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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Aluminum Ingestion Promotes Colorectal Hypersensitivity in Rodents

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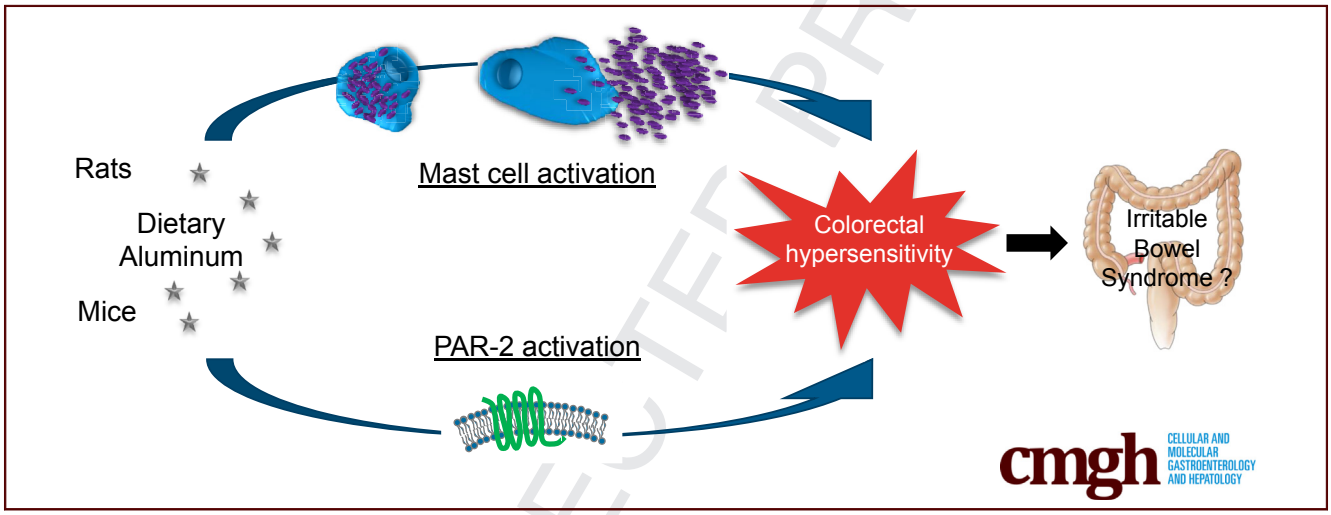

SUMMARY

Aluminum, which is commonly present in food, induces visceral hypersensitivity in rats and mice when ingested at dosages relevant to human exposure. Aluminum might be the first identified dietary risk factor for irritable bowel syndrome.

BACKGROUND & AIMS: Irritable bowel syndrome (IBS) is a multifactorial disease arising from a complex interplay between genetic predisposition and environmental influences. To date, environmental triggers are not well known. Aluminum is commonly present in food, notably by its use as food additive. We investigated the effects of aluminum ingestion in rodent models of visceral hypersensitivity, and the mechanisms involved.

METHODS: Visceral hypersensitivity was recorded by colorectal distension in rats administered with oral low doses of aluminum. Inflammation was analyzed in the colon of aluminum-treated rats by quantitative PCR for cytokine expression and by immunohistochemistry for immune cells quantification. Involvement of mast cells in the aluminum-induced hypersensitivity was determined by cromoglycate administration of rats and in mast cell-deficient mice ($Kit^{W\text{-}sh/W\text{-}sh}$). Proteinase-activated receptor-2 (PAR2) activation in response to aluminum was evaluated and its implication in aluminum-induced hypersensitivity was assessed in PAR2 knockout mice.

RESULTS: Orally administered low-dose aluminum induced visceral hypersensitivity in rats and mice. Visceral pain induced by aluminum persisted over time even after cessation of treatment, reappeared and was amplified when treatment resumed. As observed in humans, female animals were more sensitive than males. Major mediators of nociception were up-regulated in the colon by aluminum. Activation of mast cells and PAR2 were required for aluminum-induced hypersensitivity.

CONCLUSIONS: These findings indicate that oral exposure to aluminum at human dietary level reproduces clinical and molecular features of IBS, highlighting a new pathway of prevention and treatment of visceral pain in some susceptible patients. *(Cell Mol Gastroenterol Hepatol 2018;■:■–■; https://doi.org/10.1016/j.jcmgh.2018.09.012)*

Keywords: Visceral Hypersensitivity; Risk Factors; Mast Cells; PAR2.

Irritable bowel syndrome (IBS) is a chronic functional gastrointestinal disorder affecting 10–25% of the population and twice as many women as men in Western countries.[1–3] It occurs at all ages; however, 50% of patients report having had symptoms before 35 years of age. As there are no specific biomarkers, IBS is diagnosed according to symptom-based criteria. IBS is diagnosed if patients described recurring pain or discomfort in the lower abdomen accompanied by altered stool formation or frequency. According to the ROME classification, IBS patients are subcategorized as diarrhea predominant, constipation predominant, alternating, or unspecified.[4] IBS is thus a heterogeneous disorder with multiple pathophysiological mechanisms and likely different causes.[5,6] A defect in intestinal barrier defense with an increased intestinal permeability has been observed in IBS.[7] Alterations of the immune system have also been described with an abnormal activation status of immune cells, particularly mast cells or T cells. Peripheral and central modifications in brain–gut interactions are also believed to be involved in the visceral pain perception.[8] However, even if these mechanisms play a crucial role in IBS pathophysiology and the maintenance of visceral hypersensitivity, the question of the initial trigger still remains unresolved.[9] Therefore, a better understanding of the triggering factors will help to develop new therapeutic strategies. Few risk factors have been linked to IBS development; the best-documented ones are female sex, psychological factors, and preceding gastrointestinal infections.[10,11] Besides, many IBS patients identify food as a possible cause of their symptoms.[12] A broad restriction diet low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols has been suggested as a strategy to improve symptoms, irrespective of the underlying cause.[13] A more precise link between food and IBS has been demonstrated for gluten and other wheat proteins, lactose, and nickel, highlighting particular subset of IBS patients now diagnosed as nonceliac gluten/wheat sensitivity, lactose intolerance, and nickel-allergic contact mucositis.[14–17] For these subgroups of IBS patients the withdrawal of wheat, lactose, or nickel has been shown to improve symptoms.[18,19] Here, we evaluated the effect of aluminum, a common contaminant of food and water, on the abdominal pain. Aluminum is a ubiquitous element in nature, and thus it can naturally contaminate food as a result of food grown in aluminum containing soils.[20] Aluminum is also used as a food additive. It can also be taken up through contact with kitchenware or packaging.[21–23] In Europe, it was estimated that the tolerable intake of aluminum is exceeded in a significant proportion of the population, especially in children, who are more vulnerable to toxic effects of pollutants than adults.[24,25] A U.S. food additives survey calculated that most Americans ingest from 0.01 to 1.4 mg·kg body weight·d of aluminum. In the same study, it was estimated that about 5% of Americans ingested more than 95 mg/d aluminum (meaning 1.58 mg·kg·d if a 60-kg person is considered).[20] We previously reported, in a context of inflammatory bowel diseases, that aluminum ingestion in mice at a dose of 1.5 mg·kg·d altered gut homeostasis and modified tight junction proteins expressed by epithelial cells. These changes favored a leaky gut and enhanced the intensity and duration of inflammation.[26] In the present study, we showed that a 1.5 mg·kg·d ingestion of aluminum induced dose-dependent and persistent colorectal hypersensitivity in rodents. To link aluminum and IBS condition, we highlighted that aluminum triggered mechanisms involved in IBS pathophysiology. Indeed, we demonstrated that aluminum induced mast cell degranulation and activation of the proteinase-activated receptor-2 (PAR2) which were required for aluminum-induced visceral pain. Our findings indicate that oral exposure to aluminum can reproduce clinical and molecular features of IBS. We revealed a role for aluminum as a dietary factor that can promote abdominal hypersensitivity and a possible therapeutic strategy via controlled aluminum uptake or chelation.

Results

Oral Aluminum Administration Induces Visceral Hypersensitivity

In rats, CRD is the most widely used method to assess visceral pain thanks to its ease of use and robust reproducibility. In our study, the recorded parameter was a pain threshold, characterized by clearly visible abdominal contractions and elevation of the hind part of the animal's body.[27] Rats were treated orally with aluminum citrate (AlCi) at a concentration of 1.5 mg·kg·d, corresponding to the high value of dietary aluminum ingested by human, and visceral hypersensitivity was assessed.[20,21,24,25] In control animals receiving water, a mean pressure of 52 ± 1.1 mm Hg was required to induce pain (Figure 1*A*). AlCi treatment within 8 days decreased the mean pressure necessary to induce allodynia compared with control rats (54.5 ± 0.9 mm Hg vs 48 ± 1.7 mm Hg) (Figure 1*A*). This aluminum-induced nociceptive effect was maintained for the duration of administration. After 30 days of exposure, it led to a 30% increase in pain compared with control animals (Figure 1*A*). A lower concentration of AlCi of 0.5 mg·kg·d significantly decreased the pain threshold by day 8 of administration (Figure 1*B*), while with a higher dose of 3 mg·kg·d, a significant increase in visceral pain was observed as early as on the second day of treatment (Figure 1*C*). Increased pain induced by 1.5 mg·kg·d persisted significantly for 7 days after discontinuation of treatment, and 4 weeks were needed to reach the threshold of nontreated rats

§Authors share co-senior authorship.

Abbreviations used in this paper: AlCi, aluminum citrate; CRD, colorectal distension; IBS, irritable bowel syndrome; IHC, immunohistochemistry; KO, knockout; MGG, May-Grünwald Giemsa; MPO, myeloperoxidase; mRNA, messenger RNA; PAR, proteinase-activated receptor; PCR, polymerase chain reaction; WT, wild-type; ZnCi, zinc citrate.

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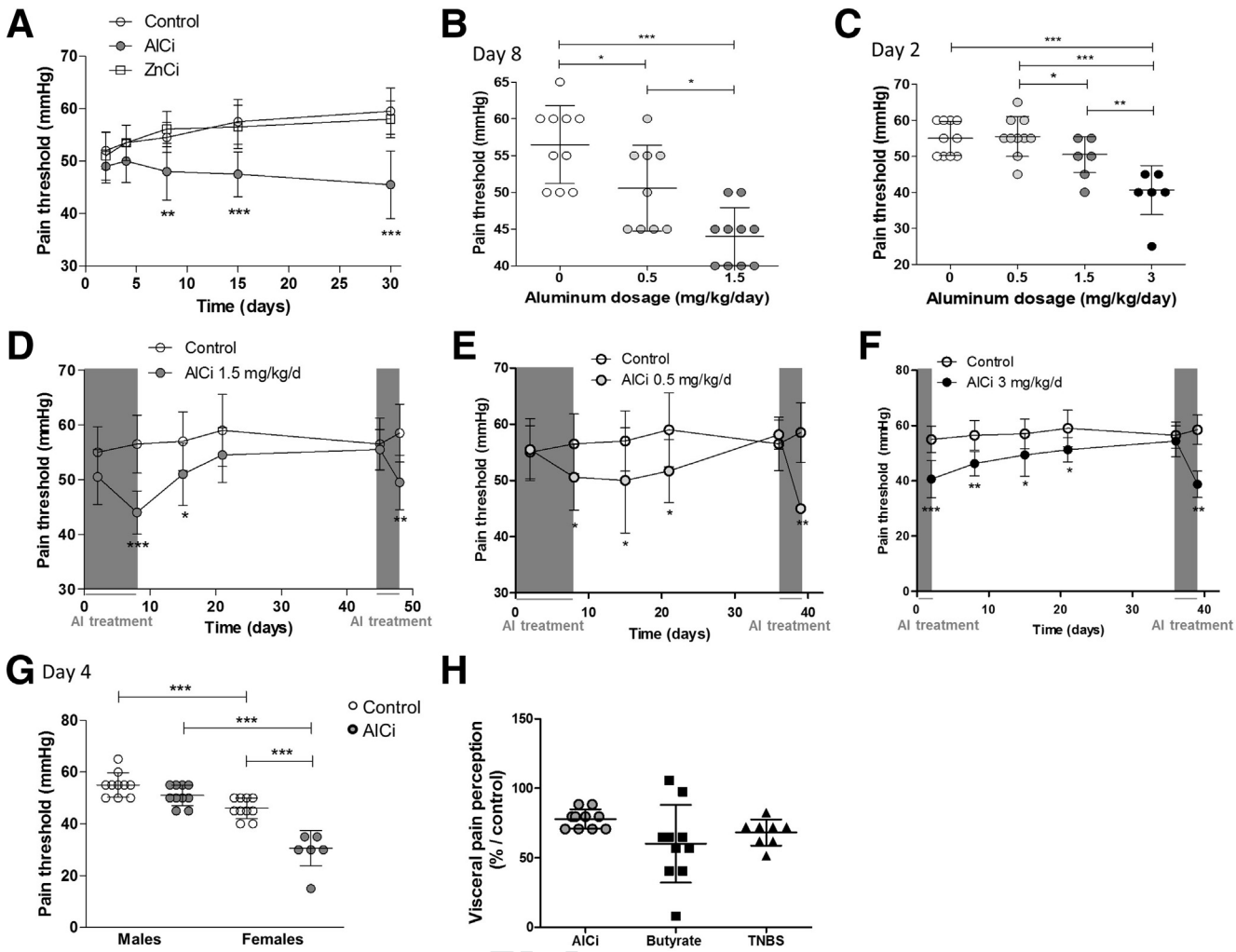

Figure 1. Aluminum-induced visceral hypersensitivity in rats. (*A*) Pain threshold in rats orally administered with water (Control), 1.5-mg·kg·d AlCi or ZnCi for 30 days (n = 10/group). Abdominal withdrawal reflex in response to CRD was measured after 2, 4, 8, 15, and 30 days of exposure. (*B*) Pain threshold in response to CRD in rats after 8 days of 0, 0.5, or 1.5 mg·kg body weight·d of AlCi administration (n = 9–10/group). (*C*) Pain threshold in response to CRD in rats after 2 days of 0, 0.5, 1.5, and 3 mg·kg·d of AlCi administration (n = 8–10/group). (*D–F*) Time course of pain threshold after administration, discontinuation, and resumption of AlCi at oral dosages of (*D*) 1.5 mg·kg·d, (*E*) 0.5 mg·kg·d, or (*F*) 3 mg·kg·d (n = 10/group). (*G*) Variation of pain threshold in response to CRD in male and female rats after 4 days of water (Control) or 1.5 mg·kg·d AlCi ingestion (n = 8–10/group). (*H*) Pain threshold variation in rats after AlCi (1.5 mg·kg·d) ingestion compared with butyrate (200 nM) and 2,4,6-trinitrobenzenesulfonic acid (150 mg/kg) administration (n = 10/group). $^{*}P < .05$, $^{**}P < .005$, $^{***}P < .0005$ using the Mann-Whitney nonparametric *U* test.

(Figure 1*D*). A second administration of AlCi at the same dose of 1.5 mg·kg·d induced pain within 2 days of administration compared with 8 days during the first administration (Figure 1*A* and *D*). Similar long-lasting effects and sensitization to repeated administration of AlCi were observed with the doses of 0.5 and 3 mg·kg·d (Figure 1*E* and *F*). Comparisons between genders showed that in each case (control or with aluminum treatment), a significantly lower pain threshold was observed among the female rats, mimicking the gender effect observed in human IBS (Figure 1*G*). Female rats were also more susceptible to aluminum than male rats as they showed a significant decrease in pain threshold after 4 days of treatment (Figure 1*G*).

To assess whether the painful impact of aluminum is a common effect to all metals or arises from the citrate complexation with aluminum, rats were treated with ZnCi at the same dosage of 1.5 mg·kg·d. Up to 30 days of treatment with ZnCi did not induce any significant variation in the pain threshold compared with control rats (Figure 1*A*).

Noninflammatory and inflammatory irritation models of colonic hypersensitivity relevant to IBS have been developed. For example, repeated butyrate enemas and intrarectal injection of 2,4,6-trinitrobenzenesulfonic acid in combination with 25–50% ethanol have been used as noninflammatory and inflammatory models of IBS, respectively.[28] We compared the effects of AlCi treatment on visceral hypersensitivity with those induced by butyrate and

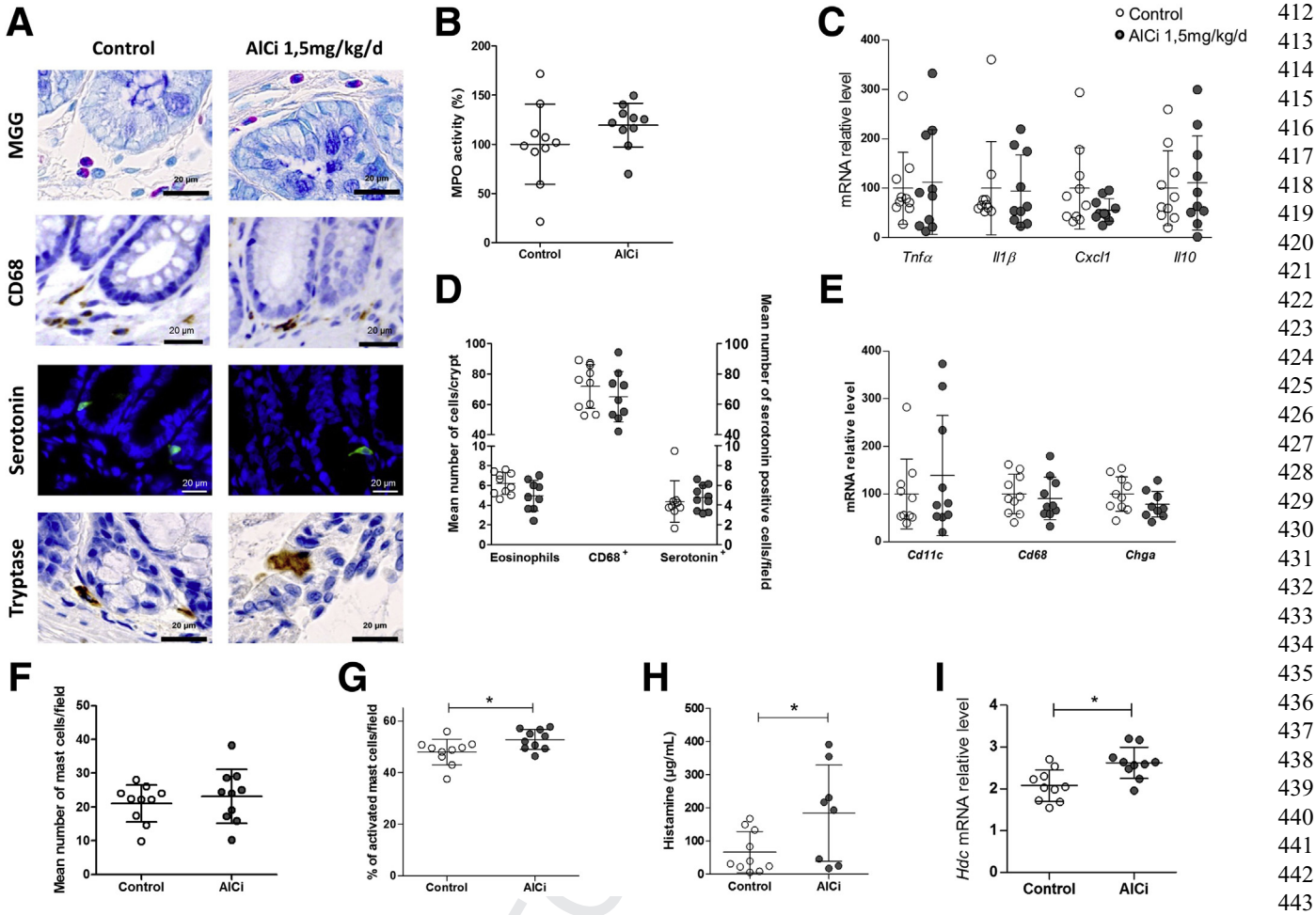

Figure 2. A dose of 1.5-mg·kg·d aluminum induced low-grade inflammation in the colon. Several inflammatory markers were analyzed in the colons of rats administered orally with water (Control) or 1.5-mg·kg·d AlCi for 1 month. (*A*) Colon MGG staining, CD68, serotonin, and tryptase immunohistochemistry. (*B*) MPO activity levels (n = 10/group). (*C*) *Tnfα*, *Il1β*, *Cxcl1*, *and Il10* transcript levels (n = 10/group). (*D*) Number of eosinophils, CD68-positive cells, and serotonin-positive cells (n = 9 or 10/group). (*E*) *Cd11c*, *Cd68*, and *Chga* transcripts levels (n = 10/group). (*F*) Total number of tryptase-positive mast cells (n = 10/group). (*G*) Percentage of degranulated mast cells. (*H*) Colon histamine levels determined by enzyme-linked immunosorbent assay kits (n = 8–10/group). (*I*) Colon *Hdc* transcript levels (n = 10/group). *$P < .05$, ***$P < .0005$ using the Mann-Whitney nonparametric *U* test.

2,4,6-trinitrobenzenesulfonic acid injections. The hypersensitivities induced in these models and by oral administration of 1.5 mg·kg·d AlCi were of similar amplitudes (Figure 1*H*).

Aluminum Activation of Mast Cells Is Necessary for Its Pronociceptive Effect

In a particular subgroup of patients, IBS symptoms might be the result of an altered immune response.[7] Signs of inflammation were assessed in the colons of rats exposed to AlCi (0, 1.5, and 3 mg·kg·d) for 1 month. Colonic histology did not show inflammatory changes (Figures 2*A* and 3*A*, MGG panel). The other evaluated parameters, colonic myeloperoxidase activity (Figures 2*B* and 3*B*) and mRNA expression of *Tnfα*, *Il1β*, *Cxcl1*, and *Il10* (Figures 2*C* and 3*C*) did not indicate any signs of inflammation. More specifically, histological evidence of low-grade inflammation was assessed by the evaluation of the recruitment of inflammatory cells. No differences were observed in stained colonic sections for the infiltration of eosinophils or macrophages (Figures 2*A* and 3*A*, MGG and CD68 panels, respectively; and Figures 2*D* and 3*D*), or by real-time PCR analysis of *Cd11c* and *Cd68* mRNA expression (Figures 2*E* and 3*E*). However, we observed that the number of serotonin-positive cells (Figure 3*A* and *D*) and *Chga* mRNA levels (Figure 3*E*) were lower in the colon after AlCi treatment at 3 mg·kg·d compared with control animals, suggesting an effect of aluminum on enteroendocrine cells. Moreover, though the total number of mast cells, assessed by tryptase immunoreactivity, was not modified by AlCi treatment, activated or degranulated mast cells were more frequent in the colons of treated rats at dosages of 1.5 and 3 mg·kg·d compared with control rats (Figures 2*A*, *F*, and *G* and 3*A*, *F*, and *G*). AlCi treatment also induced upregulation of colon histamine contents (Figure 2*H*) and *Hdc* transcripts (Figure 2*I*), indicating a mast cell activation by aluminum.

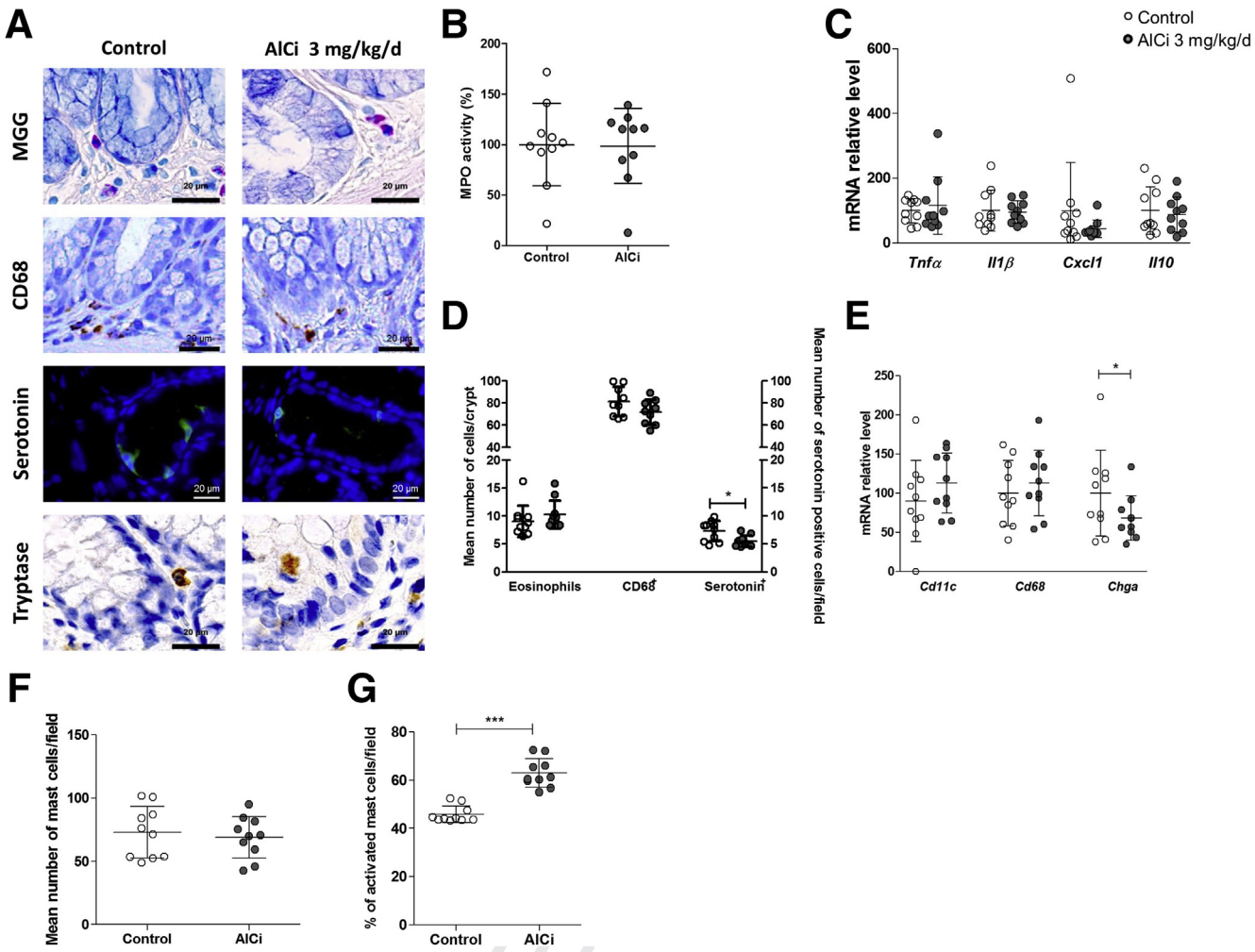

Figure 3. A dose of 3-mg·kg·d aluminum induced low-grade inflammation in the colon. Several inflammatory markers were analyzed in the colons of rats administered orally with water (Control) or 3-mg·kg·d AlCi for 1 month (n = 10/group). (*A*) Colon MGG staining, CD68, serotonin, and tryptase immunohistochemistry. (*B*) MPO activity levels. (*C*) *Tnfα*, *Il1β*, *Cxcl1*, and *Il10* transcript levels. (*D*) Number of eosinophils, CD68-positive cells, and serotonin-positive cells. (*E*) *Cd11c*, *Cd68*, and *Chga* transcripts levels. (*F*) Total number of tryptase-positive mast cells. (*G*) Percentage of degranulated mast cells. $^{*}P < .05$, $^{***}P < .0005$ using the Mann-Whitney nonparametric *U* test.

We then explore the role of aluminum-induced mast cells activation in visceral hypersensitivity. Rats were treated with water or AlCi together with cromoglycate, a compound known to prevent the degranulation of mast cells and thus the release of mast cell-derived mediators.[29] Cromoglycate administration did not modified total number of mast cells but slightly decreased mast cells activation (Figure 4*A* and *B*). However, this was not accompanied with a modification in pain threshold (Figure 4*C*). On the other hand, cromoglycate administration significantly diminished mast cells activation induced by AlCi (Figure 4*B*), which was associated with a significant increase in the mean pressure needed to induce pain in AlCi and cromoglycate co-treated rats compared with AlCi treated rats (Figure 4*C*), reflecting an inhibition of aluminum-induced hypersensitivity by cromoglycate. To explore this further, we used a mast cell–deficient mouse strain ($Kit^{W\text{-}sh/W\text{-}sh}$) harboring a reduction in c-kit tyrosine kinase–dependent signaling resulting in disrupted normal mast cell development but not in total deletion of mast cells.[30] First, and consistently with our data in rats, a significant increase of visceral motor response was observed in AlCi-exposed wild-type (WT) mice compared with control WT mice (Figure 4*D*). In WT mice, aluminum treatment also increased the number of activated mast cells (Figure 4*E* and *F*, white and gray scatterplots). Without any exogenous challenge, $Kit^{W\text{-}sh/W\text{-}sh}$ mice were as sensitive to colorectal distension as WT mice (Figure 4*G*). However, hypersensitivity induced by aluminum in WT mice was suppressed in $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Figure 4*H* and *I*). This was correlated with a significant decrease in aluminum-induced activation of mast cells (Figure 4*E* and *F*, blue scatterplots). Together, these data indicate that aluminum-induced mast cells activation is required for the observed hypersensitivity.

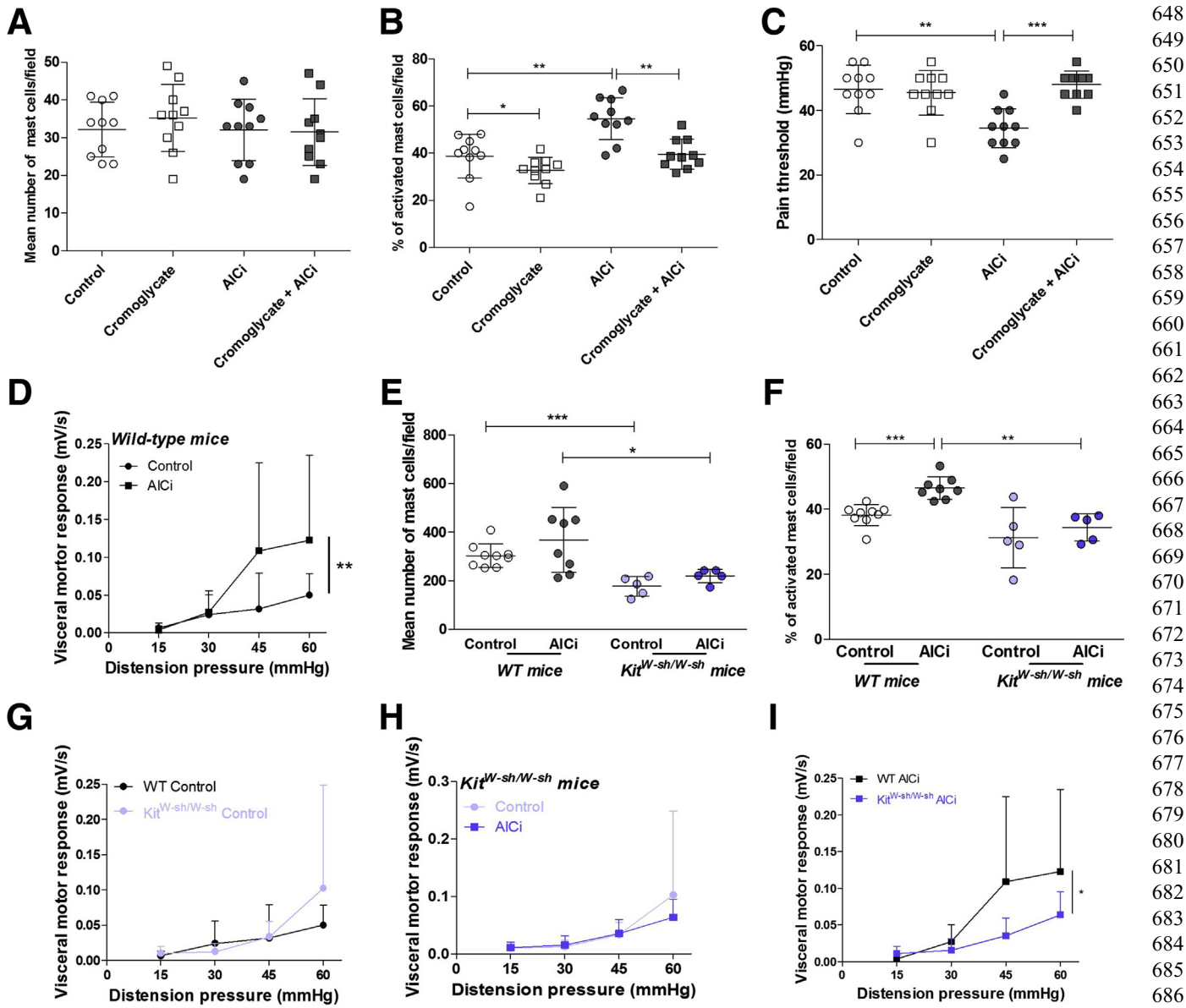

Figure 4. Involvement of mast cells in aluminum-induced hypersensitivity. (*A–C*) Rats were treated for 8 days with water (Control) or 1.5-mg·kg·d AlCi alone or concomitantly with cromolyn sodium (50 mg·kg·d intraperitoneal). (*A*) Total numbers of tryptase-positive mast cells (n = 10/group). (*B*) Percentage of degranulated mast cells (n = 10/group). (*C*) Pain threshold variation in response to CRD (n = 10/group). WT or Kit$^{W\text{-}sh/W\text{-}sh}$ female mice were orally administered with water (Control) or 1.5-mg·kg·d AlCi for 1 month and (*D*, *G–I*) pain threshold variation in response to CRD was recorded (Control: n = 13 WT, n = 10 Kit$^{W\text{-}sh/W\text{-}sh}$; AlCi: n = 14 WT, n = 10 Kit$^{W\text{-}sh/W\text{-}sh}$). (*E*) Total number of tryptase-positive mast cells. (*F*) Percentage of degranulated mast cells (Control: n = 9 WT, n = 5 Kit$^{W\text{-}sh/W\text{-}sh}$; AlCi: n = 8 WT, n = 5 Kit$^{W\text{-}sh/W\text{-}sh}$). *$P < .05$, **$P < .005$, ***$P < .0005$ using the Mann-Whitney nonparametric *U* test for panels *A–C*, *E*, and *F* and 2-way analysis of variance for *D*, *G*, *H* and *I* panels.

PAR2 Activation by Aluminum Is Required for the Induction of Visceral Pain

Several mediators have been involved in visceral nociception.[9] We observed that aluminum treatment modified the transcript levels of receptors from the cannabinoid, transient potential channel, proteinase-activated, tachykinin, and sigma-1 families in the colon of rats (Figure 5*A* and *B*). We chose to focus our attention on PAR2. As observed in rats, *Par2* mRNA was also upregulated by aluminum treatment in the colon of mice (Figure 5*C*).

To assess the role of aluminum-induced PAR2 activation in hypersensitivity, visceral pain was recorded in PAR2 KO mice. In absence of aluminum treatment, *PAR2* KO mice displayed the same response to colorectal distension as WT mice (Figure 5*D*). Once stimulated with aluminum, WT mice showed an increased visceral hypersensitivity whereas

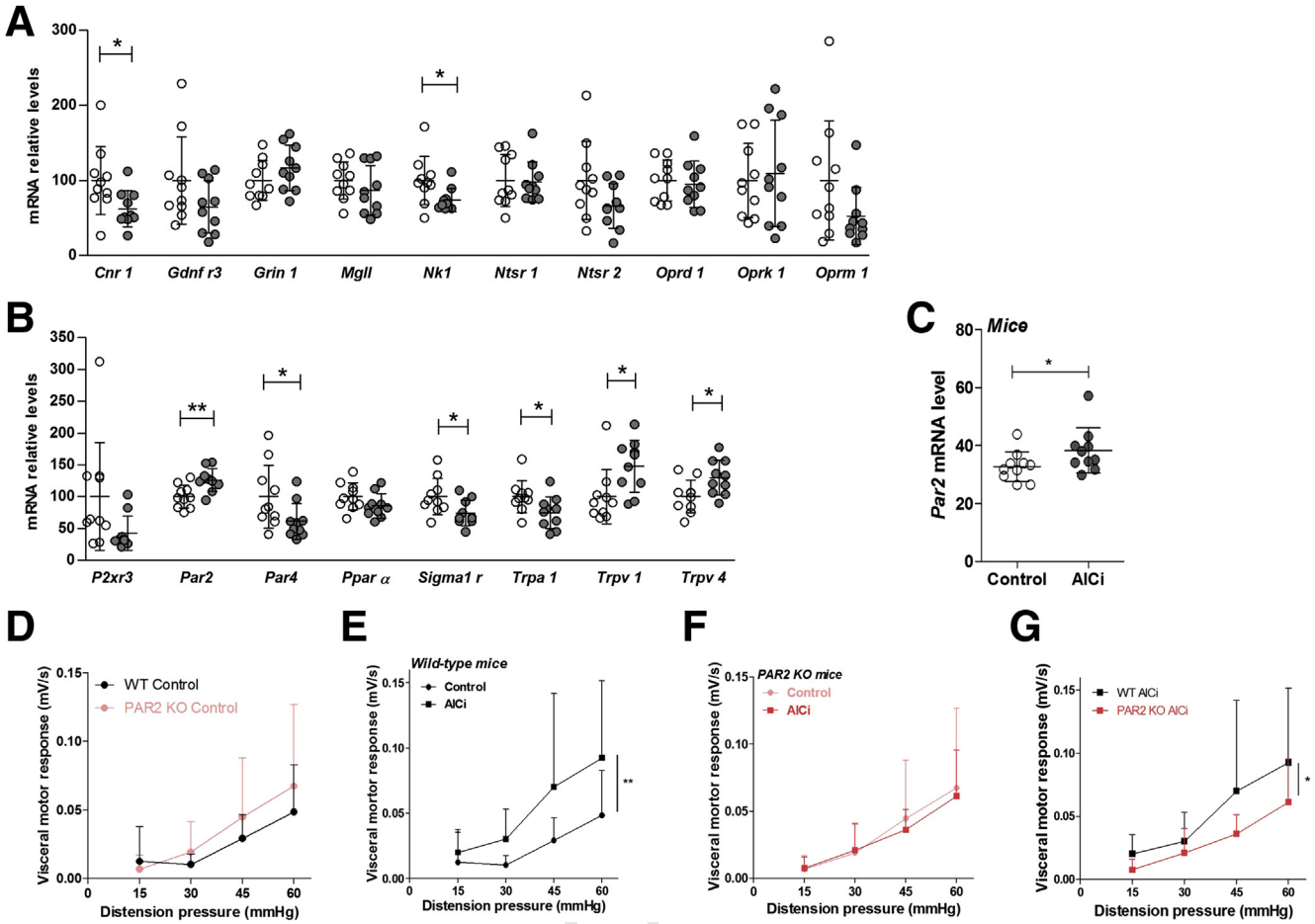

Figure 5. Involvement of PAR2 in aluminum-induced hypersensitivity. (*A*, *B*) Transcript levels of major mediators of nociception in the colons of rats orally treated with water (Control) or AlCi (1.5 mg·kg·d) for 1 month (n = 10/group). (*C*) *Par2* transcript levels in the colons of mice orally administered with water (Control) or AlCi (1.5 mg·kg·d) for 1 month (n = 10/group). (*D–G*) WT and *PAR2* KO mice were orally treated with water (Control: n = 13 WT, n = 7 PAR2 KO), or AlCi 1.5 mg·kg·d (n = 15 WT, n = 11 PAR2 KO) during 1 month. Pain threshold variation in response to CRD. *$P < .05$, **$P < .005$ using the Mann-Whitney nonparametric *U* test for panels *A–C* and 2-way analysis of variance for panels *D–G*.

PAR2 KO mice were unresponsive, describing a pain hypersensitivity dependent on PAR2 activation by aluminum (Figure 5*E–G*).

Aluminum Plays a Central Role in Mast Cells and PAR2 Activation and the Following Pain Initiation

We previously showed that mast cells activation was critical for visceral pain induced by aluminum treatment. Mast cells activation status was thus assessed in *PAR2* KO mice. In the control condition, the percentage of activated mast cells was lower in *PAR2* KO mice compared with WT mice, nevertheless this was not accompanied by a decrease in visceral hypersensitivity (Figures 5*D* and 6*A* and *B*). However, aluminum-induced mast cell activation (Figure 6*B*), histamine release (Figure 6*C*), and *Hdc* mRNA upregulation (Figure 6*D*) observed in the colon of WT mice and correlated with visceral hypersensitivity were abolished in *PAR2* KO mice, indicating a central role for aluminum in visceral pain induction.

Tryptase, released during mast cell degranulation, has been demonstrated to specifically activate PAR2 through the cleavage of its N-terminal domain.[31,32] Therefore, *Par2* expression was assessed in the colon of mast cell–deficient mice. In control condition, *Par2* mRNA levels were not modified in mast cells deficient mice. However, aluminum-induced upregulation of PAR2 in WT mice was abolished in $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Figure 6*E*). These data show that aluminum-induced mast cells activation is required for PAR2 upregulation.

Discussion

IBS is a heterogeneous condition in view of symptoms, underlying mechanisms and causes.[2,3] IBS is a lifelong disease characterized by periods of exacerbations and remissions. Current therapies do not cure the disease but rely on symptoms and quality-of-life improvement (constipation, diarrhea, pain, or depression). Elucidating triggering factors for IBS is crucial for effective treatment of

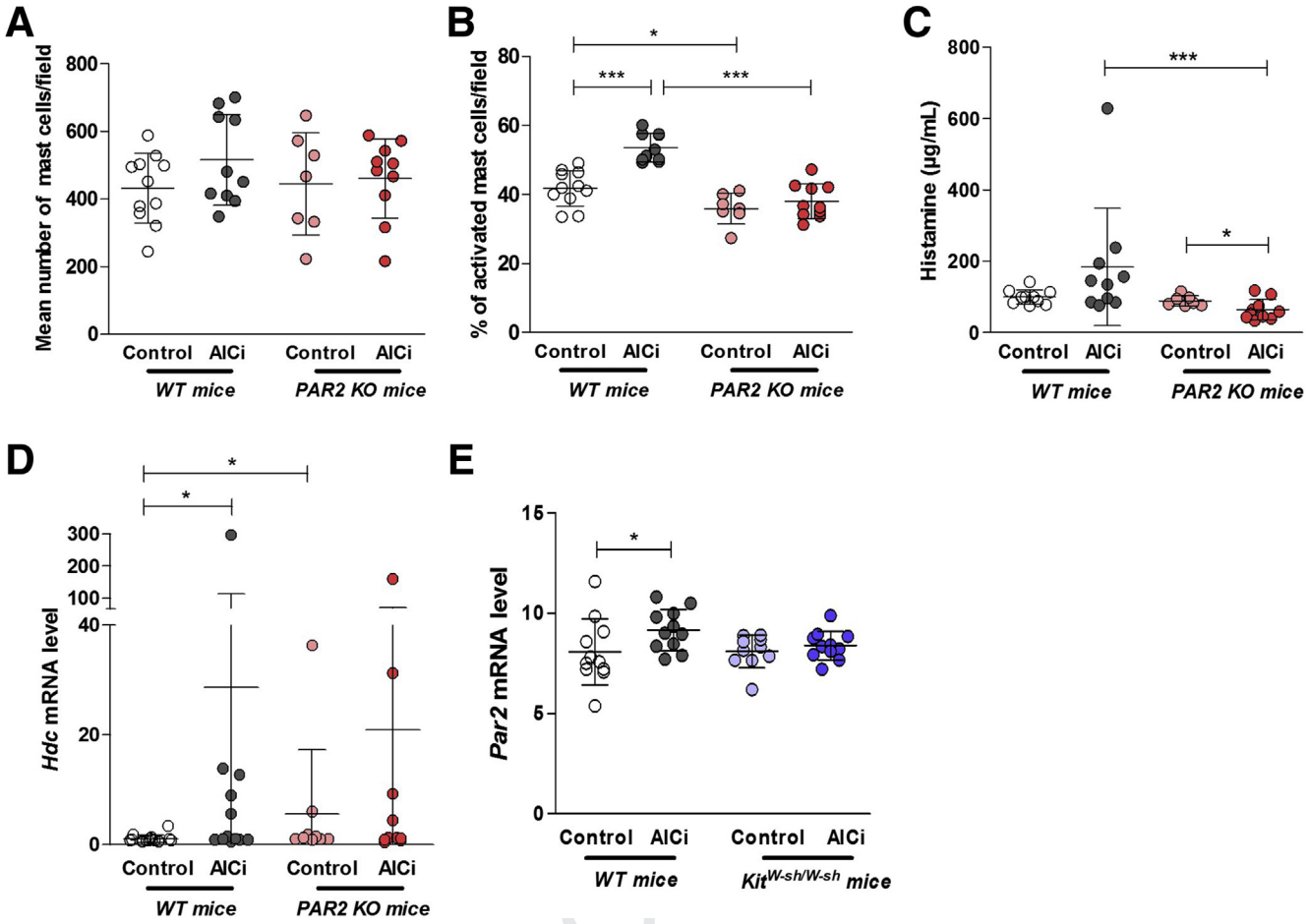

Figure 6. Central role of aluminum in mast cells and PAR2 activation and the following pain initiation. (*A*) Total number of tryptase-positive mast cells. (*B*) Percentages of degranulated mast cells (Control: n = 11 WT, n = 7 PAR2 KO; AlCi: n = 10 WT, n = 10 PAR2 KO). (*C*) Colon histamine levels determined by enzyme-linked immunosorbent assay (Control: n = 10 WT, n = 7 PAR2 KO; AlCi: n = 10 WT, n = 10 PAR2 KO). (*D*) Colon *Hdc* transcript levels (Control: n = 19 WT, n = 9 PAR2 KO; AlCi: n = 12 WT, n = 10 PAR2 KO). (*E*) *Par2* transcript levels in the colon of WT and $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Control: n = 11 WT, n = 10 $Kit^{W\text{-}sh/W\text{-}sh}$; AlCi: n = 11 WT, n = 11 $Kit^{W\text{-}sh/W\text{-}sh}$). *$P < .05$, ***$P < .0005$ using the Mann-Whitney nonparametric *U* test.

the disease. In specific subtypes of IBS patients, for which a precise trigger has been highlighted, that is gluten or wheat, lactose, or dietary nickel, a withdrawal of the causal factor ameliorated symptoms.[9,10,12,33] Here, we assessed the role, in IBS development, of a commonly found dietary contaminant, the aluminum. Aluminum is found in food products, either naturally occurring or as an additive. Aluminum can also be ingested through beverages, including water, or as a result of aluminum leaching from kitchenware or packaging.[21–23] We first showed that aluminum, at dosages relevant to human exposure, induced persistent and dose-dependent colonic hypersensitivity in rats and mice. Aluminum-induced hypersensitivity persisted over time even in case of aluminum cessation. It appeared again and amplified when aluminum treatment resumed, suggesting a sensitization phenomenon. A link to IBS triggering was evaluated according to mechanisms implicated in IBS pathophysiology that are low grade inflammation linked to aberrant neuroimmune alterations.[6,9,34–36]

We showed that AlCi treatment activated mast cells and triggered the release of tryptase and histamine in the colon of rats. We also demonstrated that stabilization of mast cells by cromoglycate administration, or deficiency of mast cells in $Kit^{W\text{-}sh/W\text{-}sh}$ mice, abolished the hypersensitivity induced by aluminum. Peripheral mast cells are often found in proximity to sensory nerve endings and vasculature, and mediators released by activated mast cells stimulate nociceptive afferents contributing to pain perception.[37] In patients with IBS, increased expression of tryptase and elevated number of mast cells in proximity to nerves have been shown and correlated with abdominal pain.[38,39] We speculate that aluminum activate mast cells to release mediators that can increase excitability of nociceptive afferences contributing to the visceral pain phenotype.

Mast cells synthesize mediators that can activate PAR2 leading to visceral pain.[40–42] Here, we showed that AlCi administration activated PAR2 in the colon of mice and rats. In addition, we demonstrated that PAR2 activation by aluminum was essential in the induction of visceral

hypersensitivity, as PAR2 KO mice were unresponsive to aluminum. We also demonstrated that PAR2 activation by AlCi was inhibited following mast cell stabilization. Moreover, mast cell activation and subsequent histamine release induced by aluminum were abolished in PAR2 KO mice, suggesting that aluminum is a key player in mast cell–and PAR2-mediated hypersensitivity.

We also found that increased visceral hypersensitivity induced by aluminum was correlated with fewer enteroendocrine cells secreting serotonin, supporting a role for these cells in aluminum-induced visceral pain. Enteroendocrine cells are specialized epithelial cells that respond to luminal stimuli by releasing various biologically active compounds. They regulate several physiological and homeostatic functions of the gastrointestinal tract, such as postprandial secretion, motility, immune responses, and sensory functions.[43] A reduced number of enteroendocrine cells has been observed in the duodenum, ileum, and colon of some patients with IBS.[44,45] and it has been speculated that this might be responsible for the visceral hypersensitivity seen in affected patients.[16] Further studies are needed to understand whether aluminum has a direct impact on enteroendocrine cell activation or differentiation. Enteroendocrine cells together with mast cells activate neurons of the enteric nervous system notably through the release of histamine and serotonin, which activate receptors located on intestinal nerves conveying pain stimulus to the brain.[46,47] Taken together, our results linked aluminum to several mechanisms implicated in IBS pathophysiology, highlighting a possible role for aluminum as a triggering factor in IBS development.

Implication of aluminum in pain development has been recently suggested. Indeed, cold allodynia associated with an elevated TRPA1 expression and aluminum accumulation in dorsal root ganglia was observed in mice intraperitoneally injected for 15 days with aluminum chloride.[48] Furthermore, decreasing aluminum concentration in dorsal root ganglia by glutathione treatment alleviated cold allodynia, opening a way for treatment in patient suffering from neuropathic pain induced by aluminium.[49]

Despite promising evidence that some treatments improved symptoms and visceral pain in IBS patients in the short term, there is no medical intervention that are effective in the long term.[9,50] The elucidation of the upstream triggers that induce and maintain the pathways involved in symptoms is needed for providing novel therapeutic strategies. Some progress have been made with patients suffering from nonceliac gluten or wheat sensitivity, lactose intolerance, and nickel-allergic contact mucositis whose symptoms have improved with an exclusion diet.[15,17–19] Similarly, in particular subgroups of IBS patients, a low-aluminum diet or aluminum chelation strategies would have an effect on IBS symptoms. Accordingly, targeting aluminum might be a promising therapeutic strategy, as suggested in neuropathic pain.[49]

Aluminum ingestion at dosages relevant to human exposure induced colonic hypersensitivity in rats and mice. Aluminum-induced visceral hypersensitivity is profound, persistent, and dose and gender dependent. It requires mast cells activation and is mediated through the PAR2. Aluminum might be the first identified dietary risk factor for IBS, implying that measures to limit aluminum dietary consumption or to chelate aluminum may represent novel pathways of prevention and treatment of IBS in some susceptible patients.

Materials and Methods

Animals and Treatments

Adult Sprague Dawley rats (100–150 g) and C57 BL/6 mice were purchased from Janvier Labs (Le Genest St Isle, France). $Kit^{W\text{-}sh/W\text{-}sh}$ and *PAR2* knockout (KO) mice were bred in the animal facilities in the Institut Pasteur de Lille and Toulouse University, respectively. Except for the comparison between males and females, only male rats were used. For experiments in $Kit^{W\text{-}sh/W\text{-}sh}$ and PAR2KO mice, female mice were used. Rats were administered orally with aluminum citrate (AlCi) (a dietary form of aluminum) (Pfaltz & Bauer, Waterbury, CT, ref. A16090) at dosages of 0.5, 1.5, and 3 mg·kg body weight·d or zinc citrate (ZnCi) (Sigma-Aldrich, Lyon, France, ref. 480762) at 1.5 mg·kg·d for different times, as detailed in the figure legends. ZnCi was used to assess whether aluminum effect is common to another metal or arose from the citrate complexation of aluminum. Some groups of rats were also treated daily with intraperitoneal cromoglycate for 8 days (50 mg·kg·d) (Sigma-Aldrich, ref. C0399). Mice were treated orally with AlCi at a dose of 1.5 mg·kg·d for 1 month.

Colorectal Distension and Visceral Sensitivity Assessment in Rats

Male and female rats were acclimatized to laboratory conditions for 1 week before each experiment. Colonic hypersensitivity was assessed by measuring the intracolonic threshold required to induce a behavioral response during colorectal distension (CRD) caused by the inflation of a balloon introduced in the colon. This response was characterized by an elevation of the hind part of the animal body and a clearly visible abdominal contraction. Distension balloons were prepared by using a 2-cm flexible latex balloon ligated to the tip of a 2-mm catheter (Vygon, Ecouen, France). Animals were lightly anesthetized with isoflurane, and the deflated flexible latex balloon was inserted intra-anally into the descending colon such that its end was 1 cm proximal to the anus. The flexible catheter was taped to the base of the tail to prevent displacement. Animals were allowed to recover for 30 minutes before CRD was initiated. The CRD tests were performed using an electronic barostat apparatus (Distender series II, G&J Electronics, Toronto, Canada) after a 5-minute retrieval period. Increasing pressure was applied continuously until pain behavior was displayed or a cutoff pressure of 80 mm Hg was reached. Butyrate (Sigma-Aldrich, ref. B5887) was administered intrarectally twice a day over 3 days (200 nM) before CRD. 2,4,6-Trinitrobenzenesulfonic acid (Sigma-Aldrich, ref. 92823) was injected intrarectally once 1 month before CRD (150 mg/kg).

CRD and Visceral Sensitivity Assessment in Mice

Three days before CRD, 2 electrodes were implanted in the abdominal external oblique musculature of mice previously anesthetized with xylazine and ketamine (Bioflex AS-631, Cooner Wire, Chatsworth, CA). Electrodes were exteriorized at the back of the neck and protected by a plastic tube attached to the skin. Electrodes were connected to a Bio Amplifier, which was connected to an electromyogram acquisition system (ADInstruments, Colorado Springs, CO). A 10.5-mm-diameter balloon catheter was gently inserted into the colon at 5 mm proximal to the rectum (Fogarty arterial embolectomy catheter, 4F, Vygon). Ten-second distensions were performed at pressures of 15, 30, 45, and 60 mm Hg acquired by inflating the balloon in a stepwise fashion with water (20, 40, 60 and 80 μL respectively) with 5-min rest intervals.[51] Electromyographic activity of the abdominal muscles was recorded and visceromotor responses were calculated using Chart 5 software (ADInstruments).

Real-Time Quantitative Polymerase Chain Reaction

Colonic tissue samples were homogenized with ceramic beads using Precellys Lysing Equipment (Bertin Technologies, Montigny le Bretonneux, France, ref. P000911-LYSK0-A). Total RNA was extracted from colonic samples with NucleoSpin RNAII kits (Macherey-Nagel, Hoerdt, France, ref. 740955). The complementary DNA was prepared with High-Capacity Complementary DNA Archive kits (Thermo Fisher Scientific, Villebon-sur-Yvette, France, ref. 4368813). Transcripts levels of genes involved in inflammation and pain transduction were quantified in the StepOne real-time polymerase chain reaction (PCR) system using a SYBR Green PCR master mix (Thermo Fisher Scientific, ref. 4385612). Relative messenger RNA (mRNA) levels were determined using the $^{\Delta\Delta}$Ct method and the values were normalized to the expression of *PolR2a* for mice and *Gapdh* for rats.[52] Primer sequences are available upon request.

Myeloperoxidase Activity Assay

Neutrophil influx in tissue was analyzed by assaying the enzymatic activity of myeloperoxidase (MPO). Rat colons were excised following euthanasia of the animals, thoroughly washed in phosphate-buffered saline, and homogenized in 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich, ref. H6269) in 50-mM phosphate-buffered saline, freeze-thawed 3 times, sonicated, and centrifuged. MPO was assayed in the clear supernatant by adding 1 mg/mL of dianisidine dihydrochloride (Sigma-Aldrich, ref. D3252) and 5 $\times$ 10^{-4}% H_2O_2. The change in optical density was measured at 450-nm wavelength. Human neutrophil MPO (Sigma-Aldrich, ref. M6908) was used as a standard. One unit of MPO activity was defined as the amount that degraded 1.0 μmol H_2O_2/min at 25°C. Readings from tissue samples were normalized to total protein content as detected by DC protein assays (Bio-Rad, Marnes-la-Coquette, France, ref. 5000111).

Histological Analysis and Immunohistochemistry

Colons were fixed in 4% paraformaldehyde overnight, processed, and embedded in paraffin wax by standard techniques. Sections (4 μm) were stained with May-Grünwald Giemsa (MGG) (Carlo-Erba, Val-de-Reuil, France, refs. E460583 and E453612). For immunohistochemistry (IHC) analysis, tissue sections were blocked with 2% goat serum (Thermo Fisher Scientific, ref. 16210064) and incubated overnight at 4°C with primary antibodies: goat anti-rat serotonin polyclonal antibody (Abcam, Cambridge, United Kingdom, ref. ab66047), mouse anti-rat CD68 monoclonal antibody (Bio-Rad [formerly Abd Serotec], Kidlington, United Kingdom, ref. MCA341R) and clone AA1 tryptase antibody (Dako, Les Ulis, France) followed by a rabbit anti-goat IgG (H+L) secondary antibody Alexa 488 (Thermo Fisher Scientific, ref. A11034), polyclonal rabbit anti-mouse immunoglobulin biotinylated antibody, and polyclonal goat anti-mouse antibody (Dako). Slides were counterstained with hematoxylin for CD68 and tryptase IHC, and with Hoechst 33258 (Thermo Fisher Scientific, ref. H3569) for serotonin IHC. Cells positive for CD68, serotonin and tryptase, and eosinophils were counted blindly by 2 investigators (5 crypts/slide, 1 slide/animal for eosinophils and CD68-positive cells; 8 fields/slide, 1 slide/animal for serotonin-positive cells; and total cells/slide, 1 slide/animal for tryptase-positive cells).

Histamine Measurement

Histamine levels were detected in colon homogenates by enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (Bertin Bioreagent, Montigny-le-Bretonneux, France, ref. A05890.96). Readings from tissue samples were normalized to total protein content as detected by DC protein assays (Bio-Rad, ref. 5000111).

Statistics

Data are expressed as mean ± SD. For Mice visceromotor response, a repeated-measures 2-way analysis of variance was performed. For all other parameters, differences between groups were compared using the Mann-Whitney nonparametric *U* test (GraphPad Prism, GraphPad Software, La Jolla, CA) (*$P < .05$, **$P < .005$, ***$P < .0005$ in the figures).

Study Approval

The animal treatment protocol was approved by the regional bioethics committee (committee no.75; authorization no.CEEA2016030317128286, May 23, 2016) and all of the animals received human care in accordance with European guidelines (Directive 86/609/EEC, European Economic Community, November 24, 1986).

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