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RESEARCH ARTICLE

Innate Immunity

IL-10 Neutralization Attenuates Mast Cell Responses in a Murine Model of Experimental Food Allergy

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ABSTRACT

IgE-mediated mast cell (MC) activation is a critical component of allergic responses to oral Ags. Several T cell–derived cytokines have been shown to promote MC reactivity, and we recently demonstrated a critical role for the cytokine IL-10 in mediating MC responses during food allergy. In this study, we further validate the role of IL-10 using Ab-mediated IL-10 depletion. IL-10 neutralization significantly attenuated MC responses, leading to decreased MC accumulation and activation, as well as inhibition of MC-mediated symptoms such as allergic diarrhea. This was accompanied by decreased Th2 cytokine gene expression, attenuated systemic T cell responses, and fewer CD4 T cells, B cells, and MCs in the spleen. Our data further confirm the role of IL-10 in driving MC responses and suggest that IL-10–responsive MCs may constitute an important player in allergic responses. *ImmunoHorizons*, 2024, 8: 431–441.

INTRODUCTION

Food allergy is a growing problem in the Western world, affecting 5–7% of children and 3–6% of adults (1–3). Mast cells (MCs) and their mediators play critical roles during food allergy, leading to various proinflammatory effects, including vasodilation, edema, smooth muscle hyperreactivity, and localized and systemic inflammation. These effects directly contribute to symptoms of food allergy such as diarrhea, vomiting, gastrointestinal dysfunction, and systemic anaphylaxis (3–6). The activation of intestinal MCs during allergic exposure to food-derived Ags depends directly on IgE-mediated signaling mechanisms that are initiated upon crosslinking of allergens with IgE Abs on MC surfaces. Furthermore, these interactions are coordinated by allergen-specific Th2 cells that induce inflammation by secreting MC-promoting cytokines and eliciting the production of Ag-specific IgE (7, 8). We and others have shown that both Th2-derived cytokines such as IL-3, IL-4, and IL-9 as well as IgE Abs can promote intestinal MC expansion and activation during allergic reactions (9–16).

Recently, we demonstrated that the immunoregulatory cytokine, IL-10, can also promote IgE-dependent MC-mediated responses during food allergy (14). MC expansion and IgE-mediated activation were abolished in the absence of IL-10 in IL-10$^{-/-}$ mice, leading to protection from food allergy development. Furthermore, adoptive transfer of either wild-type CD4 T cells or MCs restored the development of food allergy in IL-10$^{-/-}$ animals (14). Surprisingly, our data demonstrated that IL-10 could also directly enhance the proliferation, survival, expression of the IgE receptor (FcεRI) and the IgE-mediated activation of MCs, suggesting that IL-10 may act as a costimulator of MC responses. These findings have been corroborated by other studies investigating effects of IL-10 during both allergy (17) and cancer (18), and although unexpected, they are consistent

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Abbreviations used in this article: MC, mast cell; mMCP-1, murine mast cell protease-1; Treg, regulatory T cell.

with several studies demonstrating a proinflammatory role for IL-10 during allergic diseases and other chronic inflammatory conditions (19–33).

In this study, we sought to further validate the role of IL-10 via pharmacologic blockade of its activity during the acute MC-dependent phase of food allergy. Our data provide a proof of concept demonstrating that IL-10 neutralization can attenuate MC responses, resulting in suppression of food allergy symptoms and decreased expression of MC and Th2 markers. These observations not only further confirm the role of IL-10 on MCs during food allergy but suggest that inadvertent IL-10 induction may serve to promote MC-mediated allergic responses, instead of curtailing them.

MATERIALS AND METHODS

Animals

BALB/c mice were purchased from The Jackson Laboratory and Envigo. Both male and female mice were used in experiments. All mice were bred in our facilities and all animal research was performed as approved by the Institutional Animal Care and Use Committees at the respective institutions.

Food allergy regimen

To induce food allergy, BALB/c mice were i.p. immunized with 50 μg of chicken egg OVA in 1 mg of alum twice, as previously described (14, 34). Mice were challenged intragastrically with 50 mg of OVA on 6 alternating days. Control animals were i.p. sensitized but not challenged with OVA. IL-10 neutralization was performed by treating some groups of mice i.p. with 100 μg of purified anti–IL-10 15 min before each OVA challenge (both clones JES5-2A5 and JES5-16E3 from BioLegend were tested in different experiments). Additionally, in other experiments, mice were treated with 500 μg of anti–IL-10 once 16 h prior to the final challenge with OVA. Purified rat IgG1 (BioLegend) was used as a control in some but not all experiments. Mice were sacrificed 1 h after the sixth challenge with OVA, and food allergy parameters were assessed as previously described (14, 34, 35). The development of intestinal anaphylaxis was assessed as described below. Blood was collected for evaluation of Abs and murine mast cell protease-1 (mMCP-1) in serum. Jejunum was collected for histological assessment of MCs and evaluation of cytokine gene expression by RT-PCR as described above. Spleens were collected for evaluation of systemic cytokine production by T cells.

Measurement of intestinal anaphylaxis

Intestinal anaphylaxis was assessed in challenged mice by scoring the percentage of animals exhibiting allergic diarrhea for 1 h after OVA challenge (35, 36). In some experiments, the intensity of allergic diarrhea was evaluated using a scale of +, ++, and +++.

Histological analysis and enumeration of MCs

Intestinal MCs were enumerated as previously described (10). Tissue sections were stained with chloroacetate esterase, and MCs were counted in complete cross-sections of jejunum.

Spleen stimulation

Spleen cells from food allergy mice were cultured with medium, 200 μg/ml OVA, and cytokines were enumerated in supernatants as previously described (14, 34).

Quantitative PCR analysis and ELISAs

Quantitative RT-PCR was performed as previously described using TaqMan probes (14, 34). The expression of cytokine genes (IL-4, IL-5, IL-9, IL-10, IL-13, IFN-γ) was calculated relative to GAPDH transcripts. ELISAs for mMCP-1 (Thermo Fisher Scientific), IL-4, IL-5, IL-10 and IFN-γ (BioLegend), IL-13 (R&D Systems), and OVA-IgE were performed according to the manufacturers' protocols as previously described (14, 34).

Flow cytometry

Spleen cells were isolated as previously described (14, 37) and resuspended in staining medium containing 1× HBSS, HEPES buffer, and 2% FCS. For phenotypic evaluation, cells were incubated with mAbs for the detection of various markers, including CD3, CD4, CD8, B220, c-Kit, FcεRI, IgE, CD25, Foxp3, and others (BioLegend).

Statistical analysis

Data are expressed as mean ± SEM unless stated otherwise. Statistical significance comparing different sets of mice (between two groups) was determined using an unpaired Student *t* test with appropriate corrections applied, whenever applicable.

RESULTS

IL-10 neutralization attenuates MC responses during food allergy

We have previously demonstrated that IL-10 has a profound effect on MC responses, resulting in enhanced MC activation and expansion during food allergy development (14). Furthermore, the development of food allergy was attenuated in IL-$10^{-/-}$ mice and could be restored after adoptive transfer of IL-10–responding MCs. To further validate the role of IL-10, we used a well-established system of IL-10 blockade, reliant on mAb-mediated depletion of IL-10 signaling. More specifically, IL-10 activity was blocked during the acute, MC-dependent challenge phase of the food allergy model. Briefly, mice were sensitized and challenged with OVA as depicted in Fig. 1A, followed by administration of anti–IL-10 15 min prior to each oral OVA challenge. Mice were sacrificed 1 h after the final challenge and food allergy was assessed. As observed in Fig. 1B, BALB/c mice sensitized and challenged with OVA developed a robust IgE-mediated response, which was accompanied by allergic

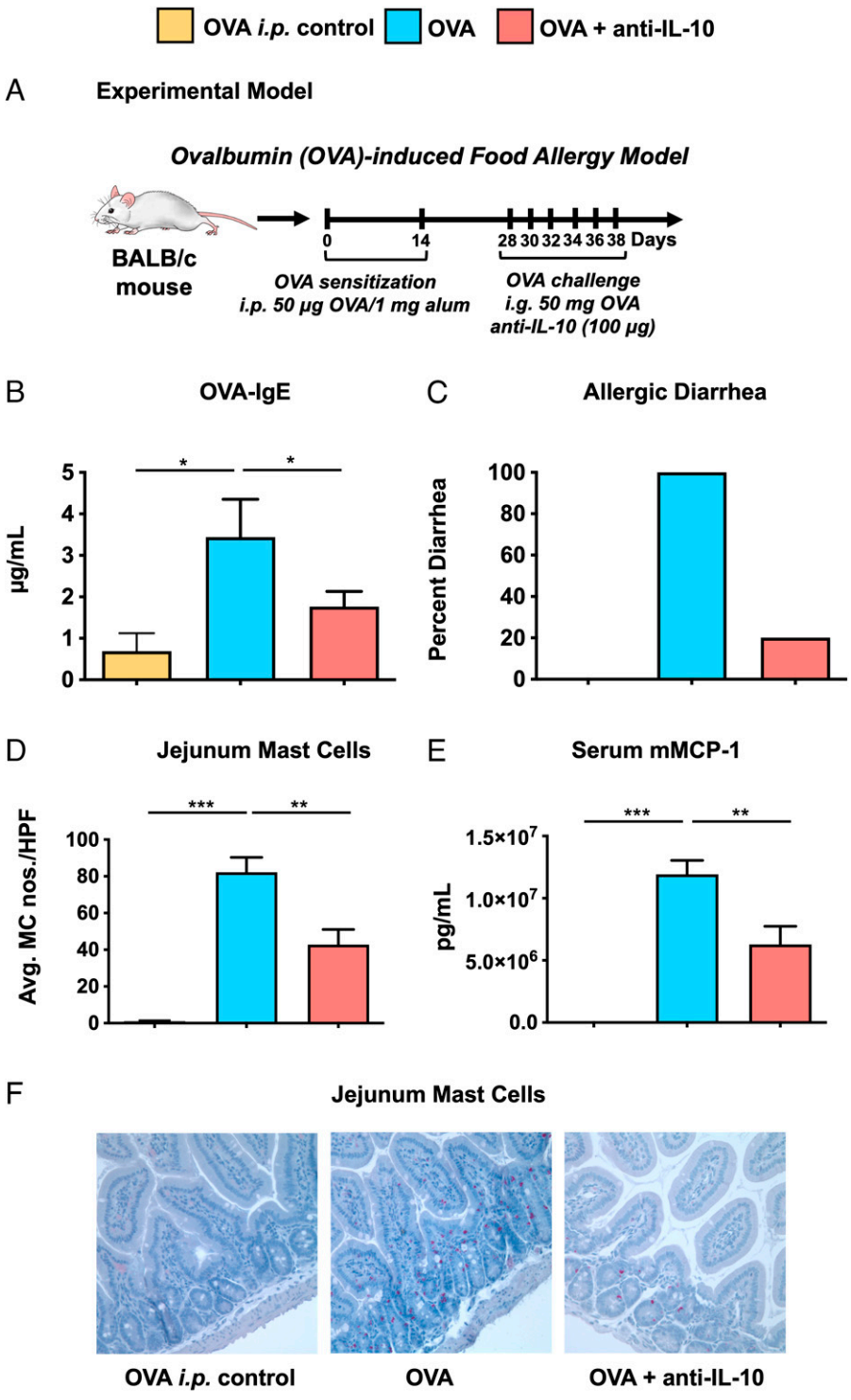

FIGURE 1. IL-10 neutralization suppresses intestinal anaphylaxis and MC responses in allergic mice. BALB/c mice were immunized and challenged with OVA as depicted in (**A**). Some mice were injected with anti–IL-10 prior to each OVA challenge. One hour later, mice were sacrificed and various indices of food allergy were measured, including (**B**) serum OVA-IgE levels, (**C**) percent of animals exhibiting diarrhea, (**D**) chloroacetate esterase $(CAE)^+$ MCs in the jejunum, and (**E**) serum mMCP-1 levels. (**F**) Histological images of MCs in the jejunum are shown (original magnification ×40). Data are representative of more than three experiments; $n = 5–7$ mice/group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (*t* test).

diarrhea (Fig. 1C), MC recruitment to the small intestine (Fig. 1D, 1F), and elevated levels of serum mMCP-1 (Fig. 1E). In contrast, IL-10 depletion signficantly reduced the induction of Ag-specific IgE, MC expansion in the small intestine, and MC degranulation as measured through the release of mMCP-1 (Fig. 1B–E). Similarly, most (80%) IL-10–depleted animals also exhibited decreased allergic diarrhea. These data demonstrate that anti–IL-10 administration can attenuate MC responses during food allergy.

IL-10 blockade decreases Th2 responses in OVA-challenged mice

To further assess the effects of anti–IL-10 in these animals, we evaluated the expression of type 2 cytokine genes in the small

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intestine of allergic animals. As previously reported (14), enteric challenge with OVA induced the secretion of several Th2 cytokines in sensitized animals, including IL-4, IL-5, IL-13, and IL-9. Furthermore, the expression of IL-10 was also enhanced. In contrast, whereas IFN-γ expression appeared to be elevated in the jejunum of allergic animals, no significant differences were observed between allergic and control animals. As observed in Fig. 2A–E, IL-10 depletion significantly attenuated the expression of IL-4, IL-5, IL-13, IL-9, and IL-10 in allergic animals, suggesting that anti–IL-10 treatment can inhibit markers of type 2 inflammation during food allergy development. Furthermore, levels of IFN-γ mRNA were not significantly different between groups, suggesting that although IL-10 depletion can suppress Th2 cytokines, it does not necessarily correlate with increases in Th1 cytokine expression (Fig. 2F).

Systemic T cell responses are decreased in IL-10–depleted mice

Next, to determine whether systemic T cell responses are also impacted by IL-10 depletion, we assessed the function of Ag-specific T cells to restimulation with OVA. As observed in Fig. 3, exposure to OVA induced the activation of Ag-specific T cells, resulting in increased secretion of IL-4, IL-5, IL-13, and IL-10 by spleen cells from allergic mice. In contrast, splenic cells from IL-10–depleted mice exhibited an attenuated phenotype, resulting in decreased secretion of these cytokines. In contrast, whereas IFN-γ secretion was decreased in splenocytes from OVA-challenged mice, no differences were observed between the control or OVA-challenged and anti–IL-10 groups.

Animals treated with anti–IL-10 exhibit decreased inflammation during food allergy development

To further evaluate the effects of anti–IL-10 treatment on immune cell populations, we next characterized various cells in the spleens of experimental animals. OVA sensitization and challenge resulted in the expansion of several immune cell populations in the spleen, compared with unchallenged controls. These included both CD4 and CD8 T cells as well as B cells (Fig. 4A–C). As we previously reported (14), a significant number of MCs were also observed (Fig. 4D, 4E). In contrast, mice

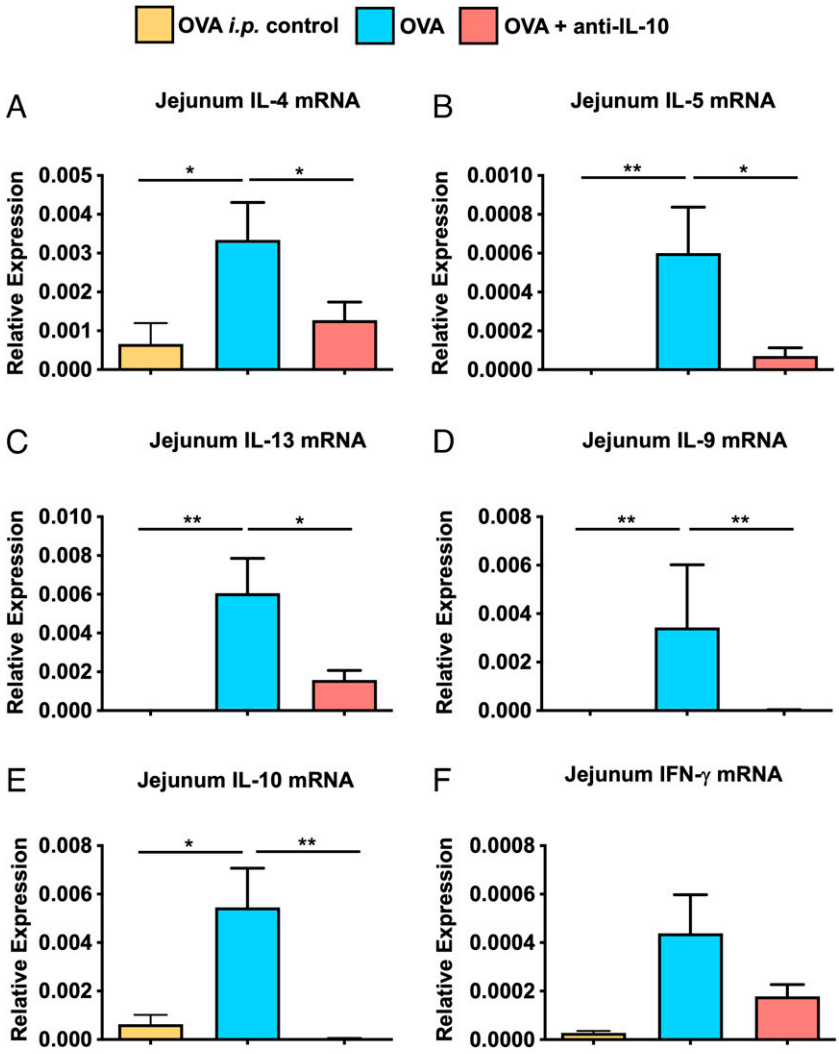

FIGURE 2. Attenuation of small intestinal Th2 cytokine expression in mice treated with anti–IL-10.

Mice were sensitized and challenged with OVA and some mice were treated with anti–IL-10 as shown in Fig. 1A. (**A–F**) Jejunal tissue was collected and mRNA expression for various Th2 cytokine genes was assessed as shown. Data are representative of two experiments; $n = 5–7$ mice/group. $^*p < 0.05$, $^{**}p < 0.01$ (*t* test).

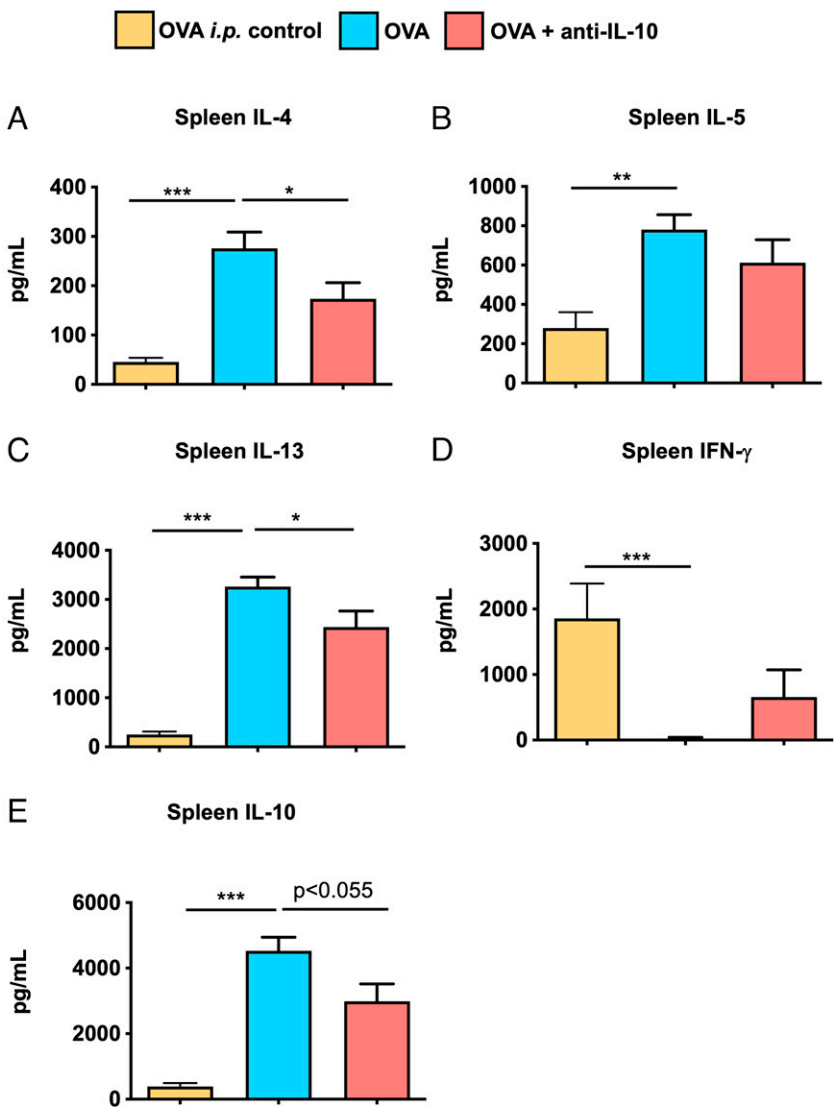

FIGURE 3. Systemic Th2 responses are decreased in mice treated with anti–IL-10.

Mice were sensitized and challenged with OVA and some mice were treated with anti–IL-10 as shown in Fig. 1A. (**A–E**) Spleen cells were collected and cultured with OVA for 96 h as described in *Materials and Methods*. Cytokines were then evaluated in supernatants. Data are representative of two experiments; $n = 5–7$ mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (*t* test).

treated with anti–IL-10 exhibited decreased numbers of splenic CD4 T cells, B cells, and MCs, compared with untreated animals. To further assess whether anti–IL-10 treatments may also modulate the numbers of regulatory T cells (Tregs) in the spleen, we also examined the frequency of Foxp3^{+} CD4 T cells. While an increased percentage of these cells was observed in the spleens of mice treated with anti–IL-10, this did not translate into a significant difference in terms of absolute numbers (Fig. 4F). These data demonstrate that IL-10 blockade not only impacts local inflammatory responses within the gut, but it can also modulate the immune cell compartment in other tissues, subsequently leading to effects on food allergy development.

IL-10 neutralization prior to final OVA challenge suppresses MC numbers and allergic diarrhea, but it has no effects on mMCP-1 release

Lastly, to assess whether IL-10 neutralization can suppress MC responses in mice with established food allergy, we treated mice with 500 μg of anti–IL-10 once prior to the final challenge with OVA. We then compared the induction of serum OVA-IgE and mMCP-1 in the same animals prior to and after anti–IL-10 administration. As observed in Figs. 5A and 5B, after the fifth OVA challenge, elevated levels of serum OVA-IgE and mMCP-1 were observed in all sensitized and challenged mice (purple circles). Anti–IL-10 treatment followed by a subsequent OVA challenge (sixth challenge) resulted in a decrease in OVA-IgE but no differences in mMCP-1 (green circles). To further assess whether anti–IL-10 treatment before the final OVA challenge may have also affected other allergic parameters, we compared these animals with mice that were subject to the entire food allergy regimen (sensitization plus six OVA challenges; light blue circles). As shown in Fig. 5C, whereas the latter group developed significant incidences of profuse diarrhea, none was observed in the group treated with anti–IL-10. Similarly, compared with untreated animals, the animals treated with anti–IL-10 had fewer intestinal MCs (Fig. 5D), which also correlated with a decreased tendency to produce Th2 cytokines

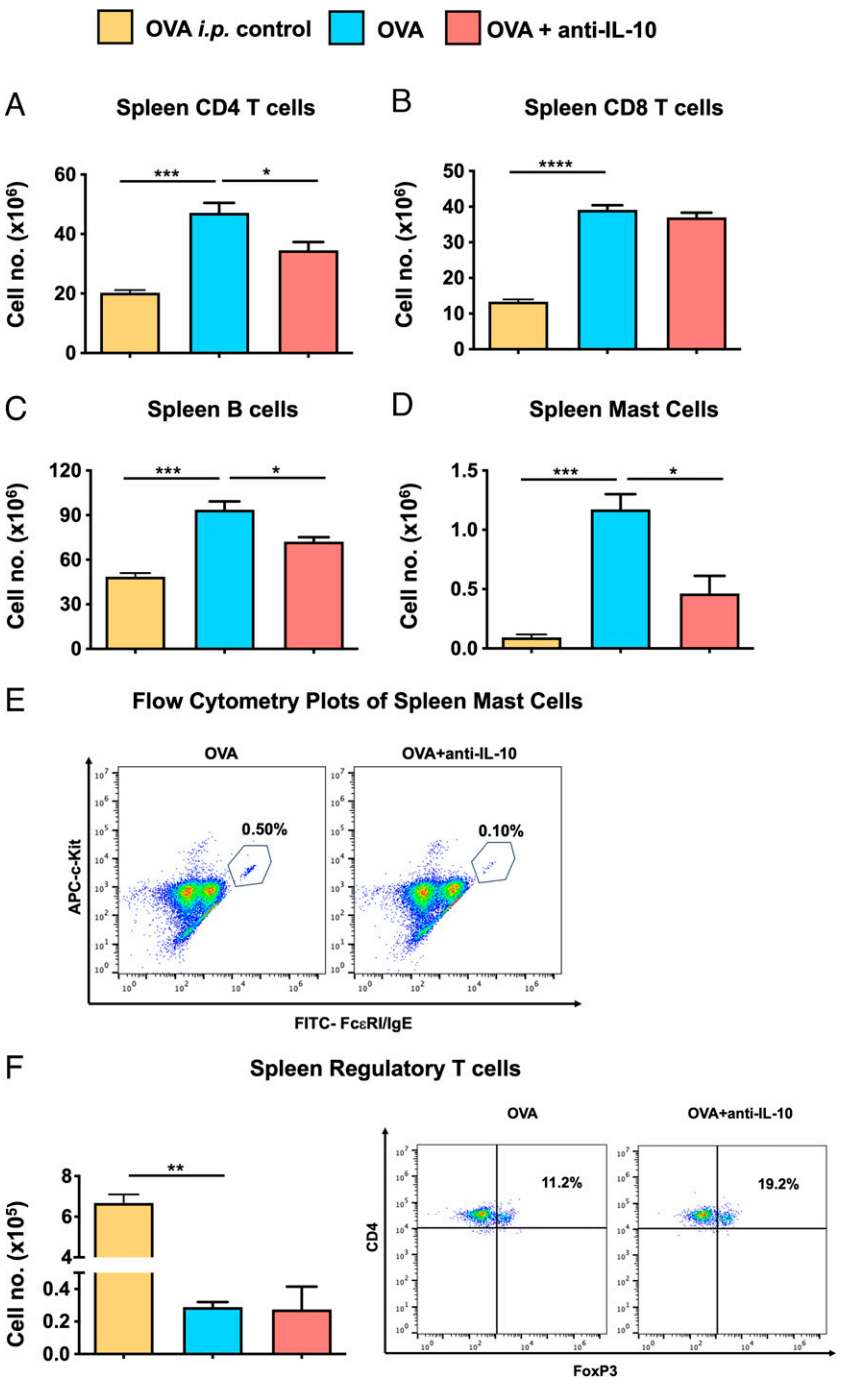

FIGURE 4. Reduced MCs and lymphocyte populations in IL-10–neutralized mice.

Mice were sensitized and challenged with OVA and some mice were treated with anti–IL-10 as shown in Fig. 1A. (**A–F**) Flow cytometry was performed on spleen cells from experimental mice and various leukocyte populations were evaluated. (A) CD4 T cells, (B) CD8 T cells, (C) B cells, (D and E) MCs, and (F) total Tregs gated on CD4 T cells are shown. Data are representative of two experiments; $n = 5–7$ mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (*t* test).

after restimulation with OVA (Fig. 5E–I). Altogether, these data suggest that IL-10 blockade in previously allergic mice may have partly beneficial effects on MC accumulation and allergic diarrhea.

DISCUSSION

In this study, we sought to further validate the role of IL-10 during IgE-mediated MC responses in food allergy. Our data demonstrating that anti–IL-10 treatment can attenuate MC responses and other markers of food allergy not only confirms our previous observations, but further highlights the role of this pleiotropic cytokine in shaping MC-mediated responses. Furthermore, considering that IL-10 induction is a prominent feature of many therapeutic regimens targeting allergic sensitization (38–40), it is possible that in some individuals, it may also have the opposite effect, and promote MC responses instead of suppressing them.

IL-10 is a highly versatile cytokine with both proinflammatory and anti-inflammatory effects and is produced by many cells including macrophages, MCs, B cells, T_H2 cells, Tregs, and

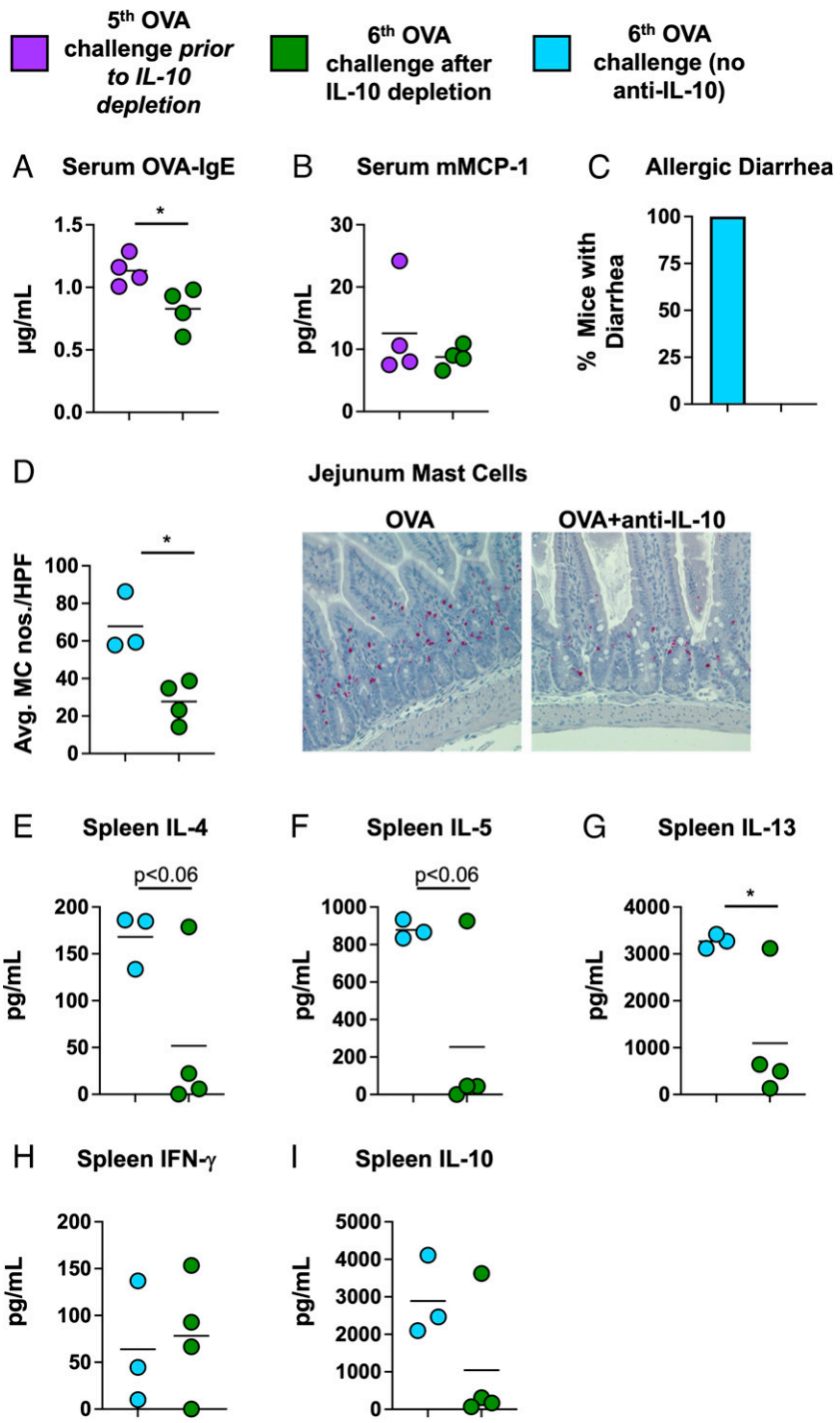

FIGURE 5. IL-10 blockade has partly suppressive effects in mice with established food allergy.

Mice were sensitized and challenged with OVA and some mice were injected with anti–IL-10 prior to the final challenge with OVA. Serum was obtained from allergic mice after the fifth OVA challenge and prior to anti–IL-10 treatment and then subsequently after anti–IL-10 treatment and final challenge with OVA. (**A** and **B**) Serum OVA-IgE and mMCP-1 levels in the same mice before and after anti–IL-10 administration; purple circles represent mice with five OVA challenges, and green circles represent the same mice after anti–IL-10 and a sixth challenge. (**C**–**I**) Mice treated with anti–IL-10 (green circles) were compared with a separate group of mice that received six OVA challenges but no anti–IL-10 (light blue circles). (C) Percent of mice exhibiting allergic diarrhea, (D) jejunal MCs (original magnification ×40), and (E–I) cytokine secretion by spleen cells after OVA stimulation are shown. *$p < 0.05$ (*t* test).

several T cell subsets (41–43). Although much of the emphasis on IL-10 research has focused on its immunoregulatory functions, several recent reports have identified critical proinflammatory roles for IL-10 during allergic responses. These include a role for IL-10 in the development of airway hyperresponsiveness, mucus metaplasia, IL-5 production, dendritic cell polarization, and eosinophilia in allergic mice (21–24, 26, 27, 33, 44–46). More recently, Kuchroo and colleagues (19) described an IL-9, IL-10–producing effector T cell subset that aggravated inflammation and had no suppressive effects. Similarly, Poholek and colleagues (47) demonstrated a critical role for IL-10 in the development of allergen-specific T_H2 cells. Finally, Qian et al. (48) demonstrated that B cell–derived IL-10 promotes allergic sensitization to asthma. These data suggest that the role of IL-10 during allergic responses may be much more nuanced than previously thought. Similar roles for other immunoregulatory cytokines such as TGF-β are also being identified, suggesting that the effects of these cytokines may be context-dependent (49).

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In this vein, we previously demonstrated a critical role for IL-10 in driving MC responses during food allergy (14). IL-10 not only enhanced IgE-mediated MC activation and cytokine release, but it also promoted MC expansion and survival, both during cell culture and in vivo (14). Furthermore, food allergy development was significantly decreased in IL-10$^{-/-}$ mice, resulting in an attenuation of MC-mediated symptoms such as allergic diarrhea, decreased activation of systemic MCs, and fewer intestinal MCs (14). Adoptive transfer of IL-10–responsive MCs or CD4 T cells restored the development of food allergy in IL-10$^{-/-}$ animals, suggesting that the attenuation of food allergy in IL-10$^{-/-}$ mice is due to a deficiency in MC responses, which can be corrected through CD4 T cell–derived IL-10 (14). These data provide strong evidence for a role for IL-10 in promoting proinflammatory functions of MC during allergic responses. This is consistent with several previous reports suggesting a proinflammatory role for IL-10 in MC function. IL-10 was initially identified as a MC stimulator and shown to promote mMCP-1 expression in MCs during helminth infections (20, 29, 30, 50–52). Similarly, another study demonstrated that IL-10 can induce IL-9 production by IgE cross-linked MCs (53). More recently, IL-10 was shown to promote IgE-mediated cutaneous anaphylaxis and enhance STAT3 and miR-155–induced IgE-mediated activation (17). Similarly, we also found that IL-10 priming can enhance IgE-mediated passive anaphylaxis in mice (14). In addition, IL-10 has also been shown to promote the upregulation of pattern recognition receptors on MCs (54) and enhance MC activation via modulation of IL-6 trans-signaling (55). Finally, Saadalla et al. (18) demonstrated that IL-10 plays a critical role in driving MC expansion during small bowel cancer. Taken together, these studies highlight a critical role for IL-10 in MC expansion and its consequent activation.

Our findings reported in this study provide further confirmatory evidence for a role for IL-10 in driving MC responses during food allergy. Using an alternative approach, relying on pharmacologic blockade of IL-10, we demonstrate that IL-10 neutralization can reduce both MC activation and systemic T cell responses during food allergy, resulting in attenuation of food allergy development. These findings directly expand on a previous report demonstrating that IL-10 neutralization can inhibit Ag-specific IgE and MC-derived histamine in mice enterally challenged with OVA (56). In this study, both IL-4 and IL-10 were shown to be critical for the induction of IgE and MC activation. Similarly, they are also consistent with more recent observations by Dent and colleagues (57) demonstrating that IL-10 together with follicular Tregs plays a critical role in inducing food Ag-specific IgE. Finally, they provide additional evidence for a role for IL-10 in wild-type mice in contrast to IL-10$^{-/-}$ animals that are known to sometimes spontaneously develop colitis (58, 59).

A number of factors may contribute to the effects of IL-10 in this system. The role of IL-10 in the amplification of IgE production is well established (22, 27). This may therefore account for one mechanism by which IL-10 modulates IgE-mediated MC activation. Similarly, IL-10 is an important regulator of Th1 responses and can directly counteract the effects of T cell–derived IFN-γ (39, 60, 61). Therefore, it is also possible that the effects of IL-10 may be indirectly mediated via antagonism of endogenous IFN-γ. As such, we have previously observed increased IFN-γ production in splenocytes from OVA-challenged IL-10$^{-/-}$ mice (14). In contrast, no differences in IFN-γ production were observed in the current study.

However, our previous findings demonstrating that IL-10 can directly promote MC proliferation and survival, as well as induce cytokine release in resting MCs, suggest that the effects of IL-10 on MCs extend beyond its effects on IgE or IFN-γ production (14). Instead, they support the proposition that IL-10 has direct effects on MC homeostasis and function, in line with previously published reports (20, 29, 30, 53). As such, we recently also found that IL-10 can also directly promote the activation and function of IL-33–stimulated MCs (62). Priming with IL-10 not only promoted the proliferation and secretion of type 2 cytokines by IL-33–stimulated BMMCs, but it also enhanced their ability to undergo IgE-mediated degranulation (62). Furthermore, in vivo treatments with recombinant IL-10 not only led to the expansion of MCs in the skin and small intestines of IL-33–treated mice but also differentially promoted MC responsiveness to IL-33 resulting in the enhancement of type 2 inflammation and the suppression of neutrophilia (62). Finally, IL-10 costimulation induced the release of mMCP-1 in both IL-33 and/or IgE-activated MCs, suggesting differentiation toward a mucosal MC phenotype (62). Interestingly, both in the current study and as we previously reported (14), increased MCs were also found in the spleens of allergic mice. A recent report suggested that splenic MCs play a critical role in the development of food allergy and that the spleen provides a unique site for the development of T cell–dependent pathogenic MCs (63). Our data demonstrating that MCs in the spleen are reduced both in IL-10$^{-/-}$ mice and after anti–IL-10 treatment suggest that IL-10 may further regulate this process. Furthermore, both our previous report as well as the data in the current study demonstrating increased IL-10 secretion in OVA-stimulated splenocytes strongly support a role for CD4 T cell–derived IL-10 in regulating MC function. Future studies aimed at characterizing the mechanisms by which this occurs will yield further insight into these interactions.

Although our findings provide further proof of concept for a role for IL-10, we did observe some variability with respect to the effectiveness of anti–IL-10 treatment, with a few mice exhibiting no decreases in MC activation or allergic diarrhea. Several factors may account for this discrepancy and need to be further investigated, including the dose of anti–IL-10, timing of treatment, age, sex, mouse strain, the gut microbiome, and experimental variation due to human error. Furthermore, unlike mice with an intrinsic deficiency in IL-10, which likely has sustained effects on the immune compartment, short-term perturbation of IL-10 signaling during acute inflammation may target beneficial cell types such as Tregs and require more precise fine tuning. This may also account for some of the divergent effects observed in Fig. 5 in mice treated with anti–IL-10 after

the development of established food allergy. In these experiments, whereas a single anti–IL-10 treatment before OVA challenge reduced allergic diarrhea, OVA-IgE levels, and intestinal MC accumulation, it had no effect on mMCP-1 release. Whether continued treatment with anti–IL-10 may further modulate this remains to be investigated. Also, a better understanding of the mechanisms by which IL-10 regulates MC activity may yield further insight into how these effects are mediated. With respect to the variables mentioned above, although we have not observed any differences between male or female mice or mice obtained from different vendors, we have not yet conducted a detailed analysis probing these and other factors in our model. In particular, the effects of the gut microbiome and mouse environment need to be further investigated, as suggested by a recent comprehensive study, which demonstrated that the gut microbiome, mouse facility, and mouse genetics all play a critical role in shaping the heterogeneity of MC responses during food allergy (64). Interestingly, these investigators also observed a direct correlation between increased IL-10 levels and mice developing anaphylaxis, suggesting that the gut microbiome may also modulate the effects of IL-10 on MC activation.

IL-10 is often used as a surrogate marker of disease improvement in the clinical management of allergic diseases (38, 39, 65–67). Our data, instead, suggest that IL-10 may have the potential to exacerbate disease development under certain conditions. This is not unwarranted, as several studies have described upregulation of IL-10 levels in allergic patients and during systemic mastocytosis (44, 68–71). Similarly, polymorphisms in the IL-10 gene have been linked to disease severity in allergic patients (72–75). Therefore, further investigation of the role of IL-10 in MC responses is much needed to ascertain the mechanisms by which it regulates MC function.

DISCLOSURES

The authors have no financial conflicts of interest.

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