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

Short running title: CyTOF analysis of CpG-based AIT in mice allergic to Fel d 1

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ACKNOWLEGMENTS

We thank Dr Hans Grönlund from Karolinska Institute, Stockholm, Sweden, for kindly providing recombinant Fel d 1. We thank U. Pechstein, G. Frache, R. Dieden and the whole the team of Materials Research and Technology of the Luxembourg Institute of Science and Technology (LIST, Belvaux, Luxembourg) for their technical assistance in ^{1}H NMR analysis of the hydrogel.

This study was supported by funding of the Ministry of Higher Education and Research of Luxembourg through the intramural research program of the Luxembourg Institute of Health. G. Montamat and M. Ollert were supported by the Luxembourg National Research Fund (FNR) through the FNR-PRIDE program NEXTIMMUNE for doctoral education (PRIDE/11012546/NEXTIMMUNE to M.O.).

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 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 $GATA3^{+}$ $FoxP3^{hi}$ 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 in the future.

KEY WORDS:

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

ABBREVIATIONS:

AIT, Allergen specific immunotherapy; APC, Antigen presenting cells; BALF, Bronchoalveolar lavage fluid; CpG, Oligodeoxynucleotides containing unmethylated CpG motifs; i.p., Intraperitoneal; PC, peritoneal cavity; pDC, plasmacytoid dendritic cell; MLN, mediastinal lymph nodes; MMI, mean metal intensity; s.c., subcutaneous; TLR, Toll-like receptor.

SUPPORTING INFORMATION:

Additional Supporting Information may be found online in the supporting information tab for this article.

(Word count 4084)

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[1,2].

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[3]. The goal of AIT in allergy is to induce long-term immune tolerance by down regulating Th2 cell-driven immune responses through allergen-specific regulatory lymphocytes[4,5]. However, only limited clinical evidence data are available for currently marketed cat AITs compared to other AITs[1]. Improving cat AIT regarding efficacy, safety, cost effectiveness, frequency of injections and duration of the treatment is thus considered an unmet need[6]. 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[7]. 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[8]. 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[9-11], which lacks induction of antibodies, was not superior over placebo in phase III trials[12].

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[13,14]. While most of the AIT studies attributed the immunotherapeutic modulation by CpG mainly to a switch from a Th2- to a Th1-type response[15], some studies suggested a possible effect of CpG in AIT via Treg activity[16,17] 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[18]. 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[19]. 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[20]. Recently, a hepatitis B vaccine containing B-type CpG received FDA approval[21].

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[18]. In addition to a higher CpG dose, we used endotoxin-free Fel d 1 as therapeutic allergen throughout the study to rule out any signal interference by a competing TLR ligand, thus already mimicking 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 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 and in a pre-clinical setting that already anticipates the endotoxin-free conditions of human trials, including the subcutaneous injection route commonly used in patients, has the capacity to induce tolerance towards an allergen and merits reconsideration as AIT adjuvant.

2 METHODS

2.1 Mice and immunization protocols

Eight-week-old female BALB/c OlaHsd mice were obtained from Envigo (Horst, Netherlands) 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 is depicted in Fig 1 and detailed in the Methods section of this article's Online Repository.

LoTox™ Natural Fel d 1 (nFel d 1, LPS content <0.03 EU/µg; Indoor Biotechnologies, Cardiff, United Kingdom) was used in immunizations and in cell cultures. The CpG oligonucleotide with the optimal murine B-Class motif (5'-tccatgacgttcctgatgct-3') was from Sanbio (Uden, Netherlands). 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 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 6 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. Differential analysis in mass cytometry was performed using the general linear model (GLM) integrated in R package lima_3.34.9. This differential analysis was applied to both population abundance as well as marker expression[22]. P-values were corrected according to the number of variables analyzed[22].

Significant p values indicated in all graphs correspond to *p<0.05; **p<0.01; ***p<0.001 and ****p<0.0001.

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 response (Figure S1A to C)[18], we designed experiments as illustrated in Figure 1A. This preclinical AIT model is characterized by i) high concentration of B-type CpG adjuvant; ii) use of endotoxin-free allergen; and iii) 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 lung function values very similar to 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 to allergic mice (Figure S2B). 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-γ) or Th-17 cytokines (IL-6, IL-17) (Figure 2). Priming with Fel d 1/CpG induced an IFN-γ response (Figure 2). Somewhat unexpectedly, while the regulatory cytokines IL-10 and TGF-β were not differentially regulated by AIT, we observed a moderate, but significant increase of TNF-α in the BALF upon AIT (Figure 2). Together, these results indicated a therapeutic effect with reduced Th2 airway inflammation and bronchial hyperresponsiveness conferred by AIT with Fel d 1/CpG in a pre-clinical model of cat allergy.

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.

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 no IL-10 secretion, which is in accordance to BALF results (Figure 2). In addition, no IFN-γ, nor IL-6 and IL-17 were 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-γ and IL-17 signal with moderate IL-6, 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 and late 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 ($CD11b^+$ $CD11c^+$ $CD317^+$ 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 ($CD3^-CD19^-$ $CD49b^-$ 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)[23], we analyzed for $CD25^+$ 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, $CD11b^+$ B1 regulatory cells (Bregs), as previously described[24], 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 hours 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-α 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[25] and for tumor-specific immunosuppression[26], we explored this immunoregulatory axis further. CyTOF data were analyzed for expression of TNF-α and its receptors TNFR1/TNFR2 at D43 (Figure S7). TNF-α was almost exclusively secreted by NK cell subtypes in AIT-treated mice only. Activated $CD62L^-$ 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-α and showed higher activation as evidenced by CD69 expression (Figure S8A). The ratio of $Tbet^+$ NK cells was increased in the PC and the MLN after AIT (Figure S8B) and $Tbet^+$ NK cells expressed more TNFR2 (Figure S7 and Figure S8C). An augmentation of TNFR2 expression was also found for the increased $CD11b^+$ 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[27]. Together, these data demonstrated that an early TNF-α 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-FcεR1 signaling axis by AIT with Fel d 1/CpG, with lower overall FcεR1 expression (Figure S10A) and less total FcεR1-positive cells and basophils in the lungs (Figure S10B). Furthermore, we observed a reduction of plasma cells ($CD45R^-$ B cells) in 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 $FoxP3^+$ Tregs and observed no changes in the conventional $CD4^+$ $CD25^+$ Treg cluster (Figure S11B). However, when analyzed for co-expression of other master

transcription factors, AIT-treated mice displayed an increased ratio of $GATA3^+$ and $FoxP3^+$ double-positive Tregs in the spleen. These double-positive Tregs (biTregs[28]) were very low in control or allergic mice (Figure 5A-B-C). The biTreg subset has been reported to be specifically equipped for counterbalancing effector cell responses[29]. Of note, $GATA3^+$ $FoxP3^+$ 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 $CD62L^+$ Th2 cells (Figure 5A-B). Both emerging clusters, $GATA3^+$ $FoxP3^+$ biTregs and $CD62L^+$ 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 up-regulated 200-fold by AIT with Fel d 1/CpG as compared to the allergic group and expressed high levels of FoxP3 and TNFR2 (Figure 5C). $GATA3^-$ Tregs showed an inverse pattern with lower FoxP3 and very low 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 $GATA3^+$ $FoxP3^+$ 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 (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 to 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 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[18] 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[30] and is in the range of tolerance-inducing CpG concentrations reported[18]. 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-1β response[31]. 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[20,21,30]. 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[21]. 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[13] 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[32], 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[33], 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[34,35].

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[36]. 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[37], 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 $CD4^+CD25^-$ T cells towards $CD4^+CD25^+FoxP3^+$ Tregs[16], which we confirmed by an increase in Tregs. CpG can also provoke B cells to proliferate, to secrete IL-10 via $CD11b^+$ Breg cells and to differentiate into plasma cells and memory B cells[15]. Despite detecting an increase in Bregs, we were unable to measure changes in IL-

10 or TGF-β secretion *in vivo*, although both cytokines have been suggested to mediate tolerance in AIT[18,38] and protective effects of CpG[16]. Our data indicated that AIT with Fel d 1/CpG induces an early TNF-α-driven response that regulates the immune tolerance-inducing TNFR2 and not the pro-inflammatory TNFR1 axis. Via TNFR2, TNF-α promotes the proliferation, differentiation and suppressive capacity of Tregs[39,40]. Indeed, we observed a unique $CD4^+CD25^+FoxP3^{hi}GATA3^+$ 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 $FoxP3^+$ Tregs is associated with superior suppressive capacity towards Th2 effector cells[29,41].

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[42]. 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[43,44], 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[8]. Other novel approaches for AIT in cat allergy also rely on a Fel d 1 only[45,46]. 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.

REFERENCES

1. Davila I, Dominguez-Ortega J, Navarro-Pulido A, Alonso A, Antolin-Amerigo D, Gonzalez-Mancebo E, et al. Consensus document on dog and cat allergy. Allergy 2018;73:1206-1222.

2. Curin M, Weber M, Thalhamer T, Swoboda I, Focke-Tejkl M, Blatt K, et al. Hypoallergenic derivatives of Fel d 1 obtained by rational reassembly for allergy vaccination and tolerance induction. Clin Exp Allergy 2014;44:882-894.

3. Pfaar O, Alvaro M, Cardona V, Hamelmann E, Mosges R, Kleine-Tebbe J. Clinical trials in allergen immunotherapy: current concepts and future needs. Allergy 2018;73:1775-1783.

4. Rondon C, Eguiluz-Gracia I, Shamji MH, Layhadi JA, Salas M, Torres MJ, et al. IgE Test in Secretions of Patients with Respiratory Allergy. Curr Allergy Asthma Rep 2018;18:67-76.

5. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. J Allergy Clin Immunol 2014;133:621-631.

6. Jutel M, Kosowska A, Smolinska S. Allergen Immunotherapy: Past, Present, and Future. Allergy Asthma Immunol Res 2016;8:191-197.

7. Gronlund H, Saarne T, Gafvelin G, van Hage M. The major cat allergen, Fel d 1, in diagnosis and therapy. Int Arch Allergy Immunol 2010;151:265-274.

8. Orengo JM, Radin AR, Kamat V, Badithe A, Ben LH, Bennett BL, et al. Treating cat allergy with monoclonal IgG antibodies that bind allergen and prevent IgE engagement. Nat Commun 2018;9:1421-1435.

9. Couroux P, Patel D, Armstrong K, Larche M, Hafner RP. Fel d 1-derived synthetic peptide immuno-regulatory epitopes show a long-term treatment effect in cat allergic subjects. Clin Exp Allergy 2015;45:974-981.

10. Worm M, Lee HH, Kleine-Tebbe J, Hafner RP, Laidler P, Healey D, et al. Development and preliminary clinical evaluation of a peptide immunotherapy vaccine for cat allergy. J Allergy Clin Immunol 2011;127:89-97.

11. Patel D, Couroux P, Hickey P, Salapatek AM, Laidler P, Larche M, et al. Fel d 1-derived peptide antigen desensitization shows a persistent treatment effect 1 year after the start of dosing: a randomized, placebo-controlled study. J Allergy Clin Immunol 2013;131:103-109.

12. Rudulier CD, Tonti E, James E, Kwok WW, Larche M. Modulation of CRTh2 expression on allergen-specific T cells following peptide immunotherapy. Allergy 2019;74:2157-2166.

13. Fonseca DE, Kline JN. Use of CpG oligonucleotides in treatment of asthma and allergic disease. Adv Drug Deliv Rev 2009;61:256-262.

14. Srivastava KD, Siefert A, Fahmy TM, Caplan MJ, Li XM, Sampson HA. Investigation of peanut oral immunotherapy with CpG/peanut nanoparticles in a murine model of peanut allergy. J Allergy Clin Immunol 2016;138:536-543.

15. Bode C, Zhao G, Steinhagen F, Kinjo T, Klinman DM. CpG DNA as a vaccine adjuvant. Expert Rev Vaccines 2011;10:499-511.

16. Gupta GK, Agrawal DK. CpG oligodeoxynucleotides as TLR9 agonists: therapeutic application in allergy and asthma. BioDrugs 2010;24:225-235.

17. Farrokhi S, Abbasirad N, Movahed A, Khazaei HA, Pishjoo M, Rezaei N. TLR9-based immunotherapy for the treatment of allergic diseases. Immunotherapy 2017;9:339-346.

18. Volpi C, Fallarino F, Pallotta MT, Bianchi R, Vacca C, Belladonna ML, et al. High doses of CpG oligodeoxynucleotides stimulate a tolerogenic TLR9-TRIF pathway. Nat Commun 2013;4:1-11.

19. Creticos PS, Schroeder JT, Hamilton RG, Balcer-Whaley SL, Khattignavong AP, Lindblad R, et al. Immunotherapy with a ragweed-toll-like receptor 9 agonist vaccine for allergic rhinitis. N Engl J Med 2006;355:1445-1455.

20. Scheiermann J, Klinman DM. Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer. Vaccine 2014;32:6377-6389.

21. Jackson S, Lentino J, Kopp J, Murray L, Ellison W, Rhee M, et al. Immunogenicity of a two-dose investigational hepatitis B vaccine, HBsAg-1018, using a toll-like receptor 9 agonist adjuvant compared with a licensed hepatitis B vaccine in adults. Vaccine 2018;36:668-674.

421 22. Nowicka M, Krieg C, Weber LM, Hartmann FJ, Guglietta S, Becher B, et al. CyTOF workflow: differential
422 discovery in high-throughput high-dimensional cytometry datasets. F1000Res 2017;6:1-53.
423 23. Hoffmann HJ, Valovirta E, Pfaar O, Moingeon P, Schmid JM, Skaarup SH, et al. Novel approaches and
424 perspectives in allergen immunotherapy. Allergy 2017;72:1022-1034.
425 24. Liu X, Jiang X, Liu R, Wang L, Qian T, Zheng Y, et al. B cells expressing CD11b effectively inhibit CD4+ T-cell
426 responses and ameliorate experimental autoimmune hepatitis in mice. Hepatology 2015;62:1563-1575.
427 25. Atretkhany KN, Mufazalov IA, Dunst J, Kuchmiy A, Gogoleva VS, Andruszewski D, et al. Intrinsic TNFR2
428 signaling in T regulatory cells provides protection in CNS autoimmunity. Proc Natl Acad Sci U S A
429 2018;115:13051-13056.
430 26. Tam EM, Fulton RB, Sampson JF, Muda M, Camblin A, Richards J, et al. Antibody-mediated targeting of
431 TNFR2 activates CD8(+) T cells in mice and promotes antitumor immunity. Sci Transl Med 2019;11:1-15.
432 27. Ticha O, Moos L, Wajant H, Bekeredjian-Ding I. Expression of Tumor Necrosis Factor Receptor 2
433 Characterizes TLR9-Driven Formation of Interleukin-10-Producing B Cells. Front Immunol 2017;8:1-14.
434 28. Kluger MA, Nosko A, Ramcke T, Goerke B, Meyer MC, Wegscheid C, et al. RORgammat expression in Tregs
435 promotes systemic lupus erythematosus via IL-17 secretion, alteration of Treg phenotype and suppression
436 of Th2 responses. Clin Exp Immunol 2017;188:63-78.
437 29. DuPage M, Bluestone JA. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease.
438 Nat Rev Immunol 2016;16:149-163.
439 30. Zent CS, Smith BJ, Ballas ZK, Wooldridge JE, Link BK, Call TG, et al. Phase I clinical trial of CpG
440 oligonucleotide 7909 (PF-03512676) in patients with previously treated chronic lymphocytic leukemia. Leuk
441 Lymphoma 2012;53:211-217.
442 31. Worah K, Mathan TSM, Vu Manh TP, Keerthikumar S, Schreibelt G, Tel J, et al. Proteomics of Human
443 Dendritic Cell Subsets Reveals Subset-Specific Surface Markers and Differential Inflammasome Function. Cell
444 Rep 2016;16:2953-2966.
445 32. Kundig TM, Klimek L, Schendzielorz P, Renner WA, Senti G, Bachmann MF. Is The Allergen Really Needed
446 in Allergy Immunotherapy? Curr Treat Options Allergy 2015;2:72-82.

447 33. Qiao M, Chen D, Ma X, Liu Y. Injectable biodegradable temperature-responsive PLGA-PEG-PLGA
448 copolymers: synthesis and effect of copolymer composition on the drug release from the copolymer-based
449 hydrogels. Int J Pharm 2005;294:103-112.
450 34. Obst R, van Santen HM, Melamed R, Kamphorst AO, Benoist C, Mathis D. Sustained antigen presentation
451 can promote an immunogenic T cell response, like dendritic cell activation. Proc Natl Acad Sci U S A
452 2007;104:15460-15465.
453 35. DuVall GA, Tarabar D, Seidel RH, Elstad NL, Fowers KD. Phase 2: a dose-escalation study of OncoGel
454 (ReGel/paclitaxel), a controlled-release formulation of paclitaxel, as adjunctive local therapy to external-
455 beam radiation in patients with inoperable esophageal cancer. Anticancer Drugs 2009;20:89-95.
456 36. Majewska-Szczepanik M, Askenase PW, Lobo FM, Marcinska K, Wen L, Szczepanik M. Epicutaneous
457 immunization with ovalbumin and CpG induces TH1/TH17 cytokines, which regulate IgE and IgG2a
458 production. J Allergy Clin Immunol 2016;138:262-273.
459 37. Mirotti L, Alberca Custodio RW, Gomes E, Rammauro F, de Araujo EF, Garcia Calich VL, et al. CpG-ODN
460 Shapes Alum Adjuvant Activity Signaling via MyD88 and IL-10. Front Immunol 2017;8:1-13.
461 38. Bacher P, Scheffold A. The effect of regulatory T cells on tolerance to airborne allergens and allergen
462 immunotherapy. J Allergy Clin Immunol 2018;142:1697-1709.
463 39. Yang S, Xie C, Chen Y, Wang J, Chen X, Lu Z, et al. Differential roles of TNFalpha-TNFR1 and TNFalpha-
464 TNFR2 in the differentiation and function of CD4(+)Foxp3(+) induced Treg cells in vitro and in vivo periphery
465 in autoimmune diseases. Cell Death Dis 2019;10:1-13.
466 40. Okubo Y, Mera T, Wang L, Faustman DL. Homogeneous expansion of human T-regulatory cells via tumor
467 necrosis factor receptor 2. Sci Rep 2013;3:1-11.
468 41. Mondoulet L, Dioszeghy V, Busato F, Plaquet C, Dhelft V, Bethune K, et al. Gata3 hypermethylation and
469 Foxp3 hypomethylation are associated with sustained protection and bystander effect following
470 epicutaneous immunotherapy in peanut-sensitized mice. Allergy 2019;74:152-164.
471 42. Gregorio J, Meller S, Conrad C, Di Nardo A, Homey B, Lauerma A, et al. Plasmacytoid dendritic cells sense
472 skin injury and promote wound healing through type I interferons. J Exp Med 2010;207:2921-2930.

473 43. Chan SK, Leung DYM. Dog and Cat Allergies: Current State of Diagnostic Approaches and Challenges.
474 Allergy Asthma Immunol Res 2018;10:97-105.
475 44. Matricardi PM, Kleine-Tebbe J, Hoffmann HJ, Valenta R, Hilger C, Hofmaier S, et al. EAACI Molecular
476 Allergology User's Guide. Pediatr Allergy Immunol 2016;27 Suppl 23:1-250.
477 45. Niespodziana K, Focke-Tejkl M, Linhart B, Civaj V, Blatt K, Valent P, et al. A hypoallergenic cat vaccine
478 based on Fel d 1-derived peptides fused to hepatitis B PreS. J Allergy Clin Immunol 2011;127:1562-1570.
479 46. Moldaver DM, Bharhani MS, Rudulier CD, Wattie J, Inman MD, Larche M. Induction of bystander tolerance
480 and immune deviation after Fel d 1 peptide immunotherapy. J Allergy Clin Immunol 2019;143:1087-1099.

FIGURE LEGENDS

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 3 times intraperitoneally (i.p., triangles) on D0, D14 and D28 with PBS $Al(OH)_3$ and got 3 nasal instillations (NI, 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 $Al(OH)_3$ priming solution and were challenged by 3 NI with Fel d 1. The CpG primed mice (orange) got 3 i.p. with Fel d 1 and CpG as adjuvant and 3 NI with Fel d 1. The specific immunotherapy group (AIT, green) were primed with Fel d 1 $Al(OH)_3$ and received 3 additional i.p. with Fel d 1 CpG-adjuvanted solution on D42, D56 and D70 and 3 NI with Fel d 1. The mice of the sham treated group (blue) got 3 primary i.p. with Fel d 1 $Al(OH)_3$ and a second round of 3 i.p. with PBS CpG solution. Nasal instillation with Fel d 1 were also administered to this group. All the mice were sacrificed at D86 after analysis of the airway hyperreactivity (Flexivent), for cell populations and cytokines secreted in BALF, immunoglobulin profile and cytokines secreted in assays with lymph node cells. **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 5 types of immunized mice according to $Gr1^+CD11c^-$ gating. Results are means +- SEM, N= 5-21, significant variations between a group of mice and the others are indicated above the groups (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$). **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.

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<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

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-γ, 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 6 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 +- 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 that group.

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 3 groups of mice. **B**, Ratio of macrophages in the myeloid compartment in MLN. **C**, t-SNE graphic representation of the myeloid compartment in the PC of control, allergic and AIT treated mice. **D**, Ratio of cDC2 in the myeloid compartment of PC, spleen and MLN of the 3 groups of mice. **E**, Ratio of $CD25^+$ $CD4^+$ T cells in the spleen T cell compartment. Results are means +- SEM, N=4-5, p-values indicated for comparison Allergic-AIT.

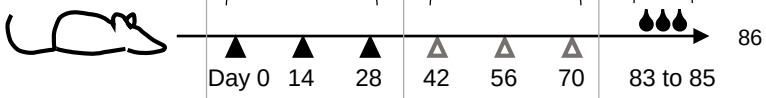
Figure 5: CyTOF analysis of the late events highlighted a shift in the Treg cell compartment. **A**, Ratio of $GATA3^+$ Tregs in the spleen and the lung and $CD62L^+$ Th2 in the T cell compartment in the spleen of the 3 groups of mice. Results are means +- SEM, N=3-4, p-values indicated for comparison Allergic-AIT. **B**, t-SNE projection of the T cell compartment of the spleen. Significant proportions

of $CD62L^+$ Th2 (#8, orange) and $GATA3^+$ Treg (#15, blue) cells arose upon AIT in treated mice. Although two other emerging subpopulations (Th2 $CD62L^+$ $GATA3^{hi}$, in green, #9, and CD8+ GATA3+ naïve cells, in violet, #19) were discernable on the t-SNE projection, the differences between the 3 groups of mice (control, allergic and AIT treated) were not significant. The $CD25^-$ Treg (dark green #11) and $CD62L^+$ $CD25^-$ Treg (fuchsia, #13) subpopulations were significantly reduced (Figure S11C). **C**, Split dot plots of $GATA3^+$ 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 $GATA3^+$ Treg population among splenic $CD3^+$ T cells. Results are means, N=2-4. The proportion of $GATA3^+$ 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 $GATA3^+$ Tregs.

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 4 types of immunized mice according to $Gr1^+CD11c^-$ 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 +- SEM, N= 3, significant variations between a group of mice and the others are indicated (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Figure 1 Leonard C et al.

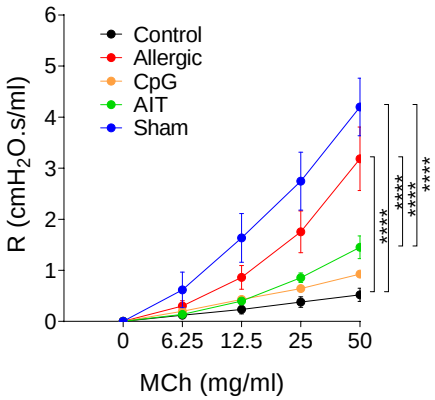
A



Control	PBS / Al(OH)$_3$	None	PBS
Allergic	Fel d 1 / Al(OH)$_3$	None	Fel d 1
CpG	Fel d 1 / CpG	None	Fel d 1
AIT	Fel d 1 / Al(OH)$_3$	Fel d 1 / CpG	Fel d 1
Sham	Fel d 1 / Al(OH)$_3$	PBS / CpG	Fel d 1

1. Airway hyperresponsiveness
2. Cells in BALF
3. Cytokines in BALF
4. Ig profiles
5. T cell assays

B



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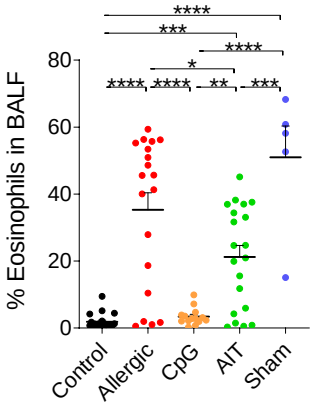

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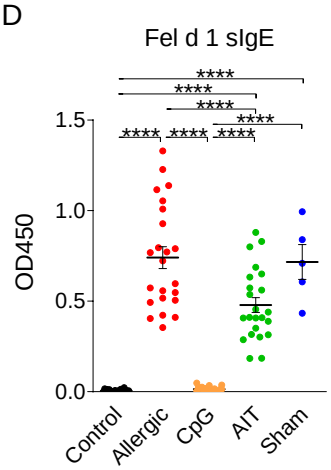

Figure 2 Leonard C et al.

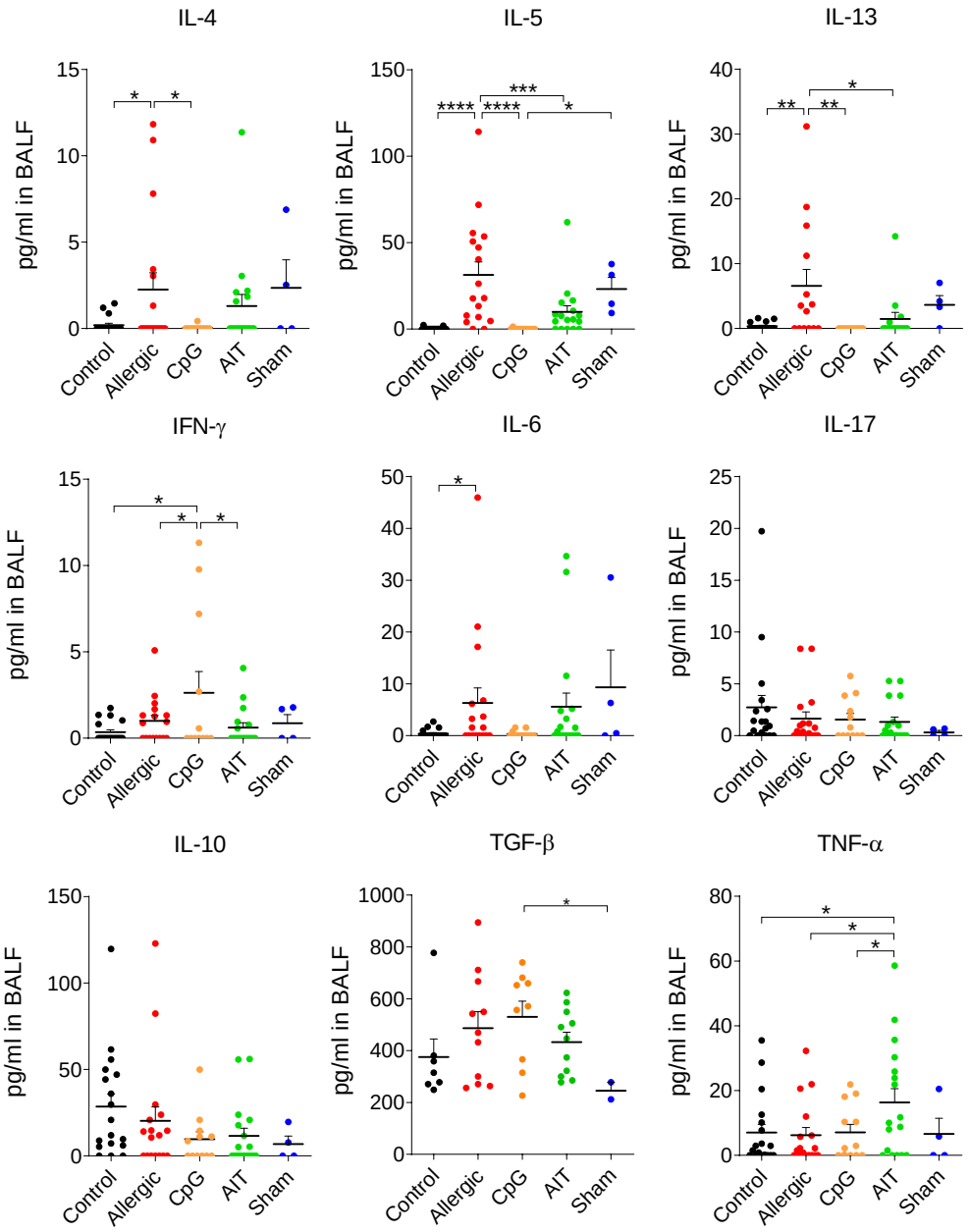

Figure 3 Leonard C et al.

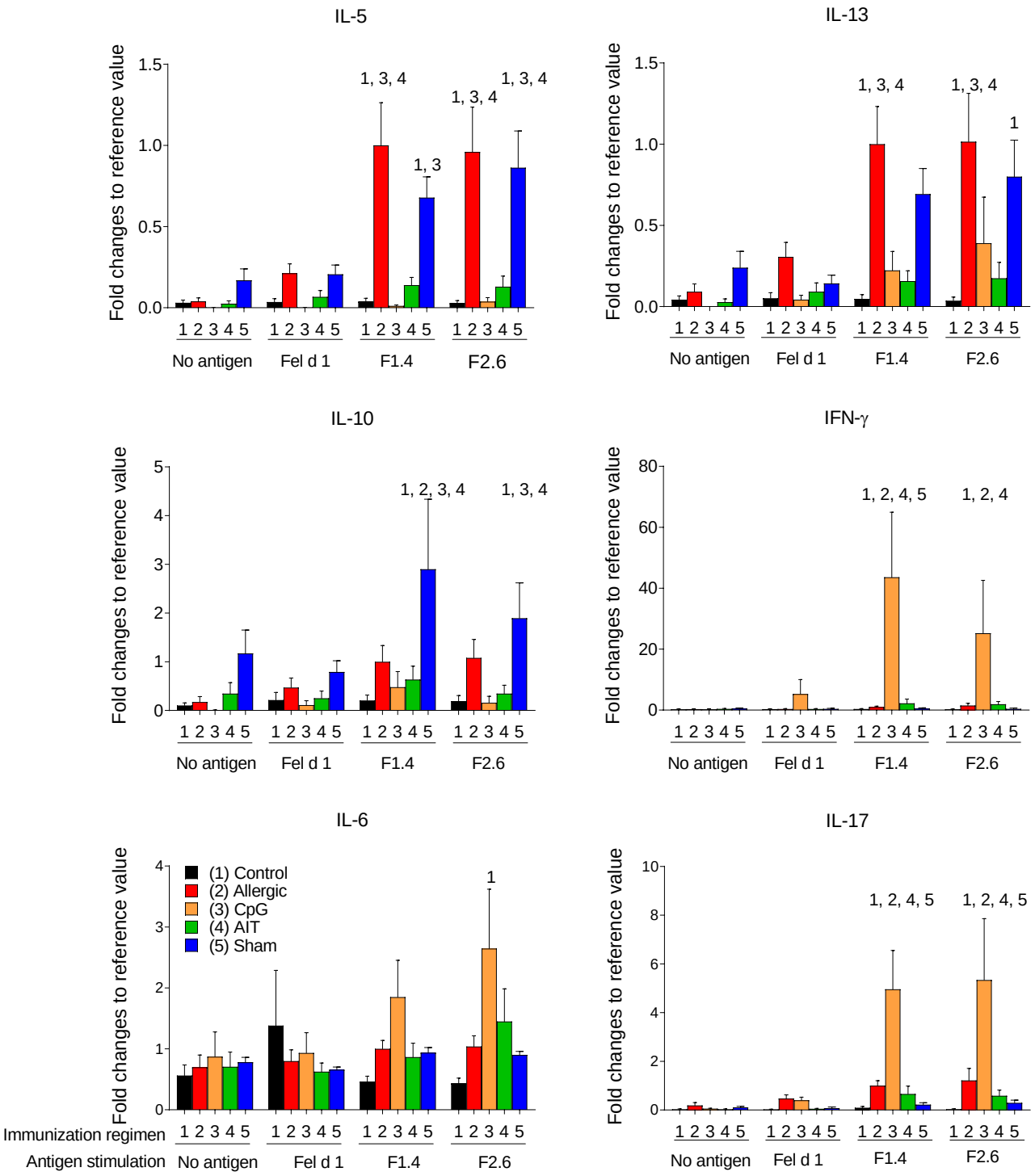

Figure 4 Leonard C et al.

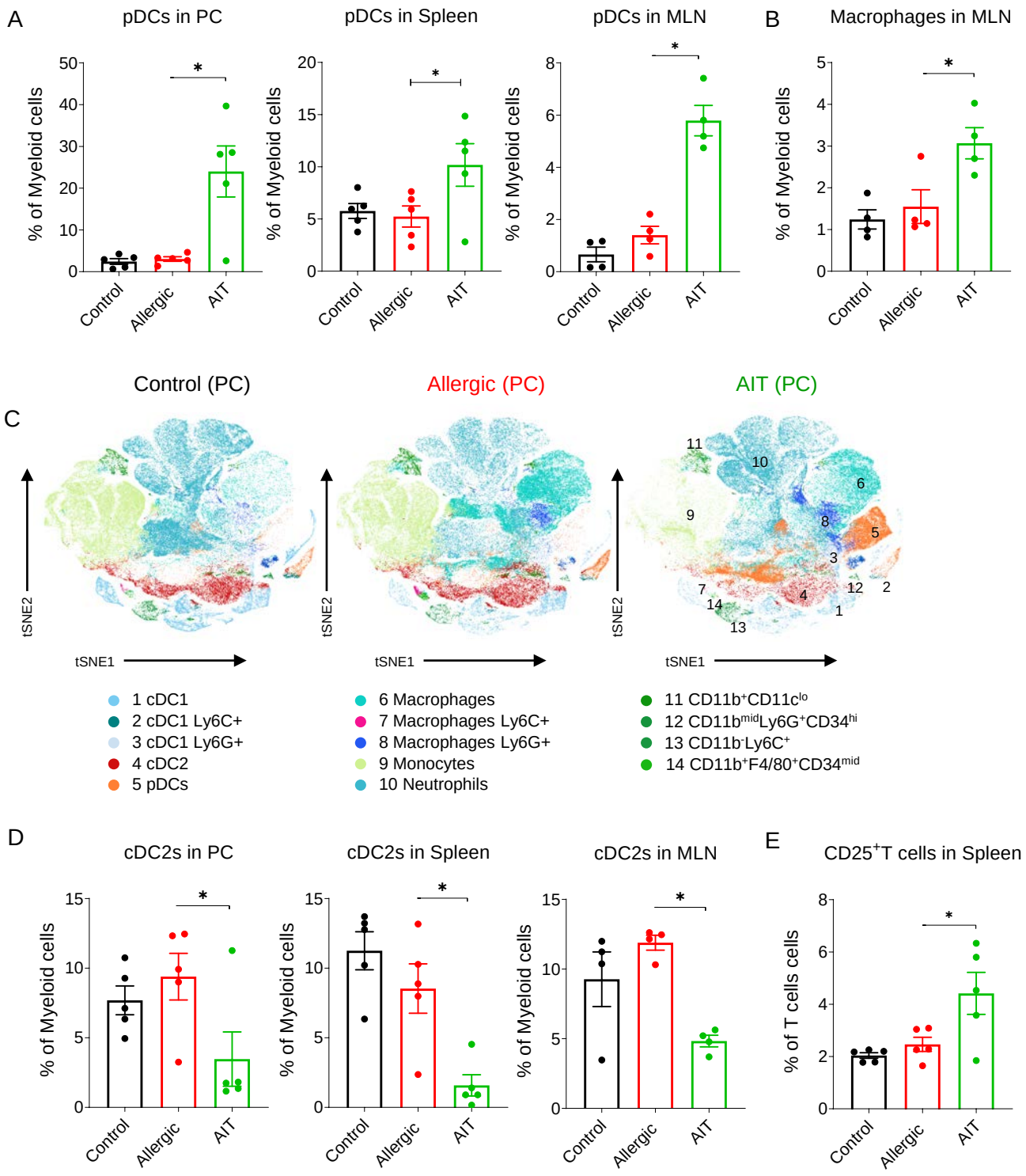

Figure 5 Leonard C et al.

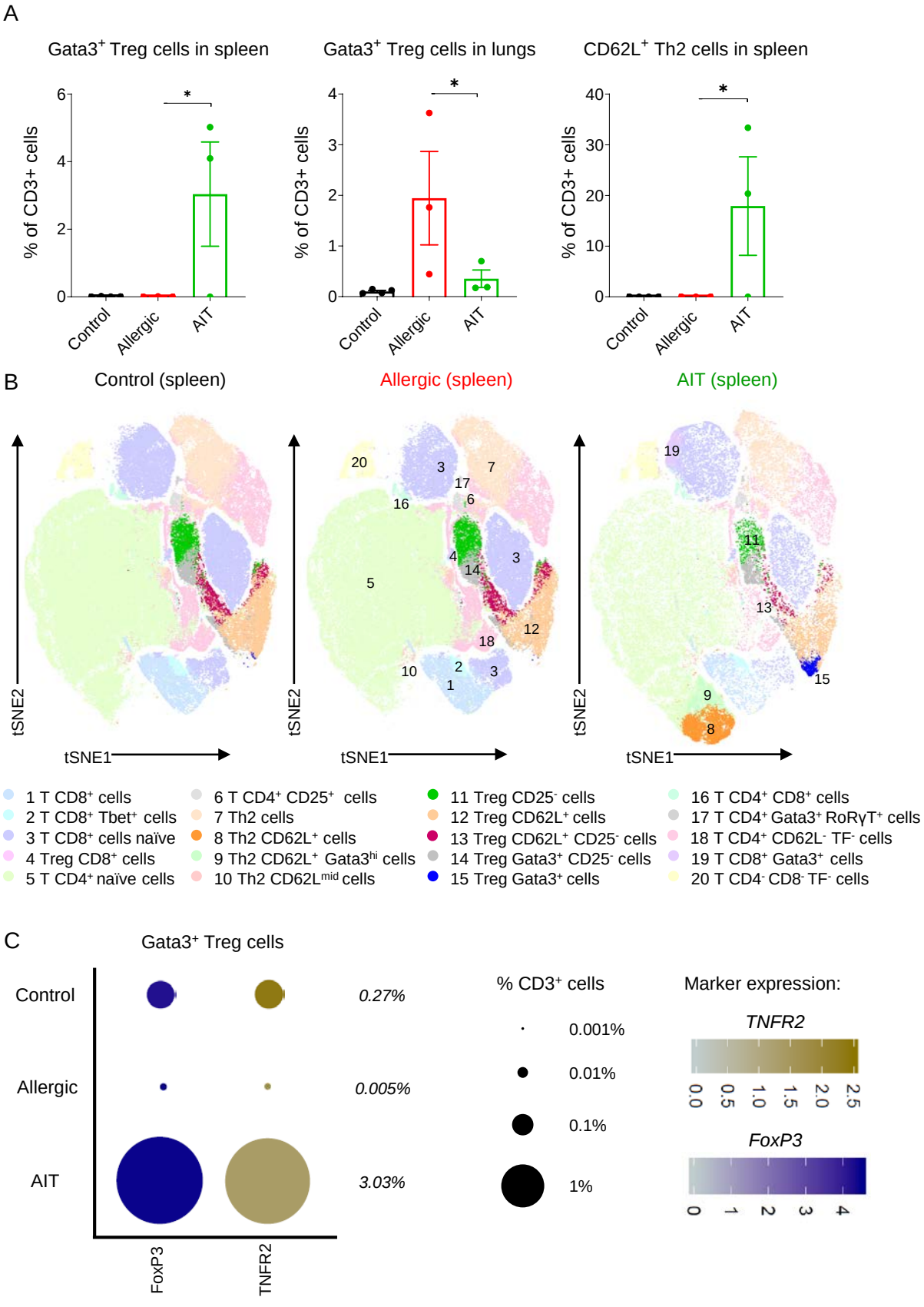

Figure 6 Leonard C et al.