

modern era, which appears to now be used for approximately one-third of patients with SCID and half of patients with SCID.

These data also reveal continued improvements in survival, which is likely multifactorial in nature. Advances in conditioning approaches, HLA typing techniques/experience, donor options, graft manipulation techniques, graft versus host disease prevention, infection prophylaxis/treatment, critical care medicine, and PID diagnostics likely all contribute. Newborn screening for SCID appears to be making a tremendous impact on transplant survival, likely by providing an opportunity for allogeneic HCT before the acquisition of infections. Expected survival following allogeneic HCT in this current era for patients with PID can be estimated on the basis of our data to be at least 70%, and is closer to 80% to 90% for many patients. It is our hope that ongoing advances in the PID and bone marrow transplant fields will continue to improve survival outcomes over the next 50 years.

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Coated microneedle-based cutaneous immunotherapy prevents Der p 1-induced airway allergy in mice



To the Editor:

Allergies are increasing worldwide, and millions of persons are struggling against a host of airway allergens.¹ Allergen-specific immunotherapy is the primary approach to cure allergies. However, allergen-specific immunotherapy is performed only after allergy symptoms have manifested in patients. A true mode of allergy vaccination in the form of "preventive immunotherapy" like that of infectious disease vaccination could be used to stop the allergy epidemic. Prevention of allergy onset in healthy subjects is called "primary prevention," and prevention in subjects who are already sensitized but have yet to develop allergy symptoms is called "secondary prevention."²

Proof of concept for secondary prevention has been clinically demonstrated. In 1 multicenter preventive allergy treatment study, children aged 6 to 14 years with seasonal rhinoconjunctivitis caused by birch and/or grass pollen were given allergen-specific subcutaneous immunotherapy (SCIT) for 3 years. Even 2 years after treatment was stopped, a significant preventive effect on asthma development was seen.³

We have previously shown that micron-sized needles widely known as microneedles can be used to deliver allergens into the skin.⁴ Microneedles are painless⁵ and can bypass the stratum corneum layer to directly deliver the allergen into the skin. As a result, unlike conventional skin patches, microneedle-based delivery is not affected by differences in skin permeabilities that naturally exist among subjects. Hence microneedles can provide better dosage control and could improve consistency of therapeutic response compared with use of a conventional skin patch for allergen delivery.

In this study, for the first time, we examined the efficacy of microneedles to deliver Der p 1, a house dust mite allergen, into mouse skin and determined its ability to prevent development of Der p 1-induced airway allergy. A direct comparison was made against SCIT. The vaccination and challenge schedule is shown in Fig 1, A, and Table E1 in this article's Online Repository at www.jacionline.org describes the different groups. Concept of coated microneedle-based allergen delivery into the skin is shown in Fig 1, B.

Der p 1 (purified natural Der p 1, >95% pure, 24 kDa; INDOOR Biotechnologies, Charlottesville, Va) was successfully coated on

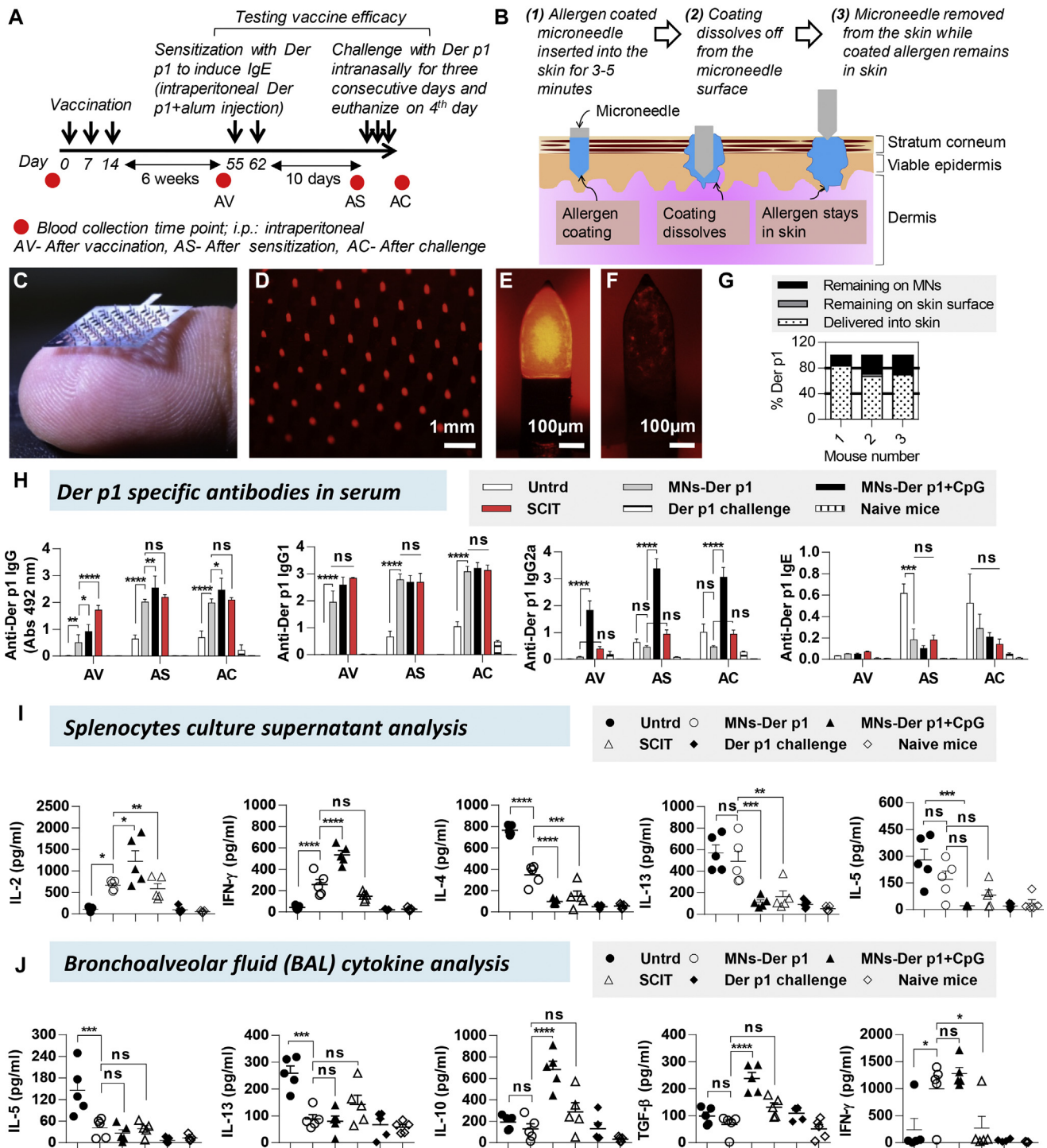


FIG 1. Microneedle (MN)-mediated preventive allergy immunotherapy. **A**, Immunotherapy schedule. **B**, Concept of allergen delivery into the skin with an allergen-coated microneedle. **C**, Digital photograph of a microneedle patch containing 57 total microneedles resting on a human fingertip. **D-F**, Fluorescence micrographs of microneedles coated with rhodamine-conjugated Der p1 (Fig 1, D), zoomed image of a single microneedle before insertion into mouse skin (Fig 1, E), and zoomed image of a single microneedle after insertion into mouse skin (Fig 1, F). **G**, Delivery efficiency from a microneedle patch coated with rhodamine-conjugated Der p1 after insertion into mouse skin. **H**, Der p1-specific antibodies in serum after vaccination (AV), after sensitization (AS), and after challenge (AC). Serum dilutions were as follows: 1:500 for IgG, IgG₁, and IgG_{2a} and 1:20 for IgE (n = 5 mice per group). **I**, Cytokine expression in splenocytes supernatants after Der p1 restimulation. **J**, Cytokine expression in BAL fluid after challenge. One-way ANOVA was used for statistical comparisons between groups. Bars denote means ± SEMs. *P ≤ .05, **P ≤ .01, ***P ≤ .001, and ****P ≤ .0001. ns, Not significant. Untrd, Untreated.

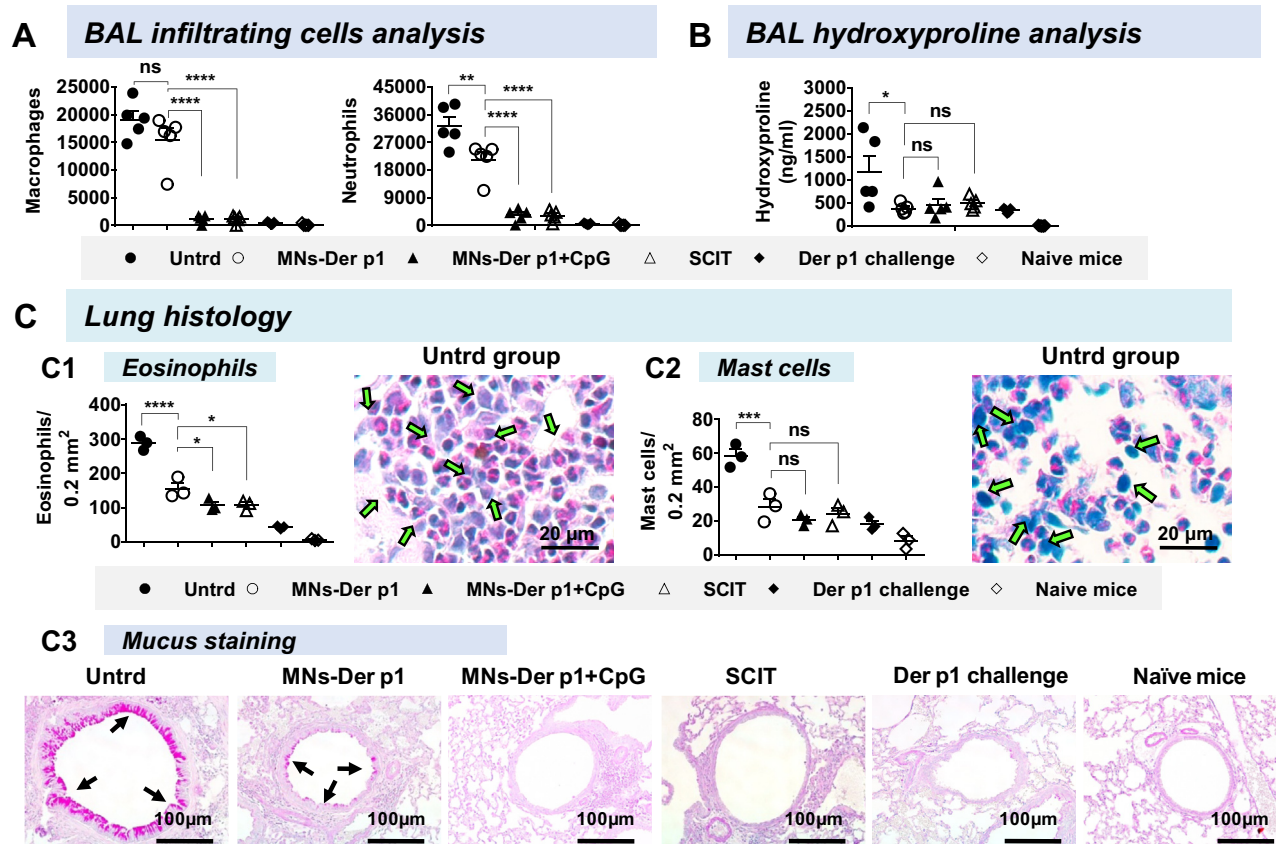


FIG 2. Lung histopathology. At the end of the allergen challenge, BAL fluid and lungs were collected for analysis. **A**, Macrophages (CD11b⁺Gr1⁻) and neutrophils (CD11b⁺Gr1⁺) in BAL fluid were counted, as described previously.⁷ **B**, Hydroxyproline content was analyzed in BAL fluid (catalog no. MAK008; Sigma Aldrich, St Louis, Mo). Each symbol represents an individual mouse (n = 5 mice per group). **C**, Lung histology. Multiple sections (7 μm thick) separated by 500 μm were obtained from the top and bottom of the left lung and stained with either hematoxylin and eosin for eosinophil analysis or May-Grünwald Giemsa stain for mast cell analysis. Each symbol represents average cells from a mouse averaged at 18 independent locations: eosinophils (C1) and mast cells (C2). Eosinophils and mast cells for other treatment groups are presented in Figs E1 and E2, respectively. **C3**, Tissue sections were stained with periodic acid-Schiff stain for mucus secretion inside the bronchioles. Bright-field micrographs are shown. Arrows point to mucus deposition. One-way ANOVA was used for statistical comparisons between the groups. Bars denote means ± SEMs. *P ≤ .05, **P ≤ .01, ***P ≤ .001, and ****P ≤ .0001. ns, Not significant. MN, Microneedle; Untrd, untreated.

the microneedle patches (Fig 1, C and D), with uniform surface coverage (Fig 1, E). Endotoxin in Der p 1 was 0.17 EU/μg, which is less than the US Food and Drug Administration–allowable limit of 14 EU/μg (assuming a 70-kg human and a 25-μg allergen dose). Any potential adjuvant effect of the endotoxin would be equally applicable to coated microneedles and SCIT groups and should not bias this study.

Hair from mouse skin was removed, and a coated microneedle patch was manually inserted, held in place for 3 minutes, and removed. A small fraction of the coating remained on the microneedle surface after removal (Fig 1, F). By using calibrated fluorescence spectroscopy, it was found that 73.3% ± 7.4% of coated Der p 1 was delivered into the skin, whereas 2.6% ± 1.1% Der p 1 was found on the skin and 24.1% ± 6.5% Der p 1 remained on the microneedle surface (Fig 1, G).

Here a microneedle patch was coated with 25 μg of Der p 1 with or without 25 μg of CpG as adjuvant. However, a microneedle patch with about 57 microneedles can be readily

coated with 100 to 200 μg of allergen and is thus adequate for human use because for most allergens, including house dust mite, the maintenance doses range between 5 and 20 μg.⁶

After vaccination, the microneedle Der p 1 (MN-Der p 1) group induced an anti-Der p 1 IgG response (Fig 1, H), which was considerably greater than that in the untreated group (untreated negative control with no Der p 1 coated on microneedles). Because purified Der p 1 protein was used, we included adjuvants in the formulations to increase its immunogenicity. We used alum in the SCIT group because it is approved for allergen immunotherapy in Europe. For microneedle coatings, we used CpG as an adjuvant because it is a known stimulator of the T_H1 pathway.

As expected, addition of CpG to Der p 1 coatings (MN-Der p 1+CpG group) increased the IgG response over that in the MN-Der p 1 group, but it was not statistically significant (Fig 1, H). SCIT induced the greatest IgG response (Fig 1, H). The anti-Der p 1 IgG₁ response was similar in the Der p 1–vaccinated groups (MN-Der p 1, MN-Der p 1+CpG group, and SCIT groups)

and was significantly greater than that in the untreated group (Fig 1, H). In contrast, the anti-Der p 1 IgG_{2a} response was found to be significantly greater in the MN-Der p 1 + CpG group compared with both the MN-Der p 1 and SCIT groups (Fig 1, H). This can be attributed to CpG, which is a known stimulator of T_H1-type immune responses. The anti-Der p 1 IgE response in all vaccinated groups was low (Fig 1, H). After sensitization, IgG, IgG₁, and IgG_{2a} antibody responses increased across all groups, but the relative trend between them did not change considerably (Fig 1, H). No major change was seen after challenge. However, after sensitization, the anti-Der p 1 IgE response in the untreated group (no Der p 1 in formulation) increased significantly compared with that in the Der p 1-vaccinated groups (Fig 1, H). This is indicative of the development of a type 1 hypersensitive reaction in the control untreated group. Naive mice and mice that received just the Der p 1 challenge (without any vaccination or sensitization) did not show any considerable anti-Der p 1 antibody response in serum across all points.

Successful allergen immunomodulation involves activation of the T_H1 pathway. Therefore, to assess the nature of the T_H pathway, splenocytes were cultured and restimulated with Der p 1. Levels of the T_H1 cytokines IL-2 and IFN- γ were significantly greater in the Der p 1-vaccinated groups (MN-Der p 1, MN-Der p 1 + CpG group, and SCIT groups) compared with those in the untreated group (Fig 1, I). In contrast, levels of the T_H2 cytokines IL-4, IL-13, and IL-5 in the MN-Der p 1 + CpG group and SCIT groups were low and similar to those in the naive control group. In the untreated group levels of the T_H2 cytokines were greater, and IgE levels were also greater. Naive mice and mice that received just the Der p 1 challenge showed low expression of both T_H1 and T_H2 cytokines.

Mice after sensitization were challenged intranasally with Der p 1 to examine the effectiveness of vaccination. After challenge, the groups vaccinated with Der p 1 (MN-Der p 1, MN-Der p 1 + CpG group, and SCIT groups) exhibited protection from development of an allergic reaction. Levels of the proinflammatory cytokines IL-5 and IL-13 in bronchoalveolar lavage (BAL) fluid were significantly less in mice vaccinated with Der p 1 than in the untreated group (Fig 1, J). Interestingly, levels of the anti-inflammatory cytokines IL-10 and TGF- β in the untreated and MN-Der p 1 groups were not different from each other (Fig 1, J); however, when CpG was added to the MN-Der p 1 coatings (ie, MN-Der p 1 + CpG group), it led to significantly greater IL-10 and TGF- β secretion compared with both untreated and MN-Der p 1 groups (Fig 1, J). This indicates a beneficial role of CpG as an adjuvant for allergy vaccination. IFN- γ levels in the lungs were significantly greater in the MN-Der p 1 and MN-Der p 1 + CpG group-vaccinated groups compared with the untreated group and even the SCIT group (Fig 1, J). This is consistent with the IFN- γ secretion pattern in splenocytes culture.

The protective nature of coated microneedle-based vaccination was reconfirmed when we analyzed effector cells (macrophages and neutrophils) in the BAL fluid. The Der p 1-vaccinated groups (MN-Der p 1, MN-Der p 1 + CpG group, and SCIT groups) had fewer macrophages and neutrophils in their BAL fluid compared with the untreated group (Fig 2, A).⁷ Of note, with the untreated group as the highest data point, the fold reduction in macrophage and neutrophil counts was greater in the MN-Der p 1 + CpG group than that in the MN-Der p 1 group, reinforcing that CpG exerts a beneficial effect as an adjuvant (Fig 2, A). Neutrophils have been

implicated in increased steroid resistance in human subjects⁸ and mice.⁹ Collagen is one of the indicators of lung remodeling. Hydroxyproline levels, which reflect collagen levels, were significantly less in BAL fluid of all groups vaccinated with Der p 1 (including the MN-Der p 1 group) and only greater in the untreated group (Fig 2, B). Lower collagen levels are indicative of a protective effect of microneedle-based Der p 1 vaccination because collagen deposition can contribute to thickening of the bronchiole wall and ultimately lead to narrowing of the airway tract and bronchial hyperresponsiveness.

Considerably lower numbers of eosinophils (Fig 2, C1, and see Fig E1 in this article's Online Repository at www.jacionline.org) and mast cells (Fig 2, C2, and see Fig E2 in this article's Online Repository at www.jacionline.org) were observed in lung sections of Der p 1-vaccinated groups compared with those in the untreated group. Furthermore, in the MN-Der p 1 and MN-Der p 1 + CpG groups, similar to the SCIT group, less mucus deposition was seen inside the bronchioles, indicating suppression of inflammatory response (Fig 2, C3). In contrast, mucus hypersecretion was noticed in the untreated group (Fig 2, C3). Mucus hyperplasia is one of the major characteristics of airway allergy wherein mucus secretion in bronchioles obstructs airflow and ultimately leads to shortness of breath.

The trend in protection generally correlated with postsensitization IgE serum levels. The untreated group, which had greater IgE levels, exhibited an allergic reaction, whereas the Der p 1-vaccinated groups, which had lower IgE levels, were better protected.

In conclusion, microneedle-based and SCIT vaccinations with Der p 1 each led to similar protective effects against sensitization in a mouse model. Because microneedles are minimally invasive, painless, and easy to apply, they can offer a more patient friendly approach of allergy vaccination compared with SCIT. Initially, allergen vaccines could be offered to subjects who are deemed to be at a greater risk of allergies caused by family history or to subjects who are already sensitized but have yet to progress to a diseased state, such as those with allergic rhinitis, to prevent their disease exacerbation to asthma.

This study provides proof of concept that microneedles have potential for allergy vaccination and immunotherapy but does not elucidate the mechanism of action. Thus follow-on studies that dissect the mechanism of action, including the contribution of regulatory T cells, are required. Future studies that focus on optimization of vaccination parameters, such as dose, frequency of administration, and number of treatments, are also required to develop this approach further.

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Cluster analysis of occupational asthma caused by isocyanates



To the Editor:

Asthma is a complex disease that includes different phenotypes. Significant advances in understanding asthma heterogeneity have been made by using unbiased multivariate approaches to group asthmatic patients into distinct clusters.¹⁻⁴ Whether such well-defined asthma phenotypes provide a better understanding of the cause of the disease remains undetermined because inciting agents in patients with common asthma are often unknown.

Occupational asthma (OA) is a form of asthma in which the causative agent is recognized. The causes of OA can be classified as high-molecular-weight agents and low-molecular-weight agents, such as diisocyanates, which are the major cause of OA in many industrialized countries.⁵

Very few investigations have been performed on clusters of OA.⁶ In the present study we sought to assess whether different clusters can be identified in patients with OA caused by a single class of chemicals, such as diisocyanates, using variables more appropriate for OA than those of the Severe Asthma Research Program.^{2,7}

Patients with a diagnosis of OA confirmed by a positive specific inhalation challenge (SIC) result with diisocyanates from 1988 to 2013 were included in our study. Positive responses to SICs included an early asthmatic reaction, a late asthmatic reaction, or both (dual reaction).⁸ All subjects signed informed consent forms to perform the procedures for the diagnosis of OA, including SICs. The local ethics committee approved use of the retrospective data of the patients (no. 3930/AO/16).

The cluster analysis was performed by using the variables reported in Table I. The number of clusters was derived both from the dendrogram obtained by using the Ward method and from the statistics cubic clustering criterion, pseudo-F, and pseudo-t². Afterward, we applied the k-means method to classify subjects into clusters. More details of the methods used are described in the Methods section in this article's Online Repository at www.jacionline.org.

Table I shows the characteristics of the 187 subjects included in the study. In a preliminary cluster analysis the 2 hexamethylene diisocyanate (HDI)-exposed patients were segregated separately from the toluene diisocyanate (TDI) and methylene diisocyanate

TABLE I. Characteristics of the patients with OA (n = 187) according to the 13-variables analysis

Age (y)	35 ± 10.8
Sex (male/female)	132/55
BMI (kg/m ²)	24 ± 3.5
Smoking status (yes/no/ex-smoker)	27/101/59
Atopy, no. (%)	52 (28)
FEV ₁ /FVC (% predicted), baseline	79 ± 8
FEV ₁ (% predicted), baseline	100 ± 13
PD ₂₀ before SIC (μg)	809 ± 573
Agent (TDI/MDI/HDI)	154/31/2
Eosinophils in peripheral blood (%)	3.9 ± 2.8
Neutrophils in peripheral blood (%)	55.8 ± 11.2
Type of reaction (early/late/dual)	38/102/47
Age at onset (y)	32 ± 10.1

Values are given as numbers and percentage or means ± SDs.

(MDI) groups. Because we preferred not to remove these 2 patients, they were forced into the TDI-exposed group in the cluster analysis presented in Table II. The reasons for this are that these patients held the same job as the TDI-exposed patients (spray painters) and the vapor pressure of HDI is closer to that of TDI than that of MDI. Furthermore, we performed an additional analysis including variables with missing data and without forcing patients from the HDI group into the TDI group, which is shown in Table E1 in this article's Online Repository at www.jacionline.org.

The largest cluster (cluster 1) contained 80 (43.0%) patients, followed by cluster 2 (n = 76, 40.5%) and cluster 3 (n = 31, 16.5%). Clusters 1 and 2 included patients sensitized to TDI, mostly with a late SIC response. Cluster 1 patients were predominantly male nonsmokers and had younger age and greater body mass index (BMI) than cluster 2 patients. Cluster 2 patients exhibited shorter exposure than patients in cluster 1. Cluster 3 included patients sensitized to MDI, mostly with an early SIC response. Atopy, pre- and post-SIC methacholine PD₂₀ FEV₁ values, and eosinophil and neutrophil percentages in peripheral blood were not significantly different among clusters.

The number of clusters we detected is smaller than that usually identified in patients with common asthma (4 or 5). Thus OA seems less heterogeneous than common asthma. One possible explanation might be the obvious lack of the early-onset phenotype. In addition, we did not identify clusters characterized by severe lung function impairment, as found in some studies of common asthma.¹⁻⁴ One reason why the severe phenotypes were not detected in patients with OA might be the short symptomatic period (average, 3 years) that might have prevented the disease progressing toward more severe functional impairment. Another possibility is the relatively short time of exposure to the specific offending agent, given that relevant exposure is limited to the working hours.

Among the different parameters considered by previous investigations on asthma heterogeneity, some variables were particularly relevant across the studies for the allocation of patients to the clusters, such as age at asthma onset, sex, atopy, and baseline lung function. Age at disease onset and baseline lung function were significantly different also within our OA clusters, whereas atopy was not. Although sex was significantly different among our clusters, we cannot tell whether this is due to sex *per se* or to a different distribution of male and female subjects among jobs with isocyanate exposure.

Eosinophils staining

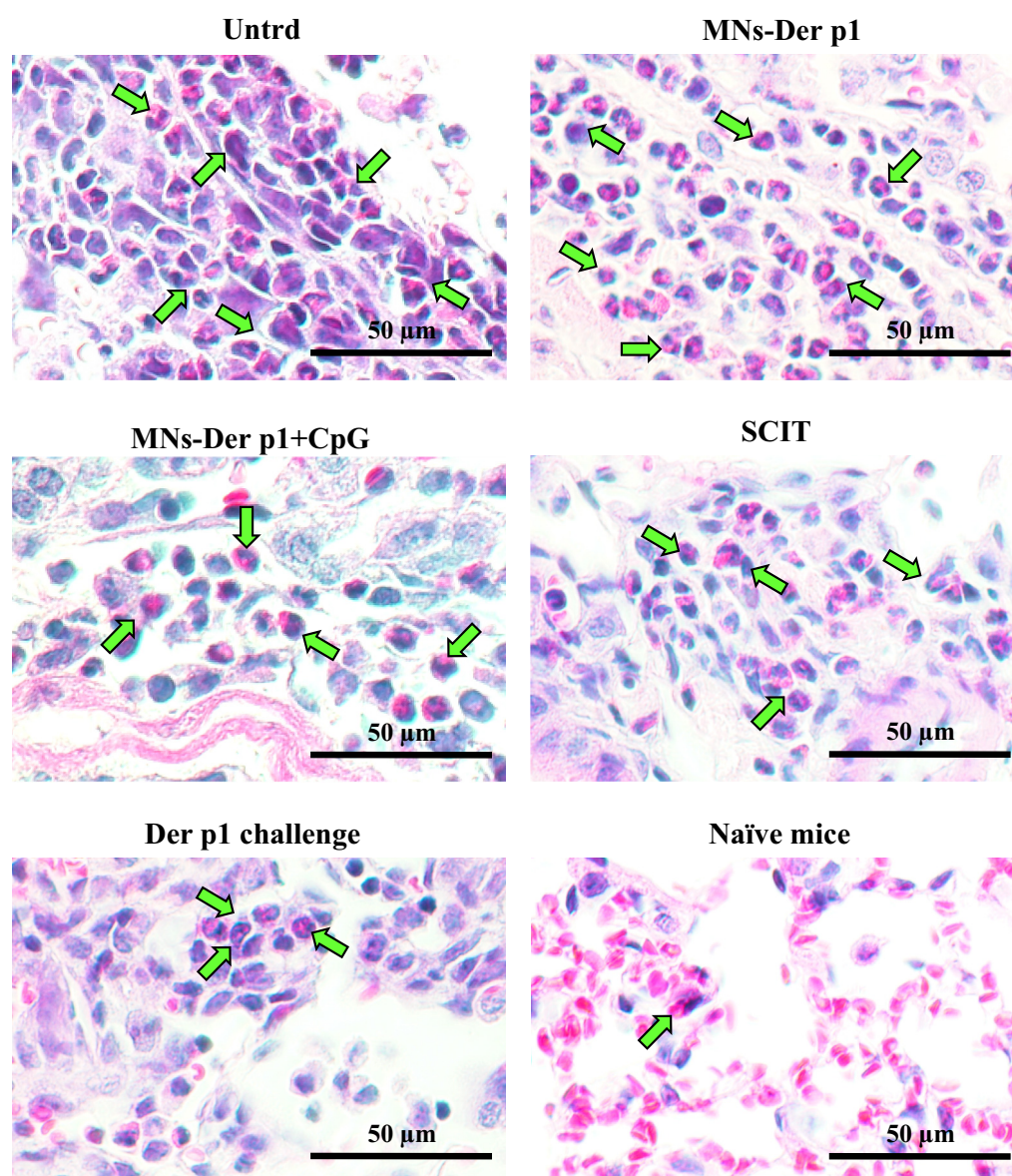


FIG E1. Hematoxylin and eosin staining for eosinophils in tissue sections of the untreated (*Untrd*), MN-Der p 1, MN-Der p 1+CpG group, SCIT, Der p 1 challenge, and naïve groups.

Mast cells staining

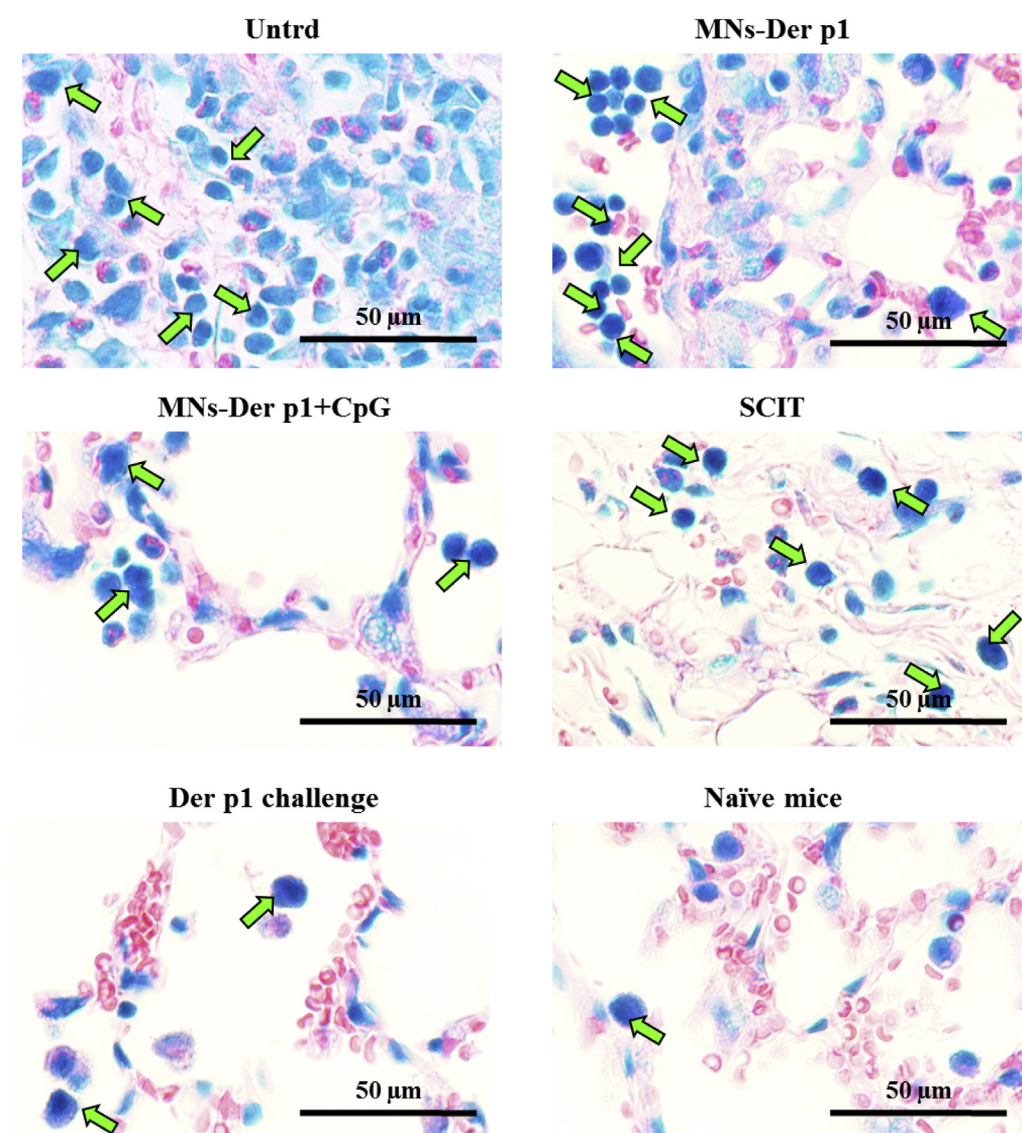


FIG E2. May-Grünwald Giemsa staining for mast cells in tissue sections of untreated (*Untrd*), MN-Der p 1, MN-Der p 1+CpG group, SCIT, Der p 1 challenge, and naïve groups.

TABLE E1. Treatment groups and doses

Groups	Vaccination	Sensitization*	Challenge†	Comment
Untreated	Untreated group that received microneedles that were not coated with Der p 1	✓	✓	Negative control, expected to induce high inflammation
MN-Der p 1	Der p 1-coated microneedles (25 µg of Der p 1)	✓	✓	Test group to study efficacy of microneedles
MN-Der p 1+CpG	Der p 1 + CpG-coated microneedles (25 µg of Der p 1 + 25 µg of CpG)	✓	✓	Test group to study effect of CpG as an adjuvant
SCIT	Der p 1 (25 µg) + alum (250 µg)	✓	✓	Positive control to mimic clinically approved subcutaneous allergy immunotherapy procedure
Der p 1 challenge	×	×	✓	Negative control to assess effect of Der p 1 challenge on naive mice
Naive	×	×	×	Negative control (without any treatment)

*Sensitization: Two doses, each containing 25 µg of Der p 1 plus 2 mg of alum administered through the intraperitoneal route at weekly intervals.

†Challenge: 50 µg of Der p 1 administered through the nasal route administered on 3 consecutive days.