IL-9 Promotes but Is Not Necessary for Systemic Anaphylaxis

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IL-9 Promotes but Is Not Necessary for Systemic Anaphylaxis

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Anaphylaxis represents an extreme form of allergic reaction, consisting of a sensitization phase during which allergen-specific IgE are produced and an acute effector phase triggered by allergen-induced degranulation of mast cells. We studied the role of IL-9, a Th2 cytokine implicated in asthma, in different models of murine anaphylaxis. Using a passive model of systemic anaphylaxis, in which anti-DNP IgE Abs were administered before challenge with DNP-BSA, we found that IL-9-transgenic mice or wild-type mice treated with IL-9 for 5 days were highly susceptible to fatal anaphylaxis. This effect was reproduced in both anaphylaxis-susceptible and -resistant backgrounds (FVB/N or [FVB/N × BALB/c] F1 mice, respectively) and correlated with increased serum concentrations of mouse mast cell protease-1 level, a protein released upon mast cells degranulation. By contrast, IL-9 did not increase the susceptibility to passive cutaneous anaphylaxis. IL-9 expression also increased the susceptibility to fatal anaphylaxis when mice were sensitized by immunization against OVA before challenge with the same Ag. In this model, serum from sensitized, IL-9-transgenic mice was more potent in transferring susceptibility to OVA challenge into naïve mice, indicating that IL-9 also promotes the sensitization stage. Finally, using IL-9R-deficient mice, we found that despite its anaphylaxis-promoting activity, IL-9 is dispensable for development of both passive and active anaphylaxis, at least in the C57BL/6 mouse background. Taken together, the data reported in this study indicate that IL-9 promotes systemic anaphylaxis reactions, acting at both the sensitization and effector stages, but is not absolutely required for this process.
promote, in synergy with IL-3, the proliferation of bone marrow-derived mast cells. In vitro, IL-9 induced the expression of transcripts encoding mast cell-specific proteases, such as mouse mast cell protease-1 (mMCP-1), mMCP-2, mMCP-4, as well as granzyme B (23, 24); up-regulated the expression of the α-chain of the high affinity IgE receptor (24); and induced IL-6 secretion (22). In vivo, IL-9-transgenic mice have intraepithelial infiltration by mast cells in gut, trachea, and kidney, but not in skin (25). This mast cell expansion correlates with elevated circulating concentrations of mMCP-1 (26). By contrast, the effect of IL-9 inhibition on mast cell responses has not been extensively studied. In a model of Schistosoma infection, a decrease in mast cells present in granulomas was reported in IL-9-deficient mice, suggesting that, at least under certain circumstances, IL-9 is required for optimal mast cell responses (27).

The effect of blocking IL-9 activity in the allergic response is controversial. Neutralizing IL-9 with an Ab decreased the IgE response, pulmonary eosinophilia, goblet cell hyperplasia, airway epithelial hyperplasia, and airway hyper-responsiveness in DBA/2 x C57BL/6 F1 mice (28). In another study, anti-IL-9 Ab administration to previously sensitized mice was able to prevent airway hyper-reactivity and lung eosinophilia upon aerosolized antigen challenge (29). By contrast, in a similar OVA-induced asthma model, IL-9-deficient mice showed no decrease in allergic pulmonary airway hyperactivity, eosinophilia, goblet cell hyperplasia, or specific IgE (30). Finally, in a typical Th2 infection model, anti-IL-9 vaccination prevented Trichuris muris expulsion and blood eosinophilia, but not the IgE response (31).

In this study we show that IL-9 can potentiate the effector and sensitization phases of the allergic reaction, but that IL-9R-deficient mice have a normal sensitivity to anaphylactic shock.

**Materials and Methods**

**Mice**

All mice were bred in our animal facility under specific pathogen-free conditions. We used 8- to 12-wk-old female mice for all experiments. Tg5 and Tg54 mice expressing a high quantity of IL-9 in all organs were described previously (15). IL-9 knockout mice (IL-9RKO) were obtained in the C57BL6 background by replacing a 2.8-kb genomic fragment containing exons 2–6 by a neomycin resistance gene (V. Steenwinkel, J. Louahed, C. Orabona, A. McKenzie, and J.-C. Renauld, manuscript in preparation). mMCP-1 (mMCP-1, 5 μg/ml; provided by H. Bazin, Universite de Louvain, Brussels, Belgium). This model was based on a protocol previously described (32). Three or 12 ng of anti-DNP IgE diluted in 10 μl of PBS or PBS alone was injected intradermally in both ears of mice with a 0.3-ml insulin syringe. One day later, 100 μg of DNP-BSA (Calbiochem) was injected i.v. in 200 μl of PBS with 0.5% Evans blue (Sigma-Aldrich). Thirty minutes after challenge, both ears were cut and incubated at 80°C in 1 ml of formamide for 2 h. The mixture was homogenized with an UltraTurrax (M. Zifferer) and centrifuged at 20,800 × g for 10 min. The absorbance of the supernatant was measured at 620 nm. The relation between Evans blue concentration and absorbance was linear, indicating that absorbance represented the quantity of Evans blue extravasation. In the first experiment, ears were weighed, and no significant difference was found between FVB/N and Tg5 animals.

**Active anaphylaxis and serum transfer**

We used two different experimental systems to evaluate active anaphylaxis. According to the first setting (9), mice were immunized i.p. on day 0 with 10 μg of OVA (Sigma-Aldrich) and 70 μl of Inject Alum (Pierce) in 200 μl of PBS. On day 14, mice were bled by periorbital puncture to harvest serum or received an i.v. injection of 0.005–1 mg of OVA in 200 μl of PBS. After the OVA challenge, the survival time after challenge was recorded for each mouse. According to the second protocol (28), mice were immunized twice i.p. with 15 μg of OVA and 45 μl of Inject alum (2 mg of aluminum hydroxide) in 200 μl of PBS on days 1 and 5. On day 14, mice were bled or injected i.v. with OVA. The survival time after challenge was recorded.

For serum transfer experiments, naive or OVA-immunized FVB/N or Tg5 mice were bled by periorbital puncture on day 14. After 2-h coagulation at room temperature, blood was centrifuged at 1600 × g for 10 min, and serum was harvested. Two hundred microliters of serum was injected i.v. in naive mice. The next day, mice were challenged with 500 μg of OVA, and survival was measured.

**ELISA**

Serum was collected after blood coagulation at room temperature and stored at −20°C before use. To detect anti-OVA IgE, 96-well plates (Maxisorp; Nunc) were coated overnight at 4°C with 50 μl of anti-IgE-specific Ab (LOME3; 5 μg/ml; provided by H. Bazin, Universite de Louvain, Brussels, Belgium) in glycine buffer containing 30 mM NaCl, pH 9.2. After washing with 0.1 M NaCl plus 0.05% Tween 20, plates were blocked for 1 h at 37°C with 150 μl of 1% BSA in PBS. Plates were washed and incubated with 50 μl of serum dilutions in 1% BSA in PBS for 2 h at 37°C. After blocking, plates were washed and incubated for 2 h at 37°C with 50 μl of 5 μg/ml biotin-conjugated OVA. After washing, plates were incubated with 50 μl of Amdex-streptavidin HRP (1 μg/ml; Amershams Biosciences). After 1 h, plates were washed and incubated with 50 μl of One-Step-Turbo-TMB ELISA (Perbio) for 15 min. The reaction was stopped with 50 μl of 2 M H2SO4, and absorbance was read at 450 nm. The titer was defined as the dilution that showed an OD of 300% of background, which was the value read at the linear range of the curve.

For measuring specific IgG responses to OVA, plates were coated overnight at 4°C with 100 μl of OVA (100 μg/ml) in glycine buffer. After washing, plates were blocked with 1% BSA in PBS at 37°C for 1 h, washed, and incubated with serial serum dilutions in 1% BSA in PBS. After 2 h at 37°C, plates were washed and incubated with goat anti-mouse IgG HRP (2 μg/ml; Santa Cruz Biotechnology) for 2 h at 37°C. Bound IgGs were detected using the same protocol as that described for the anti-IgE ELISA. The titer was defined as the dilution that showed an OD of 300% of background. Total serum mMCP-1 concentrations were measured by ELISA using a commercial kit (Moreduin Animal Health) following the manufacturer’s instructions.

**Statistical analysis**

Statistical analysis was performed using the SPSS program. Differences between survival curves were calculated by the Kaplan-Meier method and were evaluated with the log-rank test.

**Results**

**IL-9 promotes passive systemic anaphylaxis**

To test the hypothesis that IL-9 promotes type I hypersensitivity, IL-9-transgenic or control mice were injected i.v. with 5 μg of monoclonal anti-DNP IgE 1 day before challenge with DNP coupled to BSA (0.5 mg i.v.). As shown in Fig. 1, IL-9-transgenic mice (Tg5) were highly susceptible to passive systemic anaphylaxis and died within 30 min, whereas most of the control FVB/N mice survived. These results were repeatedly obtained and were reproducible in mice from two independent IL-9-transgenic lines (Tg5 and Tg54). Increasing the dose of anti-DNP IgE increased the...
that the effect of IL-9 would be dependent on the FVB/N back-
BALB/c mice to this treatment (Fig. 2). Four injections of IL-9 with those from mice that received zero to three
was used to compare survival curves from mice that received at least
DNP-BSA i.v. Survival after challenge is represented. The log-rank test
of anti-DNP IgE i.v. One day later, mice were challenged with 0.5 mg of
mortality, but five daily injections caused the death of three of five
result was obtained in more than three independent experiments. b, FVB mice were
increased to increase the susceptibility to passive anaphylaxis, we tested the
experiments. a, Six FVB/N, six BALB/c, and six (FVB/N × BALB/c) F1 mice received an i.v. injection of anti-DNP IgE (50 μg). Mice
were challenged with 1.5 mg of DNP-BSA i.v. Survival after challenge is
were observed for 2 h. Survival after challenge is represented. The log-rank test was used to com-
were challenged with 0.5 mg of DNP-BSA i.v. One day later, mice were injected i.p. for 5 days with 200 μl of PBS containing 1% normal mouse
in Fig. 1. IL-9-transgenic mice (Tg5) and 10 control (FVB/N) mice were injected i.v. with 5 μg of anti-DNP IgE. One day later, mice were
156x491]ground by comparing IL-9 transgenic (Tg5 × BALB/c) F1 and control (FVB/N × BALB/c) F1 mice. As shown in Fig. 2b, IL-9
exacerbate anaphylaxis in F1 animals, as it did in the FVB/N background, indicating that the
aggravating effect of IL-9 was not dependent on the basal sensitivity of the mouse strain.

IL-9 does not promote passive cutaneous anaphylaxis

We next investigated whether the promoting effect of IL-9 on passive systemic anaphylaxis could be extended to passive cutaneous anaphylaxis. We set up a model that enabled us to quantify fluid extravasation resulting from vascular leakage secondary to skin mast cell degranulation. We injected 3 or 12 ng of anti-DNP IgE into both ears of mice 1 day before injecting DNP-BSA and Evans blue systemically. This challenge resulted in a measurable increase in Evans blue extravasation compared with PBS-injected animals. As shown in Fig. 3 for one of four independent experiments, Evans blue extravasation was never higher in transgenic animals than in

FIGURE 2. IL-9 promotes passive systemic anaphylaxis in both susceptible and resistant mice. a, Six FVB/N, six BALB/c, and six (FVB/N × BALB/c) F1, mice received an i.v. injection of anti-DNP IgE (50 μg). Mice were challenged with 1.5 mg of DNP-BSA i.v. Survival after challenge is represented. The log-rank test was used to compare the survival curves between FVB/N and BALB/c mice or between FVB/N and F1 mice (p = 0.0005). One of three similar experiments is represented. b, Eight FVB/N, eight (FVB/N × BALB/c) F1, and eight (Tg5 × BALB/c) F1 mice received 50 μg of anti-DNP IgE i.v. One day later, mice were challenged with 1.5 mg of DNP-BSA. Survival after challenge is represented. The log-rank test was used to compare survival curves (FVB/N vs FVB/BALB, p < 0.0001; FVB/BALB vs Tg5/BALB, p = 0.0004).

FIGURE 3. IL-9 overexpression does not promote passive cutaneous anaphylaxis. We injected 3 ng of anti-DNP IgE intradermally into both ears of five FVB/N and five Tg5 mice. As a control, two FVB/N and two Tg5 mice received PBS alone, and three FVB/N and three Tg5 mice received 12 ng of anti-DNP IgE. One day later, mice were challenged i.v. with 200 μl of PBS containing 100 μg of DNP-BSA and 0.5% Evans blue. After 30 min, mice were killed by cervical dislocation, and ears were cut and incubated in formamide. The solution was homogenized with an UltraTurax. After centrifugation, absorbance of the supernatant was measured at 620 nm. Results are presented as the mean absorbance with SEM. Similar results were obtained in four independent experiments.
control mice, indicating that IL-9 overexpression does not increase sensitivity to passive cutaneous anaphylaxis.

**IL-9 promotes active systemic anaphylaxis**

After immunization with OVA and alum as adjuvant, systemic anaphylaxis can be produced by i.v. injection of the same Ag. In this model, IL-9-transgenic mice were more sensitive than control mice (Fig. 4). In a typical experiment, a dose of 5 μg of OVA was fatal for seven of 10 IL-9-transgenic mice, but for only one of 10 control mice. Similar results were obtained in two independent IL-9-transgenic lines (data not shown). In addition, OVA-specific serum IgG and IgE concentrations were significantly increased in IL-9-transgenic mice (Fig. 5).

To determine whether the effect of IL-9 was restricted to the effector phase, or whether the difference in Ig concentrations had pathophysiological significance, we transferred serum from sensitized IL-9-transgenic and control mice to naive FVB/N mice. As shown in Fig. 6, serum from IL-9-transgenic mice transferred a greater sensitivity to OVA challenge, in line with its higher concentration in Ag-specific IgE and IgG Abs. The same results were observed by transferring serum of sensitized normal or IL-9-transgenic mice into IL-9-transgenic naive mice (data not shown). Transfer of naive IL-9-transgenic serum had no effect (Fig. 6), ruling out the possibility that this effect was due to the presence of IL-9 or any other cytokine in the serum transferred from transgenic mice. Taken together, these observations indicate that IL-9 potentiates both the sensitization phase and the effector phase of anaphylaxis.

**IL-9 is not necessary for passive or active systemic anaphylaxis**

To further assess the role of IL-9 in anaphylaxis, we took advantage of mice deficient in the IL-9R. These mice were generated in the C57BL/6 genetic background, which is relatively resistant to passive anaphylaxis, with only a few animals dying upon Ag challenge even after sensitization with high IgE doses. As shown in Fig. 7, similar numbers of fatal anaphylaxis reactions were observed in control and IL-9R-deficient mice. As an alternative read-out system for anaphylaxis and mast cell degranulation, we measured the mMCP-1 protease concentration in serum. As shown in Fig. 8, serum concentrations of mMCP-1 were 100 times higher in IL-9-transgenic mice than in control mice, probably reflecting systemic mastocytosis. After systemic anaphylaxis and mast cell degranulation, the mMCP-1 concentration increased 100-fold in both FVB/N and transgenic animals. The same observation was made for (FVB/N × BALB/c) F1 and (Tg5 × BALB/c) F1 mice. However, we failed to detect any significant difference in serum mMCP-1 concentrations between IL-9R-deficient and control mice, indicating that mast cell degranulation was not affected by the absence of IL-9R (Fig. 8).

In the active anaphylaxis model, when mice were challenged 14 days after a single i.p. immunization with 10 μg of OVA, IL-9-deficient mice showed very similar sensitivity to Ag challenge (Fig. 9). In addition, OVA-specific IgG or IgE concentrations were similar in IL-9R-deficient and control mice (data not shown) in two independent experiments. In another set of experiments, mice received two OVA injections on days 1 and 5, as described in a model in which IL-9-blocking Abs decreased the IgE response (28). However, we failed to notice any difference with this experimental setup, either in two experiments based on mortality (data not shown) or in specific IgE levels (Fig. 10) between IL-9R-deficient and control mice.

**Discussion**

Anaphylaxis is classically characterized by two phases. During the sensitization phase, allergen-specific IgE Abs are produced and rapidly bind to high-affinity IgE receptors on mast cells. A second contact with the allergen induces the effector phase, resulting from mast cell degranulation and liberation of a variety of mediators. In this study we show that IL-9 promotes both phases of the reaction. For the effector phase, we observed that mice that overexpress IL-9 are highly susceptible to Ag challenge after sensitization by Ag-specific IgE. This effect of IL-9 probably results from its growth and differentiation factor activity for mast cells. IL-9-transgenic mice were indeed characterized by mast cell hyperplasia and have increased basal concentrations of mast cell proteases in serum (25). In our anaphylaxis model, in both IL-9-transgenic and control mice, Ag challenge increased the serum mMCP-1 concentration 100-fold, reflecting massive mast cell degranulation. This suggests that IL-9 induces mast cell hyperplasia, but does not affect the efficiency of IgE-dependent degranulation. As a result, IL-9 promotes a dramatic release of mast cell mediators, which are probably responsible for the increased mortality.

The same observations were made in wild-type mice receiving i.p. injections of IL-9, but, interestingly, three to five daily injections were needed for IL-9 to increase the susceptibility to anaphylaxis. This delay probably reflects the development of mast cell hyperplasia, as suggested by the observation that a few daily IL-9 injections are similarly required to increase the basal mMCP-1 concentration (unpublished observations from our laboratory). By
contrast, IL-4 and IL-13 were also reported to exacerbate anaphylaxis induced by cross-linking of IgE (11), but a single injection of these cytokines 1 h before challenge was sufficient for this activity. This effect of IL-4 and IL-13 was also found in a mast cell-independent model of anaphylaxis and correlated with an increase in vascular permeability, probably via endothelial cells. A single injection of IL-9 in this experimental model had no effect, indicating that IL-9 and IL-13 can promote the effector phase of anaphylaxis through distinct mechanisms (11). Interestingly, the effect of IL-9 on mMCP release was partially abolished in IL-4- or IL-4R-deficient mice, although we failed to demonstrate any increase in IL-4 production in IL-9-transgenic mice (data not shown). Endogenous IL-4 might either synergize with IL-9 for mast cell activation or play an indirect role in mast cell homeostasis in vivo by regulating IgE production. Indeed, IL-4-deficient mice produce very low levels of IgE, which, even in the absence of any Ag, were described to promote mast cell survival and proliferation (33, 34). By contrast, the effect of IL-9 on mMCP expression was not affected in IL-13-deficient mice (data not shown), whereas IL-13 is required for IL-9-induced goblet cell hyperplasia (18).

Contrasting with its effect on systemic anaphylaxis, IL-9 did not exacerbate cutaneous anaphylaxis. This finding is in line with the fact that IL-9-induced mast cell hyperplasia was restricted to the kidney, gut, and trachea (25), but was not observed in the skin. The reason for this particular localization remains unclear. IL-9 might act on a subpopulation of mucosal mast cells. However, IL-9-induced mast cells expressed markers of both mucosal and connective tissue-type mast cells (25). Alternatively, IL-9 could favor the localization of mast cells in these organs by inducing the expression of chemotactic factors by epithelial cells, as shown for eosinophils in the lungs (35). In the present model it is not clear whether the effect of IL-9 simply reflects an increase in mast cell numbers or whether the localization of those mast cells within vital organs, such as lungs, plays any significant role in the exacerbation of systemic anaphylaxis.

In addition to the effector phase, IL-9 enhanced the sensitization phase. After sensitization to OVA, we observed that serum from IL-9-transgenic mice was more potent in transferring susceptibility to OVA-induced shock than serum from wild-type mice. A similar exacerbation of anaphylaxis via an increase in allergen-specific IgE production was previously demonstrated for IL-13 (10). In the present study the effect of IL-9 reflected an increased OVA-specific Ab response, including IgE and IgG. Interestingly, beside the classical IgE-mast cell pathway, an alternative pathway, involving IgG, FcγRIII, macrophages, platelets, and platelet-activating factor, was reported to trigger anaphylaxis. It is therefore possible that both OVA-specific IgG and IgE are responsible for IL-9 enhancement of the sensitization phase of anaphylaxis. This could result from a direct effect of IL-9 on B lymphocytes, because IL-9 was reported to have a modest, but significant, effect on Ig production by purified B cells in vitro (19, 20). IL-9 could also indirectly promote Ig production by activating Th cells. However, we failed to detect any increase in IL-4 as CD40L expression by IL-9-transgenic CD4+ cells (data not shown).
Although these data clearly show that IL-9 promotes anaphylaxis, this factor is not required for such reactions, because the absence of IL-9 did not affect anaphylaxis in our model. By contrast, decreased mast cell infiltration after pulmonary challenge with *Schistosoma mansoni* eggs in IL-9-deficient mice suggested a nonredundant role for IL-9 in mast cell responses (30). However, it must be stressed that the anaphylaxis model does not depend on acute mast cell expansion, but, rather, on steady-state mast cell levels. In this respect, it was previously shown that IL-9 is dispensable for the generation of bone marrow-derived mast cells in vitro (30).

Conflicting results have also been reported concerning the putative requirement for IL-9 in type I hypersensitivity reactions. In line with the present data, IL-9-deficient mice developed normal allergen-induced IgE levels (27, 30) and lung inflammation upon pulmonary challenge (30). By contrast, other reports showed that anti-IL-9 Abs decreased IgE level and airway hyper-responsive-ness in B6D2F1 mice challenged with OVA (28) or airway inflammation and hyper-reactivity in BALB/c mice challenged with OVA (29).

Several potential hypotheses can be proposed to explain these discrepancies. The fact that experiments using blocking Abs or gene-targeted animals produce opposite results suggests the existence of compensatory mechanisms. Another crucial issue is the genetic background. Allergic reactions are highly dependent on the strain of mouse used for the experiments. In this respect, the IL-9R-deficient mice used in this study were generated in the C57BL/6 mouse strain, which is characterized by both a low sensitivity to allergic reactions and low levels of IL-9 production (14).

Thus, backcrossing IL-9R-deficient mice with other mouse strains, such as the high IL-9 producer DBA/2 background, might be required to unmask the role of endogenous IL-9.

Taken together, our results show that IL-9 promotes anaphylaxis both at the sensitization phase, by increasing the allergen-specific Ig response, and at the effector phase, by inducing functional mucosal mast cell hyperplasia. However, IL-9, at least in the C57BL/6 background, does not appear to be necessary for such anaphylactic reactions.

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**Disclosures**

The authors have no financial conflict of interest.

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