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Research Article

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Research Article

Per a 5-derived T-cell peptides modulate NF-kB signalling to ameliorate allergic inflammation systemically in murine model of cockroach allergic hyper-reactivity

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Abstract

Peptide immunotherapy (PIT) represents a safe and efficacious therapeutic regimen with in-consequential side-effects. The present study aims to identify T-cell epitopes of Per a 5 allergen, a delta class GST from *Periplaneta americana* and investigate effect of peptide treatment in murine model of cockroach allergen-mediated hyper-reactivity. The epitopes (TC-P1, TC-P2, and TC-P3) were identified as promiscuous MHC-II binders by MHC-Pred, ProPred, and IEDB analysis tool. Murine model of cockroach allergic hyper-reactivity was generated in Balb/c mice. A marked reduction in cellular infiltration in lungs (3-fold compared with Non-IT) was observed in T3-IT group as evidenced by total leucocyte count in BALF and histology. Specific IgE levels were reduced 3-fold in T2-IT and T3-IT compared with Non-IT with increase in IgG2a levels. IL-4 and IL-13 were reduced upto 2.5-fold in treatment groups compared with Non-IT group. Splenocytes revealed significant increase in levels of $CD4^+FoxP3^+$ T cells in TC-P1 and TC-P2 mice demonstrating a systemic shift towards Tregs. Peptide treatment downregulated NF-kB signalling in lung and enhanced the levels of immune-regulatory molecules α1-antitrypsin and elafin. Our results indicate that TC-P1 and TC-P3 alter Th2 cytokine milieu and antibody isotype ratio to suppress allergic inflammation. PIT modulates local and systemic mechanisms to resolve inflammation and possess potential for treatment in cockroach allergy.

Keywords: T-cell epitopes, immune regulation, peptide immunotherapy

Abbreviations: PIT: peptide immunotherapy;AIT: allergen immunotherapy;GST: glutathione-S-transferase;CE: cockroach extract;EPO: eosinophil peroxidase;BALF: bronchoalveolar lavage fluid.

Introduction

Allergic diseases especially allergen mediated asthma has profound public health implications in both developed and developing nations. Allergic asthma is characterized by presence of allergen-specific IgE in serum along with local and systemic activation of Type 2 proinflammatory responses. Pharmacotherapeutic interventions, though regarded as mainstay therapy, target on providing symptomatic relief without affecting immunological mechanisms. Allergen immunotherapy (AIT) is recognized as the only treatment modality with disease modifying ability resulting in long-term remission of symptoms and improved quality of life [1]. Despite the promising results of AIT, the intrinsic ability of allergens to crosslink bound IgE molecules on surface of effector cells present an enormous challenge since an approximate 5-35% of patients undergoing AIT exhibit adverse systemic reactions [2]. Additionally, variation in quality and potency of allergen extracts pose significant concerns from the viewpoint of safety and adherence to long and cumbersome AIT regimens [3].

IT with peptides derived from T-cell epitopes of allergens has been suggested as a promising candidate to improve safety of AIT. It involves administration of T-cell peptides (usually 12-20 residues), thereby lacking the size and requisite three dimensional structure to crosslink IgE bound to Fcε receptors on effector cells and thus reducing the apparent side effects of immunotherapy [4]. Treatment with T-cell epitope-derived peptides has demonstrated efficacy with cat, house dust mite, and pollen allergy with in-consequential side effects [5, 6, 7]. Previously immune-regulatory role of T-cell peptides derived from fungal Cur l 3 protein and cockroach serine protease allergen Per a 10 have been observed in murine model of allergic reactivity [8, 9].

Perennial exposure to cockroach allergens is a strong predictor of recurrent wheezing, asthma exacerbations, and associated morbidity. *Periplaneta americana* is a predominating cockroach species in tropical and sub-tropical regions worldwide. It exhibits a heterogeneous profile of multiple allergen components with varying clinical relevance depending upon co-sensitizing allergenic sources in the milieu [10]. In the

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present study, T-cell epitopes of Per a 5, a clinically relevant and cross-reactive glutathione-*S*-transferase (GST) allergen from *P. americana* were identified using in silico computational tools. Therapeutic efficacy of predicted T-cell epitopic peptides was evaluated in murine model of cockroach allergen-induced pulmonary inflammation.

Materials and methods

Identification of T-cell epitopes of Per a 5

Per a 5 homology modelled structure was used for prediction of T-cell epitopes (as described previously) [11]. Epitopes of Per a 5 were predicted for both human MHC-II by inhibitory concentration (IC_{50}) value-based quantitative prediction methods (MHCPred (http://www.ddg-pharmfac.net/mhcpred/MHCPred/) and IEDB analysis) and binding status scoring qualitative tool (ProPred (http://www.imtech.res.in/raghava/propred/). Promiscuous peptides binding to multiple HLA class II molecules were selected as T-cell epitopes. Physicochemical characterization (hydrophobicity, surface accessibility) of predicted epitopes was performed by Peptide 2.0 tool and NetSurfP2.0 server. Per a 5 epitopes demonstrating potential binding with mouse MHC-II alleles (H2-IEd, H2-IAb, and H2-IAd) were also predicted using tools in IEDB analysis server (IEDB Analysis, SMM-Align, and NN-Align).

Proteins/peptides for immunization

Cockroach extract (CE) was purified as described previously [10]. The predicted T-cell epitopes were chemically synthesized by Thermo Fisher Scientific with >95% purity. The peptide sequences were confirmed by Mass Spectrometry (Certificate of Analysis in supplementary section).

Animals

Female Balb/c mice (n = 7, 4–6 weeks old, weighing 20–22 g) were procured from Institute of Genomics and Integrative Biology (IGIB) and housed in the animal care facility of IGIB under standard laboratory conditions with ad libitum access to food and water. The animals were allowed to acclimatize 1 week prior the experiment. The study protocol was approved by animal ethics committee of IGIB, Delhi (IAEC Approval-IGIB/IAEC/20/20) following the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India. The animal studies conducted in the research article adhered to the ARRIVE guidelines.

Immunotherapy protocol

Studies by our laboratory have demonstrated allergenic relevance and cross-reactive potential of Per a 5 allergen with corresponding GST allergens in indoor allergen sources such as dust mite and fungi [10] [11],. Therefore, to evaluate immunotherapeutic efficacy of the predicted Per a 5 T-cell peptides, a model of airway allergic asthma was generated in Balb/c. Briefly, mice were intraperitoneally (i.p.) sensitized with cockroach extract (CE 20 µg) or PBS at Days 0 and 4 and intranasally (i.n.) challenged with cockroach extract (10 µg) or PBS on days 11, 12, and 14 (Fig. 2). The immunotherapy protocol was designed as described previously[8]. Immunotherapy with PBS, CE, and T-cell peptides TC-P1, TC-P2, and TC-P3 (100 µg per mice) was given by subcutaneous route on days 25, 27, 29, and 31. Mice were challenged intranasally with Cockroach extract (10 µg/mice) after 40 days of immunotherapy (days 70, 72, and 74) and sacrificed after 24 h of last challenge on 75th day.

In the mice group designated as "PBS", animals were administered with PBS in sensitization, challenge, immunotherapy, and re-challenge phases and therefore served as negative controls. Pre-IT mice were administered cockroach extract in sensitization and challenge phase followed by sacrifice on 15th day indicating the short-term effects of allergen sensitization in allergic pathophysiology. The "Non-IT" group mice received CE in sensitization and challenge phase of the protocol while PBS was administered subcutaneously in immunotherapy time points followed by CE re-challenge after 40 days.

Sample collection and processing

The trachea was exposed and BALF was collected by flushing lungs with 1.5 ml sterile PBS (flushing the lungs with 0.5 ml PBS thrice and collecting approximately 1.2 ml BAL fluid). BALF was centrifuged at 0.4 × *g* for 45 min at 4°C. RBCs were lysed using RBC lysis buffer and supernatant was collected and stored at −80°C. Lung tissue was dissected and fixed with 10% neutral buffered formalin (NBF) and paraffin embedded later for histological analysis. Blood was collected, centrifuged at 1000 rpm, sera was separated and stored at −80°C. The embedded tissue samples were sliced into 4 µm sections, stained with hematoxylin-eosin stain and were scanned using Nikon Eclipse 80i microscope attached with an in-line camera. Assessment of lung inflammation was performed by examination of cellular infiltration in peri-bronchial (in both large and small airways), perivascular regions of the lung, bronchial wall thickness and disruption of alveolar structure (by cellular infiltration). Inflammation was scored from 1 to 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 [8, 9, 12].

Determination of cellular infiltration in lungs, immunoglobulin, and cytokine analysis

Total cell count and eosinophil count was determined as described previously [13]. Cockroach allergen-specific IgE and IgG2a levels were measured in mice sera 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 per manufacturer's instructions. Mice sera was diluted 1:10 for IgE estimation and 1:50 for IgG 2a. Eosinophil peroxidase activity in BALF was measured by spectrophotometric method. Briefly, 100 µl of BALF supernatant was taken in microtiter plate in duplicates (Nunc-immuno, Denmark). One hundred microliter of substrate solution containing 1 mM hydrogen peroxide, 0.1 mM ortho-phenylenediamine-dihydrochloride and 0.1% Triton X-100 in 0.05 M Tris–HCl was added. Plate was incubated for 30 min at 37°C and reaction was stopped by adding 50 µl of 4 M sulphuric acid. Absorbance was measured at 492 nm using microplate reader (Bio-Rad Laboratories Ltd., UK). EPO activity of BALF supernatant was depicted in the term of absorbance at 492 nm. Cytokine levels (IL-4 and IL-13 in BALF) were measured by ELISA kits following manufacturer's instructions (eBioscience).

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Splenocyte isolation and staining for T regulatory cells

Briefly, spleen was harvested, washed with PBS, and minced with a syringe plunger in 2 ml of RPMI media. The obtained solution was strained through membrane filters and centrifuged at 1300 rpm for 5 min. The cell pellet was lysed with RBC lysis buffer by gentle tapping and incubated on ice for 2 min. Fresh RPMI media was added to neutralize the RBC lysis buffer and centrifuged at 1300 rpm for 5 min twice and the cell pellet was resuspended in RPMI media containing 1% foetal bovine serum (FBS). For extracellular staining, 1×10^6 cells were resuspended in FACS buffer (0.5% BSA in PBS) and stained with anti-mouse CD4-FITC antibody (BD Biosciences) as per manufacturer's instructions for 30 min at 4°C. The cells were washed twice with FACS buffer at 1300 rpm, 5 min, 4°C. Later, the cells were fixed with 2% paraformaldehyde solution for 30 min at 4°C washed at kept overnight at 4°C. To stain the intracellular levels of FoxP3, paraformaldehyde-treated cells were washed with FACS buffer twice (centrifuge at 1300 rpm, 5 min, 4°C) and then permeabilized with solution containing 0.05% Triton X-100 solution in PBS for 30 min at 4°C. The cells were washed with FACS buffer thrice (centrifuge at 1300 rpm, 5 min, 4°C) and incubated with anti-mouse FoxP3-APC antibody (BD Biosciences) as per manufacturer's instructions. The cells were washed with FACS buffer thrice, resuspended in FACS buffer and analysed flow cytometrically by BD Accuri. The data were analysed by BD Accuri C6 plus software.

Western blot analysis

The lung lobe was homogenized and protein estimation of samples was performed using the bicinchoninic acid (BCA) method. Protein (30 µg per sample) was loaded on 12.5% SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was blocked with TBS-T (Tris base saline with 0.05% tween 20) containing 5% BSA for 2 h, washed with TBS-T, and incubated with rabbit anti-mouse NF-kB (p65) antibody (Elabsciences) at 1:2000 dilution, phospho-NF-kB (ABclonal) at 1:1000 dilution, α1-antitrypsin antibody (Sigma-Aldrich) at 1:1000 dilution, anti-elafin antibody (Abcam) at 1:1000 dilution or anti-mouse β-actin antibody at 1:3000 dilution overnight at 4°C. Further, the membranes were washed with TBS-T and incubated with secondary antibody at optimized dilutions (anti-mouse IgG (1:20 000 diluted in TBS-T) or anti-rabbit IgG (1:3000 dilution for phospho-NF-kB and 1:6000 diluted in TBS-T for rest) conjugated with HRP, respectively, for 2 h at room temperature. The membrane was extensively washed with TBS-T and developed using chemiluminescence method. The protein levels of NF-kB (p65) and lung anti-proteases α1 anti-trypsin and elafin were analysed in the lung protein of mice. The intensity of bands was quantified using ImageJ software and the relative expression was expressed in comparison to β-actin house-keeping gene.

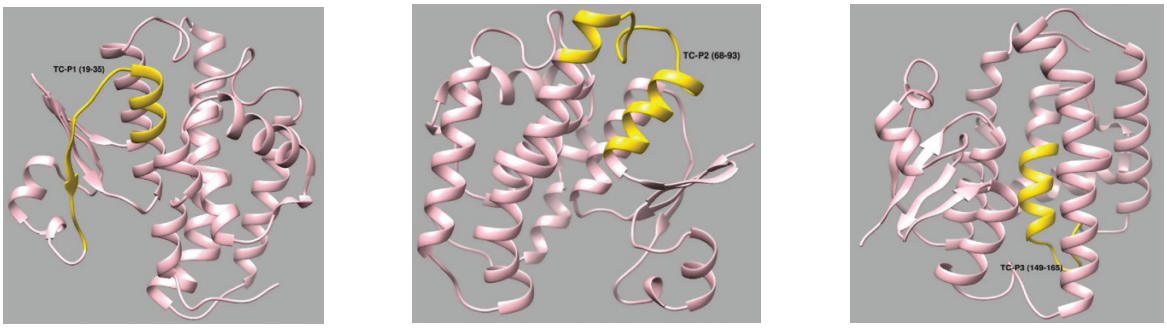
Statistical analysis

Statistical analysis of the results was done using one-way ANOVA in GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

Prediction of T-cell epitopes

T-cell epitopes of Per a 5 were predicted by utilizing MHCPred, ProPred, and IEDB analysis tool. Three T-cell epitopes encompassing 19–35 (TC-P1), 68–93 (TC-P2), and 149–165 (TC-P3) regions were identified as promiscuous human MHC



a) b) c)

HLA Distribution analysis for predicted T cell epitopes

S.no.	Epitope	HLADQ A1*01	HLADR B1*01	HLADRB 1*03	HLADRB 1*04	HLADRB 1*07	HLADRB1 *08	HLADRB 1*09	HLADR B1*11	HLADR B1*13	HLADRB1 *14	HLADRB1 *15	HLADR B3*01	HLADR B5*01
1.	TC-P1													
2.	TC-P2													
3.	TC-P3													

d)

Figure 1: Representation of predicted T-cell epitopes of Per a 5. (a) Per a 5 structure depicting TC-P1, TC-P2, and TC-P3 (a) to (c). Schematic representation of consensus HLA distribution by T-cell prediction softwares (IEDB analysis tool, ProPred. and MHCPred) exhibited by Per a 5 epitopes. Boxes filled with pink colour signify identification by all three prediction softwares, while boxes filled with yellow and blue signify identification by two and one software respectively.

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II binders. HLADRB1*01, HLADRB1*03, HLADRB1*04, HLADRB1*08, HLADRB1*11, HLADRB1*13, and HLADRB1*15 emerged as major HLA haplotypes predicted to bind promiscuously with Per a 5 T-cell epitopes. TC-P1 and TC-P2 appear to cluster in thioredoxin fold region of Per a 5 while TC-P3 occupies substrate binding site (Fig. 1). More than 50% of the residues in T-cell epitopes, TC-P1 and TC-P3 demonstrate hydrophobic character while TC-P2 demonstrates a pronounced hydrophilic nature (with 15.38% residues showing hydrophobic nature). T-cell epitopes (TC-P1 and TC-P3) demonstrate propensity to occupy buried regions of protein (Percentage of residues with RSA values >25%: 35.39% and 17.64%, respectively) with an exception of TC-P2 that occupies surface exposed regions of the protein (percentage of residues with RSA values >25%: 76.92%) (Table 1). Computational identification of T-cell epitopes possessing binding affinity with mouse MHC-II alleles was performed using IEDB analysis server. The predicted epitopic regions demonstrated significant overlap with predicted T-cell epitopes for human MHC-II alleles (TC-P1, TC-P2, and TC-P3) (Supplementary Table S5).

IT with T-cell peptides leads to resolution of pulmonary inflammation

To determine the effect of immunotherapy on generated murine model of cockroach allergic inflammation, mice were subcutaneously administered with CE, Per a 5-derived T-cell peptides and PBS (CE-IT, T1-IT, T2-IT, T3-IT, and non-IT groups, respectively) in immunotherapy phase of the protocol. Analysis of total leucocyte count in BALF indicates high levels of cellular infiltration in lung of Pre-IT and Non-IT mice groups. In comparison, CE-IT and Peptide IT groups were able to reduce lung inflammation. Maximum reduction in total cellular infiltration was observed in T3-IT group (3.2-fold change with respect to Non-IT) followed by T1-IT and CE-IT mice (2.5- and 2.25-fold reduction with respect to Non-IT). Eosinophil count in BALF demonstrates significant reduction in IT administered groups in comparison to Pre-IT and Non-IT mice groups. Lung histological examination by Hematoxylin and eosin (H&E) staining exhibit cellular infiltration and modest disruption of alveolar architecture (due to cellular infiltration in alveolar regions) in Pre-IT and Non-IT mice groups. IT with peptide TC-P1

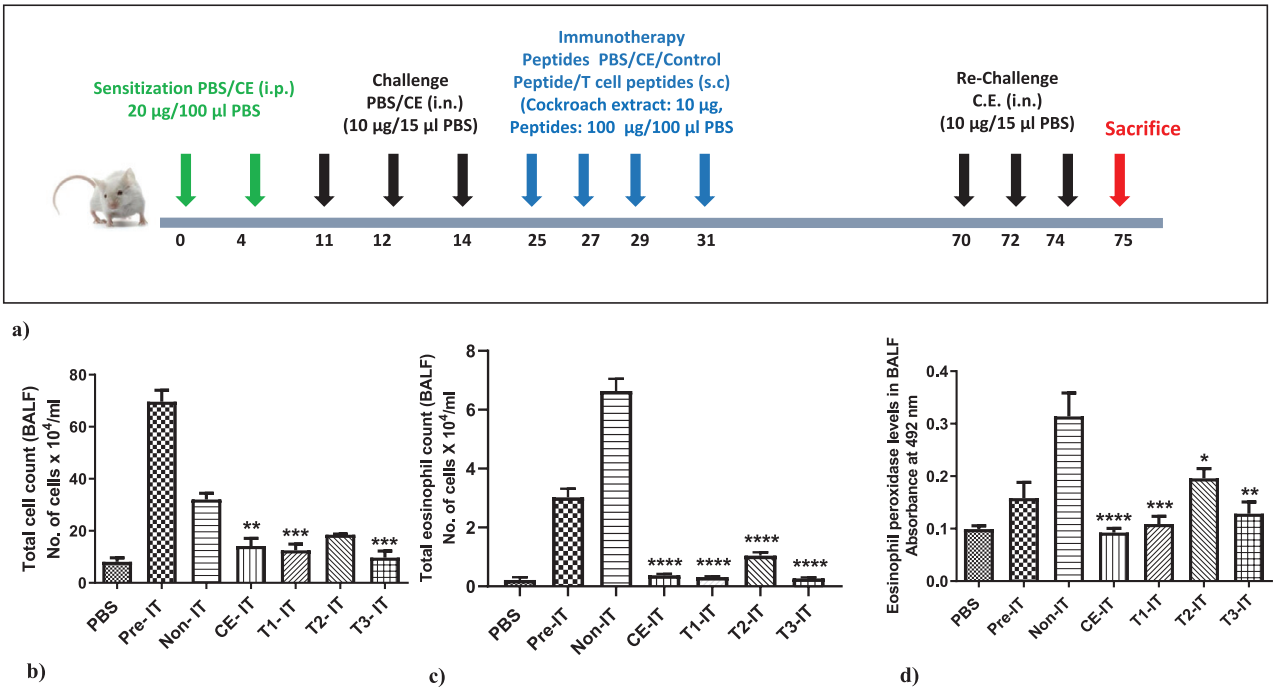

Figure 2: Assessment of pulmonary inflammation in murine model of cockroach allergy: (a) Schematic representation of immunization protocol followed for evaluation of immunotherapeutic potential of Per a 5-derived T-cell epitope peptides. Effect of administration of Per a 5-derived T-cell peptides on lung inflammation was observed by (b) Total leucocyte count, (c) total eosinophil count in BALF, and (d) Eosinophil peroxidase levels in BAL fluid.

Table 1: Physico-chemical analysis of Per a 5 T-cell epitopes.

S. no.	Peptide	Peptide sequence	Region	% Hydrophobic residues	% Acidic residues	% Basic residues	% Neutral residues	% of residues above threshold relative surface accessibility (RSA >25%)
1.	TC-P1	LAAKAIGVDLNLKVTN	19-35	56.25	6.25	12.5	25	35.39
2.	TC-P2	RAILSYLADQYGKDDSLYRRDAKKRA	68-93	15.38	15.38	26.92	26.92	76.92
3.	TC-P3	VAGNKLTIADLAIVSSV	149-165	58.82	5.88	5.88	29.41	17.64

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(T1-IT) lead to maximal resolution in inflammation followed by TC-P2 (T2-IT) as evidenced by reduced inflammation score (Fig. 3a and b).

Allergic inflammation is characterized by eosinophil dominant infiltration in lung, therefore, levels of eosinophil peroxidase (EPO) were analyzed in BALF (Fig. 2b). EPO is an oxidoreductase enzyme present in secretory granules of eosinophils and is a marker of eosinophilic infiltration. High levels of EPO were observed in Non-IT and Control-IT, in comparison immunotherapy with CE was able to reduce EPO levels significantly (3.3-fold change when compared with Non-IT). Among the peptides, IT with TC-P1 and TC-P3 lead (2.8- and 2.4-fold with respect to Non-IT group) to reduction in EPO levels signifying reduction in eosinophilic infiltration in lungs upon peptide immunotherapy (Fig. 2c).

Per a 5-derived T-cell peptides modulate serum cockroach allergen-specific IgE and IgG2a levels

Serum IgE is principal mediator of allergic reactions and therefore the effect of immunotherapy was examined on cockroach allergen-specific IgE. A robust decline in cockroach allergen-specific IgE antibody was observed in CE-IT and T-cell peptide IT groups. In comparison to Non-IT group, three times reduction in cockroach-specific IgE levels was observed in T2-IT group followed by T3-IT and CE-IT groups (2.7- and 2.3-fold reduction, respectively). Analysis of blocking serum IgG2a antibodies revealed maximum upregulated levels in T3-IT group (1.8-fold) followed by T1-IT group (1.4 times) (Fig. 4).

Peptide immunotherapy leads to suppression of Th2 immune responses and induce T regulatory cells systemically

Administration of T-cell peptides TC-P2 and TC-P3 (T2-IT and T3-IT groups, respectively) was able to reduce the levels of Th2 cytokines significantly demonstrating an overall shift from Th2-dependent pro-inflammatory microenvironment (with more than 5-fold reduction in IL-4 levels and more than 2.5 times reduction in IL-13 cytokine levels). To ascertain the same, analysis of T regulatory cells (CD4+FoxP3+ cells) was carried out in splenocyte population. An increase in T regulatory cell population was observed in peptide immunotherapy groups with TC-P1 and TC-P2 administered mice (1.46 and 1.28 times increment, respectively) signifying towards induction of systemic adaptive regulatory mechanisms (Figs. 5–6).

Peptide immunotherapy downregulates NF-kB signalling and upregulate the levels of immune-regulatory anti-proteases in lung

To determine the underlying cellular mechanisms involved in mediating suppression of allergic pathophysiology, western blot analysis of lung samples was performed. Levels of

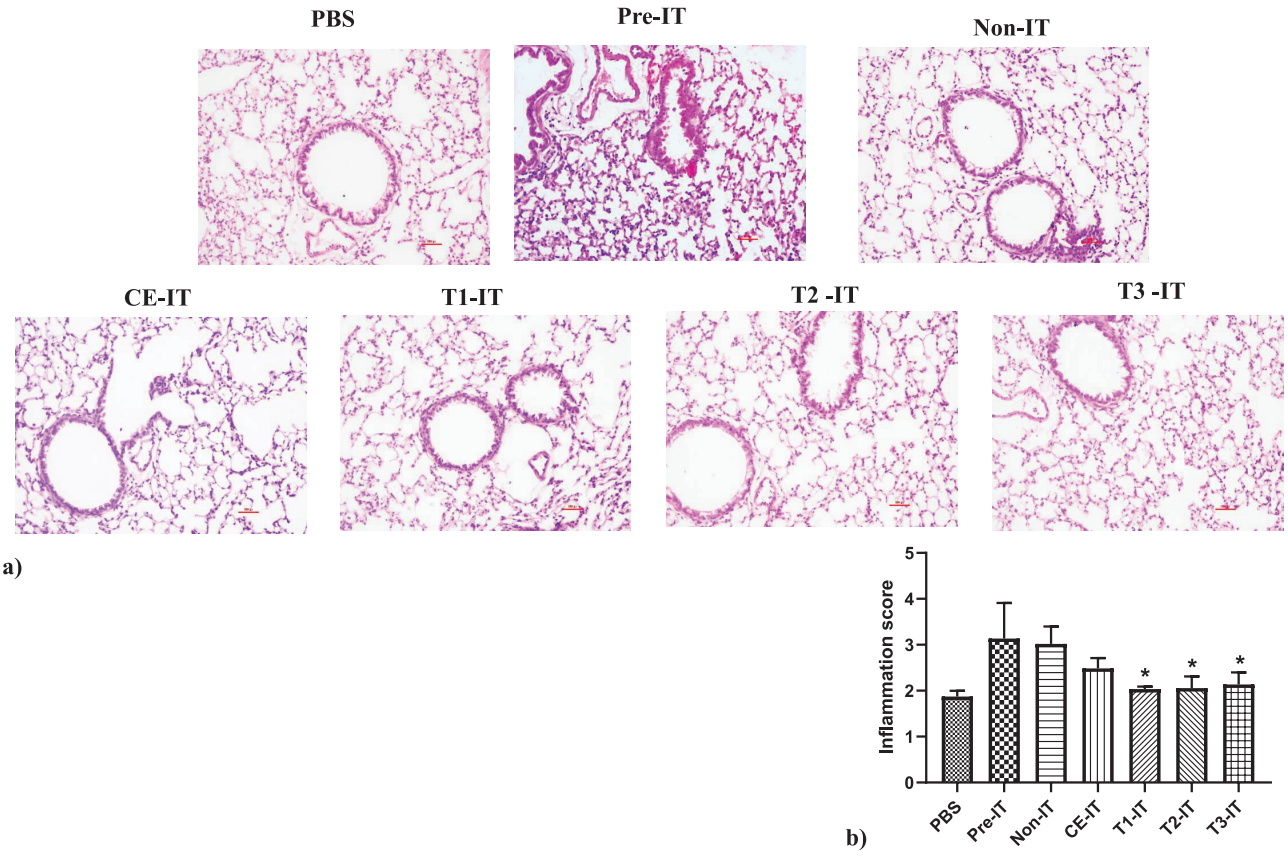

Figure 3: Histological examination of H&E staining of lung tissues was performed using Nikon Eclipse 80i and captured using NIS Elements F 4.6 software. (a) Representative lung histology (H&E staining) of PBS, Pre-immunotherapy (Pre-IT), Non-immunotherapy (Non-IT), Control peptide IT, Cockroach allergen immunotherapy (CE-IT), and Per a 5-derived T-cell peptide immunotherapy (T1-IT, T2-IT, and T3-IT). Resolution of inflammation was observed in mice receiving immunotherapy with peptides. (b) Inflammation core representative of mean ± SEM of 5-6 mice per group. *$P < 0.05$ when compared with non-immunotherapy group; ns: not significant; determined by one way ANOVA.

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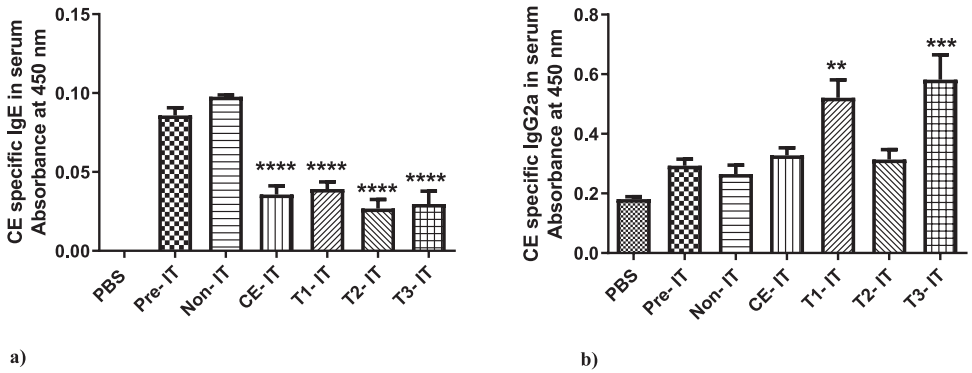

Figure 4: Determination of cockroach allergen specific IgE and IgG2a levels in serum of murine model of cockroach allergy: Levels of (a) specific IgE and (b) IgG2a levels against cockroach allergen were estimated using indirect ELISA. Data are presented as mean ± SEM of 5-6 mice per group. *$P < 0.05$ when compared with non-immunotherapy group; ns: not significant; determined by one-way ANOVA.

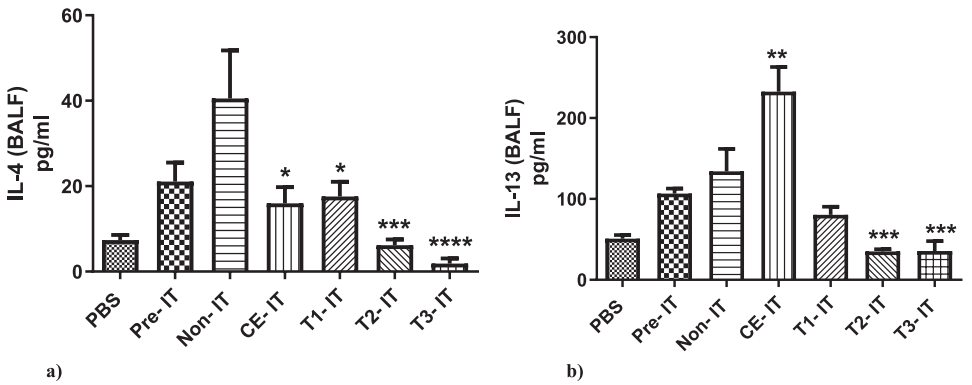

Figure 5: Th2 cytokine analysis: analysis of Th2 cytokines (a) IL-4 and (b) IL-13 was performed in BAL fluid by ELISA. Data are presented as mean ± SEM of 5–6 mice per group. *$P < 0.05$ when compared with non-immunotherapy group; ns: not significant; determined by one-way ANOVA.

pNF-kB (p65)/NF-kB were significantly downregulated in immunotherapy group (both CE-IT and PIT mice groups). Maximal suppression was observed in TC-P1 administered mice followed by TC-P2 and CE-IT group. Further, levels of intrinsic lung protease inhibitors α1-anti-trypsin and elafin were investigated in lung samples. A significant upregulation of α1-anti-trypsin was observed in TC-P2 and TC-P3 administered mice while elafin levels were elevated in TC-P2 and TC-P3 administered mice (Fig. 7).

Discussion

Intrinsic HLA-binding degeneracy of allergen T-cell epitopes and ability of CD4+ T cells to recognise an epitope in context of multiple HLA class II molecules position T-cell peptide therapy as a promising candidate for allergy therapeutics [14, 15]. With expansion of allergen sequencing data and machine learning algorithms, the prediction accuracy of computational tools has increased manifold [16]. Considering the inherent diversity of HLA allelic variants, in silico algorithms trained on vast repertoire of epitope sequences represent a cost effective and fairly less tedious approach for preliminary screening of T-cell epitopes. In the present study, a combination of IC_{50} value-based quantitative prediction tool (MHCPred) and binding status scoring qualitative tools (ProPred and IEDB analysis tool) were employed for determination of promiscuous T-cell epitopes of Per a 5 allergen. Consensus amongst the predicted data revealed presence of three T-cell epitopes, namely TC-P1, TC-P2, and TC-P3 spanning the regions 19-35, 68-93, and 149-165 amino acids of Per a 5 allergen, respectively. In contrast to nominal antigens which are presented in context of HLA-DR molecules, allergen epitopes are also presented in context of highly conserved HLA-DP and HLA-DQ subtypes [17]. Amongst, the predicted T-cell epitopes, TC-P1, TC-P2, and TC-P3 demonstrated binding with multiple HLA-DR molecules while TC-P2 exhibited binding affinity with HLA-DQ A1 as well. Structural and physico-chemical analysis of epitopes reveal TC-P1 and TC-P3 as stretches of largely hydrophobic amino acid residues with low surface accessibility. The epitope TC-P3 occupies the most inaccessible region of Per a 5 protein while TC-P2 exhibits hydrophilicity and high surface accessibility (Fig. 1c).

To harness the potential of T-cell epitopes as promising tool for alleviating allergic manifestations, their safety and efficacy needs to be evaluated. Therefore, a murine model of cockroach allergic hyper-reactivity was generated. The murine model demonstrated cardinal features of airway allergic inflammation characterized by allergen induced airway infiltration (predominately eosinophilic in nature) and increased cockroach allergen-specific serum IgE (Pre-immunotherapy group). Treatment with allergen (CE) and peptides TC-P1 and TC-P3 was able to significantly reduce infiltration of proinflammatory cells in lungs as evidenced by total leucocyte count, eosinophil count, and lung histological examination. A concomitant reduction in eosinophil peroxidase (EPO, an indicator of eosinophil-mediated pathogenicity and asthma severity) levels in BALF was observed in treatment groups indicative of rescued pulmonary inflammation [18].

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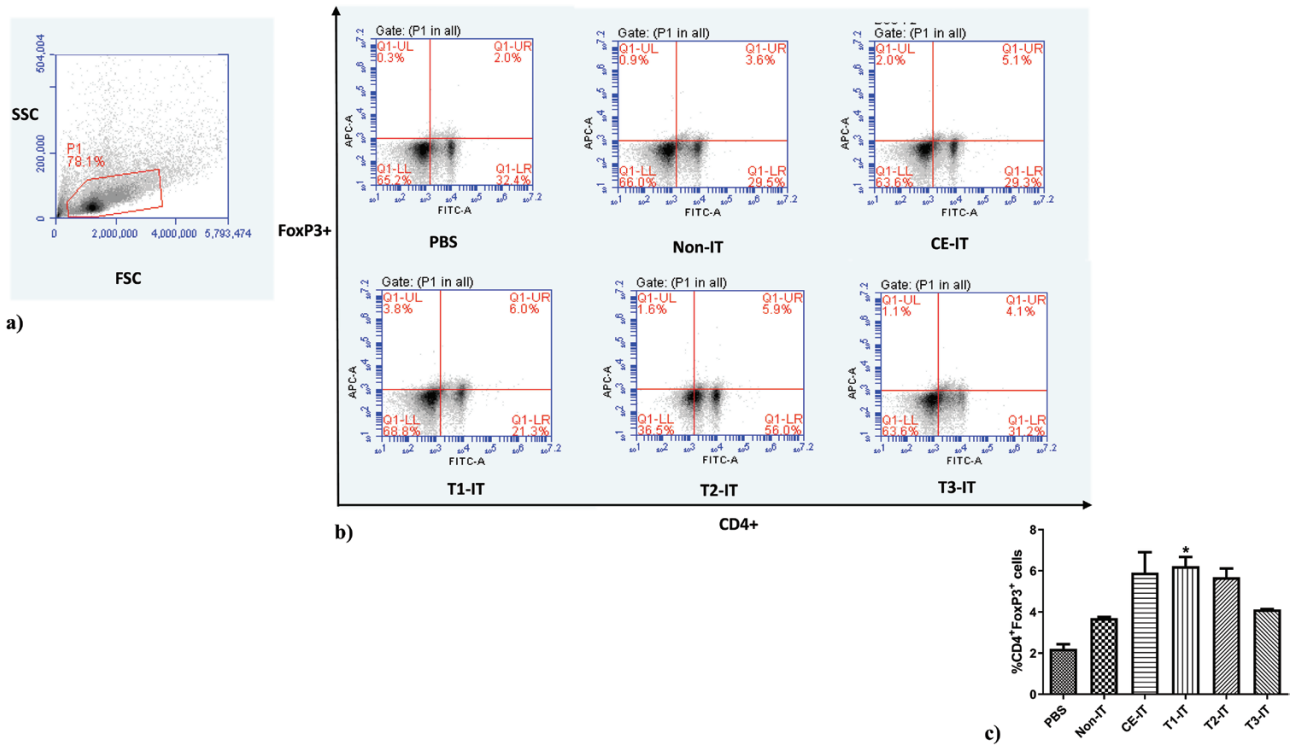

Figure 6: Analysis of T regulatory cells in splenocytes. Flow cytometric analysis of splenocyte demonstrated increase in CD4+FoxP3+ T regulatory cell population in peptide administered groups. (a) FSC vs SSC plot, (b) representative dot plots mice groups depicting CD4+FoxP3+ T regulatory population and (c) bar graph showing T regulatory cells in mice splenocytes. Data are presented as mean ± SEM of 5-6 mice per group. *$P < 0.05$ when compared with non-immunotherapy group; ns: not significant; determined by one-way ANOVA.

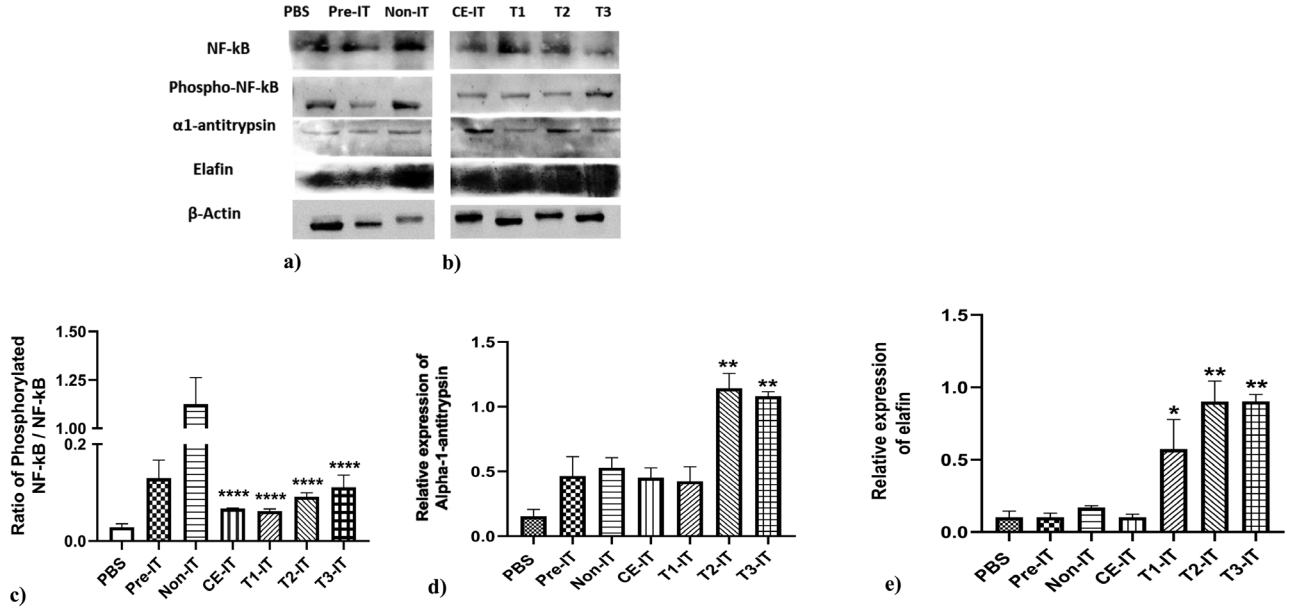

Figure 7: Expression analysis of NF-kB (p65) and intrinsic anti-proteases in lung. Expression of NF-kB, α1-antitrypsin, and elafin was analysed by western blot analysis of mice lung. (a) and (b) Representative western blot images of NF-kB, phospho-NF-kB, α1-antitrypsin, elafin, and β-actin. Bar graph depicting densitometric analysis of (c) ratio of phospho-NF-kB to NF-kB, (d) α1-antitrypsin, and (e) elafin in comparison to β-actin. Data are represented as mean ± SEM of 3–5 samples per group. Statistical significance was determined using one-way ANOVA in comparison to non-immunotherapy administered group.

Long-term resolution of inflammation in AIT is achieved through reduction in allergen-specific IgE levels with concomitant production of blocking IgG2a antibodies in serum. Earlier studies on T-cell epitopes of PLA2 have demonstrated robust shift in cytokine profile and antibody isotype ratio favouring towards a less pronounced Th2 cytokine milieu and elevated blocking antibody production upon peptide treatment [19]. In the cockroach allergic model, the AIT and

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PIT groups demonstrated reduction in IgE; however, maximal reduction in serum IgE levels was observed in TC-P2 IT group followed by IT with TC-P3. An increase in serum IgG2a levels was observed in treatment groups, with maximum increase in TC-P3 IT and TC-P1 IT groups.

The success of conventional allergen immunotherapy relies on initiating immunological mechanisms to subvert proinflammatory microenvironment thereby leading to sustained resolution of inflammation. Administration of TC-P3 peptide was able to downregulate Th2 cytokines IL-4 and IL-13 most significantly. Immunotherapy has been shown to induce regulatory cells indicative of generation of peripheral tolerance. T regulatory cells have been observed to be induced in SCIT treatment [20]. Therefore, $CD4^{+}FoxP3^{+}$ T regulatory cell populations were observed in splenocytes. Flow cytometric analysis demonstrates an increase in T regulatory cells in TC-P1 and TC-P2 administered mice [18] groups. Thus, the in vivo studies indicate towards immunotherapeutic efficacy of Per a 5-derived T-cell peptides in murine model of cockroach allergy. These peptides are able to resolve lung inflammation and induce T regulatory cell population systemically.

Sustained generation of a state of tolerance is of paramount importance for success of immunotherapy. Earlier studies have indicated essential role of NF-kB activation in promoting allergic inflammation [21]. Moreover, positive correlation in nasal symptom score and NF-kB activation has been observed in pollen allergic patients. Western blot analysis reveals high NF-kB (p65) levels in Pre-IT and Non-IT mice groups. Therapy with allergen extract as well as Per a 5-derived peptides was able to significantly downregulate NF-kB levels in lung. Earlier, IT with grass pollen has been shown to downregulate NF-kB activation in neutrophils when compared with untreated patients [22].

Allergen encounter has been known to disrupt protease-antiprotease homeostasis in lungs. Since, cockroach allergen is a rich source of potent protease allergen, therefore, we were interested to observe the effect of cockroach allergen exposure on lung intrinsic anti-proteases α1-antitrypsin and elafin. These are potent elastase inhibitors and play a crucial role in recovering from proteolytic damage initiated by allergen exposure and subsequent mediators of inflammation. In our study, western blot analysis of lung samples revealed marginal upregulation of α1-antitrypsin in non-immunotherapy group. In comparison, therapy with peptide demonstrated upregulation in α1-antitrypsin and elafin. Apart from their direct role of maintaining protease-antiprotease homeostasis, these proteins play important immune-regulatory functions as well. For instance, α1-antitrypsin blocks proliferation of Th1, Th2, and Th 17 cells and enhances T regulatory cells [23]. While, lung protective functions of elafin, mediated by inhibiting NF-kB signalling, have been recently observed in lung injury model of hyperoxia [24]. Interestingly, sustained expression of α1-antitrypsin is associated with reduced inflammatory phenotype in autoimmune diseases and T regulatory cell expansion [25].

Earlier studies with Fel d 1-derived peptides demonstrated robust tolerance generation comparable to protein [26]. Similarly, subcutaneous administration of Bet v 1 T-cell epitope peptide at supraoptimal concentration is effective in reducing T-cell reactivity to whole allergen [27]. This phenomenon of linked suppression by T-cell-derived peptides can be harnessed to provide therapeutic benefits to immunologically modify allergic features and provide long-term remission of pathological symptoms. Epitopic regions encompassing TC-P1 and TC-P3 occupy relatively inaccessible regions of Per a 5 by virtue of high hydrophobic character. Examination of HLA binding data reveals high promiscuity in binding multiple HLA alleles as well (11 of 13 and 10 of 13 HLA alleles tested by consensus analysis for TC-P1 and TC-P3, respectively). Evaluation of histopathological features in allergic hyper-reactivity model indicates efficacious resolution of inflammation by TC-P1 and TC-P3. Earlier studies with immunodominant T-cell peptides of serine protease cockroach allergen Per a 10 (T-P8 and T-P10) also demonstrated suppression of Th2 immune responses accompanied with modulation of local and systemic immune responses [9]. It may be therapeutically advantageous to administer immunodominant T-cell epitope peptides from different cockroach allergens; however, future studies are warranted to evaluate the combinatorial efficacy of these epitopes.

Conclusions

Therapeutic relevance of PIT was assessed in murine model of cockroach allergic hypersensitivity. TC-P1 and TC-P3 resolved features of cockroach allergen-induced pulmonary inflammation by modulating local cytokine milieu. Resolution of airway allergic parameters was associated with systemic expansion of T regulatory cells, downregulation of NF-kB signalling and upregulation of immune-regulatory proteins in lung.

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Conflicts of Interest

The authors declare no conflict of interest.

Ethical Approval

The study was approved by Institutional Animal Ethics Committee. (IAEC Approval- IGIB/IAEC/20/20). The animal studies conducted in the research article adhere to the ARRIVE guidelines.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Author Contributions

Swati Sharma (SS): Conceptualization, Methodology, Experiments, Data Analysis Writing- Original draft

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preparation, Ekta Nagar (EN): Methodology, Experiments and Data Analysis & Naveen Arora: Conceptualization, Writing-Review and Editing, Funding Acquisition. SS and EN contributed equally in the manuscript.

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