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T cell epitopes of Per a 10 modulate local-systemic immune responses and airway inflammation by augmenting Th1 and T regulatory cell functions in murine model

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ABSTRACT

Peptide immunotherapy (PIT) represents a safe and efficacious therapeutic modality for allergic diseases. Present study evaluates immunotherapeutic potential of T cell peptides of major cockroach allergen, Per a 10 in murine model of airway allergy. Treatment with peptides T-P8 and T-P10 demonstrated maximal resolution of pathophysiological features such as reduced recruitment of inflammatory cells to airways, lowered specific IgE, induction of IgG2a antibodies in serum, immune deviation towards Th1 cytokine milieu, suppression of Th2 cytokines in BALF and splenocyte culture supernatant and resolution of lung inflammation. A significant increase in $CD4^+Foxp3^+$ cells in spleen indicate towards induction of T regulatory cell mediated peripheral tolerance characterized by shift in cytokine milieu from Th2 to T regulatory cytokines. PIT modulates regulation of immune responses at both local and systemic levels, contributes towards holistic improvement in allergic features in mice and thus demonstrate potential for safe, specific and efficacious treatment for cockroach allergy.

1. Introduction

Allergen immunotherapy (AIT) is the only disease modifying therapeutic approach and involves the induction of tolerance towards the offending allergen via administration of allergen extracts in small doses over a period of time. AIT leads to decreased disease severity and provides long term curative effects (Akdis and Akdis, 2015). Allergen extracts are complex heterogeneous mixtures with variable composition of individual allergenic proteins, biological potency and batch to batch variation. Immunotherapy with allergen extracts carries an inherent risk of sensitizing the individual with other allergens and may lead to adverse allergic reactions with a potential risk of anaphylactic shock in severe allergic patients. IT with purified allergen may cause adverse reactions due to IgE crosslinking on mast cell surfaces and subsequent release of mediators (Pomés et al., 2017).

To improve the safety and efficacy of IT, various strategies have been employed such as chemical modification of allergens to form allergoids and use of modified or unmodified recombinant allergens (Keskin et al., 2006; Marth et al., 2014; Fonseca and Kline, 2009). IT with immunodominant T cell epitopes of allergens is another strategy to reduce the potential side effects. Peptide immunotherapy (PIT) involves administration of short peptides comprising immunodominant T cell epitopes of the major allergenic proteins. These peptides are designed to lack the conformational structure required to crosslink IgE on surface of mast cells and basophils while possessing their immunological ability to interact and modulate allergen specific T cell responses (Hoffmann et al., 2017). T cell epitopes display extensive HLA class II binding degeneracy therefore offer a widespread clinical utility without individual endotyping (O'Hehir et al., 2016). Also, tolerance generated by peptide immunotherapy is associated with linked suppression, in which, administration of supraoptimal concentrations of single dominant peptide in vivo results in non-responsiveness to whole allergen as well, thereby positioning it as a safer and efficacious option to treat allergic diseases (Campbell et al., 2009).

Studies in murine models and clinical trials have demonstrated the induction of peripheral T cell tolerance by immunodominant peptides of house dust mite allergen Der p 1, cat allergen Fel d 1 and bee venom phospholipase A_2 (PLA_2) (Hoyne et al., 1993; Briner et al., 1993; Müller et al., 1998a). A study on cross reactive cytochrome c allergen Cur l 3 from *Curvularia lunata* demonstrated pronounced immunotherapeutic potential of short T cell peptides in mouse model of allergy (Sharma et al., 2011). Recent studies on Fel d 1 and grass allergen derived

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peptide immunotherapy regimen SPIRE showed long term and sustained therapeutic effects in cat and grass pollen allergic patients respectively (Patel et al., 2013; Couroux et al., 2015; Ellis et al., 2017).

In our previous study, immunodominant epitopes of Per a 10 allergen were predicted using *in silico* tools and the peptides T-P8 and T-P10 demonstrated reduced IgE binding and significant T cell proliferation in PBMCs (Govindaraj et al., 2016). The current study was undertaken to evaluate immunotherapeutic potential of T cell peptides in the murine model of allergic disease.

2. Material and methods

2.1. Animals

Female Balb/c mice (n = 36, 4–6 weeks old, weighing 20 to 22 g) were procured from National Institute of Nutrition (NIN, Hyderabad) and housed in the animal care facility of Institute of Genomics and Integrative Biology (IGIB), Delhi under standard laboratory conditions with ad libitum access to food and water. The animals were allowed to acclimatize one week prior the experiment. The study protocol was approved by animal ethics committee of IGIB, Delhi, following the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India.

2.2. Proteins/Peptides for immunization

Cockroach extract (CE) was prepared in 0.1 M PBS and Per 10 was purified from cockroach extract by affinity chromatography as described previously (Sudha et al., 2008). Synthetic peptides of T cell epitopes T-P8, T-P9 and T-P10 were synthesized by the manufacturer (Thermo fisher Scientific, Germany) with purity of ≥95% and sequences were confirmed by mass spectrometry (MALDITOF) analysis (Supplementary data S4 to S6). An unrelated control peptide of fungal origin was used as control peptide (Govindaraj et al., 2016).

2.3. Immunotherapy protocol

To evaluate immunotherapeutic efficacy of the predicted T cell peptides a model of airway hyper-reactivity was generated in Balb/c mice. Briefly, mice were i.p. sensitized with cockroach extract (CE 10 μg) or PBS at Day 0 and 14 and i.n. challenged with purified cockroach allergen Per a 10 (10 μg) or PBS on day 17, 19 and 21. (Fig. 1a). The immunotherapy protocol was designed as described previously (Sharma et al., 2011). Immunotherapy with PBS, Control peptide and T cell peptides T-P8, T-P9 and T-P10 (100 μg per mice) was given by subcutaneous route on day 24, 26, 28 and 30. Mice were challenged with Per a 10 (10 μg) after 40 days of immunotherapy (on day 70, 72 and 74) and sacrificed after 24 h of last challenge on 75th day (Fig. 1a). (Refer Table 1 for sequence of T cell epitopes and Table 2 for detailed Immunotherapy mice groups).

2.4. Sample collection and processing

Blood was collected from mice and sera was separated and stored at −70 °C. The trachea was exposed, and bronchoalveolar lavage fluid (BALF) was obtained by flushing lungs thrice with 0.5 ml sterile PBS (total of 1.5 ml). BALF was centrifuged and the supernatant was collected and stored at −70 °C. BALF (100 μl) was spread on the slide, fixed and stained with Leishman's stain for differential cell counts. Lung tissue was dissected from one lobe and fixed in 10% NBF (v/v) for histological analysis. Spleens were collected, washed with sterile PBS and used for splenocyte isolation and cell culture.

2.5. Immunoglobulin analysis

Per a 10 (whole allergen) specific IgE and IgG2a were measured in serum samples of all the mice using biotinylated anti-mouse IgE (2 μg/ml, BD Pharmingen, USA) and IgG 2a-peroxidase (1:1000 dilution, BD Pharmingen, USA) by indirect ELISA as described elsewhere (Sharma et al., 2015). Mice sera was diluted 1:10 for IgE estimation and 1:50 for IgG 2a.

2.6. Splenocyte isolation and culture

Spleens collected from the mice were minced separately, passed through sieve and incubated in RBC lysis buffer, followed by sterile PBS. The single cell suspensions were prepared in RPMI 1640, (Sigma Aldrich) supplemented with 10% fetal calf serum (FCS), 1% sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin. For staining T regulatory cells, the splenocyte cell suspension was stained with FITC labelled anti- CD 4 and PE labelled anti- Foxp3 antibodies (eBiosciences). The cells were acquired using FACS Calibur Flow cytometer (BD Biosciences) and analysed using software BD CellQuest Pro. The splenocytes of different groups were cultured in 12 well plates (1×10^6 per well) and stimulated with 10 μg of Per a 10. The cells were allowed to proliferate at 37 °C in CO_2 incubator for 72 h and supernatant was collected for cytokine analysis.

2.7. Cytokine analysis

IL-4, IL-5, IL-10 (BD Pharmingen, USA and R & D, MN, USA), TGF-β and IFN-γ (eBioscience) were determined in BALF and splenocyte culture supernatant by ELISA following manufacturer's instructions.

2.8. Histopathology

The fixed lung tissues were paraffin embedded and sliced to 4 μm sections. The sections were stained with hematoxylin-eosin (HE) and scanned using light microscope. The images were captured by Nikon Eclipse 80i microscope with an in-line camera and assembled into multipanel figures using Photoshop software (Adobe version 7). Inflammation was scored from 1-5. 'one' inflammatory reaction affecting < 20% of the airways, 'two' as 20–40% of the airways affected, 'three', 40–60%, 'four', 60–80% and 'five', > 80% of the airways affected. Inflammation score was presented as the mean ± SD of the mice in each group.

2.9. SDAP analysis of peptides

Peptide similarity match was performed for peptides T-P8, T-P10 and control peptide to determine their cross reactive potential. Sequences were submitted to peptide similarity tool in Structural Database of Allergenic Proteins server (SDAP server). It determines similar peptide sequences in other allergen entries in SDAP with similar overall physico-chemical properties. The results were retrieved and cutoff value was assigned using histogram for best protein-peptide similarity index statistics (Schein et al., 2007).

3. Bioinformatic analysis of Per a 10 T cell epitopes for mouse MHCII

T cell epitope prediction data of Per a 10 allergen for mouse MHC Class II alleles was performed using bioinformatic tools. Per a 10 peptide sequence was subjected to prediction tools NetMHCIIpan 3.2 server, NN align and SMM Align. Strong binders from NetMHCIIpan 3.2 (default threshold cut off for strong binders taken as 2% of percentile), SMM align and NN Align (cut off IC_{50} value below 500 nM) were included for analysis . A consensus was taken amongst the predicted epitopes and T cell epitope regions were predicted.

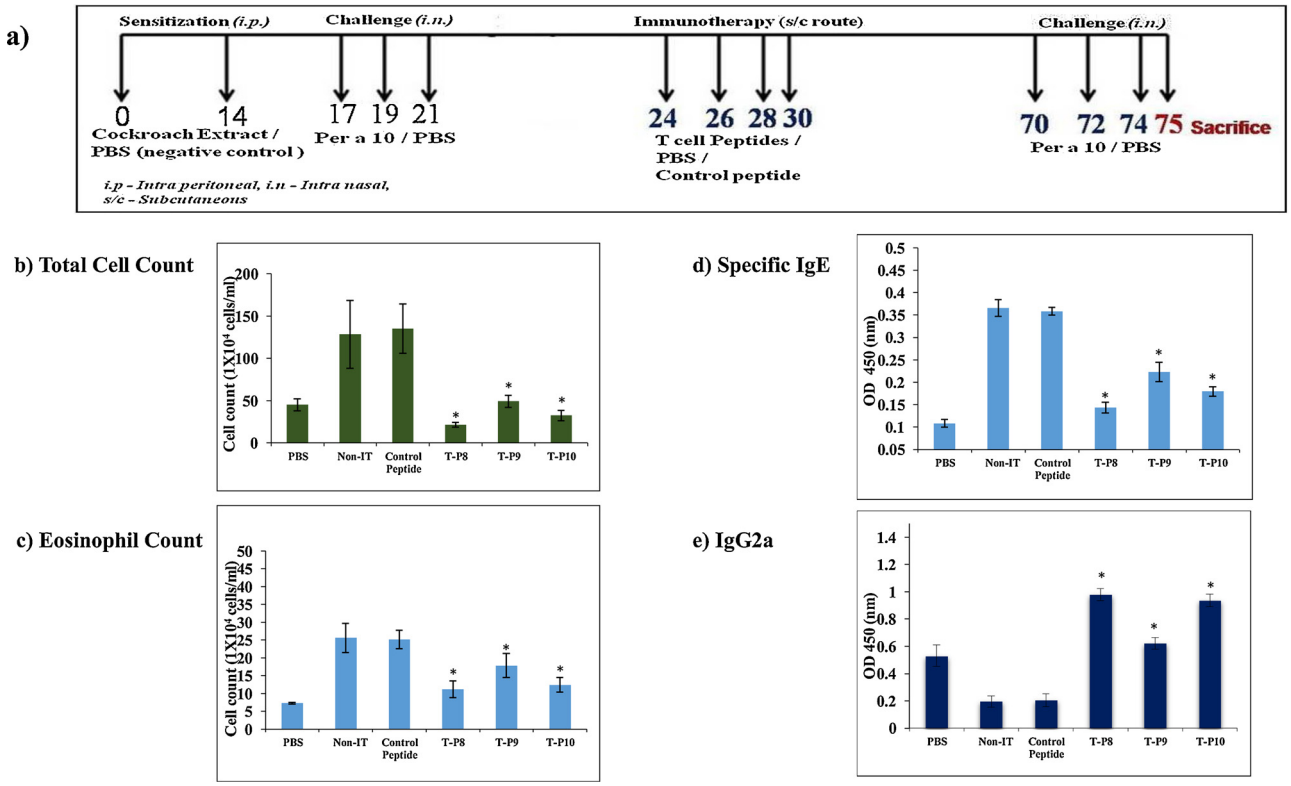

Fig. 1. a) Balb/c mice were intraperitoneally sensitised with cockroach extract (CE 10 µg) or PBS at Day 0 and 14. Mice were intranasally challenged with purified cockroach allergen Per a 10 (10 µg) or PBS on day 17, 19 and 21. Immunotherapy with PBS, Control peptide and T cell peptides T-P8, T-P9 and T-P10 (100 µg per mice) was given by subcutaneous route on day 24, 26, 28 and 30. Challenge with Per a 10 (10 µg) was given after 40 days of immunotherapy (on day 70, 72 and 74). Mice were sacrificed on 75th day and BALF, blood, spleen, and lungs were harvested for experiments. **b)** Cellular infiltration in BALF determined by total cell count employing trypan blue method. **c)** Eosinophilic infiltration was observed by smearing BALF cell suspension on slides and staining them with Leishman's stain. The slides were observed under the microscope. **d)** Serum levels of Per a 10 specific IgE and **e)** IgG2a were determined by indirect ELISA as described. Data is presented as mean ± SD (with n = 6 per group) and $p < 0.05$ compared to control peptide was considered significant. * represents $p < 0.05$ compared to control peptide (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1
List of sequences of Per a 10 derived T cell peptides.

Peptide	Sequence	Size (mer)	Position in Per a 10 protein
T-P8	YLVLASLIACS	11	4–14
T-P9	LVVNGQ	6	214–219
T-P10	SNVASLKGFIT	11	240–250
Control Peptide	YVGNRKDSSEAIEF	14	–

Table 2
List of mice groups employed for Immunotherapy protocol.

S.no.	Group title	Sensitization	Challenge	Therapy	Post therapy challenge
1.	PBS	PBS	PBS	PBS	PBS
2.	Non IT	CE	Per a 10	PBS	Per a 10
3.	Control Peptide	CE	Per a 10	Control Peptide	Per a 10
4.	T-P8	CE	Per a 10	T-P8	Per a 10
5.	T-P9	CE	Per a 10	T-P9	Per a 10
6.	T-P10	CE	Per a 10	T-P10	Per a 10

4. Statistical analysis

Data are presented as mean ± standard deviation. Statistical analysis of results was done by using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The statistically significant difference was determined using student *t*-test between Per a 10 challenged, control peptide treated mice group and different treatment groups. The p value less than 0.05 was considered as significant.

5. Results

5.1. IT with T cell peptides effectively suppressed cellular infiltration in lungs and leads to higher Per a 10 specific IgG2a and lower IgE levels in mice

Murine model of allergic hypersensitivity was generated by intraperitoneal sensitization with cockroach allergen and intranasal challenge with purified Per a 10. Per a 10 induced the influx of eosinophils in the BALF of mice treated with PBS (non-IT group) and control peptide. Immunotherapy with peptides T-P8, T-P9 and T-P10 suppressed the total cellular infiltration by 84%, 63% and 75% respectively in treated mice as compared to control peptide treated group (Fig. 1b). Allergic hypersensitivity is characterized by increased eosinophilia in lungs, administration of the T cell peptides was associated with 55%, 28.9% and 50% reduction of eosinophils in the treated groups respectively (as compared to control peptide). PBS treated and control peptide treated groups showed high levels of allergen specific IgE and reduced IgG 2a. Meanwhile, T-P8 and T-P10 treated mice demonstrated reduction of 60% and 49% in IgE levels respectively with a consequent 4.5 fold increase in IgG2a levels. T-P8 administered mice showed most pronounced therapeutic effects in reduction of both cellular infiltration and allergen specific IgE (Fig. 1b to 1e) followed by T-P10 and T-P9 ($p < 0.05$ as compared with control peptide treated group).

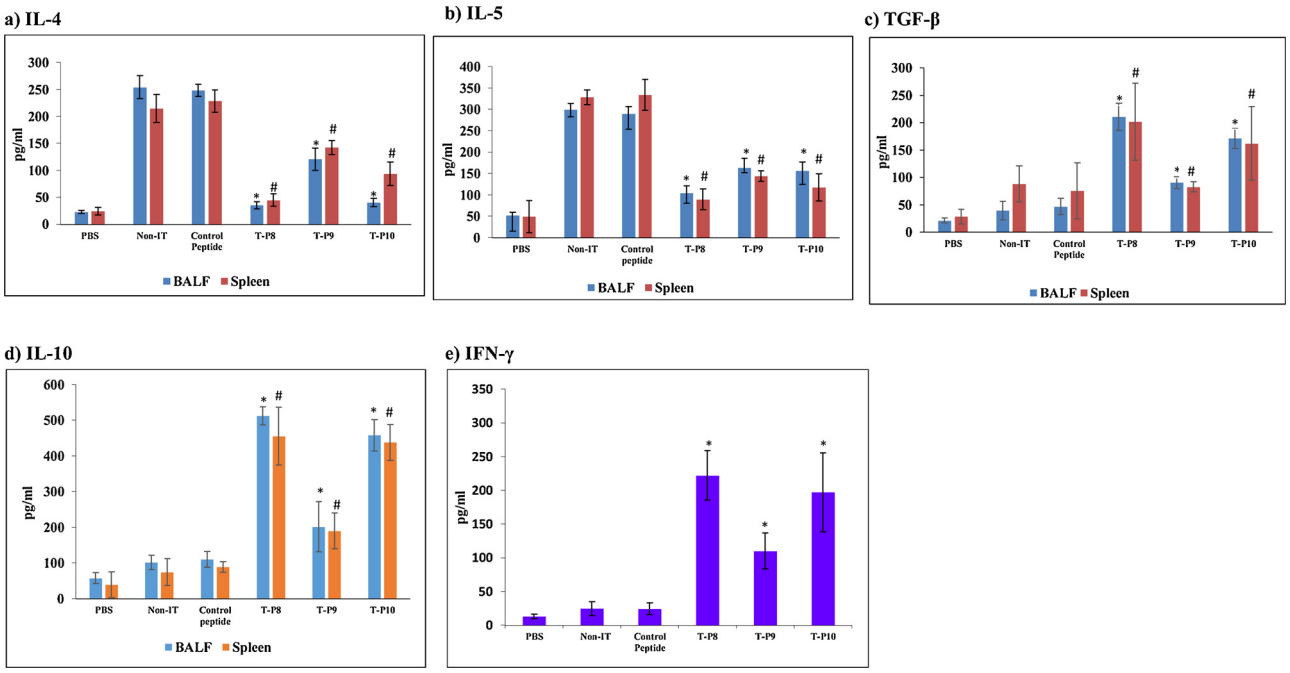

Fig. 2. Cytokine analysis in Bronchoalveolar lavage fluid (BALF) and Splenocyte culture supernatant. BALF was centrifuged and supernatant used for cytokine analysis. Splenocytes from individual mice were seeded in 12 well plates (1×10^6 cells per well), challenged with 10 μg of Per a 10 and cultured for 72 h at 37 °C at 5% CO_2. Supernatant was collected after 72 h and analysed for cytokines: **a)** IL-4, **b)** IL-5, **c)** IL-10, **d)** TGF- β and **e)** IFN-γ (levels in BALF) were estimated following manufacturer's instructions. Data is presented as mean ± SD (with n = 6 per group).* and # symbols represent $p < 0.05$ compared to control peptide for BALF and splenocyte culture supernatant respectively.

5.2. PIT leads to reduced Th2 and increased T regulatory cytokine secretion in vivo and in allergen stimulated splenocyte culture supernatant

Proinflammatory Th2 cytokines, IL-4 and IL-5 were elevated in BALF and splenocyte culture supernatant derived from PBS and control peptide treated groups while the T regulatory cytokine IL-10 and TGF-β were low (Fig. 2a, b, d and e). Peptide immunotherapy caused reduction in the levels of IL-4 and IL-5. Treatment with T-P8 resulted in approximately 80% reduction in IL-4 levels, while 64% and 73% reduction was observed in the levels of IL-5 in BALF and spleen respectively. Upon treatment with peptides T-P8 and T-P10, approximately four fold increase in TGF-β and IL-10 levels were observed in BALF. Similarly, splenocyte culture supernatant demonstrated approximately two fold increase in TGF-β and fivefold increase in IL-10 levels. Levels of Th1 cytokine IFN-γ were measured in BALF supernatant, a significant induction of IFN-γ was observed in T-P8 and T-P10 demonstrating shift in cytokine profile from Th2 to Th1.

5.3. Peptide immunotherapy exerts its effects via increased levels of Treg cells in vivo

Elevated levels of IL-10 and TGF-β in BALF as well as splenocyte culture supernatant in peptide treated mice groups indicate towards a possible increase in T regulatory cell number in vivo. To ascertain this possibility, splenocytes were isolated from individual mice and stained with fluorochrome labelled antibodies (FITC-CD4 + and PE-Foxp3) and double positive cells were visualized by flow cytometry (Fig. 3). T-P8 and T-P10 administered mice demonstrated robust increase in T regulatory cells in vivo with an approximate six fold and threefold increase in cell number respectively.

5.4. T cell peptide immunotherapy rescues allergic inflammation in mice lungs

HE staining of lung sections from mice of different groups was performed to determine the pathophysiological changes upon allergen exposure and treatment with respective peptides in lung tissue of mice. Lung sections of mice sensitized, challenged and treated with PBS showed normal lung epithelium with negligible infiltration of cells. However, re-exposure of Per a 10 allergen to mice model of allergic asthma (sensitized and challenged with cockroach extract and Per a 10 respectively without any treatment, non-IT group) showed high inflammation as evidenced by increased recruitment of inflammatory cells and disruption of alveolar integrity. Treatment with T cell derived peptides of Per a 10, was able to rescue inflammation in mice lung, post 40 days of immunotherapy signifying their therapeutic potential (Fig. 4a). The effect observed on mice lungs was specific as treatment with control peptide did not show reduction in inflammation. Maximal resolution of inflammation, was observed in the following order, T-P8, followed by T-P10 and T-P9 (represented as inflammation score Fig. 4b).

5.5. Peptide similarity match using SDAP

SDAP analysis was performed to evaluate cross reactivity potential of T-P8, T-P10 and control peptide (Table S1 to S3). T-P8 demonstrated high similarity with chitinase Ziz m1, cysteine protease from house dust mite Der p 1, Der f 1 and seed storage protein Ara h1 (PD sequence similarity index 5.44, 6.2, 7.67 and 6.97 respectively). T-P10 demonstrated highest similarity with Der p1 (PD sequence similarity index 5.32) followed by defensin like protein Amb a 4 (short ragweed) and trypsin like protease Tyr p 3. It also showed similarity to various food allergens such as thaumatin like protein Mal d 2 (apple), alkaline serine protease Cuc m1 (muskmelon), papain proteinase 1 (papaya). Control peptide showed highest similarity (PD sequence similarity index 2.29) with alcohol dehydrogenase Cand a 1 (*Candida albiacans*) and lower similarity to some fungal proteins from mitogillin, metalloprotease and catalase family.

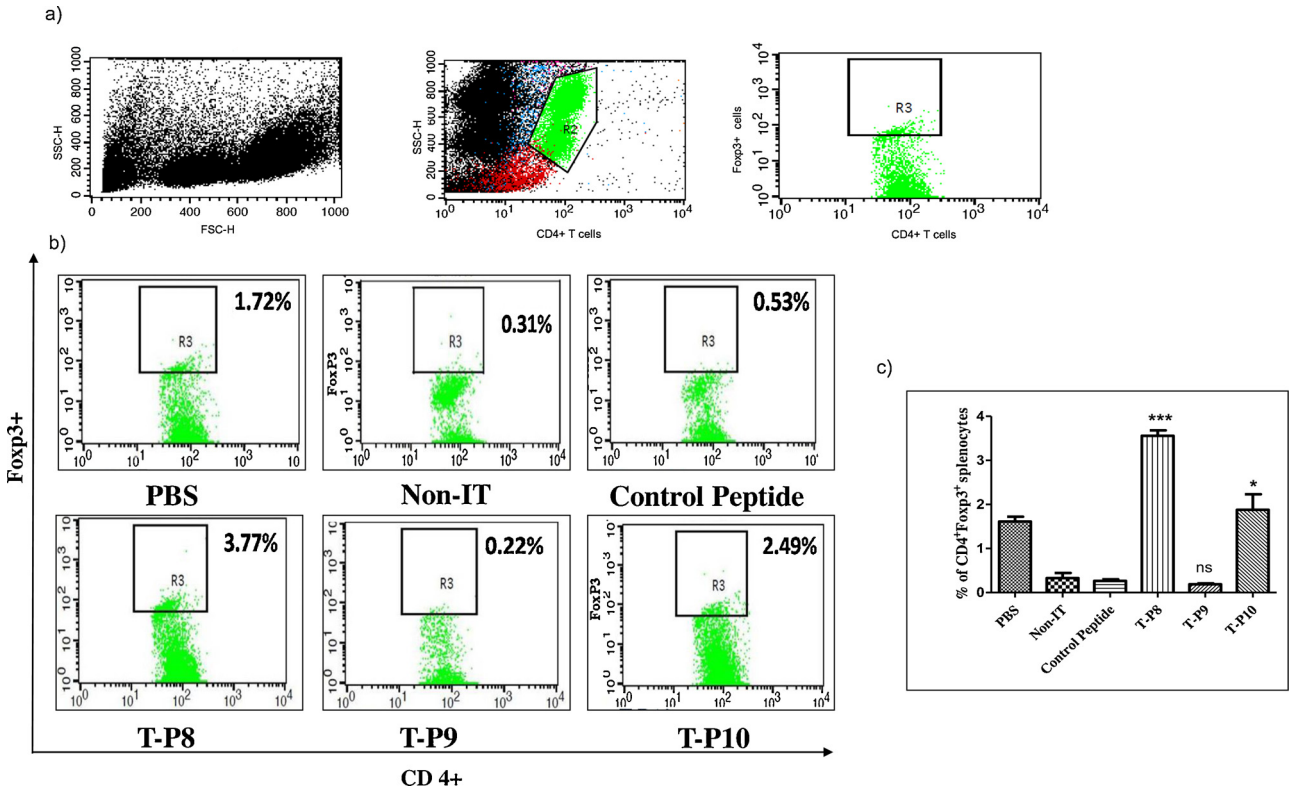

Fig. 3. Single cell suspensions prepared from spleen of mice were stained with FITC labelled anti CD4 and PE labelled anti Foxp3 antibodies. A) Gating scheme to identify Foxp3+ CD4 + T cells. CD4 + cells were gated and analyzed for expression of Foxp3. The cells ranging from 10^1 to 10^2 magnitude on Y axis were taken as foxp3 negative by comparing it with the unstained control. Acquisition was performed using FACS Calibur Flow cytometer and analyzed using software BD CellQuest Pro. b) Representative data of CD4+Foxp3+ T cells among different groups. Data is representative of one of the six mice per group. c) Percentage of CD4+ Foxp3+ cells in the mice groups. Data is presented as mean ± SD (with n = 6 per group) and $p < 0.05$ compared to control peptide was considered significant compared to control peptide.

5.6. Bioinformatic analysis of Per a 10 T cell epitopes for mouse MHCII

In silico analysis of T cell epitopes of Per a 10 for mouse MHC II was performed using NetMHCIIpan 3.2 server (Jensen et al., 2018) and IEDB analysis tools SMM Align (Nielsen et al., 2007) and NN align (Nielsen and Lund, 2009). Consensus was taken amongst the predicted epitopes and four epitope regions were selected (Fig S7 in supplementary data).

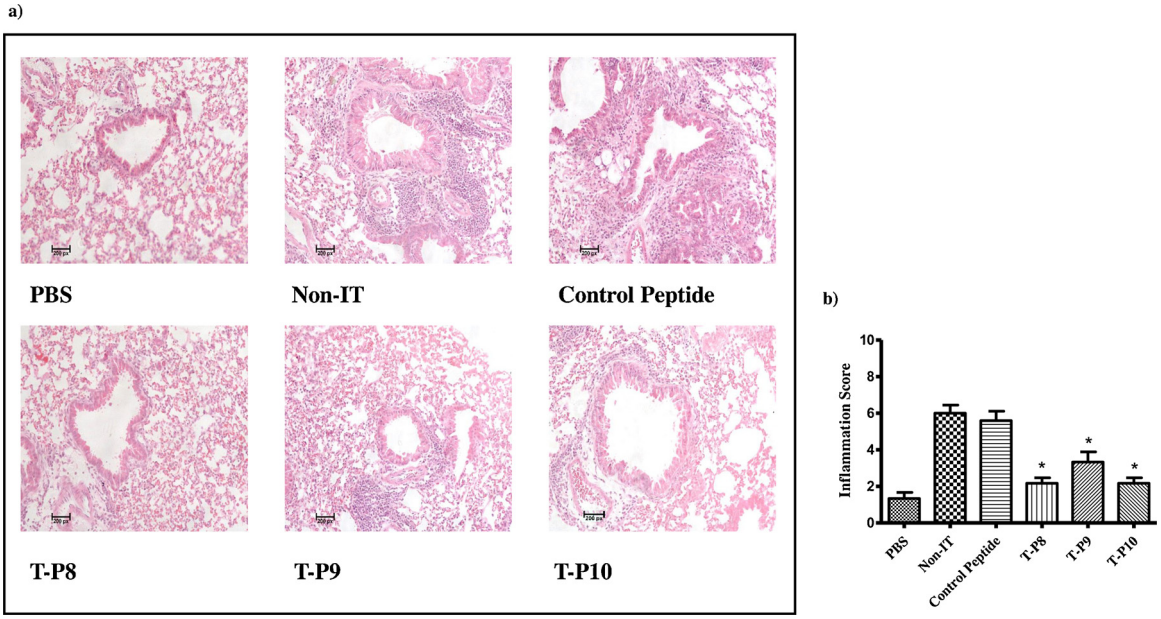

Fig. 4. a) Lung Histology and inflammation score in different treatment groups were observed by Haematoxylin and Eosin staining of paraffin embedded lung sections. Representative images per group are shown above. b) Inflammation score is representative of mean ± SD (with n = 6 per group). *symbol represents $p < 0.05$ compared to control peptide and PBS treated mice groups. Br. Represents bronchiole in lung section.

6. Discussion

Allergen immunotherapy (AIT) is an established therapeutic practice for management of allergic diseases. It is the only therapy that modifies the natural course of allergic reaction and provides long term curative benefits (Durham et al., 1999). Despite being the most efficacious therapeutic strategy, prolonged and frequent dosing regimens, inherent risk of adverse IgE mediated reactions and potential anaphylaxis warrant the need to have alternative therapeutic modalities (Pom?s et al., 2017). IT with short, synthetic peptides corresponding to T cell epitopes of allergen offer advantages over traditional approaches in terms of safety and efficacy.

In the present study, a model of airway allergic disease was generated by sensitization with cockroach extract and challenge with purified protease allergen Per a 10. To ascertain the binding affinity of Per a 10 derived peptides T-P8, T-P9 and T-P10 (previously predicted on human MHC II dataset (Govindaraj et al., 2016)) in the mice model, binding affinity of Per a 10 T cell epitopes towards mouse MHC II alleles was in silico predicted (supplementary data Fig S7). The prediction softwares employed in the study rely on analysis of potential binding affinities of peptides and are based on artificial neural network framework trained on immune epitope database.

An immunotherapy protocol was developed wherein mice were treated with Per a 10 derived T cell peptides and a final challenge was given after 40 days of IT. Mice without IT (sensitized and challenged by allergens CE and Per a 10 respectively and PBS administered during IT phase of the protocol) demonstrated lung inflammation characterized by high total cellular infiltration, specifically airway eosinophilia, along with high Per a 10 specific serum IgE, low IgG 2a and elevated proinflammatory Th2 cytokines and reduced T regulatory cytokines in BALF and splenocyte culture supernatant. Similar inflammatory phenotype was observed upon treatment with an unrelated control peptide.

Peptide treatment resulted in diminished recruitment of inflammatory cells to the airways as evidenced by low total cell count and airway eosinophilia in the treated groups. The peptides T-P8 and T-P10 rescued the pathophysiological features in the mice model to a great extent and demonstrated effective therapeutic potential. A significant reduction in eosinophilia may contribute towards restoration of airway function as proinflammatory mediators secreted by eosinophils (such as eosinophil peroxidase and eosinophil cationic proteins) contribute extensively towards airway epithelial damage, mucus hypersecretion and airway hyper-responsiveness (Possa et al., 2013).

Studies on T cell epitopes of PLA2 have demonstrated a shift in cytokine profile with reduced Th2 cytokines (Oldfield et al., 2002) and changes in antibody isotype ratio favoring towards elevated levels of IgG4 antibodies in subjects (Müller et al., 1998b; O'Hehir et al., 2016). In our study, robust induction of Th1 type cytokine (IFN-γ) and suppression of Th2 cytokine milieu was observed upon treatment with T-P8 and T-P10. Modulation of Th1/Th2 cytokine ratio has been shown to be important for IgG2a blocking antibody production during immunotherapy (Ebner et al., 1997). Reduction in specific IgE with a consequent increase in IgG2a antibodies was also observed implying active regulation of memory B cell responses upon peptide immunotherapy.

In our study, a significant increase in Th1 cytokine IFN- γ as well as T reg cytokines IL-10 and TGF-β was observed upon peptide treatment with an overall improvement in pathophysiological features of allergy suggesting the combinatorial action of Th1 and Treg cytokines to be the crucial driving forces in diminishing inflammatory Th2 responses in airways. However, upon peptide treatment the switch from Th2 to Th1 immune response needs to be well coordinated, since an uncontrolled bias towards Th1 milieu may possibly lead to inflammation in the airways and prove to be counterproductive in allergy treatment (Cui et al., 2005; Jatakanon et al., 1999). Induction of T regulatory cells upon PIT may provide the necessary regulation and suppress excessive proliferation of both Th1 and Th2 cells (due to its immune suppressive actions on both T cell subsets) and may contribute towards safe and efficacious treatment (Dehzad et al., 2011; Jutel et al., 2006). A combination of immune deviation (towards Th1) and T regulatory responses has earlier been shown to play a crucial role in Fel d 1 derived peptide immunotherapy in cat allergic individuals (Alexander et al., 2005). Our study further reinforces the need of maintaining the fine balance in Th1/Treg /Th2 ratios to be of utmost importance for success of PIT.

$CD4^+$ T cells play a pivotal role in driving immune hyper-responsiveness towards allergen and therefore are prime targets of immunotherapeutic interventions (Tanabe, 2007). Studies conducted on murine models have shown that administering supra-optimal concentration of dominant T cell epitopes is marked by a transient increase in Th2 cytokines (hyper-activation), followed by induction of anergy and a subsequent decline in synthesis of major Th2 cytokines (IL-4 and IL-5) and sustained production of IL-10 (O'Hehir et al., 2016). Peptide immunotherapy with Fel d 1 peptide demonstrated IL-10 dependent tolerance generation and linked suppression in the murine model (Campbell et al., 2009). Induction of anergic state depends on IL-10 levels upon IT with allergen PLA2 as well as T cell epitope containing peptides of PLA2 (Akdis et al., 1998). In our study, T-P8 and T-P10 demonstrated increase in levels of IL-10 and TGF-β in both BALF and splenocyte culture supernatant. Earlier, IL-10 and TGF-β have been shown to act in a co-operative manner in the suppression of immune responses upon SIT in house dust mite patients (Jutel et al., 2003).

Induction of peripheral tolerance is considered as a hallmark of AIT and is characterized by generation of T regulatory cells (T regs) (Akdis et al., 2014). T regs control and modify allergic immune responses via multitude of activities such as inhibition of activation of Th2 cells, mast cells, basophils and eosinophils. T reg cells exert their direct effect on B cells and suppress the production of allergen specific IgE and increase in IgG4. Studies on grass pollen immunotherapy demonstrate induction of T regulatory cells upon immunotherapy (Francis et al., 2003). To explore the mechanism of reduction in Th2 proinflammatory cytokines in the treated groups, splenocytes from each group were isolated and analyzed for the presence of $CD4^+$ $Foxp3^+$ T cells in splenocytes. The number of T regulatory cells was significantly increased in T-P8 and T-P10 administered group demonstrating their role in improved pathophysiological features in the mice model.

Initial studies on grass pollen allergic patients undergoing immunotherapy revealed an increase in IL-10 expressing T regulatory cells in PBMCs demonstrating induction of peripheral T cell tolerance (Francis et al., 2003). Local induction of T regulatory cells in nasal mucosa upon SCIT has been associated with high clinical efficacy in rhinitis patients (Radulovic et al., 2019). T-P8 and T-P10 led to decline in Th2 cytokines with a consequent induction of IL-10 and TGF-β in both BALF and splenocyte culture supernatant, thereby implying towards robust induction of both local and systemic immune responses upon PIT.

As per the in silico MHC binding data, the peptide region encompassing T-P8 emerged as the strongest binder (amongst the three predicted peptides), while T-P10 demonstrated weak MHC-II binding in Net MHCIIPan 3.2, for H2-I-Ad and H2-IEd haplotype expressed in Balb/c mice (supplementary data Fig S7 c). This trend is in accordance with the in vivo results obtained in our murine model. In our earlier in vitro study with human PBMCs, the peptide T-P9 did not show convincing results as well, which might be attributed to its small size (a six-mer) and ability to present peptides to MHCII (Govindaraj et al., 2016). In the in vivo mice model of allergy, IT with T-P9 showed minimal effects (highest being T-P8 followed by T-P10), however it did not show MHC binding in the in silico studies. Given the complexities of MHC-II peptide interaction, it seems the effects (although lower most amongst the three predicted peptides) of T-P9 treatment seem to be less dependent on its MHC-II binding affinity.

The maximal resolution of pathophysiological features of allergic hypersensitivity, immune deviation and T cell tolerance were

demonstrated by peptide immunotherapy with T-P8 and T-P10 in cockroach allergic hypersensitivity model. Therapy with T-P8 and T-P10 effectively modulates the B and T cell responses towards the allergen, demonstrates safety and efficacy in murine model of allergic hypersensitivity and possess potential for treatment of cockroach allergy. As per the SDAP data, peptides T-P8 and T-P10 demonstrate similarity with known serine and cysteine protease allergens from dust mite, seed storage proteins and certain food allergens. Treatment with these peptides may prove to be beneficial in case of patients cross reactive to other protease allergens as well.

In conclusion, peptide immunotherapy using T cell epitopes by T-P8 and T-P10 demonstrates efficacy in murine model of allergic hypersensitivity and possess potential for treatment of cockroach allergy. Peptide treatment is associated with synergistic action of immunological responses including Th2 to Th1 switching, production of blocking antibodies as well as regulatory pathways involving T regulatory function.

Conflict of interests

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.01.003.

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