



## ORIGINAL ARTICLE

## Allergen-Specific Immunotherapy and Biologics

# Comprehensive mapping of immune tolerance yields a regulatory TNF receptor 2 signature in a murine model of successful Fel d 1-specific immunotherapy using high-dose CpG adjuvant

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## Abstract

**Background:** The prevalence of allergy to cat is expanding worldwide. Allergen-specific immunotherapy (AIT) has advantages over symptomatic pharmacotherapy and promises long-lasting disease control in allergic patients. However, there is still a need to improve cat AIT regarding efficacy, safety, and adherence to the treatment. Here, we aim to boost immune tolerance to the major cat allergen Fel d 1 by increasing the anti-inflammatory activity of AIT with the established immunomodulatory adjuvant CpG, but at a higher dose than previously used in AIT.

**Methods:** Together with CpG, we used endotoxin-free Fel d 1 as therapeutic allergen throughout the study in a BALB/c model of allergy to Fel d 1, thus mimicking the conditions of human AIT trials. Multidimensional immune phenotyping including mass cytometry (CyTOF) was applied to analyze AIT-specific immune signatures.

**Results:** We show that AIT with high-dose CpG in combination with endotoxin-free Fel d 1 reverts all major hallmarks of allergy. High-dimensional CyTOF analysis of the immune cell signatures initiating and sustaining the AIT effect indicates the simultaneous engagement of both, the pDC-Treg and B-cell axis, with the emergence of a systemic GATA3<sup>+</sup> FoxP3<sup>hi</sup> biTreg population. The regulatory immune signature also suggests the involvement of the anti-inflammatory TNF/TNFR2 signaling cascade in NK and B cells at an early stage and in Tregs later during AIT.

**Conclusion:** Our results highlight the potential of CpG adjuvant in a novel formulation to be further exploited for inducing allergen-specific tolerance in patients with cat allergy or other allergic diseases.

**Abbreviations:** AIT, allergen-specific immunotherapy; APC, antigen-presenting cells; BALF, Bronchoalveolar lavage fluid; CpG, oligodeoxynucleotides containing unmethylated CpG motifs; i.p., intraperitoneal; MLN, mediastinal lymph nodes; MMI, mean metal intensity; PC, peritoneal cavity; pDC, plasmacytoid dendritic cell; s.c., subcutaneous; TLR, toll-like receptor.

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**Present address**

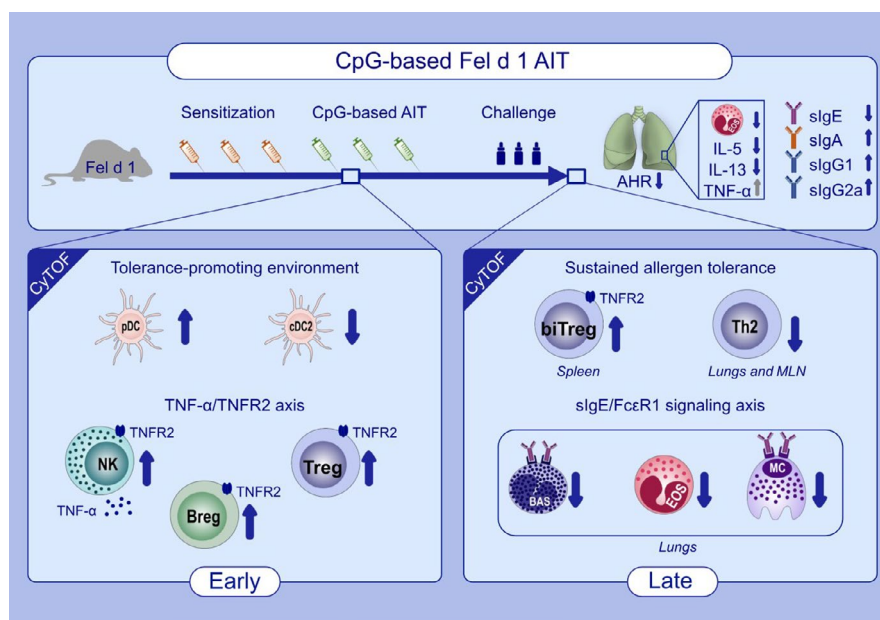
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**KEYWORDS**

allergen immunotherapy, biTregs, CpG-ODN, Fel d 1, TNFR2

**GRAPHICAL ABSTRACT**

AIT adjuvanted with a high and safe dose of CpG reverts major hallmarks of cat allergy without inducing any Th1/Th17 profile. The use of endotoxin-free Fel d 1 and B-Type CpG in AIT induces a tolerance-promoting immune reaction through an early pDC-NK cell-Breg-Treg axis, characterized by a sustained TNFR2 expression. A novel double positive FoxP3<sup>+</sup> GATA3<sup>+</sup> Treg subpopulation appears upon AIT.

Abbreviations: AHR, airway hyper reactivity; AIT, allergen specific immunotherapy; biTreg, double positive FoxP3<sup>+</sup> GATA3<sup>+</sup> Treg; CyTOF, cytometry by time-of-flight; CpG, oligodeoxynucleotides containing unmethylated CpG motifs; Fel d 1, Felis domesticus 1, major cat allergen; TNFR2, tumor necrosis factor receptor 2

**1 | INTRODUCTION**

As cat ownership is rising, allergic sensitization and diseases such as rhinitis and asthma due to cat allergy are increasing worldwide. Avoidance measures for cat-allergic patients are difficult to implement since persistent airborne cat allergens are widespread and exposure even occurs in public places, where cat allergens have been transferred to by cat owners.<sup>1,2</sup>

While pharmacotherapy is an option for milder forms of cat allergy, only allergen-specific immunotherapy (AIT) can provide causal treatment with the promise of effective disease control in patients with moderate to severe cat allergy.<sup>3</sup> The goal of AIT in

allergy is to induce long-term immune tolerance by downregulating Th2 cell-driven immune responses through allergen-specific regulatory lymphocytes.<sup>4,5</sup> However, only limited clinical evidence data are available for currently marketed cat AITs compared with other AITs.<sup>1</sup> Improving cat AIT regarding efficacy, safety, cost-effectiveness, frequency of injections, and duration of the treatment is thus considered an unmet need.<sup>6</sup> Novel cat AIT products with the potential to solve these unmet needs would fill a missing gap in the expectations of allergy specialists and cat-allergic patients.

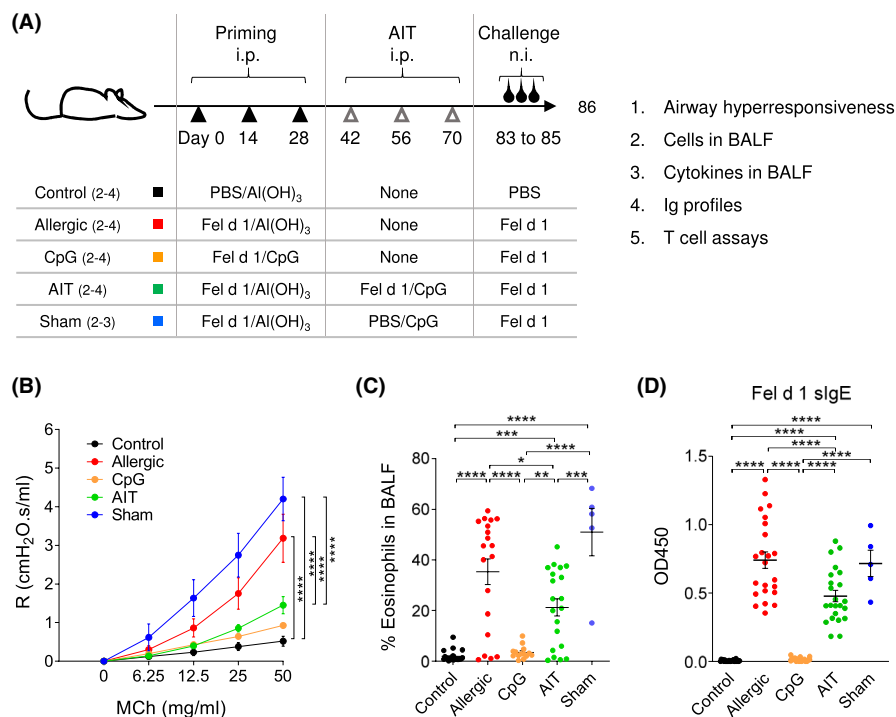
As dominant allergen in cat allergy, Fel d 1 is an ideal target for AIT.<sup>7</sup> Blocking Fel d 1 through passive immunotherapy by injecting a single dose of two monoclonal IgG4 antibodies successfully mitigated

acute symptoms in cat-allergic patients.<sup>8</sup> Consequently, novel approaches for cat AIT inducing a sustainable blocking antibody response against Fel d 1 appear to be promising strategies for long-term cure of cat allergy. In contrast, tolerance-inducing peptide AIT based on overlapping Fel d 1-derived T-cell epitopes,<sup>9-11</sup> which lacks induction of antibodies, was not superior over placebo in phase III trials.<sup>12</sup>

We thus hypothesized that the most effective cat AIT may be achieved by optimizing regulatory T- and B-cell responses with induction of blocking antibodies against Fel d 1 through immune adjuvants. CpG oligonucleotides, which signal via TLR9, were previously considered as a promising adjuvant candidate for AIT.<sup>13,14</sup> While most of the AIT studies attributed the immunotherapeutic modulation by CpG mainly to a switch from a Th2- to a Th1-type response,<sup>15</sup> some studies suggested a possible effect of CpG in AIT via Treg activity<sup>16,17</sup> through a TLR9-IDO cascade along the pDC-Treg axis. This activity is dose-dependent, with higher doses of CpG promoting immune tolerance and lower doses of CpG supporting Th1/Th17-driven inflammation.<sup>18</sup> This dose-dependence may be an explanation why AIT with CpG had only

limited success in previous trials in ragweed allergy, as the CpG doses used then were below the tolerance-promoting concentration range.<sup>19</sup> In general, B-type CpGs are considered safe in humans and have thus been tested as vaccine adjuvant for infectious diseases and cancer in multiple clinical trials.<sup>20</sup> Recently, a hepatitis B vaccine containing B-type CpG received FDA approval.<sup>21</sup>

Based on the above considerations, we sought to evaluate CpG as AIT adjuvant in a pre-clinical model of cat allergy, at a novel dose that is sufficiently high to favor the simultaneous engagement of the pDC-Treg and the B-cell axis.<sup>18</sup> In addition to a higher CpG dose, and to rule out any signal interference by a competing TLR ligand, we used sterile and endotoxin-free reagents, including Fel d 1 allergen, throughout the study in all injected solutions. Thus, we already mimicked the conditions of human AIT trials, where the use of endotoxin-free therapeutic allergens is mandatory. Using this well-defined pre-clinical model of AIT, we showed by multidimensional immune phenotyping including mass cytometry that a regulatory immune signature characterized



**FIGURE 1** Preventive (primary immunization) and curative (AIT) effects of CpG on airway hyper-reactivity. (A) Immunization regimens. Mice of the control group (black) were injected three times intraperitoneally (i.p., triangles) on D0, D14, and D28 with PBS Al(OH)<sub>3</sub> and got 3 nasal instillations (n.i., drops) on D83, D84, and D85 with PBS. The allergic mice (red) were sensitized to Fel d 1 by 3 i.p. with Fel d 1 Al(OH)<sub>3</sub> priming solution and were challenged by 3 n.i. with Fel d 1. The CpG-primed mice (orange) got 3 i.p. with Fel d 1 and CpG as adjuvant and 3 n.i. with Fel d 1. The specific immunotherapy group (AIT, green) were primed with Fel d 1 Al(OH)<sub>3</sub> and received 3 additional i.p. with Fel d 1 CpG-adjuvanted solution on D42, D56, and D70 and 3 n.i. with Fel d 1. The mice of the sham-treated group (blue) got 3 primary i.p. with Fel d 1 Al(OH)<sub>3</sub> and a second round of 3 i.p. with PBS CpG solution. Nasal instillation with Fel d 1 was also administered to this group. All the mice were sacrificed at D86 after analysis of the airway hyper-reactivity (Flexivent), for cell populations and cytokines secreted in BALF, immunoglobulin profile, and cytokines secreted in assays with lymph node cells. Mice were grouped as described in supplementary methods. (B) Airway resistance upon challenges with increasing concentrations of methacholine (0; 6.25; 12.5; 25 and 50 mg/ml). Results are means ± SEM. N = 5–13; significant *p*-values are indicated on the side of the graphs. (C) Proportions (%) of eosinophils among living cells detected in BALF of the five types of immunized mice according to Gr1<sup>+</sup>CD11c<sup>-</sup> gating. Results are means ± SEM, N = 5–21, significant variations between a group of mice and the others are indicated above the groups (\**p* < .05; \*\**p* < .01; \*\*\**p* < .001; \*\*\*\**p* < .0001). (D) Relative quantification (OD 450 nm) of Fel d 1 specific immunoglobulins E (sIgE) in the sera of the immunized or treated mice by ELISA

by expression of TNF receptor 2 (TNFR2) was induced de novo, regulatory T- and B-cell immune responses were activated and all major hallmarks of the allergic response were reverted. Our results demonstrate that B-type CpG adjuvant, when applied at higher doses than previously suggested for AIT, has the ability to induce tolerance toward an allergen. This capacity of CpG was evaluated in a pre-clinical model under endotoxin-free conditions, which correspond to the conditions of clinical trials, and via the subcutaneous injection route commonly used in patients. Based on our data, CpG merits reconsideration as AIT adjuvant in humans.

## 2 | METHODS

### 2.1 | Mice and immunization protocols

Eight-week-old female BALB/c OlaHsd mice were obtained from Envigo and kept in a specific pathogen-free animal facility with unlimited access to food and water. Animal handling procedures met the European directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the National Animal Research Authority. The allergic sensitization and main AIT protocol are depicted in Figure 1 and detailed in the Methods section of this article's Online Repository. To note, endotoxin-free reagents and buffers were used for the preparation of solutions for injections in mice, in order to prevent co-activation of any other TLR than TLR9, and to mimic the Good Manufacturing Practice (GMP) conditions required for human testing.

LoTox™ Natural Fel d 1 (nFel d 1, LPS content <0.03 EU/μg; Indoor Biotechnologies) was used in immunizations and in cell cultures. The CpG oligonucleotide with the optimal murine B-Class motif (5'-tccatgacgttctctgatgct-3') was from Sanbio. The high-dose CpG corresponds to an injection of 21 μg CpG in a final volume of 200 μl per injection. The thermosensitive hydrogel used for subcutaneous AIT injections was synthesized as described in the Methods section of this article's Online Repository at a lactic acid/glycolic acid molar ratio of 15/1 and a PEG mass ratio of 30%.

### 2.2 | Ig profiles by ELISA

For Fel d 1-specific antibody analyses, the serum of each mouse was tested individually. Recombinant Fel d 1 was coated at a concentration of 0.5 μg per 100 μl PBS in Maxisorp 96-well plates (Thermo Fischer Scientific). Sera were analyzed at dilutions of 1/250 for IgA, IgE, and IgG3; 1/10,000 for IgG2a and 1/800,000 for IgG1.

### 2.3 | Cytokine measurements

IL-4, IL-5, IL-6, IL-10, IL-13, IL-17a, IFN-γ, and TNF-α were measured in BALF by Cytometric Bead Array (CBA; Becton Dickinson, limits of

detection 10–2500 pg/ml). Data were recorded on a Fortessa flow cytometer (Becton Dickinson) and analyzed with FCAP array software. Similarly, supernatants of DC-T cell cultures were collected at day 4 to assess the concentration of IL-5, IL-6, IL-10, IL-13, IL-17a, and IFN-γ. Active TGF-β secreted in the BALF was measured separately with Human/Mouse TGF-β 1 Ready-SET-Go ELISA (Thermo Fisher Scientific, limits of detection 8–1000 pg/ml).

### 2.4 | Cell preparation for mass cytometry

Mice were sensitized as for the main AIT experiments (see Figure 1), comparing three conditions: allergic, AIT, and untreated healthy control. Mass cytometry of isolated cells was performed at day 43 (D43, early events) 24 h after the first AIT injection, or on D86 (late events), after the third and final allergen challenge (Figure S5A). Cells were recovered from the peritoneal cavity (PC), the spleen, and the mediastinal lymph nodes (MLN) on D43; and from the spleen, the MLN, and the lungs on D86, as described in the Methods section of this article's Online Repository.

### 2.5 | Cellular staining for mass cytometry

All the markers used for the staining are listed in Table S1 (for further details, see Methods section of this article's Online Repository, together with staining protocols). Most of the markers were from Fluidigm, except for a few antibodies that were self-stained using the Maxpar® Metal-Labeling Kit (Fluidigm) as detailed in Table S1.

### 2.6 | Data acquisition and processing for mass cytometry

On every acquisition day, the mass cytometer (HELIOS; Fluidigm) was calibrated using CyTOF Tuning Solution (Fluidigm) and calibration beads (Maxpar Four Elements EQ Beads; Fluidigm) to ensure reproducibility between the different acquisition runs on different days. Samples were run between 1 and 3 days after the staining at a concentration of  $0.5 \times 10^6$  cells/ml and a flow of 30 μl/min. The integrated mass data (IMD) files generated were transformed and normalized using CyTOF software v6.5 (Fluidigm) to FCS files (Figure S5B).

### 2.7 | Statistics and bioinformatics tools

Data collected for BALF cell populations, BALF cytokines, lung airway resistance, and Ig concentrations were compared with each other using one-way ANOVA and Bonferroni tests. Cytokine concentrations obtained from DC-T cell assay supernatants were normalized for each of the six independent experiments to the values obtained for the allergic mice when stimulated with the F1.4 main

Fel d 1 epitope. The comparison was made by 2-way ANOVA and Bonferroni tests. For the CyTOF data, the normality in the distribution of the different groups was first tested using standard normality tests and methods (Shapiro-Wilk test, Kolmogorov-Smirnov test, and visual support via non-quantitative QQ plot graphical observation). Unless otherwise stated, the data followed a normal distribution, and the parametric general linear model (GLM) integrated in R package `lima_3.34.9` was applied. This differential analysis was applied to both population abundance as well as marker expression.<sup>22</sup> *p*-values were corrected according to the number of variables analyzed.<sup>22</sup> When data failed the normality test but groups showed equal variance, the non-parametric Kruskal-Wallis test was applied (indicated in the legend). When data failed both the normality test and the equal variance test, neither parametric nor non-parametric tests were applicable, and no statistical indications were displayed

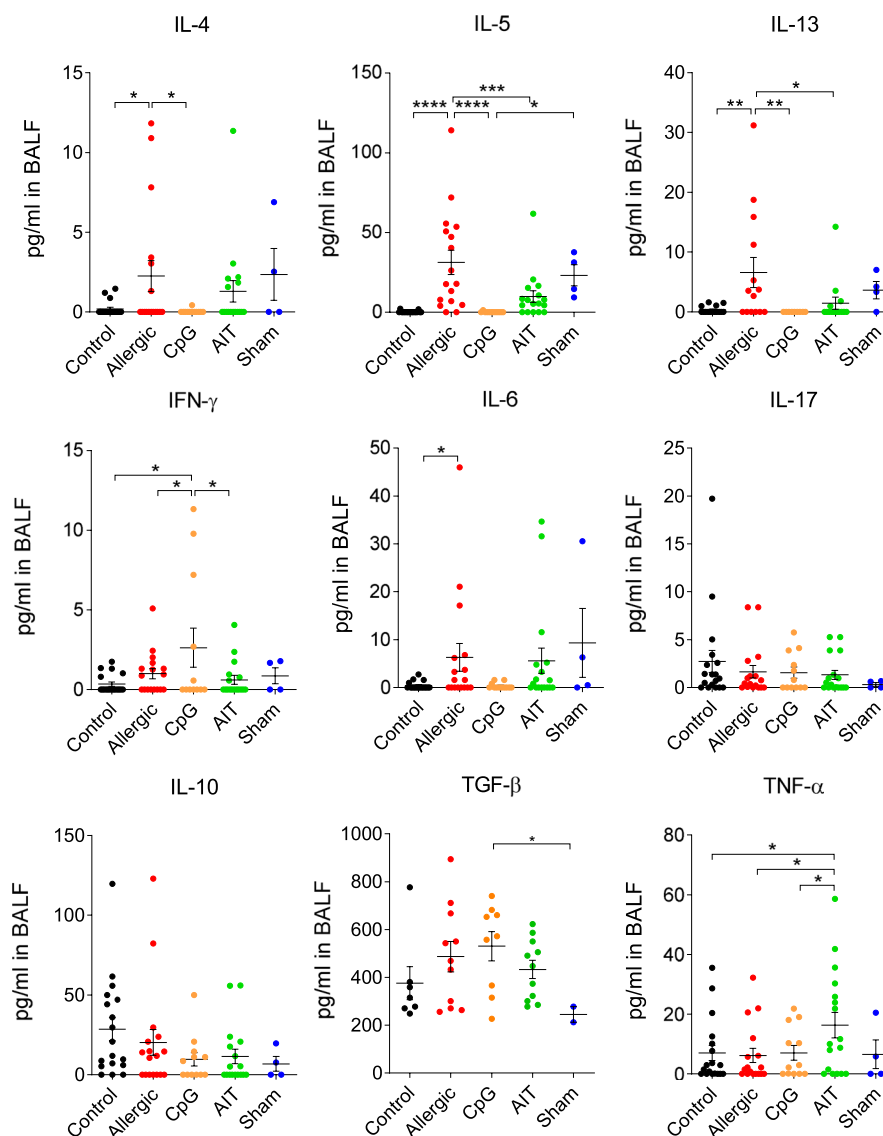
on the graph (indicated in the legend). Significant *p*-values indicated in all graphs correspond to \**p* < .05; \*\**p* < .01; \*\*\**p* < .001; and \*\*\*\**p* < .0001.

Full methods are provided in online Appendix S1.

### 3 | RESULTS

#### 3.1 | Fel d 1-specific AIT with high-dose CpG adjuvant efficiently improves airway parameters and promotes changes in serum antibody profiles

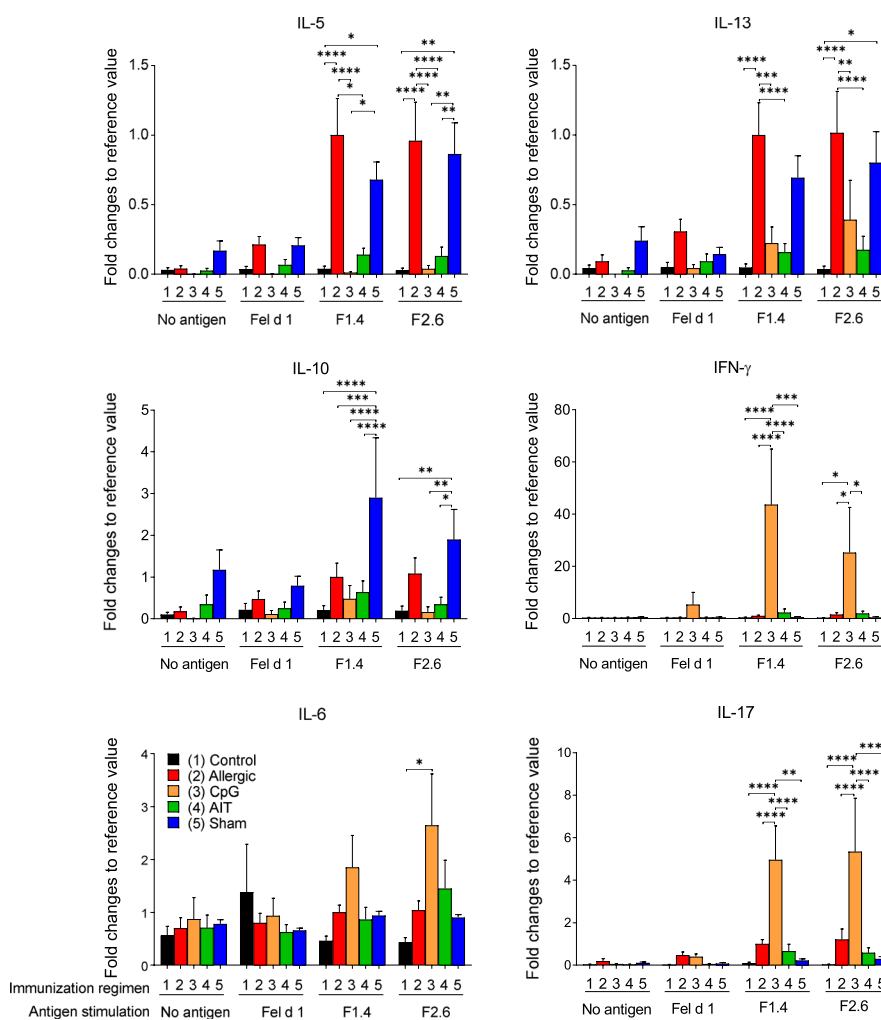
Based on initial results confirming that CpG adjuvant used at high doses primarily induces a regulatory cytokine (IL-10) response (Figure S1A-C),<sup>18</sup> we designed experiments as illustrated in



**FIGURE 2** Reduction of Th2-type cytokines and absence of classical regulatory cytokines in BALF upon AIT. IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFN-γ, and TNF-α cytokines were measured via BD™ CBA. TGF-β was measured by ELISA. Results are means ± SEM, *N* = 5–18 (TGF-β, *N* = 2–11); significant variations between a group of mice and the others are indicated above the group (\**p* < .05; \*\**p* < .01; \*\*\**p* < .001; \*\*\*\**p* < .0001)

Figure 1A. This pre-clinical AIT model is characterized by (a) high concentration of B-type CpG adjuvant; (b) use of endotoxin-free allergen; and (c) focus on the most relevant cat allergen Fel d 1. To assess AIT with Fel d 1 and high-dose CpG (Fel d 1/CpG), we first analyzed airway hyper-responsiveness after allergen and methacholine challenge (Figure 1B). AIT with Fel d 1/CpG significantly improved lung resistance to a level of non-allergic mice. As expected, allergic mice that received either no AIT or sham AIT with CpG only had the highest airway resistance. Of note, mice that were primed with Fel d 1/CpG in a preventive vaccination approach showed no difference in lung function values compared with healthy controls. These results fully matched with airway eosinophilia in BALF (Figure 1C). None of the groups developed neutrophilic or other inflammatory airway responses (Figure S2A). Cellular BALF results correlated with histological analyses of lungs, showing a reduction in inflammation and mucus production in AIT-treated compared with allergic mice (Figure S2B). Fel d 1-specific antibodies were measured at the end

of AIT after allergen re-exposure (D86). Allergic mice showed the highest Fel d 1-specific IgE levels. The levels of sIgE were lower in the AIT-treated than in the allergic group (Figure 1D). AIT-treated mice were also distinguishable by higher levels of sIgA and sIgG1 (Figure S3). Fel d 1/CpG-primed mice, similarly to control mice, synthesized very low levels of Fel d 1-specific IgE, IgG1, and IgA. In line with TLR9 ligand effects, AIT-treated and Fel d 1/CpG-primed mice showed a stronger IgG2a response (Figure S3). The sham group showed antibody profiles similar to the allergic group. Thus, Fel d 1/CpG-AIT induced changes in antibody profiles, such as a modified Th2 response with reduced IgE and increased IgG1 together with elevated sIgA and sIgG2a. The investigation of cytokines in the BALF further underlined the strong anti-inflammatory effect induced by AIT with Fel d 1/CpG, with a significant reduction of Th2 cytokines (IL-4, IL-5, IL-13), but no increase in Th1 (IFN- $\gamma$ ) or Th-17 cytokines (IL-6, IL-17; Figure 2). Priming with Fel d 1/CpG induced an IFN- $\gamma$  response (Figure 2). Somewhat unexpectedly, while the regulatory



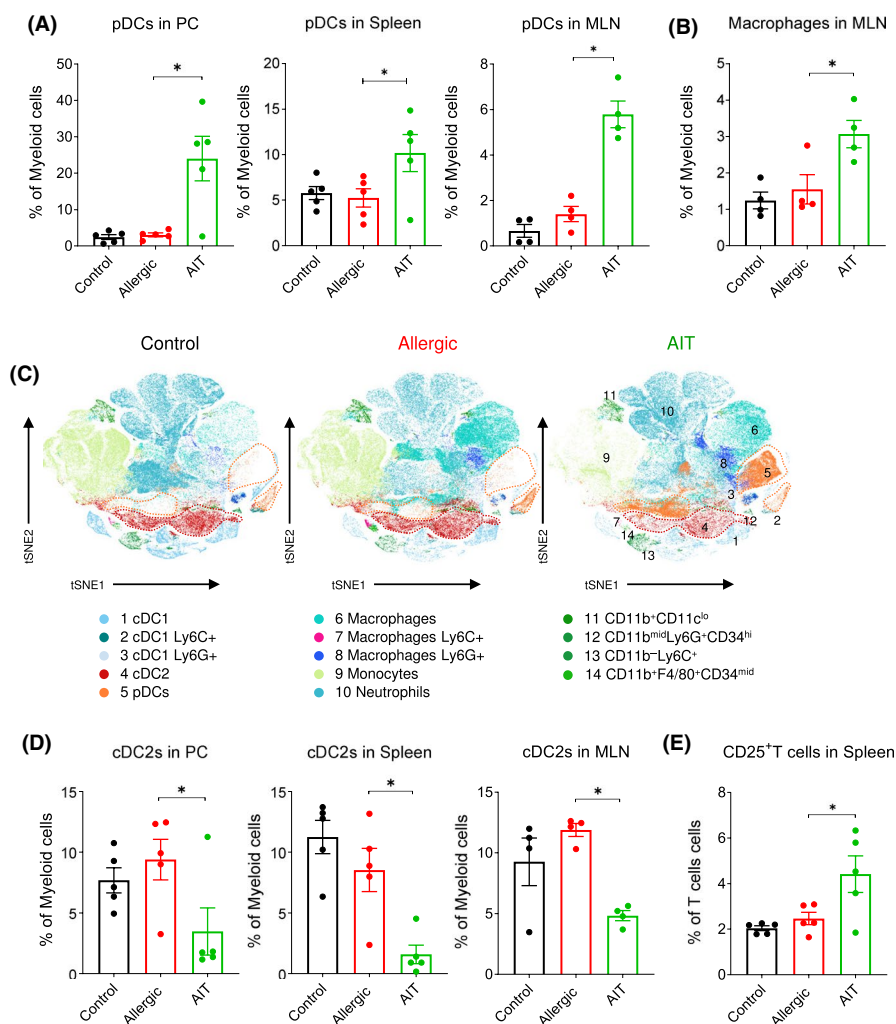
**FIGURE 3** Cytokine profiles obtained in dendritic cell-T-cell assays with T cells isolated from mice of the different groups. IL-5, IL-13, IL-10, IFN- $\gamma$ , IL-6, and IL-17 secreted by cells from mediastinal lymph nodes of each group of mice upon re-stimulation by dendritic cells pulsed with no antigen, Fel d 1 or its 2 major epitopes, F1.4 and F2.6, were measured in the supernatants by BD™ CBA. Data from six independent experiments were normalized to the value obtained from cells isolated from allergic mice stimulated by F1.4 peptide (most reactive epitope in allergic mice). Shown are fold changes (mean  $\pm$  SEM),  $N = 5-18$ , significant variations (2-way ANOVA followed by Bonferroni multiple comparison) between a group of mice and the others are indicated above the group (\* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ ; \*\*\*\* $p < .0001$ )

cytokines IL-10 and TGF- $\beta$  were not differentially regulated by AIT, we observed a moderate, but significant increase of TNF- $\alpha$  in the BALF upon AIT (Figure 2). Together, these results indicated a therapeutic effect with reduced Th2 airway inflammation and bronchial hyper-responsiveness conferred by AIT with Fel d 1/CpG in a pre-clinical model of cat allergy.

### 3.2 | Induction of distinct Fel d 1-specific T-cell responses by high-dose CpG under AIT or vaccination conditions

Having shown that AIT with Fel d 1/CpG can effectively reduce allergic airway responses (Figures 1 and 2), we further assessed the immune mechanisms by analyzing effector T cells isolated

from the lung-draining MLN (D86). MLN cells were co-cultured with bone marrow-derived DCs from naïve mice, which were pre-pulsed with either Fel d 1 or with two dominant T-cell epitopes from Fel d 1 (F1.4, F2.6, see Figure S4). While cytokine secretion was very low in control mice, cells from allergic mice showed a Th2-type response with prominent IL-5 and IL-13 secretion, especially when re-stimulated with the two peptides (Figure 3). AIT with Fel d 1/CpG completely abolished this Th2 bias, but induced only insignificant IL-10 secretion, which is in accordance with BALF results (Figure 2). In addition, no differential regulation of IFN- $\gamma$ , IL-6, and IL-17 was detectable in the AIT group, suggesting that AIT with Fel d 1/CpG is capable of controlling the Th2 response without engaging Th1 or Th17 activity. Quite strikingly, however, the vaccination-like priming of naïve mice with Fel d 1/CpG induced a strong IFN- $\gamma$  and IL-17 signal with moderate IL-6,



**FIGURE 4** Deep analysis of cell changes induced by the first AIT injection. Mass cytometry analysis of early events revealed high APC activation and a concomitant T-cell activation by the treatment. (A) Ratio of pDC in the myeloid compartment in PC, spleen, and MLN of the three groups of mice. (B) Ratio of macrophages in the myeloid compartment in MLN. The non-parametric Kruskal-Wallis statistical test was applied as normality tests failed. (C) t-SNE graphic representation of the myeloid compartment in the PC of control, allergic, and AIT-treated mice. Dotted lines surround the cDC2 and pDC subpopulations. (D) Ratio of cDC2 in the myeloid compartment of PC, spleen (using non-parametric Kruskal-Wallis statistical test in both cases) and MLN of the three groups of mice. (E) Ratio of CD25<sup>+</sup> CD4<sup>+</sup> T cells in the spleen T-cell compartment. Results are means  $\pm$  SEM,  $N = 4-5$ ,  $p$ -values indicated for comparison Allergic-AIT

suggesting a mixed Th1/Th17 response. Sham-treated mice expressed a Th2 response similar to allergic mice, but with a stronger IL-10 secretion (Figure 3). Together, these T-cell cytokine results indicated that the Th2 bias of allergic mice could be reverted by AIT with Fel d 1/CpG. However, vaccination-like priming of naïve mice with Fel d 1/CpG induced an allergy-protective Th1/Th17 signature, while no such signature was detectable under the Th2-modifying AIT conditions.

### 3.3 | Fel d 1-specific AIT with high-dose CpG adjuvant induces an early myeloid and regulatory lymphocyte response

To further analyze the regulatory immune mechanisms behind the successful Fel d 1/CpG-based AIT, we designed high-dimensional immune phenotyping experiments using mass cytometry with subsequent unsupervised data analysis of three groups (Figure S5). Immune cells were collected from three anatomical sites early (PC, spleen, and MLN) and late (spleen, MLN, and lungs) in AIT. A panel of 34 phenotypic and functional markers was applied (Tables S1 and S2). Early in AIT (D43), the ratio of plasmacytoid dendritic cells (CD11b<sup>+</sup> CD11c<sup>+</sup> CD317<sup>+</sup> pDCs; complete definition see Table S2) was increased in all analyzed tissues of AIT-treated mice (Figure 4A), and the proportion of macrophages elevated in MLN (Figure 4B). For a complete visualization of AIT-induced cellular changes at the injection site (peritoneal cavity, PC), a t-SNE analysis of the myeloid cell compartment (CD3<sup>-</sup> CD19<sup>-</sup> CD49b<sup>-</sup> cells) was performed (Figure 4C). Parallel to the increase in pDCs, we observed a reduction of cDC2 cells in all tissues (Figure 4C,D). Since successful AIT has been associated with an early involvement of regulatory T cells (Tregs),<sup>23</sup> we analyzed for CD25<sup>+</sup> T cells and found them expanded in the spleen of AIT-treated mice (Figure 4E), but not in the PC or in MLN (Figure S6A). Furthermore, CD11b<sup>+</sup> B1 regulatory cells (Bregs), as previously described,<sup>24</sup> were found to be increased in the PC of the AIT group, but not in spleen or MLN (Figure S6B). These data showed that 24 h after the first AIT injection with Fel d 1/CpG, the ratio of Th2-promoting cDC2s was reduced, both locally and systemically, in favor of a tolerance-promoting DC environment dominated by pDCs. These changes promoted an early activation of T- and B-regulatory lymphocyte responses.

### 3.4 | Fel d 1-specific AIT with high-dose CpG adjuvant induces an early and sustained protective immune response with a characteristic TNF receptor-2 signature

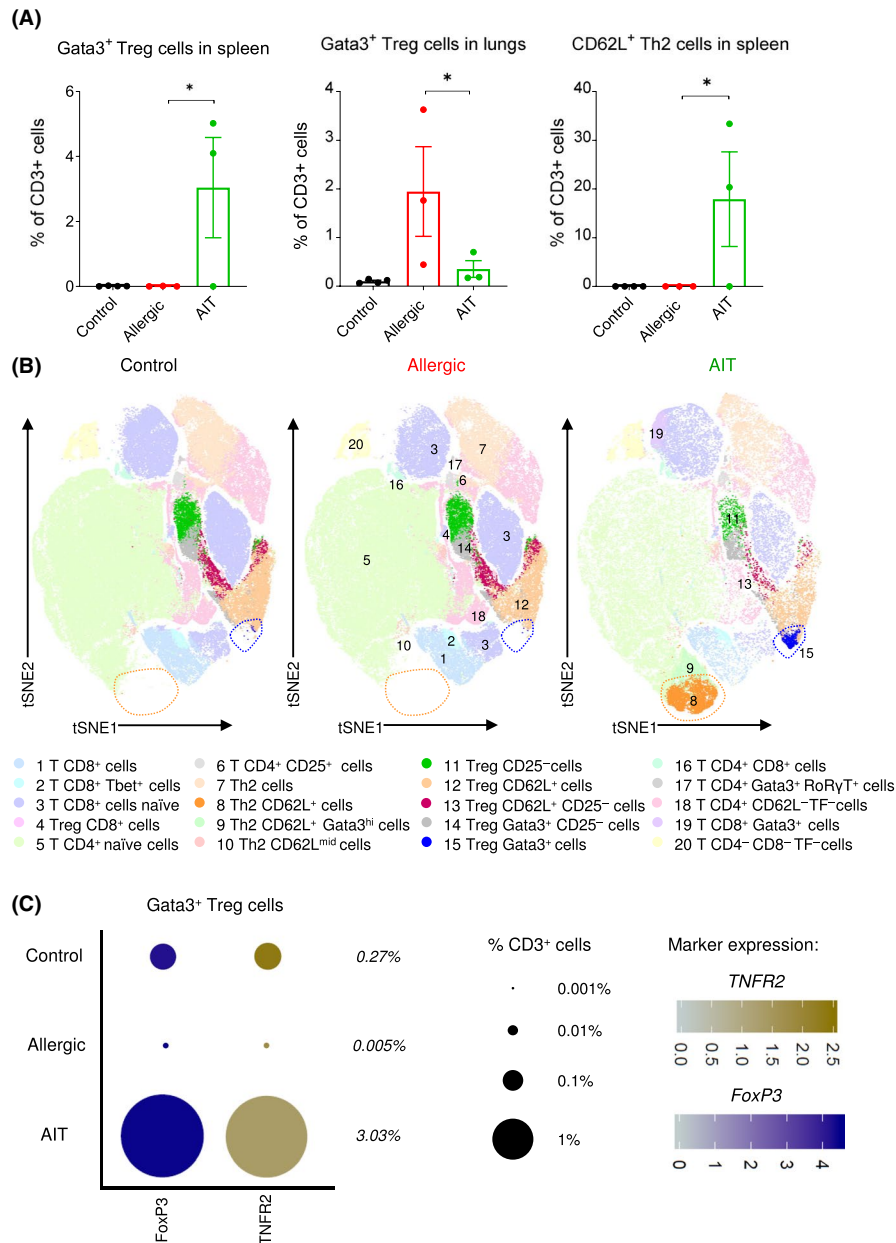
We detected an unexpected increase in the secretion of TNF- $\alpha$  in the BALF of AIT-treated mice (Figure 2). Since the TNF/TNFR2 signaling cascade has already been demonstrated to be a key pathway for immune tolerance in autoimmunity<sup>25</sup> and for tumor-specific

immunosuppression,<sup>26</sup> we explored this immunoregulatory axis further. CyTOF data were analyzed for expression of TNF- $\alpha$  and its receptors TNFR1/TNFR2 at D43 (Figure S7). TNF- $\alpha$  was almost exclusively secreted by NK cell subtypes in AIT-treated mice only. Activated CD62L<sup>-</sup> NK cells in the spleen of AIT-treated mice seemed to be particularly relevant. Although their overall ratio was not increased, these cells expressed significantly higher levels of TNF- $\alpha$  and showed higher activation as evidenced by CD69 expression (Figure S8A). The ratio of Tbet<sup>+</sup> NK cells was increased in the PC and the MLN after AIT (Figure S8B), and Tbet<sup>+</sup> NK cells expressed more TNFR2 (Figures S7 and S8C). An augmentation of TNFR2 expression was also found for the increased CD11b<sup>+</sup> B-cell population (Figure S6B) in the PC of AIT-treated mice (Figures S7 and S8D), thus indicating the presence of B cells with regulatory potential early in AIT.<sup>27</sup> Together, these data demonstrated that an early TNF- $\alpha$  response was induced by AIT with Fel d 1/CpG in pDCs, NK cells, and Bregs, which only involved the tolerance-promoting TNFR2 and not the inflammatory TNFR1 axis.

To shed light on the sustained effects of AIT with Fel d 1/CpG, we analyzed tissues by mass cytometry at the end of AIT (D86). These analyses confirmed that AIT reduced all effector cells of the allergic response in the lungs to levels close to controls (Figure S9A,C,D). In addition, Th2 cells were significantly reduced in the lungs and in MLN (Figure S9B). CyTOF analyses also showed a modulation of the IgE-Fc $\epsilon$ R1 signaling axis by AIT with Fel d 1/CpG, with lower overall Fc $\epsilon$ R1 expression (Figure S10A), and less total Fc $\epsilon$ R1-positive cells and basophils in the lungs (Figure S10B). Furthermore, we observed a reduction of plasma cells (CD45R<sup>-</sup> B cells) in the lungs upon AIT (Figure S10C). No Th17 polarization was seen in the lungs (Figure S11A) or other tissues analyzed.

Next, we focused on AIT-induced FoxP3<sup>+</sup> Tregs and observed no changes in the conventional CD4<sup>+</sup> CD25<sup>+</sup> Treg cluster (Figure S11B). However, when analyzed for co-expression of other master transcription factors, AIT-treated mice displayed an increased ratio of GATA3<sup>+</sup> and FoxP3<sup>+</sup> double-positive Tregs in the spleen. These double-positive Tregs (biTregs<sup>28</sup>) were very low in control or allergic mice (Figure 5A-C). The biTreg subset has been reported to be specifically equipped for counterbalancing effector cell responses in general<sup>29</sup> and of the Th2 type in particular.<sup>30</sup> Of note, GATA3<sup>+</sup> FoxP3<sup>+</sup> biTregs were also increased in the lungs of allergic mice, most likely as a consequence of allergic inflammation in the effector organ, but were reduced after AIT to a very low level comparable to control mice (Figure 5A). Another T-cell population emerging de novo under AIT with Fel d 1/CpG was a subset of non-activated, naïve-like CD62L<sup>+</sup> Th2 cells (Figure 5A,B). Both emerging clusters, GATA3<sup>+</sup> FoxP3<sup>+</sup> biTregs and CD62L<sup>+</sup> Th2 cells, were clearly detectable in the t-SNE plot of splenic T cells (Figure 5B, clusters #8 and #15), as were other regulated T-cell clusters (Figure S11C). A split dot plot analysis showed that the biTreg cluster #15 was upregulated 200-fold by AIT with Fel d 1/CpG as compared with the allergic group and expressed high levels of FoxP3 and TNFR2 (Figure 5C). GATA3<sup>-</sup> Tregs showed an inverse pattern with lower FoxP3 and very low



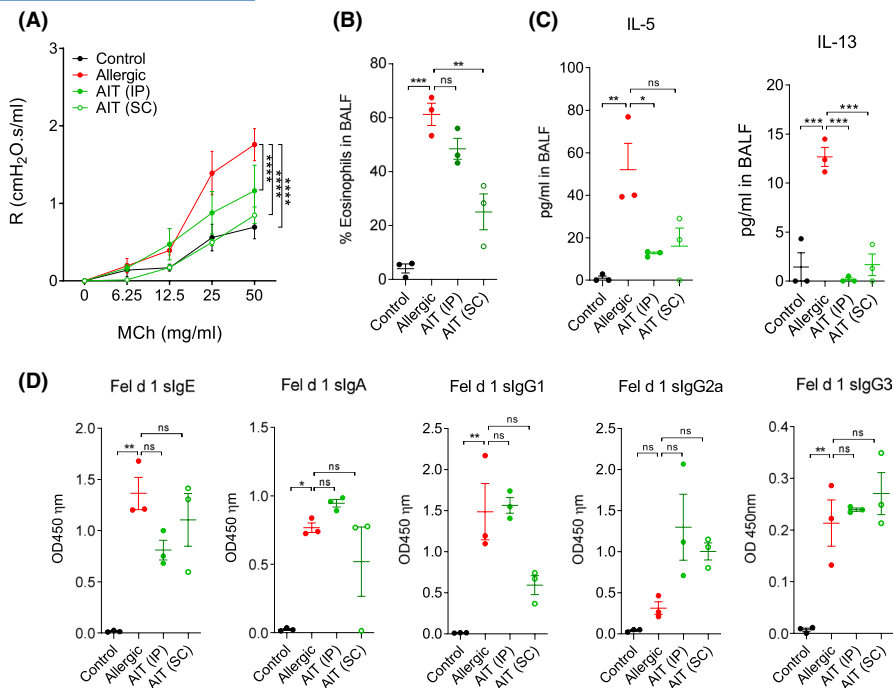


**FIGURE 5** CyTOF analysis of the late events highlighted a shift in the Treg-cell compartment. (A) Ratio of GATA3<sup>+</sup> Tregs in the spleen and the lung and CD62L<sup>+</sup> Th2 in the T-cell compartment in the spleen of the three groups of mice. Results are means  $\pm$  SEM,  $N = 3-4$ ,  $p$ -values indicated for comparison Allergic-AIT. (B) t-SNE projection of the T-cell compartment of the spleen. Dotted lines surround the CD62L<sup>+</sup> Th2 (#8, orange) and GATA3<sup>+</sup> Treg (#15, blue) cells that arose upon AIT treatment. Although two other emerging subpopulations (Th2 CD62L<sup>+</sup> GATA3<sup>hi</sup>, in green, #9, and CD8<sup>+</sup> GATA3<sup>+</sup> naïve cells, in violet, #19) were discernable on the t-SNE projection, the differences between the three groups of mice (control, allergic, and AIT-treated) were not significant. The CD25<sup>-</sup> Treg (dark green #11) and CD62L<sup>+</sup> CD25<sup>-</sup> Treg (fuchsia, #13) subpopulations were significantly reduced (Figure S11C). (C) Split dot plots of GATA3<sup>+</sup> Treg cells from the spleen of the control, allergic and treated mice on day 86. The size of the dots is proportional to the ratio (%) of the GATA3<sup>+</sup> Treg population among splenic CD3<sup>+</sup> T cells. Results are means,  $N = 2-4$ . The proportion of GATA3<sup>+</sup> Tregs is strongly increased in the treated mice. The color of the plots reflects the expression of FoxP3 or TNFR2 (MMI: mean metal intensity), both concomitantly expressed in these specific GATA3<sup>+</sup> Tregs

TNFR2 expression (Figure S11D). These data indicated that the positive effect of AIT with Fel d 1/CpG in controlling allergic inflammation coincided with major changes in Treg subpopulations, both locally and on a systemic level, amongst which the emergence of splenic GATA3<sup>+</sup> FoxP3<sup>+</sup> biTregs, expressing high levels of the immune tolerance checkpoint receptor TNFR2, was the most striking finding.

### 3.5 | Subcutaneous Fel d 1-specific AIT with high-dose CpG adjuvant in a hydrogel-based delivery system successfully reverts major hallmarks of allergy

The ease of accessibility for immune cell analyses at the AIT injection site led us to investigate the effects and mechanisms of Fel d 1-specific AIT with high-dose CpG adjuvant first in a model using the intraperitoneal



**FIGURE 6** Adaptation to subcutaneous injection for Fel d 1/CpG specific AIT. Mice of the AIT (i.p.) group received 3 AIT intraperitoneal injections. Mice of the AIT (s.c.) group received 3 subcutaneous injections with the AIT solution mixed with thermogelling hydrogel. (A) Airway resistance upon challenges with increasing concentrations of methacholine (0; 6.25; 12.5; 25; and 50 mg/ml). (B) Proportions (%) of eosinophils among living cells detected in BALF of the four types of immunized mice according to Gr1<sup>+</sup> CD11c<sup>-</sup> gating. (C) Reduction of Th2 type cytokines IL-5 and IL-13 in the BALF. (D) Immunoglobulin profile (Fel d 1 specific-IgE, -IgA, -IgG1, -IgG2a, -IgG3) upon sensitization to Fel d 1 and AIT (i.p. or s.c. with hydrogel). Results are means  $\pm$  SEM,  $N = 3$ , significant variations between a group of mice and the others are indicated (\* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ ; \*\*\*\* $p < .0001$ )

(i.p.) route for AIT, which showed that the allergic phenotype could be successfully reverted. To translate these findings forward to human application in a pre-clinical setting, we developed a delivery system that allowed for subcutaneous (s.c.) injection of Fel d 1-specific AIT with high-dose CpG and compared its effect with i.p. AIT (experimental design, Figure S12). Aiming to maintain a high local concentration of CpG and Fel d 1 allergen at the injection site, the Fel d 1/CpG-containing AIT solution was mixed with a triblock copolymer hydrogel (NMR structure, Figure S13) that is thermo-sensitive and forms a gel upon temperature increase after injection. The functional lung data indicated that s.c. injection of AIT in the hydrogel induced a significant improvement of lung resistance to the level of non-allergic control mice, comparable with i.p. AIT (Figure 6A). In addition, airway eosinophilia was reduced in the s.c. AIT group, even more pronounced than in the i.p. AIT group (Figure 6B). The anti-allergic effect of s.c. AIT with Fel d1/CpG in the hydrogel was also evident on the level of Th2 cytokine secretion. IL-5 and IL-13 were equally reduced in both AIT-treated groups (Figure 6C). Interestingly, s.c. AIT induced an antibody response slightly different than i.p. AIT, with less modulation of sIgE, sIgG1, and sIgA, but a similar increase of sIgG2a and sIgG3 (Figure 6D). Altogether, these data suggested that Fel d 1-specific AIT with high-dose CpG adjuvant, for which we have provided a high-dimensional analysis of the underlying immune mechanisms in an i.p. injection AIT model, could be successfully adapted to a hydrogel delivery system in a pre-clinical s.c. AIT model optimized for future use in translational studies.

## 4 | DISCUSSION

In this study, we evaluated a novel role of CpG as tolerance-inducing adjuvant for AIT in a pre-clinical model of cat allergy. We used a CpG dose that is sufficiently high to favor the simultaneous engagement of the pDC-Treg and the B-cell axis<sup>18</sup> and that has so far not been evaluated for AIT. Another novelty of our study was that we used endotoxin-free Fel d 1 as therapeutic allergen throughout, to rule out interference with the TLR9 signaling cascade targeted by CpG through the presence of other TLR ligands. All the major hallmarks of the allergic response were reverted by AIT combined with high-dose CpG and a regulatory immune signature was induced de novo.

The dose of CpG used for successful AIT with Fel d 1 in our pre-clinical model of cat allergy corresponds to the maximum dose of B-type CpG tolerated in humans<sup>31</sup> and is in the range of tolerance-inducing CpG concentrations reported.<sup>18</sup> An integrated transcriptomic and proteomic data set showed that human pDCs, in contrast to other human DCs, lack caspase-1 and express low levels of other inflammasome proteins, thus being unable to mount an IL-1 $\beta$  response.<sup>32</sup> Thus, B-type CpG might generate very limited systemic adverse effects in humans. In our study, pDCs were one of the major myeloid cell subsets regulated early on by Fel d 1-specific AIT with high-dose CpG. Based on the favorable safety profile of B-type CpG immune adjuvants, multiple clinical trials in cancer and infectious diseases have been initiated.<sup>20,21,31</sup> In hepatitis B prevention, a

vaccine with B-type CpG adjuvant showed superior seroprotection compared to an alum-based vaccine, which led to the recent FDA approval of a first vaccine containing CpG adjuvant for hepatitis B.<sup>21</sup> These safety considerations together with our pre-clinical results in a murine model of cat allergy support a reconsideration of B-type CpG as AIT adjuvant. Our data showed that B-type CpG, when applied at higher doses than previously suggested for AIT<sup>13</sup> and in a pre-clinical setting that already anticipates the conditions of human trials, is very effective in inducing allergen-specific immune tolerance. Although it has been occasionally suggested that CpG alone without allergen could be sufficient for immunotherapy of allergic diseases,<sup>33</sup> our results indicated no curative effect by CpG in the absence of allergen. Thus, cat-allergic patients with medium to severe rhinitis and/or asthma could be targeted by this novel approach via Fel d 1-specific AIT with high-dose CpG adjuvant in a thermosensitive hydrogel drug delivery system that allows for subcutaneous AIT injection of Fel d 1 and CpG. The hydrogel is based on biodegradable thermogelling PLGA-PEG-PLGA triblock polymers,<sup>34</sup> which are in a dissolved liquid state at lower temperatures and rapidly transform into a gel as the temperature increases after injection. Biodegradable thermogelling PLGA-PEG-PLGA triblock polymer hydrogels, which have already been used in clinical trials for other medical indications, can serve as a depot for high concentrations of allergens and immune adjuvants, allowing a sustained release of components over several days to induce efficient APC-T cell priming responses.<sup>35,36</sup>

One major novel finding of our study was that the immune response induced by B-type CpG adjuvant varies according to whether it is applied in a vaccination-like approach under naïve immune conditions or under already established Th2-driven allergic conditions, such as in AIT. Surprisingly, while priming naïve mice with Fel d 1/CpG induced an allergy-protective Th1/Th17 profile, a regulatory signature with characteristic TNFR2 expression along a pDC-NK cell-Breg-Treg axis was detectable under the Th2-modifying AIT conditions, but no Th1/Th17 response as described by others in an AIT model using CpG.<sup>37</sup> These differential results allow major novel insights for the future design of preventive and curative allergy vaccines using CpG adjuvant. Similar to what has been suggested by others,<sup>38</sup> we propose that the use of endotoxin-free Fel d 1 prevented TLR4-driven co-inflammatory responses through LPS, thus allowing the exclusive induction of a tolerance-promoting cascade via CpG and TLR9.

In our study, we found a significant increase of pDCs at the AIT injection site and in the lymphoid system early in AIT with CpG. Plasmacytoid DCs are able to skew naïve CD4<sup>+</sup> CD25<sup>-</sup> T cells toward CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> Tregs,<sup>16</sup> which we confirmed by an increase in Tregs. CpG can also provoke B cells to proliferate, to secrete IL-10 via CD11b<sup>+</sup> Breg cells, and to differentiate into plasma cells and memory B cells.<sup>15</sup> Despite detecting an increase in Bregs, we were unable to measure changes in IL-10 or TGF- $\beta$  secretion *in vivo*, although both cytokines have been suggested to mediate tolerance in AIT<sup>18,39</sup> and protective effects of CpG.<sup>16</sup> During the early phase of CpG-based AIT, we found a rapid induction of NK cells with a TNF- $\alpha$ /TNFR2 profile. Although their role in AIT has not been investigated in depth, NK cells, as early providers of cytokines, were suggested to play a role in

allergen-specific immune suppression by skewing the T-cell response through changing the cytokine milieu.<sup>40</sup> Our data indicated that AIT with Fel d 1/CpG induces an early TNF- $\alpha$ -driven response that regulates the immune tolerance-inducing TNFR2 and not the pro-inflammatory TNFR1 axis. Via TNFR2, TNF- $\alpha$  promotes the proliferation, differentiation, and suppressive capacity of Tregs.<sup>41,42</sup> Indeed, we observed a unique CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>hi</sup> GATA3<sup>+</sup> Treg subpopulation that was also high in TNFR2 and appeared *de novo* upon AIT. It has been suggested that the co-expression of IRF4 or GATA3 in FoxP3<sup>+</sup> Tregs is associated with superior suppressive capacity toward Th2 effector cells.<sup>29,43,44</sup> In addition, the co-expression of GATA3 and FoxP3 in Tregs is key in regulating intestinal immune tolerance<sup>45</sup> and in suppressing pro-fibrotic immune response in the skin.<sup>46</sup>

In summary, this study investigated for the first time the potential of a higher dose of B-type CpG in successfully modulating the allergic response to Fel d 1 in a pre-clinical AIT model of cat allergy and analyzed the underlying immune mechanisms at endotoxin-low conditions similar to human AIT trials. One of the key immune cells activated early in CpG-driven immune responses are pDCs, which represent a rare population of circulating cells, normally absent from peripheral tissues, including the skin. They can, however, rapidly invade the skin, both in humans and mice, and sense nucleic acids through TLR7 and TLR9.<sup>47</sup> Thus, the successful transfer of Fel d 1-specific AIT with high-dose CpG adjuvant to a thermo-sensitive hydrogel delivery system, already optimized for future subcutaneous use in possible human trials, has the potential to be further exploited for inducing allergen-specific tolerance in patients. An important deliberation in the development of any novel AIT for cat allergy will be the selection of allergens for AIT. Although crude cat dander extracts are complex mixtures containing at least 8 allergens,<sup>48,49</sup> it was recently shown that tackling cat allergy by targeting the dominant cat allergen Fel d 1 through passive antibody immunotherapy is a valuable strategy.<sup>8</sup> Other novel approaches for AIT in cat allergy also rely on a Fel d 1 only.<sup>50,51</sup> We demonstrated here that a molecular AIT approach based on Fel d 1, combined with a well-tolerated immunostimulatory CpG adjuvant under endotoxin-free conditions, strikingly improves all hallmarks of allergy, both on a local level in the exposed airways and on a systemic level with the induction of a regulatory lymphocyte signature.

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## CONFLICT OF INTEREST

Dr. C. Leonard reports a patent WO2019/076478A1 pending, and a patent WO2019/076477A1 pending. G. Montamat, C. Davril, O. Domingues, O. Hunewald, D. Revets and Dr. C. Guerin: nothing to disclose. Dr. S. Blank reports non-financial support from ALK-Abellø,

grants, personal fees and non-financial support from Bencard Allergie GmbH, personal fees from Teomed AG, grants from Leti Pharma, grants and personal fees from Thermo Fischer Scientific, grants from Allergy Therapeutics, outside the submitted work. J. Heckendorn, G. Jardon and Dr. F. Hentges: nothing to disclose. Dr. M. Ollert reports that he is a cofounder of Tolerogenics SARL, Luxembourg, during the conduct of the study; personal fees from Hycor Diagnostics, personal fees from Thermo Fischer/Phadia, outside the submitted work; In addition, Dr. Ollert has a patent WO2019/076478A1 pending, and a patent WO2019/076477A1 pending.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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