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WILEY CONTACT DERMATITIS

ORIGINAL ARTICLE

Increased expression of interleukin-9 in patients with allergic contact dermatitis caused by *p*-phenylenediamine

Marie Baeck[1,2] | Anne Herman[1,2] | Laurence de Montjoye[1,2] | Emilie Hendrickx[3] | Paméla Chéou[3] | Perrine M. Cochez[3] | Laure Dumoutier[3]

[1]Department of Dermatology, Cliniques universitaires Saint-Luc, Brussels, Belgium

[2]Institut de Recherche Expérimentale et Clinique (IREC Pôle Pneumologie, ORL, Dermatologie), Université catholique de Louvain, Brussels, Belgium

[3]de Duve Institute, MEXP Unit, Université catholique de Louvain, Brussels, Belgium

Correspondence
Professor Baeck Marie, Department of Dermatology, Cliniques universitaires Saint-Luc, U.C.L. Avenue Hippocrate, 10, B-1200 Brussels, Belgium.
Email: marie.baeck@uclouvain.be

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Background: Allergic contact dermatitis has been described as a type IV reaction caused by antigen-specific T cells. Central roles for $CD8^+$ cytotoxic T cells as effector cells and $CD4^+$ T cells as regulatory cells have been suggested. T helper (Th) 2 and Th1 cytokines have been implicated; however, the nature of the allergen influences the Th response.

Objective: To determine the types of T cells and cytokines expressed in patients allergic to *p*-phenylenediamine (PPD).

Methods: Serial skin biopsies of areas with positive patch test reactions in 29 PPD-sensitized patients were collected. T cell markers and cytokine expression were analysed by flow cytometry and quantitative reverse transcription polymerase chain reaction in both skin and peripheral blood mononuclear cells (PBMCs) of sensitized patients.

Results: We observed increased expression of T cell markers and Th2/Th9-associated cytokines in both skin and stimulated PBMCs of PPD-allergic patients. Moreover, interleukin (IL)-9 was mainly produced by Th9 cells, in both skin and PBMCs. Further investigations showed that *Il9r*-deficient mice were more affected in a PPD contact hypersensitivity model than wild-type mice.

Conclusion: We did not confirm the preclinical presence of $CD8^+$ T cells. However, the expression of different T cell markers positively correlated with patch test reactions. IL-9 expression was strongly upregulated and directly related to patch test severity. In addition, we showed that IL-9 has an anti-inflammatory role in a mouse model of PPD contact hypersensitivity.

KEYWORDS

allergic contact dermatitis, IL-4, IL-9, *p*-phenylenediamine, Th2, Th9

1 | INTRODUCTION

Contact allergy is a complex disease, and a considerable challenge for public health and immunology. Allergic reactions caused by oxidative hair dye ingredients such as *p*-phenylenediamine (PPD) constitute a special concern, owing to their severity and the widespread use of such ingredients. The prevalence of PPD sensitization in Europe has been estimated to be 4% in a patch tested population[1] and 0.8% in the general population.[2]

Allergic contact dermatitis (ACD) is usually described as a type IV reaction mediated by antigen-specific T cells, whereas the other types of hypersensitivity reaction are mediated by antibodies. Type IV reactions require a prior sensitization phase in which an exogenous allergen (hapten) comes into contact with the epidermis. This allergen then binds to self-proteins to form a new antigen that can be recognized and processed by dendritic cells. Specific T cells are then activated, following which the individual acquires a specific immunological memory of the contact sensitizer at the T cell level. During the sensitization phase, upon renewed contact with the sensitizing substance at any skin location, the often lifelong memory will result in the mounting of an immune response, resulting in eczema. The immune response usually takes >12 hours to become manifest, and peaks at approximately 24 to 72 hours. The molecular mechanisms that determine whether an individual becomes sensitized are still largely unknown.[3]

Anne Herman and Laurence de Montjoye contributed equally.

The respective roles of $CD4^+$ and $CD8^+$ cells remain a subject of debate.[3–7] Vocanson et al[8,9] suggested that $CD8^+$ T cells have an effector function, because these cells seem to be present in the skin of allergic patients at a very early stage, before the onset of clinical symptoms. In addition, these authors showed a possible modulatory role for $CD4^+$ T cells, which become the predominant T cells when clinical lesions develop. Despite the increasing evidence that the main effector cells of ACD are $CD8^+$ cytotoxic T cells, the nature of the antigen and/or its exposure route may possibly contribute to determining the cell type involved in the inflammatory response.[3,6] In a recent study, we showed, in patients with ACD caused by corticosteroids, that the inflammatory infiltrate was composed of $CD3^+$ T cells with a predominant T helper (Th) 2 cytokine profile.[10] We showed that increasing numbers of $CD3^+$, $CD4^+$ and, to a lesser extent, $CD8^+$ T cells correlated with patch test positivity. Through immunostaining and quantitative reverse transcription polymerase chain reaction (qRT-PCR), we found that dermal T cell infiltration did not precede the onset of skin lesions, but that $CD3^+$ T cell recruitment rapidly increased with the positivity of the patch test sites. The predominance of $CD8^+$ T cells as effector cells was therefore absent in patients with allergy to corticosteroids. However, the presence of $CD4^+$ T cells appeared to be associated with the onset of clinical inflammation. Furthermore, we showed that the onset of the skin lesions correlated with strong induction of interleukin (IL)-4 expression, even at early time points, as well as significant IL-5 production.

In the same vein, a role for Th2 cytokines has already been shown in mouse models of ACD caused by other allergens such as PPD, nickel, and other metals.[11–15] For instance, in allergy to PPD, ear swelling induced by topical PPD application was shown to be lower in *STAT6*$^{-/-}$ mice deficient for Th2 cytokines than in wild-type (WT) mice, showing that Th2 cytokines play an essential role in ACD induction.[15] Conversely, other authors failed to induce a Th2 response following repeated topical PPD application in mice, and concluded that exposure to hair dyes was not associated with relevant Th2 induction.[16]

Some importance has also been attributed to Th17 cells, which constitute a subpopulation implicated in the pathogenesis of various allergic disorders, including ACD.[17,18] In a murine model of allergy to PPD, a trend towards a systemic increase in IL-17A expression was observed.[19] In addition, a recent study by Caiazzo et al corroborated that IL-26, a Th17-associated cytokine, was implicated in keratinocyte damage during ACD.[20] However, we were not able to confirm a Th17-associated response in ACD caused by corticosteroids.[10] IL-9, a cytokine that is considered to be associated with the newly described Th9 lymphocyte subset, might likewise play an essential role in the pathogenesis of skin inflammation, and in particular in ACD.[21] Indeed, the level of IL-9 was found to be increased in skin samples from positive patch test reactions in patients with ACD caused by various allergens.[22,23] A high level of IL-9 secretion was also observed after allergen stimulation of peripheral blood mononuclear cells (PBMCs) from patients allergic to PPD and nickel as compared with non-allergic control subjects.[11,23] However, the exact origin and role of IL-9 in ACD remain unclear.

The aim of this study was to explore the nature of the T cells involved in allergy to PPD in comparison with the previous observations made with corticosteroids. Therefore, we analysed the T cell recruitment and cytokine production profile in positive patch test reactions of 29 PPD-sensitized patients. We then measured the expression of different cytokines associated with Th populations in stimulated PBMCs from patients allergic to PPD.

2 | MATERIALS AND METHODS

2.1 | Patients

Overall, 29 patients (25 women and 4 men) with prior positive patch test reactions to PPD were included in this study, as were 16 healthy control subjects. The patients were otherwise healthy, and were investigated when clinically free from dermatitis. All of them had a history of ACD after using hair dyes or after temporary henna tattoos. The study and data collection were conducted with the approval of the hospital and faculty institutional review board (Commission d'Ethique Biomédicale Hospitalo-Facultaire) of Université catholique de Louvain, Belgium (NCT 340320084407). Informed consent for all diagnostic procedures was obtained from all study subjects. The data from all of the PPD-sensitized and control subjects are summarized in Table S1. To be eligible as controls, the 16 healthy subjects (12 women and 4 men) had to meet the criteria of having never shown a hypersensitivity reaction after using hair dyes or after temporary henna tattoos, and to have negative patch test results with PPD.

2.2 | Patch tests

All subjects were physically examined and patch tested with PPD 1% pet. (Chemotechnique Diagnostics, Vellinge, Sweden), as included in the European baseline series. Three series of PPD patch tests were applied to patients 1 to 14. The patch test materials used were IQ Ultra chambers (Chemotechnique Diagnostics) applied to the buttocks with Fixomull Stretch (Smith and Nephew, Zaventem, Belgium). The 3 series of patches were removed after 8, 24 and 48 hours, respectively, and the patch test reactions were evaluated at the same time points, as well as on day (D) 4 and D7. For patients 15 to 29, the patch test was removed after 48 hours (on D2), with readings being performed at the same time point as well as on D4 and D7. The patch test reactions were evaluated according to ESCD guidelines.[24]

2.3 | PBMC isolation and stimulation (patients 9 to 29)

Blood samples were collected before patch testing and at 48 hours after application. Total human PBMCs were purified from the blood of controls and allergic patients by centrifugation (20 minutes-1082g) on a Lymphoprep gradient (Elitech, Puteaux, France). The cells were then washed with phosphate-buffered saline (PBS)/1 mM EDTA, and resuspended in autologous RPMI medium (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts) containing 10% patient plasma. The PBMCs were stimulated for 48 hours at 37°C with anti-CD3/anti-CD28 beads (Life Technologies, Thermo Fisher Scientific, Waltham, Massachusetts; 500 000 beads to 10^6 cells), phytohaemagglutinin (PHA) (Sigma, Merck, Darmstadt, Germany; 5 μg/mL), or PPD (Sigma;

2.5 μg/mL). After this incubation, the cells were harvested for flow cytometry or RNA extraction.

2.4 | Skin biopsies (patients 1 to 14)

In order to capture the preclinical stage, we decided to take skin biopsies at three different time points. Therefore, 3-mm and 6-mm punch biopsies were collected from both positive and negative patch test sites at 8, 24 and 48 hours following PPD application. Normal skin was also biopsied before patch testing (at 0 hours). For dermal cell isolation, skin biopsies were incubated overnight at 4°C in dispase II at 2.5 U/mL (Roche, Merck, Darmstadt, Germany) in PBS before separation of the epidermis and dermis with a forceps. Dermal cells were further isolated by digestion with collagenase type III (Whortington Biochem, Lakewood, New Jersey) at 200 U/mL for 2 hours at 37°C. The cells were then filtered through 40-μm nylon filters to obtain a single-cell suspension, and stimulated with anti-CD3/anti-CD28 beads (Life Technologies). For RNA extraction, skin biopsies were deep-frozen in liquid nitrogen.

2.5 | RNA extraction and qRT-PCR

Total RNA was isolated from the skin biopsies or PBMCs with the TriPure isolation reagent (Roche). Reverse transcription was performed on 1 μg of total RNA with oligo(dT) primer (Roche) and Moloney murine leukaemia virus reverse transcriptase (Life Technologies). Quantitative PCR amplifications were performed with cDNA from 25 ng of total RNA with primer sets and TaqMan probes for human EF-1, CD3, CD4, CD8, Foxp3, TCRG, interferon (IFN)-γ, IL-17, IL-4, and IL-9, and qPCR TaqMan MasterMix Taqman (Eurogentec, Liège, Belgium). The sequences of the primers and probes used are shown in Table S2. Samples were first heated for 10 minutes at 95°C. cDNA was amplified as follows: 40 cycles of a two-step PCR protocol for 10 seconds at 95°C and for 1 minute at 60°C. A standard curve with various concentrations of a cloned cDNA fragment was constructed for each gene for accurate quantification.

2.6 | Flow cytometry on dermal cells of patch test-positive skin biopsies

Dermal cells were incubated for 3 hours with BD GolgiStop Solution (BD Biosciences, Erembodegem, Belgium), and then with the LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Life Technologies) and 2 μg/mL antibodies for extracellular staining for 1 hour at 4°C. For intracellular cytokine staining, the cells were fixed and permeabilized by use of the Cytofix/Cytoperm Plus Kit (BD Biosciences) and stained. The cells were analysed with a FACS Fortessa (BD Biosciences). Gating included forward and side scatter and viability analysis. Data were analysed with FLOWJO (Flow Jo, Ashland, Oregon). The following antibodies were used: anti-γδ (B1), anti-IL-4 (8D4-8), anti-IL-9 (MH9A4), anti-IFN-γ (B27), anti-CD3 (UCHT1) and anti-CD4 (OKT4) from Biolegend (San Diego, California), and anti-CD8 (RPA-T8) from BD Biosciences.

2.7 | IL-9 enzyme-linked immunosorbent assay

IL-9 production was measured in PBMC supernatants after 48 hours of PHA stimulation. An enzyme-linked immunosorbent assay specific for human IL-9 was performed with mouse antibodies generated in our laboratory (MH9A4 and biotinylated MH9A3), as previously described.[25] Biotinylated antibody was detected with streptavidin-horseradish peroxidase (VWR, Radnor, Pennsylvania). Absorbance was read at 450 nm, with a 96-well plate spectrophotometer (VERSAmax; Molecular Devices, San Jose, California). IL-9 concentrations were calculated by means of a standard curve generated via the use of calibrated standards.

2.8 | Contact hypersensitivity model

The IL-9 receptor α knockout mice (*Il9r*$^{-/-}$) used in the contact hypersensitivity (CHS) model were generated on the C57BL/6 background as described previously.[26] These were sensitized with a solution of 5% wt/vol PPD (CAS no. 106-50-03; Sigma) and 3% hydrogen peroxide (H_2O_2) diluted in acetone/olive oil (4:1). The solution was applied to shaved back skin (50 μL) and the dorsal sides of ears (25 μL/ear) on days 0 and 5. Five days after the last sensitization, challenges were performed every day. The first challenge was performed by applying PPD (5%) and H_2O_2 (3%) in acetone/olive oil to the dorsal sides of ears (25 μL/ear). Subsequent challenges were performed with PPD (5%) in acetone/olive oil. Control mice received a vehicle solution (including 3% H_2O_2 for the two sensitizations and the first challenge). All solutions were freshly prepared. Ear thickness was measured with a micrometer before each application of PPD and 24 hours after the last challenge (Mitutoyo, Aurora, Illinois).

2.9 | Haematoxylin and eosin and immunohistological staining

One skin sample was fixed in formaldehyde and embedded in paraffin for histological and immunohistological staining. The paraffin sections were cut (5 μm), deparaffinized, rehydrated, and then sequentially incubated with the primary monoclonal antibodies anti-CD3 (lot 128; Dako, Santa Clara, California), anti-CD8 (clone C8/144B; Dako) and anti-CD4 (clone 4B12; Novocastra, Leica Biosystems, Diegem, Belgium). Peroxidase-conjugated goat anti-mouse immunoglobulins or anti-rabbit IgG were used as secondary antibodies when appropriate, and revealed by incubation with 3-amino-9-ethylcarbazole. The sections were counterstained with haematoxylin. For negative controls, the primary antibodies on the control tissue sections were omitted. The stained slides were digitized by means of automated whole-slide image capture with a Mirax Midi scanner (Carl Zeiss MicroImaging, Oberkochen, Germany). Mouse epidermal thickness was measured at different locations of the section with the Panoramic Viewer measuring tool (3DHISTECH, Budapest, Hungary). Crust percentages were calculated by measuring the length of the crusts and dividing it by the length of the section. Two evaluators performed analysis of the staining.

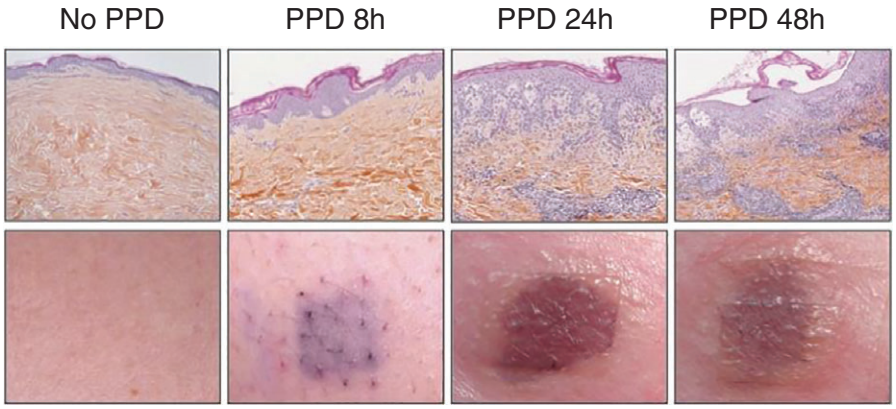

FIGURE 1 Clinical response and haematoxylin and eosin staining of healthy skin and patch test-positive biopsies (at different time points) of an allergic patient. PPD, *p*-phenylenediamine

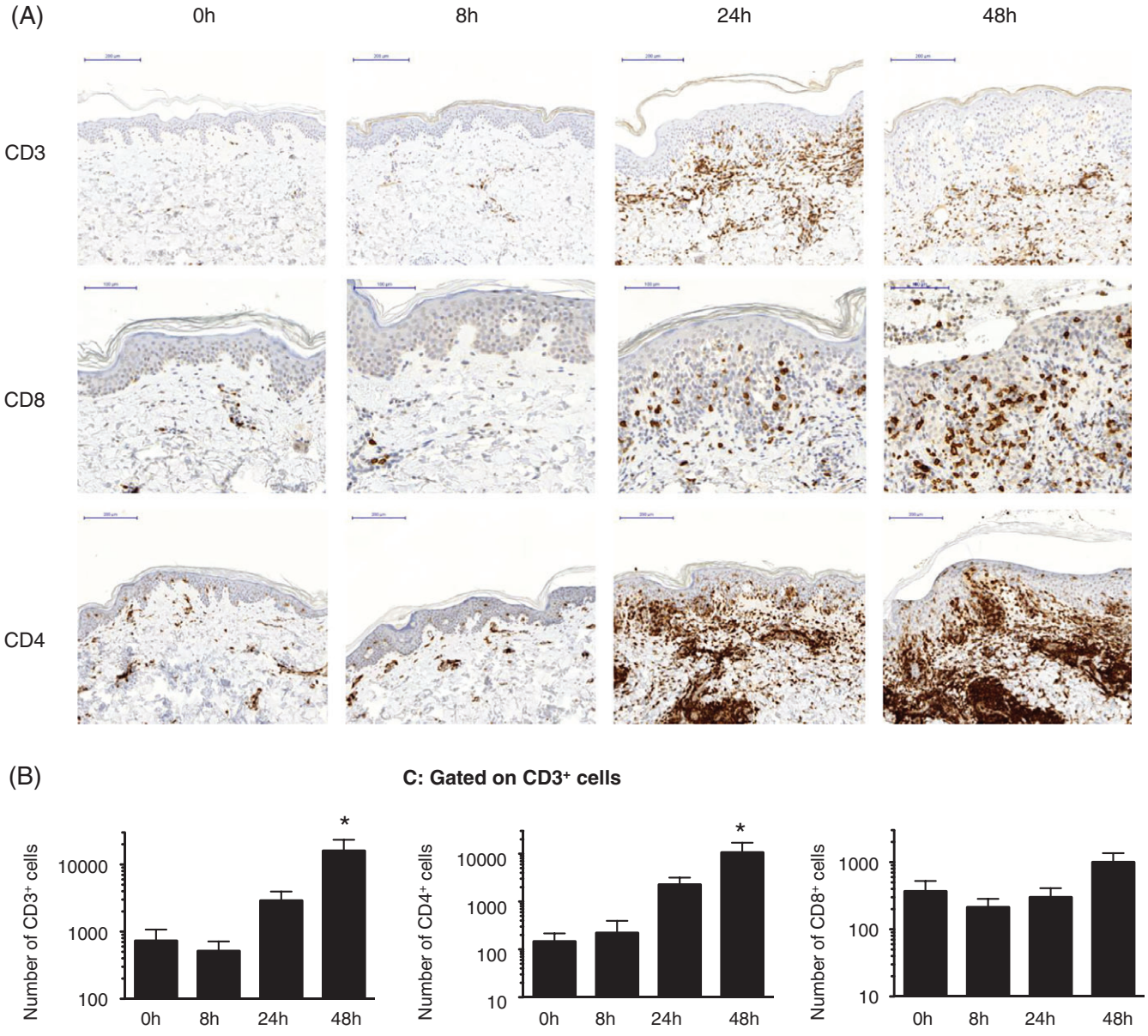

FIGURE 2 T cell infiltrate. (**A**), Representative immunohistochemistry on skin biopsies of allergic patients at the indicated time points in *p*-phenylenediamine patch testing. Skin sections were labelled with anti-CD3, anti-CD8 and anti-CD4 antibodies. (**B**) and (**C**), Flow cytometry on dermal cells from healthy skin and patch test-positive biopsies (at the indicated time points) of allergic patients. Cells were gated on living cells. B, $CD3^+$ staining on total cells. C, $CD8^+$ and $CD4^+$ staining on gated $CD3^+$ T cells (mean ± standard error of the mean for 6 patients) (*$P < .05$; Friedman test, Dunn's multiple comparison test vs healthy skin)

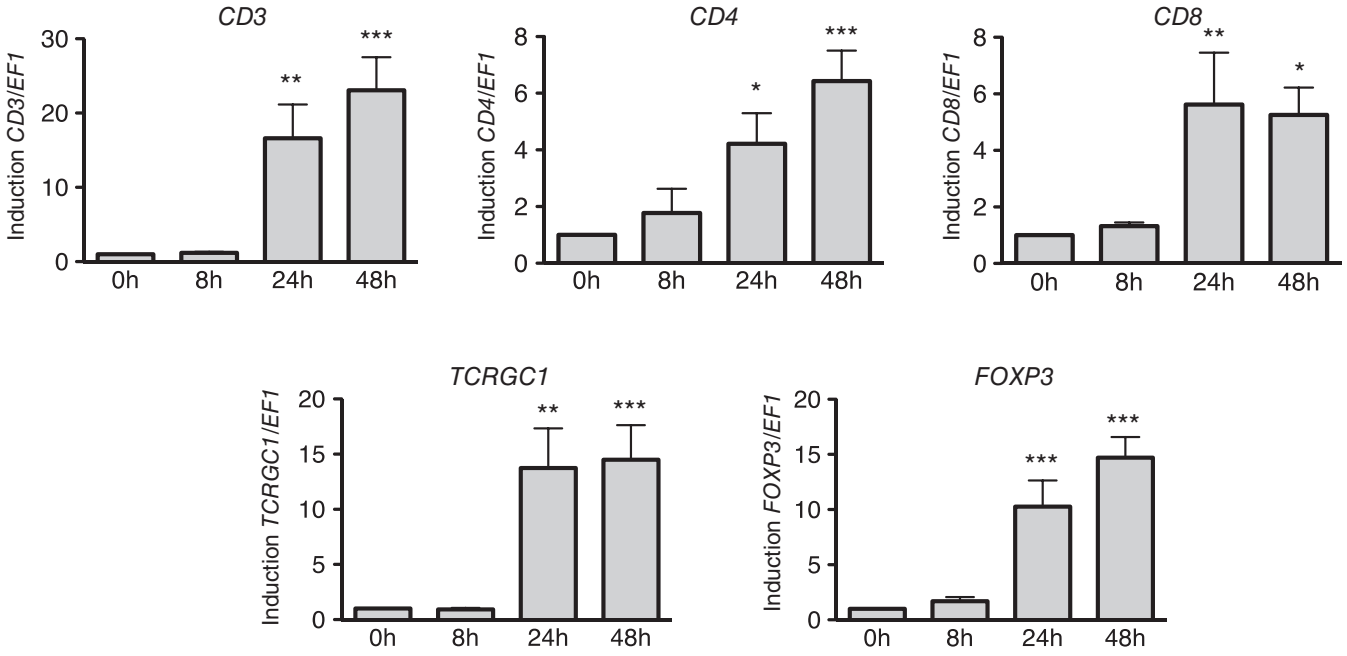

FIGURE 3 T cell markers. RNA was isolated from skin biopsies taken at 0, 8, 24 and 48 hours from *p*-phenylenediamine-sensitized patients (n = 13). Quantitative reverse transcription polymerase chain reaction analysis for *CD3*, *CD4*, *CD8*, *TCRGC1*, *FOXP3* and *EF1* mRNA expression. (*P < .05, **P < .01, ***P < .001; repeated measures ANOVA, Bonferroni's multiple comparison test vs healthy skin)

2.10 | Statistical analysis

Statistically significant differences between PPD-sensitized and control subjects were assessed with INSTAT (GraphPad Software, La Jolla, California) by means of a one-tailed unpaired Student's t test or one-way ANOVA with Tukey's or Bonferroni's multiple comparison test.

3 | RESULTS

3.1 | Clinical responses to PPD patch testing and histological staining

Among patients 1 to 14 evaluated at 8, 24 and 48 hours (D2) and on D4 and D7, 11 patients showed rapid positive test reactions at 24 hours, 2 showed positive test reactions on D2, and 1 showed a positive test reaction on D4. No clinical responses were observed at 8 hours following application (Table S1). Among patients 15 to 29, who were evaluated only on D2, 11 patients reacted positively on D2, 1 reacted positively on D4, and 3 reacted positively on D7. Sixteen of 29 patients (64%) had severe vesiculobullous reactions (+++) according to the ESCD guidelines. Most patients (n = 17) were sensitized through the use of hair dyes, and 4 were sensitized through exposure to black henna tattoo. This explains why patients mainly had contact eczema of the face or the scalp. Patch test results, sensitization routes and clinical symptoms are summarized in Table S1. Cross-reactions with other aromatic amines (toluene-2, 5-diamine, *p*-aminophenol, *m*-aminophenol, benzocaine, diaminodiphenylmethane, Disperse Orange 1, and Disperse Orange 3) were observed in a large proportion of patients.

At 8 hours, in contrast to the absence of clinical response, histological analysis showed small changes in most cases, with mild mononuclear cell infiltration, mainly in the dermis, and often in a perifollicular distribution. At 24 hours, histological analysis showed dermal infiltration with epidermal changes such as spongiosis and exocytosis. At 48 hours, significant dermal infiltration was observed in most patients, and correlated with the intensity of the clinical response (Figure 1).

3.2 | Tissue infiltration by T cells

To identify the T cell infiltrate associated with the allergic response to PPD, we collected skin biopsies at different time points: specifically, before the onset of the skin lesion (8 hours), and when clinical symptoms were present (24 and 48 hours). $CD3^+$, $CD4^+$ and $CD8^+$ T cells were analysed by immunohistological staining and flow cytometry. Surprisingly, we did not observe T cell infiltration before the onset of the clinical reaction. Indeed, $CD3^+$, $CD4^+$ and $CD8^+$ T cells infiltrated the dermis only when the first clinical symptoms had appeared (redness, papules, and/or vesicles) (Figure 2A-C), suggesting that $CD8^+$ T cells probably do not play an effector role in this reaction. As observed in ACD caused by corticosteroids, we found increases in the numbers of $CD3^+$ and $CD4^+$ T cells, and, to a lesser extent, an increase in the number of $CD8^+$ T cells, which correlated with patch test positivity (Figure 2C). Gene expression analysis of *CD3* confirmed the increase in the number of $CD3^+$ T cells, which correlated with the expression profile of *CD4* and *CD8* (Figure 3). In addition, *TCRGC1* and *FOXP3* expression had also increased at 24 hours after patch application, suggesting that γδ T cells and regulatory T cells infiltrated the dermis as well (Figure 3).

3.3 | IL-9 expression is increased in skin biopsies

To evaluate the expression of Th1, Th2, Th9 and Th17 cytokines, qRT-PCR was performed on RNA isolated from skin biopsies collected at different time points from 14 PPD-allergic patients. We observed

huge increases in *IL4* and *IL9* expression, and, to a lesser extent, increases in *IFNG* and *IL17* expression. This increase coincided with the clinical response (Figure 4A). In contrast to *IL17*, *IL4* and *IFNG*, *IL9* expression was significantly higher in the 9 patients who showed rapid severe reactions (++/+++ at 24 hours, according to the ESCD guidelines) than in patients who reacted later and/or less intensely (?+ at 24 hours) (Figure S1). This shows that *IL9* expression is significantly correlated with the severity of the patch test reaction. To identify whether IL-9 was produced by Th2, Th9, or "non-T" cells, cells from the dermis of allergic patients were isolated and subsequently stimulated, in vitro, with anti-CD3 and anti-CD28 antibodies. Surface and intracellular staining for IL-9 and IL-4 were then evaluated by flow cytometry. The percentages of IL-9$^+$ and IL-4$^+$ cells increased after in vitro stimulation (Figure 4B). These cytokines were mainly produced by CD3$^+$ cells (Figure 4C,D). Among the CD3$^+$ cells, both cytokines were mainly produced by CD4$^+$ cells, although IL-9 was also produced by some CD8$^+$ and γδ$^+$ cells (Figure 4E). These results suggest that Th cells were the main sources of IL-9 and IL-4 in ACD caused by PPD. In addition, the IL-9$^+$ cells were most probably a Th9 subset, and not a Th2 subset, because they did not express *IL4* (Figure 4B).

3.4 | IL-9 expression is increased in PBMCs after stimulation

Finally, we also evaluated the expression of *IL4*, *IL9*, *IL17* and *IFNG* in purified and restimulated PBMCs from 24 PPD-allergic patients and 16 healthy controls. After PHA/IL-2 stimulation, the induction of *IL9* expression was higher in allergic patients than in healthy controls, whereas the expression of the other 3 cytokines remained unchanged (Figure 5A,B). The induction of *IL9* expression more than doubled when PBMCs were stimulated with PPD alone in 4 allergic patients (Figure 5C), in contrast to PBMCs from healthy controls. Using flow cytometry, we confirmed that IL-9 was produced after PBMC stimulation with PHA/IL-2 and not in unstimulated PBMCs (Figure 5D). As in

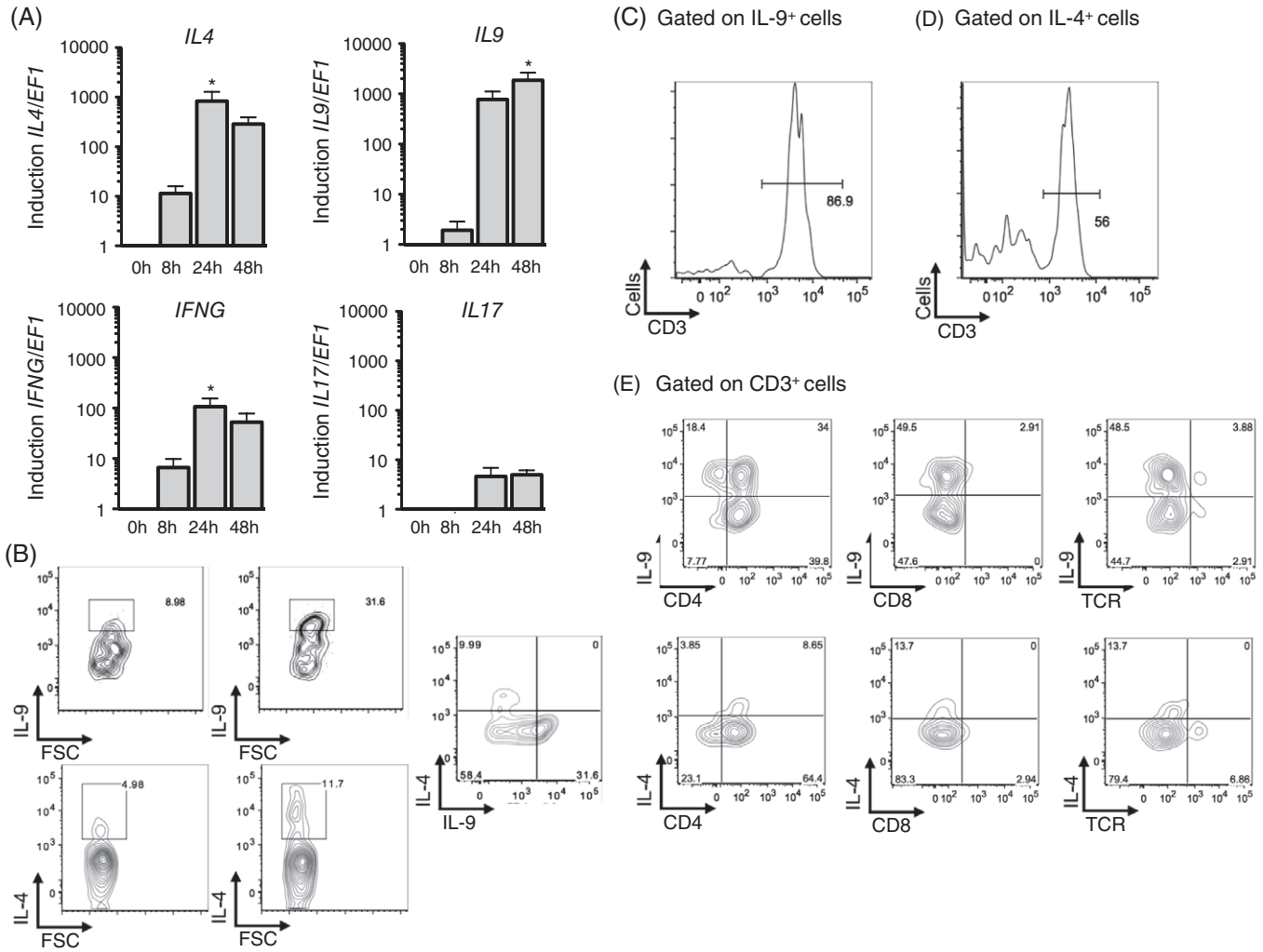

FIGURE 4 Expression of T helper-related cytokines and characterization of *IL9*$^+$ cells in the skin. (**A**), RNA was isolated from skin biopsies taken at 0, 8, 24 and 48 hours from *p*-phenylenediamine-sensitized patients (n = 13). Quantitative reverse transcription polymerase chain reaction analysis was performed for *IL4*, *IL9*, *IFNG*, *IL17* and *EF1* mRNA expression. (*P < .05, **P < .01; repeated measures ANOVA, Bonferroni's multiple comparison test vs healthy skin.). (**B-E**), Dermal cell suspension from healthy skin and patch test-positive biopsies (at 48 hours) of allergic patients that was stimulated or not stimulated with anti-CD3/anti-CD28 and monensin for 4 hours at 37°C. (**B**), Cells were gated on living CD45$^+$ cells, and the expression of interleukin (IL)-9 and IL-4 was analysed by flow cytometry on unstimulated cells (left panel) or stimulated cells (middle and right panels). (**C**) and (**D**), Staining for CD3 on IL-9$^+$ cells (C) and IL-4$^+$ cells (D). (**E**), Cells were gated on CD3$^+$ cells, and staining for IL-9, IL-4, CD4, CD8 and γδ was performed on allergic patients. The image is representative of three allergic patients. FSC, forward scatter; IFN, interferon

skin samples, $CD3^+$ cells, and particularly $CD3^+$ $CD4^+$ cells, were the main sources of IL-9 (~68%) (Figure 5E). The remaining $CD3^+$ cells were $CD8^+$ cells (~21%) (Figure 5E). Again, the $IL\text{-}9^+$ cells were not a Th2 subpopulation, because they did not express IL-4 (Figure 5D). Overall, these results indicated that IL-9 was produced by Th9 cells in PPD-allergic patients, in both skin and PBMCs.

3.5 | IL-9 plays a protective role in a model of PPD-induced ACD

To examine the role of IL-9 in PPD-induced ACD, we used a mouse model adapted from a previous model.[16] We treated *Il9r*-deficient mice with PPD, and they showed aggravated development of CHS, with more ear swelling than PPD-treated WT littermates (Figure 6A). This aggravation was accompanied by more acanthosis and higher crust percentages (Figure 6B-D). These observations suggested that IL-9R, which is the receptor for IL-9, plays a protective role in this model.

4 | DISCUSSION

The present results confirm that PPD is a strong contact sensitizer causing rapid, severe responses, with reactions appearing within 24 hours after contact with the allergen in more than two-thirds of patients, and involving vesiculobullous reactions graded +++ according to the ESCD guidelines in more than half of patients.

As already shown in ACD caused by corticosteroids, no T cell infiltration was observed before the onset of the clinical reaction.[10] Moreover, $CD3^+$, $CD4^+$ and $CD8^+$ T cell infiltration directly correlated with the occurrence of clinical symptoms (redness, papules, and vesicles). *TCRGC1* and *FOXP3* expression also suggested that γδ T cells and regulatory T cells were additionally implicated. Although the nature of the antigen or its exposure route may influence the cell type involved in the inflammatory process, serial skin biopsies collected very early and at different time points, including during the preclinical stages, did not show any effector role for $CD8^+$ T cells or any modulatory role for $CD4^+$ cells in this reaction.

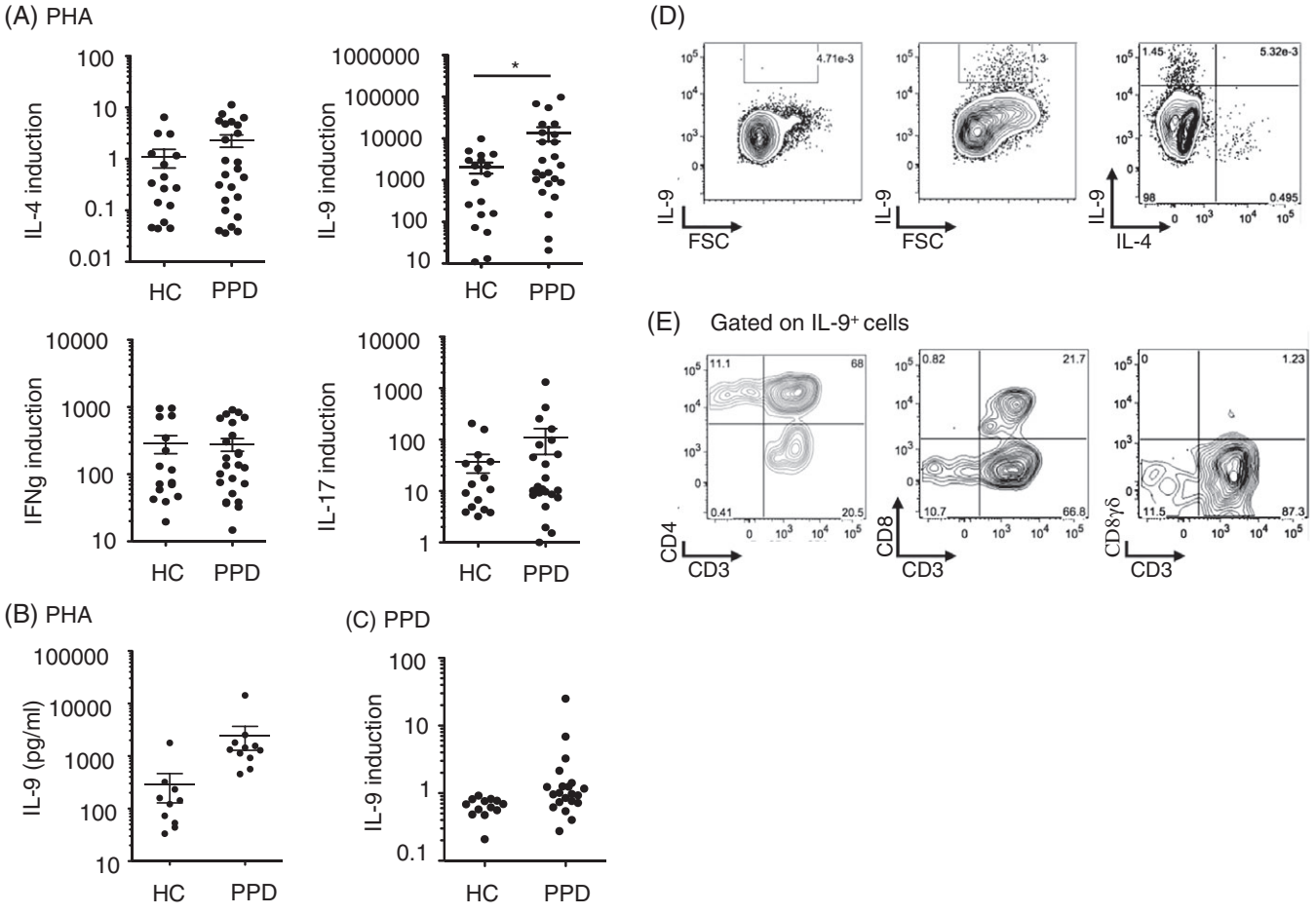

FIGURE 5 Expression of T helper cytokines in restimulated peripheral blood mononuclear cells (PBMCs) and characterization of interleukin (IL)-9^+ PBMCs. (**A-C**), RNA was isolated from PBMCs of healthy controls (HC, $n = 16$) and *p*-phenylenediamine (PPD)-allergic patients (PPD, $n = 24$). PBMCs were stimulated with phytohaemagglutinin (PHA) (A, B) or PPD (C) for 48 hours. A and C, Quantitative reverse transcription polymerase chain reaction analysis for *IL4*, *IL9*, *IFNG*, *IL17* and *EF1* mRNA expression. Induction was calculated by comparing the expression of cytokines in stimulated cells with that in unstimulated conditions. B, An IL-9 enzyme-linked immunosorbent assay was performed on the supernatant of PHA-stimulated PBMCs. The limit of detection was 12 pg/mL. Each point represents one patient. (*$P < .05$, **$P < .01$; unpaired *t* test with Welch's correction). (**D**) and (**E**), PBMCs from allergic patients were stimulated or not stimulated with anti-CD3/anti-CD28 for 48 hours at 37°C, and then incubated with monensin for 4 hours. D, Cells were gated on living $CD45^+$ cells, and the expression of IL-9 and IL-4 was analysed by flow cytometry on unstimulated cells (left panel) or stimulated cells (middle and right panels). E, Cells were gated on $IL\text{-}9^+$ cells, and staining for CD3, CD4, CD8 and γδ was performed. The results are representative of four allergic patients. FSC, forward scatter; IFN, interferon

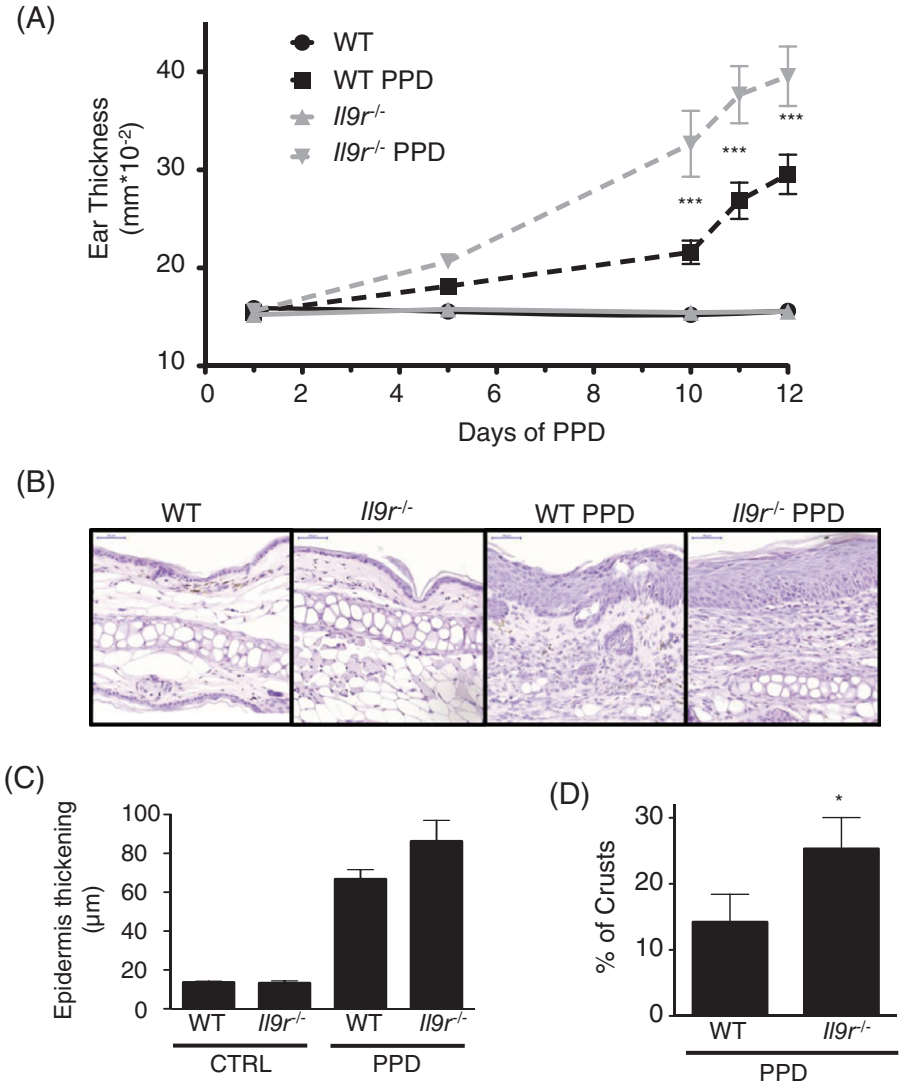

FIGURE 6 *p*-Phenylenediamine (PPD)-induced contact hypersensitivity (CHS) is aggravated in *Il9r*$^{-/-}$ mice. (**A**), Ear thickness was measured before each PPD treatment and 24 hours after the last challenge with a micrometer to gauge the development of CHS. Data are expressed as mean ± standard error of the mean (SEM) (n = at least 4 for control groups; n = least 7 for PPD-treated groups), and represent at least three independent experiments (***P < .001; two-way ANOVA, Bonferroni's multiple comparison test). (**B**), Hematoxylin eosin staining of ear skin sections from wild-type (WT) and *Il9r*$^{-/-}$ mice, treated or not treated with PPD, 24 hours after the third challenge. One representative image is shown for each treatment regimen. (**C**), Acanthosis was evaluated by measuring epidermal thickness at six different locations with the Panoramic Viewer measuring tool. (**D**), Crust percentages were calculated by dividing the length of the crust by the length of the section. Data indicate mean ± SEM (n = 4 for control groups; n = 8 for PPD-treated groups), and represent 2 independent experiments (*P < .05, **P < .01; Mann-Whitney test for comparison of treated mice). Histological analysis was performed by two evaluators. CTRL, control

In PPD-allergic patients, we observed huge increases in *IL4* and *IL9* expression, and, to a lesser extent, an increase in *IFNG* expression. This increase was concomitant with the clinical response, suggesting that these Th1/Th2/Th9-related cytokines play an essential role in the mechanism of ACD caused by PPD. On the other hand, the level of IL-17, which is a Th17-related cytokine, did not significantly increase after PPD application. This contrasts with the cellular response associated with nickel allergy, in which several authors found high levels of IL-17 in the supernatant of activated T cells,[27] and others showed enhanced expression of IL-26, which is a Th17-related cytokine, in both the skin and PBMCs of allergic subjects.[20] Our results show that the nature of the antigen influenced the T cell response.

IL4 expression also increases in response to many other allergens, such as fragrances and methylisothiazolinone.[28] The detrimental role of Th2 cytokines has already been shown in different models of ACD caused by PPD and other allergens. *STAT6*$^{-/-}$ mice, which are deficient in Th2, are almost totally protected against ear swelling, histopathological skin changes and inflammatory infiltrates induced by PPD application.[29]

In addition to increased *IL9* expression in skin samples of positive patch test reactions, a high level of IL-9 secretion was observed after allergen stimulation of the PBMCs of patients with ACD caused by PPD. Moreover, *IL9* expression was higher in patients who showed rapid, severe reactions than in patients who reacted later or less intensely. Thus, *IL9* expression significantly correlated with the

severity of the patch test reaction. Our results are in line with those of Gutin et al, who showed massive *IL9* expression in 5 biopsies from patients with bullous patch test reactions to PPD.[22] Similarly, the PBMCs of PPD-allergic patients secreted IL-9 after PPD stimulation, whereas those of tolerant individuals did not. Increased *IL9* expression is not restricted to PPD, but can be a common response to different allergens. Indeed, IL-9 expression is likewise increased in biopsies of positive patch test reactions to nickel and in the PBMCs of nickel-allergic patients.[22] We showed that IL-9$^+$ cells were mainly CD3$^+$ CD4$^+$ cells, which suggests that Th cells were the major sources of IL-9. In addition, the IL-9$^+$ cells were not a Th2 subpopulation, as they did not express IL-4.

A proinflammatory effect of IL-9 has been widely described in various allergic disorders, including asthma and allergic rhinitis.[30,31] The fact that the IL-9 level strongly correlates with the severity of the patch test reaction[22] suggests that IL-9 similarly promotes an inflammatory reaction in ACD. The proinflammatory role of IL-9 was previously confirmed in rodents, as ear swelling in *Il9*$^{-/-}$ mice was less pronounced in a model of CHS caused by 2,4-dinitrofluorobenzene (DNFB) than in WT mice.[22] However, our study clearly showed that *Il9r*$^{-/-}$ mice sensitized to PPD developed more severe contact eczema than WT mice. This indicates that, in fact, IL-9 plays an anti-inflammatory role in PPD-induced CHS. Liu et al similarly observed that IL-9 reduced Th1-dependent cytokine production in their model of DNFB-mediated CHS.[22] In addition, the production of IFN-γ by the PBMCs of nickel-allergic patients was blocked by an anti-IL-9 antibody.[23] The anti-inflammatory function of IL-9 was also observed in arthritis and experimental autoimmune encephalomyelitis (EAE), in which IL-9 was involved in the resolution of inflammation.[32,33] In those models, the absence of IL-9 or its receptor impaired the activation of regulatory T cells, causing chronic arthritis with excessive cartilage destruction or severe EAE, respectively. This was corroborated by treatment with IL-9, which promoted regulatory T cell activation and brought about the resolution of inflammation.

The kinetics of IL-9 production in our model differed from those of other proinflammatory cytokines. Indeed, IL-4 expression and IFN-γ expression increased very rapidly (at 8 hours after application), peaked at 24 hours, and were already falling at 48 hours, whereas IL-9 expression, like that of the regulatory marker FOXP3, was still increasing at 48 hours. It is tempting to hypothesize that IL-9 plays mainly a regulatory role in PPD-mediated ACD.

5 | CONCLUSION

To conclude, in both humans and a mouse model of ACD caused by PPD, we have suggested the essential role of IL-9 in the pathogenesis of this inflammatory process. Indeed, the increased *IL9* expression in the purified, restimulated PBMCs of PPD-allergic patients as compared with control subjects, as well as the regulatory role of IL-9R in PPD-induced CHS models, provides significant information on *IL9* in ACD.

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Conflict of interest

The authors declare no potential conflict of interests.

ORCID

Marie Baeck https://orcid.org/0000-0003-0499-7939
Anne Herman https://orcid.org/0000-0001-7000-1672

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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