

Imatinib mesylate alleviates diarrhea in a mouse model of intestinal allergy

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Abstract

Background When sensitized epicutaneously and challenged orally with ovalbumin, Balb/c mice develop allergen-induced diarrhea. As mast cells play important roles in diarrhea, we studied whether allergic diarrhea could be alleviated with imatinib mesylate. **Methods** Balb/c mice were sensitized and challenged with ovalbumin and treated orally with imatinib. Cytokine mRNA expressions were determined with quantitative RT-PCR and numbers of small intestinal mast cells determined by staining for chloroacetate esterase and mucosal mast cell protease-1. Immunofluorescence staining was used to assess the intestinal CCL1 expression. **Key Results** Ovalbumin-sensitized and challenged Balb/c mice developed diarrhea, which was associated with increased number of mast cells and expression of interleukin (IL)-4 and -13, and chemokines CCL1 and CCL17 in the small intestine. Treatment with imatinib reduced the incidence of diarrhea, inhibited the development of mastocytosis and jejunal mRNA expression of IL-13, CCL1, CCL17 and CCL22. Mast cell-deficient W/W^V mice, and surprisingly, also their mast cell-competent control (+/+) littermates failed to develop diarrhea as a response to ovalbumin. This strain-dependent difference was associated with the inability of +/+ and W/W^V mice to increase the number of intestinal mast cells and expression of IL-4, IL-13, CCL1 and CCL17 after ovalbumin challenge.

Conclusions & Inferences Development of allergic diarrhea is associated with the ability of mice to develop intestinal mastocytosis. Imatinib inhibited the development of intestinal mastocytosis, reduced the incidence of diarrhea, and reduced the expression of IL-13, CCL1, and CCL17. Targeting intestinal mast cells could be a feasible approach to treat allergic diarrhea.

Keywords CCL1, diarrhea, food allergy, imatinib, mastocytosis.

Abbreviations: CAE, chloroacetate esterase; CCL, chemokine ligand; IL, interleukin; MMCP-1, mucosal mast cell protease-1; OVA, ovalbumin; W/W^V, mast cell deficient mouse strain (*Kit^W/Kit^{W-v}*); WBB6F1 (+/+), WBB6 mouse is a control littermate of W/W^V mouse and a hybrid of the genetic background WB x C57BL/6 F1.

INTRODUCTION

Food allergies have become more prevalent and are currently a major health concern world-wide. Despite significant advances in the knowledge of mucosal immune system, the pathogenesis of food allergy has remained incompletely understood.

To study the mechanisms of food allergy mouse models of intestinal allergy have been developed. Studies using these models have revealed that mast cells are important effector cells in the development of allergic diarrhea. Intestinal mastocytosis and increased serum IgE are prerequisites for development of allergic diarrhea.¹ Repeated allergen challenge results in activation of intestinal mast cells and release of mucosal mast cell protease-1 (MMCP-1).¹ Studies with parasite-infected MMCP-1 knock-out mice have shown that MMCP-1 increases permeability of the intestinal barrier by disrupting the cell-cell tight junctions.² Allergic diarrhea is associated with local Th2-type of cytokine environment in the intestine.³ In particular IL-4 has been shown to

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play a key role in the development of intestinal mastocytosis and diarrhea.³ Th2 type of cytokines IL-4 and IL-13 have also been shown to promote smooth muscle hypercontractility of the intestine.^{4,5} Increased intestinal permeability occurs during parasite infection and endogenously produced IL-4 and/or IL-13 have shown to have direct permeability augmenting effects on intestinal epithelial cells.⁶ Increased intestinal permeability is reflected as increased serum ovalbumin level.⁷

Imatinib mesylate (Gleevec) is a selective tyrosine kinase inhibitor which is used in the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors. In addition to the oncogenic Bcr-Abl, imatinib also inhibits some other tyrosine kinases, which include platelet-derived growth factor (PDGF) receptor and KIT which is encoded by the proto-oncogene, *c-kit*.^{8,9} Importantly, mast cells express high levels of KIT on their surface,¹⁰ and inhibition of KIT activation has been shown to result in the apoptotic death of human mast cells.¹¹

Imatinib has been reported to attenuate pulmonary hyperreactivity in a model of allergic asthma¹² which suggests that imatinib may have mast cell inhibiting activity also *in vivo*. As mast cells and development of intestinal mastocytosis play central role in the development of allergic diarrhea we hypothesized that targeting mast cells with imatinib could alleviate intestinal food allergy. We have previously shown that when Balb/c mice are first epicutaneously sensitized and then orally challenged with ovalbumin they develop diarrhea which is associated with increased levels of intestinal mast cells and expression of Th2-type of cytokines.¹³ By using this model we have now studied further the role of mast cells in allergic diarrhea and studied whether intestinal food allergy could be alleviated by inhibiting the function of intestinal mast cells with imatinib treatment.

MATERIALS AND METHODS

Chemicals and preparation of drug

Imatinib mesylate (Novartis, West Sussex, UK) was aseptically prepared by removing tablet film cover, dissolved into ethanol and diluted into endotoxin-free saline buffered with 100 $\mu\text{mol L}^{-1}$ Na-acetate (pH 3.2). Solution was centrifuged three times at 10 000 $\times g$ to collect the dissolved active compound, kept at 4 °C. Fresh drug solutions were prepared weekly.

Epicutaneous sensitization

Seven-week-old female Balb/c mice were purchased from the National Laboratory Animal Center of Taconic (Lille Skensved, Denmark). Mast cell-deficient mice W/W^V and their wild type littermates +/- were from the Jackson Laboratory (Maine, NE, USA). These littermates are hybrids of the genetic background WB $\times$ C57BL/6 F1, and they were from the same colony as the W/W^V mice.

All mice were maintained on an ovalbumin-free diet. The mice were epicutaneously sensitized with 100 μL of ovalbumin (1 mg mL⁻¹, Fraction V, Sigma-Aldrich: St. Louis, MO, USA) and the controls with saline. After anesthetization with 4% isoflurane, the backs of the mice were shaved, and the skin was lightly abraded by using injection tape (Tegaderm™, 3M, Borken, Germany). Twice a week ovalbumin or saline was placed on gauze which was secured with tape to mouse back skin (Fig. 1).

Intragastric challenges and treatment with imatinib

Starting from day 38, mice in all study groups received 10 ovalbumin challenges (50 mg mL⁻¹, 200 μL) without prior fasting. Administration of ovalbumin was provided with a feeding needle (AgnTho's AB, Lidingö, Sweden).

Sham-sensitized and ovalbumin-sensitized groups of Balb/c mice also received imatinib (2 mg 100 μL^{-1}) orally on days 1, 4, 18, and 22 during the sensitization phase and simultaneously with ovalbumin doses during the challenge phase. Endotoxin-free saline served as vehicle control. W/W^V and +/- mice were treated identically except omission of imatinib/vehicle (Fig. 1).

Evaluation of diarrhea, tissue, and blood sampling

Mice were placed individually in empty boxes and their diarrhea consistency was evaluated visually 0, 20, 40, and 60 min after each

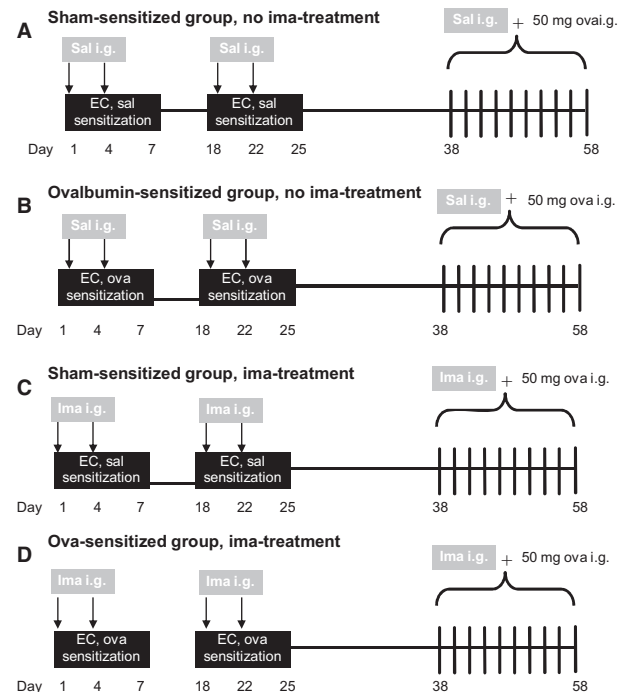


Figure 1 Sensitization and challenge protocol of Balb/c mice. All mice received 10 oral doses of ovalbumin during the challenge phase (vertical lines between days 38–58). Two groups were treated with imatinib (C and D). In groups A, B, C, and D, the numbers of mice are eight, eight, seven, and six, respectively. I.g., intragastric dose; sal, saline; ova, ovalbumin; EC, epicutaneous; ima, imatinib. In studies with +/- and W/W^V mice, similar sensitization and challenge protocol was used except that no imatinib or its vehicle was given.

oral dose between the 6th and 9th ovalbumin dose. At each evaluation point the boxes were emptied from the stools. To be interpreted as diarrhea the stools had to be clearly watery. At sacrifice the small intestine from pylorus to cecum was excised and divided into two segments of equal length. From both sides a 1 cm segment was used for the jejunal samples, those for duodenum were excised directly after the antrum of stomach. Serum samples were collected 1 h after the challenge from the abdominal aorta.

Histology

Duodenal and jejunal specimens were submerged in formaldehyde and paraffin embedded. Mast cells were stained for CAE as described¹⁴ with modifications.¹⁵ Briefly, paraffin sections were dewaxed in toluene and rehydrated with alcohol series. New Fuchsin (4 %, Sigma-Aldrich, USA) was dissolved into HCl and added to 4% NaNO₂. Phosphate buffer and naphthol AS-D chloroacetate (Sigma-Aldrich) were added, and the sections were incubated for 20 min.

Light microscopy of MMCP-1 was performed by using rabbit antibody (MMCP-1 kit, Moredun Scientific Ltd, Midlothian, UK) as described previously.¹³ After antigen retrieval endogenous peroxidase activity was blocked with 5% H₂O₂. Next, HRP-conjugated rabbit anti-mouse MMCP-1 antibody (1 : 100 dilution) was added and detected by diaminobenzidine tetrachloride (DAB) (Vector Laboratories, Burlingame, CA, USA). Hematoxylin (Sigma-Aldrich) was used for background staining. Stained cells were calculated from transsectional segments of at least 0.75 mm² under light microscope equipped with Olympus Soft Imaging Solutions Cell[^]P software (version 2.6: Billerica, MA, USA).

Apoptotic MMCP-1⁺ cells were evaluated using TUNEL technique with ApopTag green fluorescence (Chemicon, Millipore, Billerica, MA, USA) together with goat anti-rabbit Alexa 546 (Invitrogen). A DNA-binding dye 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) served as background. Stained samples were evaluated with Nikon Eclipse E600 microscope (Nikon Co., Tokyo, Japan), and cross sectional area of the gut was measured by computer-assisted planimetry (Image-Pro Plus, version 4.5, Media Cybernetics Inc., Silver Spring, MD, USA).

For immunofluorescence double staining of MMCP-1 (Moredun ELISA kit sheep antibody, 3.3 µg mL⁻¹) and CCL1 (R & D Systems; Clone 148113, 20 µg mL⁻¹, Minneapolis, MN, USA) were used. As isotype control rat IgG_{2A} (Caltag Laboratories; 0.5 µg mL⁻¹, Burlingame, CA, USA) was used.

Serum MMCP-1 and immunoglobulin measurements

Quantification of serum MMCP-1 was assayed by ELISA kit (Moredun Scientific Ltd). To determine ovalbumin-specific IgE, a rat anti-mouse IgE (clone R35-72, 2 µg mL⁻¹, BD Pharmingen) was used for coating. The IgE samples were diluted 1 : 10 and 1 : 20 in 1% BSA-PBS. For ovalbumin-specific IgG₁/IgG_{2A} ELISAs, the plates were ovalbumin coated (2 µg mL⁻¹, fraction V, Sigma) and the serum samples were diluted for IgG₁ (1 : 10 000 and 1 : 30 000) and IgG_{2A} (1 : 50 and 1 : 200) in 1% BSA-PBS. For detection of IgG₁/IgG_{2A}, a biotin-labeled isotype-specific secondary antibody was used at 2 µg mL⁻¹ final concentration (clone A85-3, BD Pharmingen, San Diego, CA, USA). Biotinylated ovalbumin-specific IgE was generated according to manufacturer's instructions (EZ-Link[™] Sulpho-NHS-Biotinylation Kit, Pierce, Rockford, IL, USA), the excess label was removed by dialysis (Slide-A-Lyzer Dialysis Cassette, Pierce), and diluted to 1 : 1000. For visualization (OD 405 nm) streptavidin-horseradish peroxidase (BD Pharmingen, Gaithersburg, MD, USA) and peroxidase

substrate reagents (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) were used. Samples representing each research group equally in number were selected to the microtiterplates and measured simultaneously.

Analysis of gene expression

Jejunal tissues were analyzed for IL-4, IL-13, CCL1, CCL17, and CCL22 mRNA levels by real-time quantitative PCR technique as previously described.¹⁶ Briefly, jejunal tissues were homogenized with Ultra-Turrax T8 homogenizator (IKA Labortechnik, Staufen, Germany). Total RNA was isolated according to the manufacturer's instructions (GIBCO, Gaithersburg, MD, USA) and converted into respective cDNA. For PCR, the pre-designed primers and fluorogenic TaqMan probes were from PE Applied Biosystems. The samples were amplified in duplicate using ABI Prism 7500 Sequence Detector System (PE Applied Biosystems, Foster City, CA, USA): a 10 min preincubation at 95 °C, and 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. The data were normalized relative to the ribosomal protein 18S as an endogenous control.

Ethical permissions

All experiments were carried out and approved by the Norwegian Animal Research Authority.

Statistics

Calculations were performed with GraphPad Prism 5.02 program (GraphPad Software, Inc., San Diego, CA, USA) by using Chi-squared statistics, Student's *t*-test, or Mann-Whitney's *U*-test when appropriate. Data are presented as mean ± standard deviation (SD). Values of *P* < 0.05 were considered statistically significant.

RESULTS

Imatinib alleviates diarrhea in ovalbumin-sensitized and challenged Balb/c mice

Balb/c mice, which were first sensitized with dermal ovalbumin and then challenged orally with ovalbumin, developed diarrhea during the oral challenge period (Fig. 1). The development of diarrhea was associated with increased number of intestinal mast cells and increase of serum MMCP-1 indicating degranulation of mast cells. Imatinib mesylate is a tyrosine kinase inhibitor which inhibits also KIT, the most important growth factor receptor of mast cells. As shown in Fig. 2A, imatinib treatment of ovalbumin-sensitized and challenged mice significantly reduced the incidence of diarrhea (*P* = 0.0018). Diarrhea lasted longer in the vehicle-treated group than in the imatinib-treated group (Fig. 2B).

Sensitization to ovalbumin is not affected by imatinib treatment of Balb/c mice

In the Balb/c mice, sensitization with allergen results in parallel increase in the levels of allergen-specific

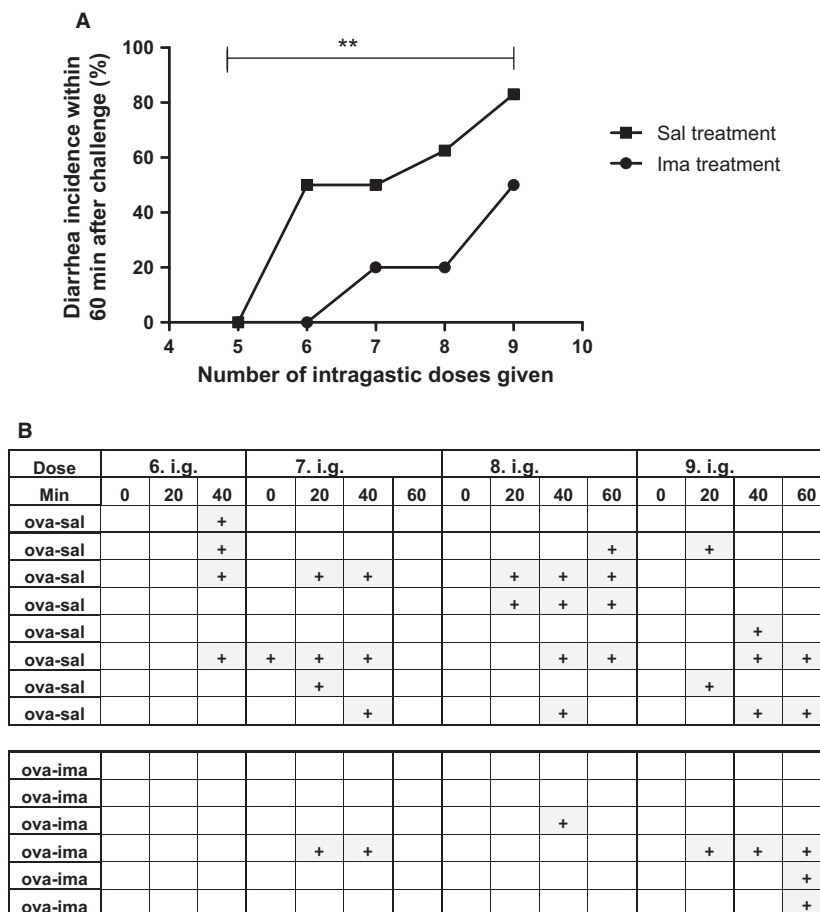


Figure 2 Imatinib treatment alleviates the incidence (A) and duration (B) of diarrhea in ovalbumin-sensitized Balb/c mice. Fecal samples were evaluated by visual inspection. Diarrhea appeared in most cases 20–40 min, but never 80 min or later after the ovalbumin dose. Dichotomized grading: no diarrhea (no markings) or diarrhea (+). No diarrhea was observed in sham-sensitized groups of sal-sal ($n = 8$) or sal-ima ($n = 8$) (data not shown). Number of mice in ova-sal group was eight and in ova-ima group it was six. When difference between saline and imatinib-treated mice were compared, $P = 0.0018$ for all cases (Chi-squared statistics).

IgE and IgG₁. To study whether the effect of imatinib was mediated by attenuation of the sensitization process, we assessed the magnitude of the serological response. In ovalbumin-sensitized Balb/c mice IgE and IgG₁ levels were clearly increased (Table 1). Importantly, treatment of the mice with imatinib had no effect on these immunoglobulin levels, revealing that the sensitization to ovalbumin was not inhibited by imatinib.

The effect of imatinib on the number of intestinal mast cells in Balb/c mice

Mast cells have been implicated in the pathogenesis of allergic diarrhea, and therefore we studied whether imatinib had effect on the densities of intestinal CAE⁺ and MMCP-1⁺ mast cells in the Balb/c mice (Fig. 3). Mast cells were detected by demonstrating the chloro-

acetate esterase (CAE)-positivity and the presence of the mast-cell specific neutral protease MMCP-1. In ovalbumin-sensitized and challenged mice the number of duodenal CAE⁺ mast cells was significantly lower in imatinib-treated mice than in control mice (Fig. 3A). Similarly, imatinib treatment tended to lower the numbers of duodenal and jejunal MMCP-1⁺ cells, but the effect was not statistically significant (Fig. 3B and D). There was also a tendency for lower serum MMCP-1 levels in imatinib-treated mice (saline- vs imatinib-treated mice 14173 ± 11246 vs 5923 ± 4413 ng mL⁻¹, respectively, $P = 0.09$).

Imatinib does not induce apoptosis of MMCP-1⁺ mast cells in ovalbumin-sensitized Balb/c mice

Imatinib has been shown to induce apoptosis of human mast cells.¹⁷ Therefore we assessed whether

Table 1 IgE and ovalbumin-specific IgG₁ levels in different groups of mice. Increase of serum ovalbumin-specific immunoglobulin levels in sensitized mice confirms successful sensitization to ovalbumin in all of the groups studied

Treatment groups	IgE 1 : 20		IgG1 1 : 10 000	
	Average	SD	Average	SD
Balb/c sal-sal	0.071	0.013	0.150	0.083
Balb/c ova-sal	0.412 [†]	0.358	1.144	0.890
Balb/c sal-ima	0.063	0.004	0.187	0.070
Balb/c ova-ima	0.605 [‡]	0.362	1.623 [‡]	0.241
W/W ^{-v} sal	0.095	0.087	1.397	0.898
W/W ^{-v} ova	1.019 [§]	0.530	2.839 [§]	0.832
+/+ sal	0.079	0.120	0.839	0.145
+/+ ova	0.569 [¶]	0.261	3.154 [¶]	0.087

Asterisks indicate *P* values of <0.05 (*) and <0.01 (**), *n* = 6–8. [†]*P* = 0.01 when compared to Balb/c sal-sal (*). [‡]*P* = 0.003 when compared to Balb/c sal-ima (**). [§]*P* = 0.006 when compared to W/W^{-v} sal (*). [¶]*P* = 0.005 when compared to +/+ sal (**). ova-ima, ovalbumin-sensitized and imatinib-treated; ova-sal, ovalbumin-sensitized and saline-treated; sal-ima, sham-sensitized and imatinib-treated; sal-sal, sham-sensitized and saline-treated; SD, standard deviation.

the lower mast cell numbers in the intestine of the imatinib-treated allergic mice were due to imatinib-induced apoptosis of mast cells. However, double immunofluorescence staining for MMCP-1 and TUNEL showed no increase in the number of apoptotic MMCP-1⁺ cells in imatinib-treated Balb/c mice (data not shown).

Imatinib inhibits the expression of chemokines and IL-13 in Balb/c mice

The lack of apoptosis suggested that the reduced number of mast cells could be a consequence of decreased local proliferation and/or defective recruitment of mast cell progenitors into the intestinal wall. Therefore we next assessed the effect of imatinib on the expression of chemokines and cytokines known to induce proliferation of mast cells. As demonstrated in Fig. 4, ovalbumin sensitization and challenge clearly increased mRNA expression of CCL1, CCL17, CCL22, and IL-13 mRNA. Treatment with imatinib almost totally prevented the sensitization-dependent strong up regulation of mRNA expression of all of these cytokines and chemokines.

Development of allergic diarrhea depends on the ability of mice to induce intestinal mastocytosis: comparison of mast cell-deficient and mast cell-competent mice

Imatinib inhibits KIT, which is the most important regulator of mast cell viability. To assess the role of KIT-signaling in the development of mastocytosis and allergic diarrhea, we studied the development of diarrhea in W/W^{-v} (*Kit*^W/*Kit*^{W-v}) mice, which have a defective KIT-receptor, and are therefore deficient in

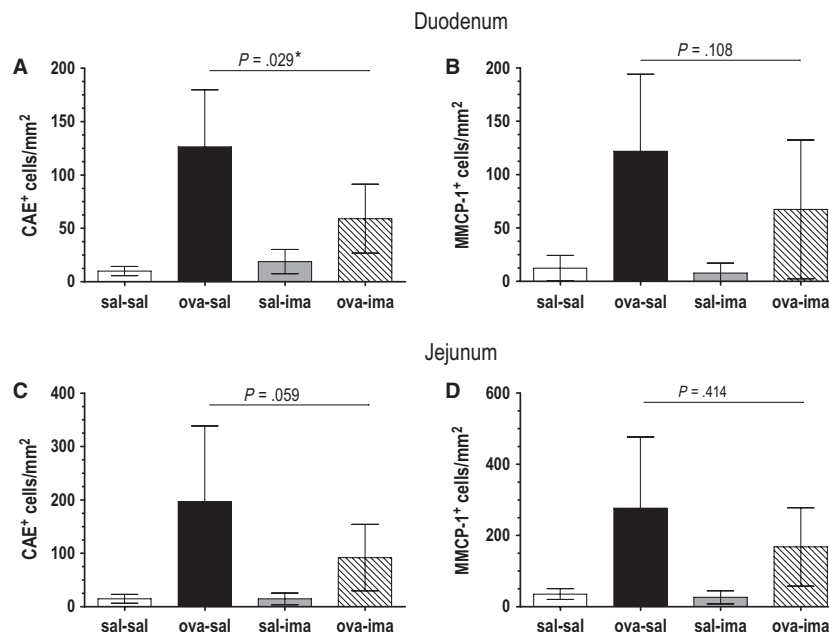


Figure 3 Effect of imatinib treatment on the number of duodenal and jejunal CAE⁺ and MMCP-1⁺ cells in Balb/c mice. Data from CAE mast cell staining (A, C) and MMCP-1 (B, D); sal-sal, sham-sensitized and saline-treated; ova-sal, ovalbumin-sensitized and saline-treated; sal-ima, sham-sensitized and imatinib-treated; ova-ima, ovalbumin-sensitized and imatinib-treated. Statistically significant when *P* < 0.05 (*), *n* = 5–8.

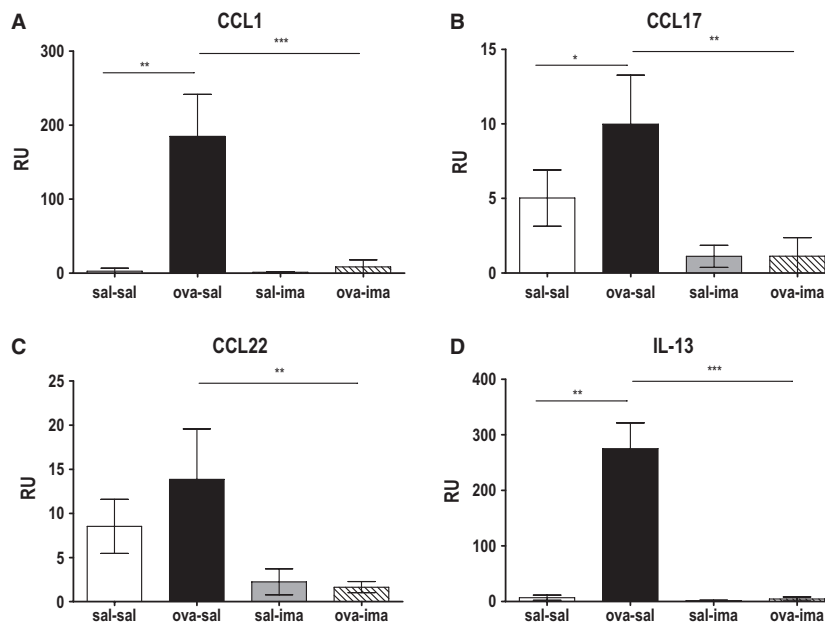


Figure 4 Imatinib treatment inhibits jejunal mRNA expression of CCL1 (A), CCL17 (B) and CCL22 (C) and IL-13 (D) in Balb/c mice. RU = relative units. Statistically significant *, ** and *** when $P < 0.05$, 0.01, and 0.001, respectively.

mast cells, and in their wild type littermates, the WBB6F1-(+/+) mice, which are mast cell-competent.

As expected, the ovalbumin-sensitized mast cell-deficient W/W^V mice did not develop diarrhea after ovalbumin challenge. Somewhat unexpectedly their control littermates, the +/+ mice, which have normal numbers of intestinal mast cells, also failed to develop diarrhea as a response to ovalbumin sensitization and challenge (data not shown). The +/+ have been shown previously to develop diarrhea when stimulated with bacterial antigens.¹⁸ The striking difference between the two mast cell-competent strains, the Balb/c and the +/+ mice, made it possible to study additional aspects of the mechanism of allergic diarrhea. Ovalbumin sensitization of the W/W^V and the +/+ mice clearly increased ovalbumin-specific IgE and IgG₁ levels in both strains (Table 1), suggesting that resistance of these strains to develop diarrhea was not related to ovalbumin sensitization.

Compared to Balb/c mice, the plasma histamine and MMCP-1 levels were lower in +/+ mice (data not shown). Therefore, we next assessed the intestinal mast cell densities in these mice. As expected, few mast cells could be detected in ovalbumin or sham-sensitized W/W^V mice. In sham-sensitized +/+ mice, mast cell density was comparable to that seen in the Balb/c mice. However, a striking difference was observed between Balb/c and +/+ mice after they had

been sensitized and challenged with ovalbumin (Fig. 5). Thus, in Balb/c mice the number of jejunal CAE⁺ mast cells was more than 20-fold and that of MMCP-1⁺ cells 35-fold higher after ovalbumin challenge as compared to the +/+ mice (Fig. 6). This suggests that in W/W^V and +/+ mice, the resistance to develop diarrhea was most likely due to the inability of these strains to increase the number of their intestinal mast cells as a response to ovalbumin sensitization and challenge.

Ovalbumin sensitization and challenge increases jejunal inflammation in Balb/c, but not in +/+ mice or W/W^V mice

To explore why Balb/c mice developed intestinal mastocytosis and diarrhea, while the +/+ mice failed to do so, we studied the expression of inflammatory cytokines and chemokines in the jejunum of these mouse strains.

In Balb/c mice, the IL-4 and IL-13 mRNA expression levels were significantly higher in the ovalbumin-sensitized than in the sham-sensitized controls (Fig. 7A and B). In contrast, in W/W^V and +/+ mice, ovalbumin sensitization and challenge did not affect IL-4 or IL-13 expression (Fig. 7A and B). This suggests that ovalbumin sensitization and challenge resulted in a Th2-type of response in Balb/c mice, but not in the +/+ mice.

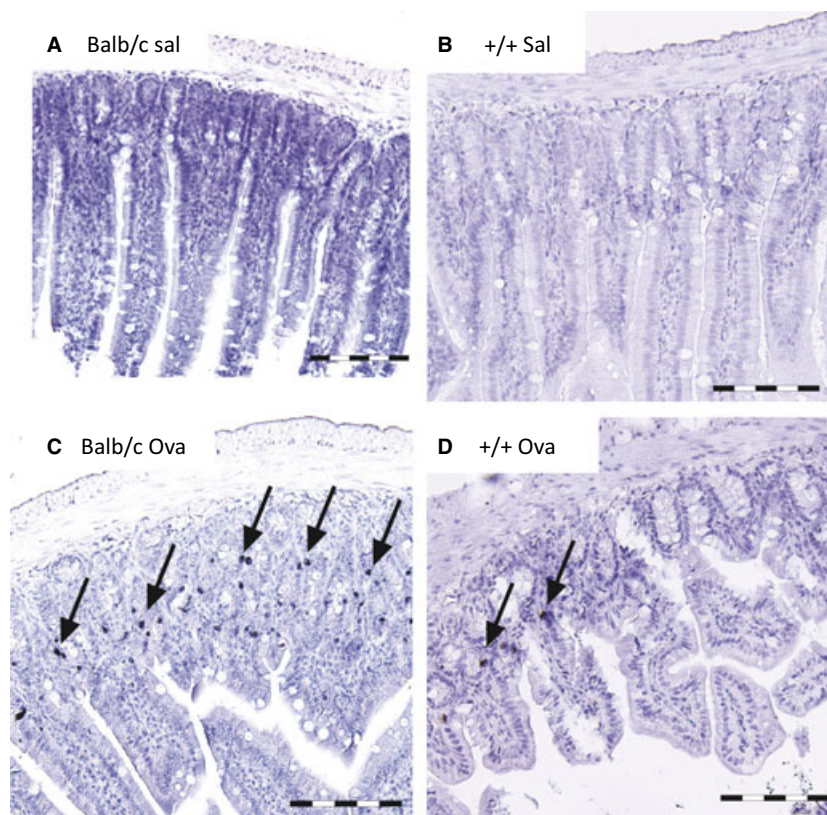


Figure 5 Ovalbumin sensitization and challenge increases the number of mast cells in Balb/c mice but not in $+/+$ mice. Balb/c mice sensitized with saline (A), or ovalbumin (C). In the ovalbumin-sensitized $+/+$ mice (D), few MMCP-1 $^{+}$ cells can be seen when compared to sham-sensitized $+/+$ mice (B). Scale length is 100 μ m.

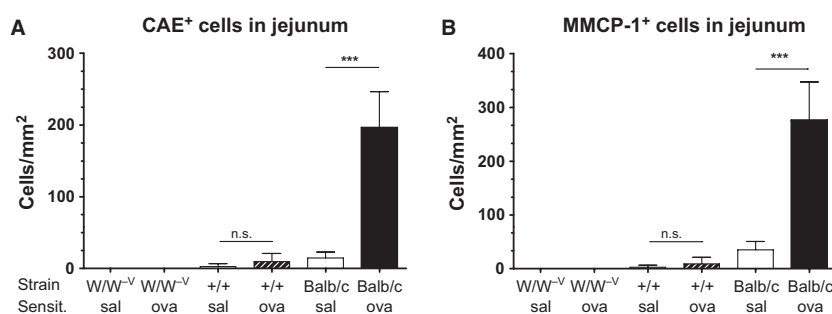


Figure 6 In Balb/c mice ovalbumin-sensitization and challenge induce pronounced mastocytosis whereas in W/W $^{-V}$ or $+/+$ mice no significant mastocytosis was observed as a response to ovalbumin, ($n = 6-8$ mice per group).

Significant differences were evident also in the chemokine expression profiles between Balb/c and W/W $^{-V}$ and Balb/c and $+/+$ strains. A marked induction of CCL1 expression was observed after ovalbumin-sensitization and challenge in Balb/c mice whereas no such changes were observed in W/W $^{-V}$ or $+/+$ mice (Fig. 7C). A two-fold increase in the mRNA expression of CCL17 was observed in ovalbumin-sensitized Balb/c mice compared to sham-sensitized controls whereas no

induction of CCL17 was observed in the W/W $^{-V}$ or $+/+$ mice (Fig. 7D).

CCL1 is expressed in the small intestine exclusively by mast cells

CCL1 is a ligand for CCR8, expressed by a subset of T-cells, and thus CCL1-CCR8 axis has been suggested to represent a link between innate and adaptive

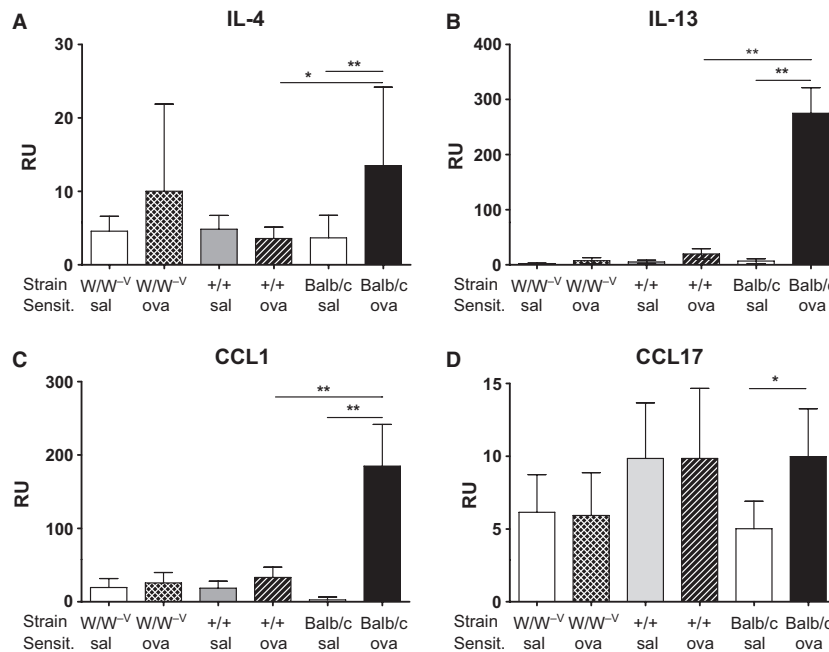


Figure 7 Jejunal mRNA expression profiles show significant induction of inflammatory cytokines and chemokines in Balb/c mice but not in +/- or W/W^{-V} mice. (A) IL-4, (B) IL-13, and (C) CCL1, (D) CCL17 mRNA expression, $n = 6-8$, sensit., sensitization.

immunity. CCL1 has been shown to be produced by several cell types; therefore we assessed its cellular origin in the intestine.

As demonstrated in Fig. 8, in sham-sensitized Balb/c mice the protein expression of CCL1 was localized to the MMCP-1⁺ cells. In fact, all the MMCP-1⁺ cells showed faint expression of CCL1⁺, and all the CCL1⁺ cells were also MMCP-1⁺. In ovalbumin-sensitized and challenged Balb/c mice, the expression of CCL1 was clearly more pronounced. The number of CCL1⁺ cells remained unchanged but in addition an abundant number of MMCP-1⁺, but CCL1⁻ mast cells also appeared. In +/- mice the expression of CCL1 was seen only in occasional cells (Fig. 8).

DISCUSSION

Dermal sensitization followed by repeated oral ovalbumin challenges induced intestinal mastocytosis and diarrhea in Balb/c mice, consistent with the results of previous studies using this model.^{13,16} Here we report that oral treatment with imatinib mesylate alleviated significantly the development of diarrhea. Imatinib treatment also attenuated the allergen-induced small intestinal mastocytosis, suggesting that the effect of imatinib is mediated at least partly by its effects on intestinal mast cells.

Imatinib has been shown to induce apoptosis of human mast cells¹⁷ through inhibition of KIT, the

main growth factor receptor of mast cells. However, the reduced number of intestinal mast cells in imatinib-treated mice was not due to apoptosis as the proportion of apoptotic cells was not affected by imatinib treatment (data not shown). KIT-mediated signaling is however, important for the development of intestinal mastocytosis in the mouse.¹⁹ W/W^{-V} mice, which lack functional KIT-receptor, have very few mast cells. Furthermore, expression of class I_A PI-3K is a prerequisite for appearance of gastrointestinal mast cells.²⁰ Impaired KIT-signaling has been reported in PI3K^{-/-} knock-out mice supporting the essential role of KIT in intestinal mast cell colonization.¹⁹ It is thus conceivable that the reduced number of intestinal mast cells in the imatinib-treated Balb/c mice is at least partly explained by inhibition of KIT-signaling resulting in decreased influx of circulating mast cell progenitors and/or attenuation of local proliferation of mast cells in the intestine. Imatinib also reduced the mRNA expression of IL-13 and several chemokines i.e. CCL1, CCL17 and CCL22 in the intestine which can also affect the local proliferation of mast cells and development of diarrhea. Interstitial cells of Cajal (ICC) are c-Kit positive and these cells may contribute to gut motility. Thus it is possible that imatinib may also directly reduce the gut motility through having an effect on ICC.

To further assess the role of mast cells and c-KIT signalling in allergic diarrhea, we studied mast cell

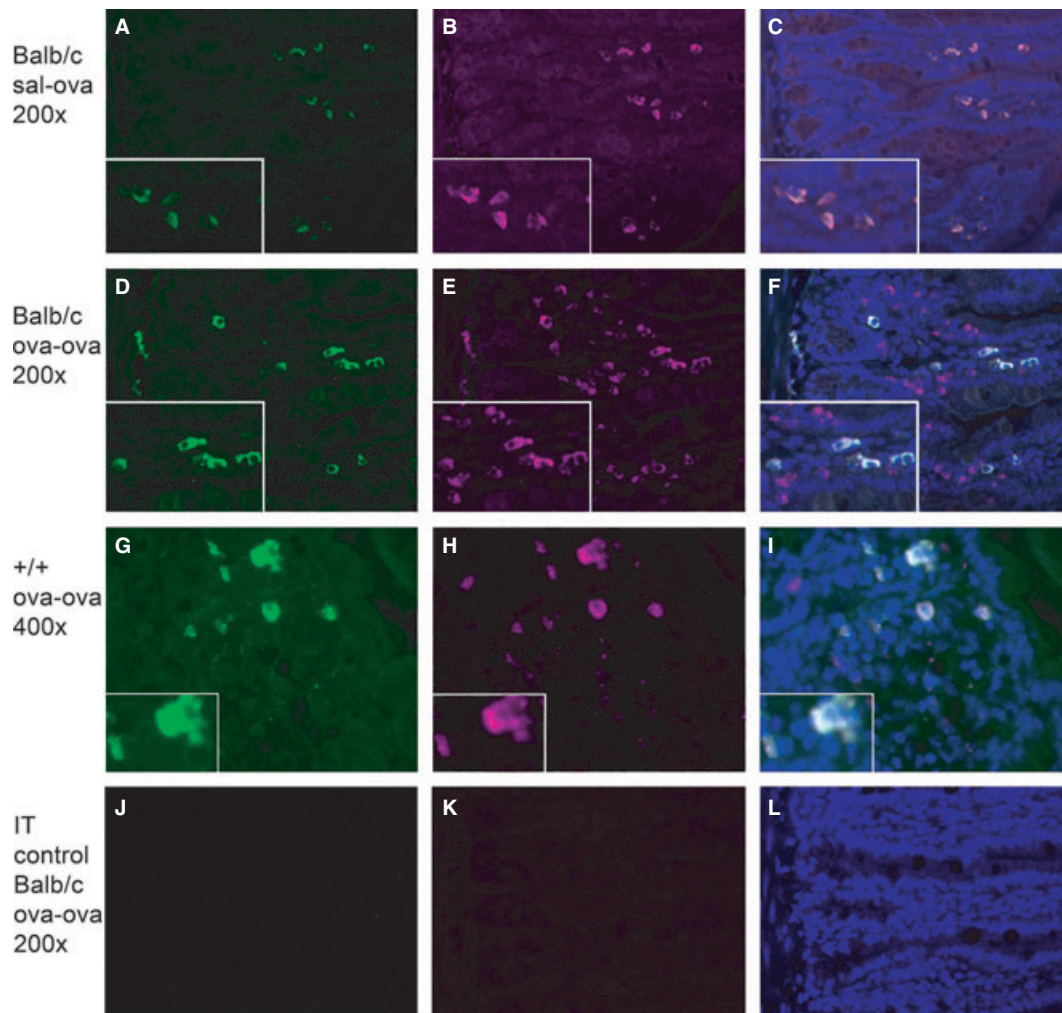


Figure 8 MMCP-1 and CCL1 staining of jejunal mucosa. From left to right the columns are CCL1⁺ (green), MMCP-1⁺ (magenta) and merge in which double positive mast cells become white or whitish. In Balb/c and +/+ mice mast cells are consistently double positive. Strong expression of CCL1⁺ can be seen in Ova sensitized Balb/c mast cells. In ovalbumin-sensitized +/+ mice mast cells are CCL1 positive but compared to Balb/c only a few mast cells can be seen. (J-L) Isotype controls (IT) for CCL1 and MMCP-1. Magnifications are 200 × or 400 ×.

deficient W/W^V mice and their control +/+ littermates, which are mast cell-competent. The sensitized W/W^V mice did not develop diarrhea after oral ovalbumin challenge. This would suggest that mast cells have a significant role in the development of allergic diarrhea. In addition to mast cells, W/W^V mice also lack ICC cells in the gastrointestinal tract which may be a contributing factor to the lack of diarrhea in these mice. However, the +/+ littermates, which have a normal intestinal mast cell and ICC-cell population, did not develop diarrhea either, a phenomenon which has also been observed by Brandt *et al.* in another model of allergic diarrhea.¹ This striking strain-dependent difference between Balb/c mice and the W/W^V or +/+ offered an opportunity to study the mechanisms of

allergic diarrhea in more detail. Both strains developed high IgE and IgG₁ levels suggesting that there was no difference in the sensitization to ovalbumin. Also the mast cell-deficient W/W^V mice developed high ovalbumin specific IgE-levels which suggests that mast cell-derived cytokines, such as IL-4 and IL-13, do not significantly contribute to IgE switch and generation of serum IgE. Although in some *in vitro* studies mast cells have been shown to be able to induce IgE switch²¹ this may not take place to a significant degree *in vivo*. This notion is further supported by the study of Cheng and coworkers, in which they observed that lack of FcεRI expressing cells did not have any significant effect on serum IgE-levels.²² The principal difference between the diarrhea-developing Balb/c

mice, and the diarrhea-resistant (either W/W^V or $+/+$) mice, was that only Balb/c mice developed intestinal mastocytosis (Fig. 6). In Balb/c mice the number of jejunal CAE⁺ mast cells increased more than 20- and that of MMCP-1⁺ cells 35-folds compared to either W/W^V or $+/+$ mice. This suggests that the development of diarrhea depends on the ability of the mice to induce intestinal mastocytosis.

In Balb/c mice ovalbumin sensitization and challenge resulted in clearly increased jejunal IL-4, IL-13, CCL1 and CCL17 mRNA expression (Fig 7). In contrast, in $+/+$ mice ovalbumin sensitization and challenge induced only moderate expression of IL-13 and CCL1. This Th2 type of cytokine profile in Balb/c mice is in accordance with a previous study which showed that Balb/c mice are prone to develop Th2 type of immune response.²³ Th2 type of cytokine profile has also been associated with hypercontractility of the gastrointestinal tract.^{4,5} Thus the development of a more robust Th2-type immune response in Balb/c mice can be one factor which explains the development of mastocytosis and diarrhea in Balb/c but not in $+/+$ mice.

The receptor of CCL1 is CCR8. In the lung CCR8 expression is associated with 70% of CD4⁺ cells.²⁴ When CCR8⁺CD4⁺ cells are activated *in vitro* they produce Th2-type of cytokines IL-4, IL-5, IL-9 and IL-13.²⁵ Thus, increased expression of CCL1 in the intestine is likely to lead into recruitment of CCR8⁺CD4⁺ T-cells and production of IL-4 and IL-13. Marked increase of CCL1 expression has been described in mast cells after IgE-mediated activation²⁶ but CCL1 expression has been described also in monocytes and lymphocytes. The present results revealed that in the intestine CCL1 is localized almost exclusively to mast cells (Fig. 8). In control sham-sensitized mice all the MMCP-1⁺ cells were CCL1⁺ and all the CCL1⁺ cells were MMCP-1⁺. In ovalbumin-sensitized and challenged Balb/c mice the number of CCL1⁺ cells remained unchanged, but the expression of CCL1 was more pronounced. In addition a large population of MMCP-1⁺ but CCL1⁻ cells appeared (Fig 8). Imatinib treatment also significantly reduced the expression of CCL1 in the small intestine. Thus the reduction of CCL1 most likely leads into reduced influx of CCR8⁺CD4⁺ cells into intestine. This in turn would lead into decreased expression of Th2 type of cytokines by T-cells and into decreased proliferation of mast cells.

Taken together the results suggest that in sensitized Balb/c mice ovalbumin challenge induces production of CCL1 by intestinal mast cells, which in turn leads to recruitment of CCR8 and CD4⁺ T-cells

with ensuing production of the Th2-type cytokines IL-4 and IL-13. These cytokines could further stimulate the proliferation of mast cells resulting in intestinal mastocytosis and diarrhea. The suggested interplay between mast cells and CCR8⁺ T-lymphocytes actually resembles a similar scenario shown to take place in the asthmatic lung.²⁴ When present in the above-described cytokine environment, also CD4⁺ T cells transferred from ovalbumin-sensitized mice may induce diarrhea in naïve mice after ovalbumin challenge.²⁷

To conclude, treatment with imatinib mesylate decreased the incidence of diarrhea in Balb/c mice in adjuvant-free model of intestinal allergy. Alleviation of diarrhea was associated with reduced induction of intestinal mastocytosis and inhibition of expression of Th2-type cytokines and chemokines. Significant strain-dependent differences were observed between Balb/c and W/W^V and $+/+$ mice regarding the development of mastocytosis and diarrhea in response to ovalbumin sensitization and challenge. This striking strain-specific difference should be taken into consideration when mast cell-deficient mice are utilized to study the role of mast cells in the pathogenesis of diseases. The present results support also the notion that development of small intestinal mastocytosis is a critical factor in the pathogenesis of allergic diarrhea. Therefore, intestinal mast cells and intestinal chemokines responsible for the generation of intestinal mastocytosis could represent potential targets for novel therapies.

Highlights

Allergic diarrhea is dependent on the ability of mice to induce intestinal mastocytosis. Symptoms of allergic diarrhea can be alleviated by targeting intestinal mast cells by using the tyrosine kinase inhibitor imatinib mesylate.

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DISCLOSURE

The authors have no competing interests and have no conflict of interests.

AUTHOR CONTRIBUTIONS

KV designed the study, carried out the animal experiments, and prepared the manuscript; JL was responsible for the qPCR analy-

sis, carried out the experiments, performed the statistical analysis; AHL carried out histological and animal experiments and prepared the manuscript; MIM was responsible for immunofluorescence imaging and prepared the manuscript; PTK offered financing of the facilities, and prepared the manuscript; AB coordinated the study, offered financing of the facilities, and prepared the manuscript; KKE designed the study, offered financing of the facilities, and prepared the manuscript; All authors read and approved the final manuscript.

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