-IgLC-immunized mice 30 min after TNBS challenge. A, mMCP-1 levels in plasma were assessed 30 min after TNBS challenge. Results are expressed as mean $\pm$ SEM. * $p < 0.05$ compared with PBS-immunized mice. $n = 6$ mice/group (Student t test). B, Colonic vascular permeability changes measured by Evans blue leakage from blood into colonic tissue. Results are expressed as mean microliters of plasma exudated/milligram dry weight colon $\pm$ SEM. $n = 6$ mice/group (Student t test). * $p < 0.05$ compared with PBS-treated mice.

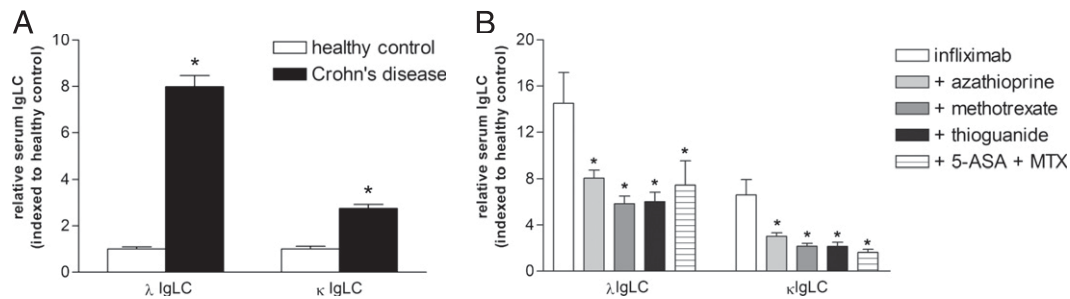


FIGURE 6. Human serum IgLC concentrations are increased in patients with CD. **A**, Concentrations of λ and κ IgLC in serum of patients with CD ($n = 91$, all treatments included; closed bars) were significantly increased with regard to serum IgLC levels of control subjects ($n = 11$; open bars). Concentrations are depicted as relative concentration indexed to IgLC serum levels of healthy controls. Relative concentrations represent ratio of serum IgLC levels in patients with CD to healthy controls. $*p < 0.05$ compared with healthy controls (two-way ANOVA and a Bonferroni multiple comparison test). **B**, Categorization of analyzed serum samples according to the medication received by patients with CD. Patients with CD received infliximab (mouse anti-TNF- α mAbs) medication either without ($n = 25$) or with ($n = 66$) comedication with immunosuppressive drugs. Both λ and κ IgLC serum levels of patients with CD receiving infliximab with comedication azathioprine ($n = 29$), methotrexate ($n = 23$), thioguanide ($n = 8$), or 5-ASA and methotrexate ($n = 6$) are significantly decreased compared with subjects receiving infliximab only. $*p < 0.01$ compared with patients receiving infliximab only (one-way ANOVA followed by a Bonferroni multiple comparison test).

decreased, but infiltration and proliferation of these cells are not affected by F991 treatment.

Passive immunization with TNP-specific IgLC was shown to induce in vivo mast cell activation and to induce increased vascular permeability after local intrarectal TNBS challenge. Increased vascular permeability can be induced by mast cell-derived histamine, serotonin, lipid mediators, and cytokines and will permit plasma extravasation, allowing macromolecules and inflammatory cell to infiltrate into the interstitium (26). This is one of the major events of the early phase of inflammation. These results

are in correspondence with previous observations in a murine nonatopic asthma model in which IgLC was also responsible for the mast cell-induced airway hyperresponsiveness and mucosal exudation (19).

We presume that mast cell activation results from crosslinking of receptor-bound IgLC. Although there are several lines of evidence suggesting the presence of a putative receptor on mast cells for IgLC, it has not been identified yet, but studies using knockout mice for γ -chain-associated receptors, such as Fc ϵ RI, Fc γ RIII, and paired Ig-like receptor-A, demonstrated that these receptors are not involved in IgLC-mediated mast cell activation (18). Currently, a mast cell membrane-associated protein that interacts with IgLC is a further subject of investigation in our laboratory.

CD is a disorder not accompanied by concomitant plasmacytosis (i.e., infiltration of plasma B cells) (27). However, increased levels of IgLC in serum of patients suffering from CD compared with healthy controls and an intense λ IgLC staining in intestinal tissue specimens suggest a polyclonal B cell response, because these cells are the source for IgLC. In contrast, UC is a disorder associated with increased basal plasmacytosis (28, 29). Unfortunately, serum IgLC levels of patients with UC could not be determined due to restricted access to human serum samples. However, an intense staining specific for IgLC in intestinal tissue specimens as seen in Fig. 7 suggests elevated levels of circulating IgLC as well. The staining observed in patients with UC was more pronounced compared with patients with CD. These results suggest a functional importance for B and plasma cells in the initiation and exacerbation of IBD. Currently, it is being investigated if λ IgLC colocalizes with mast cells and/or B lymphocytes in colonic tissue of patients with IBD.

Immunosuppressive drugs like azathioprine, methotrexate, and thioguanine are widely used to dampen the inflammatory response in IBD (30). Both azathioprine and thioguanine are purine antagonists, whereas methotrexate is a folic acid antagonist. The action of these compounds is predominantly based on the abrogation of lymphocyte proliferation by inhibiting DNA synthesis. This could also lead to suppression of the production of IgLC. As has been demonstrated in this study, in patients with CD receiving immunosuppressive drugs comedicated with infliximab, significantly reduced serum concentrations of IgLC were found compared with treatment with infliximab alone. From the lack of effect on serum IgLC levels of infliximab alone, it can be concluded that the release of TNF- α is downstream from IgLC-induced mast cell degranulation in IBD.

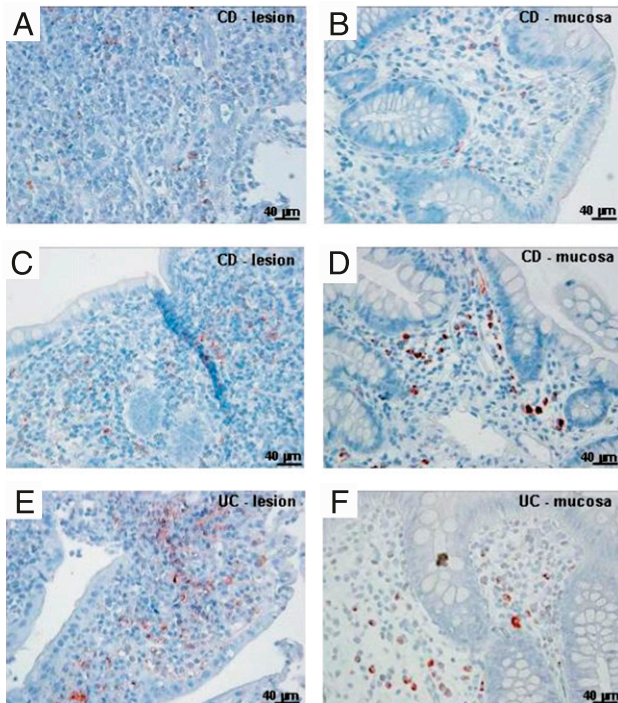


FIGURE 7. Immunohistochemical staining for λ IgLC of human colon biopsies of patients with IBD (original magnification $\times 400$). **A** and **B** are representative pictures of λ IgLC staining of colon biopsies of patient suffering from CD. **C** and **D** are representative pictures of λ IgLC staining of ileum biopsies of patient suffering from CD. **E** and **F** are representative pictures of λ IgLC staining of colon biopsies of patient suffering from UC. **A**, **C**, and **E** correspond to lesion/lymphoid structures in the human colon and ileum, whereas **B**, **D**, and **F** are biopsies of colon or ileum of pathological inflamed mucosa.

Since the early 1980s, several groups have demonstrated a strong correlation between increased IgLC levels in cerebrospinal fluid and disease activity in patients suffering from multiple sclerosis. IgLC quantification has recently been proposed as a rapid and reproducible biomarker supporting current MS diagnostic criteria (31). We published earlier that serum levels of IgLC of patients with nonatopic asthma are increased (19). Also, in other chronic inflammatory conditions, such as rhinitis, dermatitis, and rheumatoid arthritis, increases in serum or local IgLC have been demonstrated (32–34).

To our knowledge, we are the first to describe that IgLC may be associated with mast cell activation in the development of IBD. Our data in an experimental model for IBD support the presence of increased IgLC levels in serum and tissue from patients with IBD, whereas treatment with immunosuppressive drugs decreased serum IgLC. Our study points out that further research is warranted to investigate the potential value of IgLC as a diagnostic and therapeutic target in the pathogenesis of IBD.

Disclosures

The authors have no financial conflicts of interest.

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