Cellular Physiology and Biochemistry

Cell Physiol Biochem 2015;36:831-842

DOI: 10.1159/000430259
Published online: May 27, 2015

Accepted: April 23, 2015

1421-9778/15/0363-0831$39.50/0

Original Paper

Identification and Characterization of a Novel IL-4 Receptor α Chain (IL-4Rα) Antagonist to Inhibit IL-4 Signalling

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Key Words
Allergy • Phage display • IL-4 signalling pathway • IL-4 receptor • IgE • ELISA • HEK-Blue cell line

Abstract
Background/Aims: In recent times, allergy has become a financial, physical and psychological burden to the society as a whole. In allergic cascades, cytokine IL-4 binds to IL-4 receptor (IL-4R), consequently producing allergen-specific IgE antibodies by B cells. In addition, among other functions, IL-4 is also responsible for B and T cell proliferation and differentiation. Hence, characterization of novel antagonists that inhibit IL-4 signalling forms the overall aim of this study. ***Methods:*** Phage display was used to screen a random 12-mer synthetic peptide library with a human IL-4Rα to identify peptide candidates. Once identified, the peptides were commercially synthesized and used for *in vitro* immunoassays. ***Results:*** We have successfully used phage display to identify M13 phage clones that demonstrated specific binding to IL-4Rα. The peptide N1 was synthesized for use in ELISA, demonstrating significant binding to IL-4Rα and inhibiting interaction with cytokine IL-4. Furthermore, the peptide was tested in a transfected HEK-Blue IL-4 reporter cell line model, which produces alkaline phosphatase (AP). QUANTI-Blue, a substrate, breaks down in the presence of AP producing a blue coloration. Using this colorimetric analysis, >50% inhibition of IL-4 signalling was achieved. ***Conclusion:*** We have successfully identified and characterised a synthetic peptide antagonist against IL-4Rα, which effectively inhibits IL-4 interaction with the IL-4Rα *in vitro*. Since IL-4 interaction with IL-4Rα is a common pathway for many allergies, a prophylactic treatment can be devised by inhibiting this interaction for future treatment of allergies.

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Introduction

There has been a marked increase in prevalence of atopic disease in regions such as Western Europe, the US, Australasia and Asia Pacific during recent years, especially in industrialized nations. A 20-fold increase has been experienced by western countries and as much as 40% of the population suffers from this disease [1-6]. A recent statistical study carried out has shown that a rise in allergy trend has taken place over the last 20 years [7]. A rise in trend has also been studied in 2007 by the Australasian Society of Clinical Immunology and Allergy. These studies indicate an alarming situation and require a more thorough and aggressive approach to resolve this disease.

In atopic individuals, an immune response is mounted by T cells that are activated by allergens, promoting T helper Type 2 (Th2) variant of cells. Once stimulated, these cells subsequently produce cytokines such as interleukin-4 (IL-4) and interleukin-13 (IL-13). Unlike the T helper Type 1 (Th1) cells in non-atopic individuals, an excessive production of cytokines IL-4 and IL-13 takes place from Th2 cells in atopic individuals [8-12]. When the allergen comes into contact with antigen presenting cells (APC), the allergens are processed and presented to naïve T cells, which mature into Th2 cells and release cytokines such as IL-4, causing IgE-mediated immune response. Chemical mediators that are released as a result cause symptoms like sneezing, wheezing and eczema [8, 13-16].

IL-4 and IL-13 play key roles in Th2 immunity and asthma pathogenesis. The function of these cytokines is partially linked through their shared use of the interleukin-4 receptor-alpha (IL-4Rα) chain. The Type I receptor comprising IL-4Rα and the common γ-chain is expressed by hemopoietic cells and is exclusively responsive to IL-4. In contrast, the Type II receptor comprising IL-4Rα and interleukin-13 receptor-Alpha1 (IL-13Rα1) is responsive to both IL-4 and IL-13 [12, 17-20]. Therefore, the concept that IL-4 and IL-13 can be targeted simultaneously is of great importance because of their shared dependence on the IL-4Rα chain of the receptor. Hence, this research targets the common IL-4Rα with a novel synthetic antagonist. Here, we report the findings on the potential effect of the antagonist on IL-4 signalling.

Although a range of techniques has been employed to down regulate the interaction between IL-4/IL-13 with IL-4Rα (e.g. monoclonal antibodies, antagonists and soluble receptors), the efficiency of these approaches still remains in doubt and under scrutiny [20-23]. For example, mouse models have been designed to improve mouse monoclonal antibodies against human IL-4 receptor to study the effects of T and B cell differentiation [20]. However, research in this area is still incomplete and demands a more thorough and complete investigation to make it a reality [21, 22, 24].

In a study carried out by Holtzman and colleagues, it was found that administration of soluble IL-4R (sIL-4Rα) had no clinical efficacy in treating asthma and the development of this project has been stopped altogether [23]. Similarly, soluble receptors such as the recombinant human IL-4R (RhIL-4R) have also been tested to bind with IL-4 cytokine and antagonise its effects on allergy. However, the allergic symptoms occur from combined effects of IL-4 and IL-13 cytokines, the disadvantage of this system is that it does not affect IL-13 but only IL-4. Therefore, this remains an unreliable method of curing allergy. In other miscellaneous cases, soluble receptors have also shown to be toxic to patients, which have resulted in deaths. The reason for toxicity is still unknown [25-27]. Thus, phage display technology may be useful in the quest to identify novel peptide inhibitors of IL-4 signalling, for the first time, for the possible future treatment of allergy.

Materials and Methods

General description of the M13 phage library

Phage display libraries, PhD-12, in which 12-mer random peptides are expressed at the amino terminus of protein pIII of the filamentous bacteriophage were purchased from *New England BioLabs Inc.*

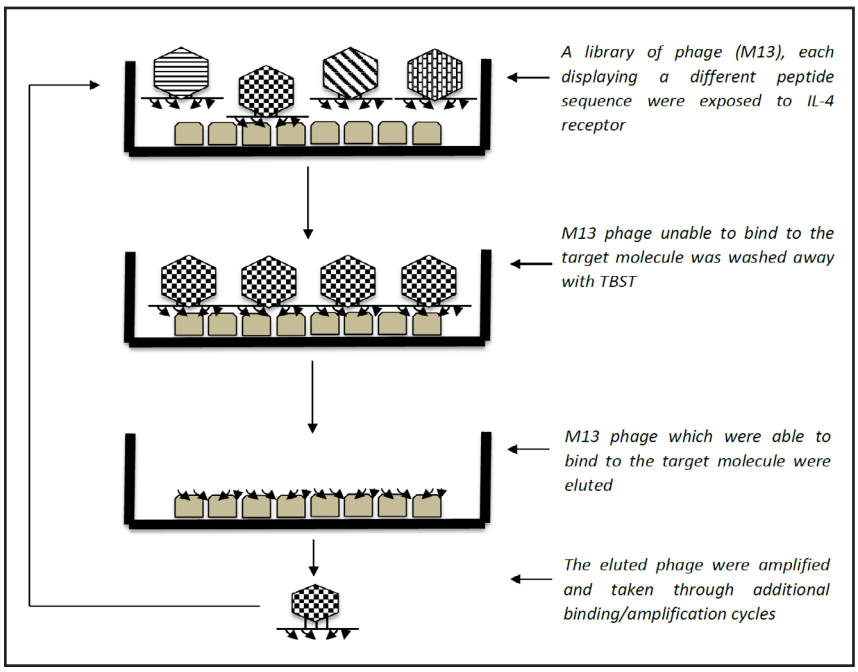

Fig. 1. Schematic diagram of steps taken to elute the phage and perform biopanning. Purified recombinant human IL-4Rα was used as the target antigen.

Table 1. Input phage titers for subsequent rounds of biopanning and appropriate calculations

Biopanning rounds	Initial pfu	Input titer of phage	Input volume for next round
Round 1	2.25×10^{11}	$2 \times 10^{11}/2.25 \times 10^{11}$	0.88 µl
Round 2	2.3×10^{10}	$2 \times 10^{11}/2.3 \times 10^{10}$	8.8 µl
Round 3	5.8×10^{11}	$2 \times 10^{11}/5.8 \times 10^{11}$	0.344µl
Round 4	1.3×10^{11}	$2 \times 10^{11}/1.3 \times 10^{11}$	1.3 µl
Round 5			Last round, hence no further calculations required

(Beverly, MA USA) as a kit. All experiments were carried out under strict sterile conditions using a Class II laminar flow cabinet (Fig. 1).

Biopanning

Aliquots of the phage libraries were screened with recombinant human IL-4Rα (*Abcam*); coated on flat-bottomed 96 microtiter microplates (*Thermo Scientific*), following the manufacturer's instructions. In the first and second rounds of panning, the coating concentration of protein was 50nM. To increase the stringency of the panning, the wells were coated with decreasing concentrations of protein and increasing doses of TBST. In the fifth round of panning, the concentration of the protein was 10nM. Table 1 shows the calculations done to obtain input volumes for subsequent panning rounds.

Amplification and sequencing of phage clones

Amplification of 10 individual plaques from the fifth round of panning was subjected to amplification and sequencing by following the manufacturer's instructions and our established methods [28]. Sequencing was performed by the *Micromon DNA Sequencing Facility* at Monash University (Melbourne, Australia). Once the DNA sequences for all 10 phage clones were obtained, they were further analysed using the *FINCH TV* sequence analysis software. This helped ascertain which sequences out of the 10 were most suitable to carry out further immunoassays. The flanking regions were used to locate the 36 base pairs of DNA of interest, which encoded the 12-mer peptide sequence displayed on the M13 phage surface. The hybridization positions of the -28 and -96 sequencing primers were indicated as well. Short peptide designated N1 (Primary Sequence: *XXXXXXXXXXXX*) along with the biotinylated version were chemically

synthesised by *AUSPEP* (Melbourne, Australia) and received in a lyophilized condition with >90% purity. The biotin was added to the *N*-terminal of the peptide. The quality of all the peptide was assessed by high performance liquid chromatography (HPLC) and confirmed by mass spectrometry analysis (purity >90%). Further analysis included alignment of 12-mer sequences with IL-4 and IL-13 cytokines using *ClustalW2* multiple alignment software. The sequence of the peptide has not been shown due to intellectual property reasons with Deakin University (Waurn Ponds, VIC, Australia).

M13 binding ELISA

Four rows of a 96-well microtiter plate were coated with 90 µl of the target IL-4Rα (10nM) protein and the M13 ELISA was performed according to manufacturer's instructions and our established methods [28]. The colour development on the plates was read by using *Thermo Labsystems Multiscan* plate reader, set at 492 nm of wavelength.

Synthesis of the biotinylated peptide

All 10 phage clones isolated in this study gave a strong amino acid sequence consensus, only one 12-mer peptide was sent for synthesis, denoted as N1 (*AUSPEP*, Australia). A biotinylated version of the peptide was also synthesized (N1 biopep). The purity of the peptides was >90% and the total peptide synthesized was 5 mg. Dilution of biotinylated peptide: 1 mg of the N1 biopep was taken from the 5 mg stock and dissolved in 500 µl of dH_20 (sterilized). The peptide dissolved immediately; hence, another 500 µl dH_20 was added to make up to the required 1mg/ml of peptide concentration.

Direct ELISA

Immobilization of 90 µl target IL-4Rα (10 nM) was carried out on a 96-well plate overnight. The plate was sealed with a parafilm and placed in a humidified container with agitation. Following day, the plate was slapped face down on a paper towel to get purged of target solution. 220 µl of blocking buffer (BSA + NaN_3 blocking solution) was added to the wells. The plate was incubated at 4°C for 1-2 h. The blocking buffer was removed by slapping the plate face down on the paper towel, and gently washed 3-4 times with 280 µl/well of 0.3% TBST. Next, 100 µl of biotinylated N1 peptide was added to each designated well of the ELISA plate. The plate was incubated for 1-2 h at RT with agitation on a rocker. Negative controls were setup in 3 separate wells without the addition of N1 peptide. The wells were subjected to dH_20 only, with the exception of the peptide. This was followed by 3-4 washes with 280 µl/well of 0.3% TBST. Finally, 150 µl HRP-conjugated streptavidin (*Sigma Aldrich*) (1:500 dilution in blocking buffer) was added to the wells and incubated for 1 h with agitation on a rocker. Once again, the plate was gently washed 3-4 times with 280 µl/well of 0.3% TBST. A developing solution was prepared, by dissolving a single tablet of phospho-citrate buffer with sodium-perborate in 100 ml of dH_2O, then 1 tablet of HRP substrate ortho-phenyl diamine (OPD; *Sigma Aldrich*, Australia) was dissolved in 7.5 ml of this solution. 200 µl of this solution was added to each well; the plates were wrapped in foil and incubated for 30 min at room temperature, with agitation on a rocker. The reaction was stopped by adding 50 µl of 4M H_2SO_4. The colour development on the plates was read by using *Thermo Labsystems® Multiscan* plate reader, set at 492 nm of wavelength. Statistical analysis was performed on the data obtained from the ELISA.

Inhibition ELISA

Initially, the same protocol was followed, as described in the direct ELISA protocol above. After the blocking of the target IL-4Rα, IL-4 cytokine was pipetted into the wells and was incubated for 1-2 hours at room temperature, with agitation on a rocker, followed by 3-4 washes with 250 µl 0.3% TBST. Following this, 120 µl of N1 biopep (0.2mg/ml) was added to each designated well of the ELISA plate. This was then developed as described in the section above. Further, statistical tests were performed on the results obtained from the ELISA.

Human cytokine reporter cells

HEK-Blue IL-4/IL-13 cell kit was purchased from *InvivoGen (San Diego, CA, USA)*. These cells allow the detection of bioactive IL-4 and IL-13 through the activation of STAT6 pathway. These cells are derived from a stable transfection of human embryonic kidney cells HEK293 with human STAT6 gene to obtain a fully active STAT6 pathway. The cells are also transfected with another stable STAT6 inducible secreted

embryonic alkaline phosphastase (SEAP) reporter gene. Lastly, the cells have been used as an expression tool for recombinant IL-4R, which spans the cell membrane of the HEK-Blue cell line [29]. Upon stimulation from either IL-4 or IL-13 cytokine, the cells secrete SEAP. The levels of SEAP production can be detected using the QUANTI-Blue substrate, which turns purple/blue in the presence of SEAP.

Cell culture

HEK-Blue cells were cultured in DMEM media (*Invitrogen*), following the manufacturer's instructions. The effect of peptide concentration on cell viability was also tested to confirm the occurrence of apoptosis. Therefore, the highest concentration of peptide N1 (225 μM) was chosen for our studies.

Inhibition assay of cytokine IL-4 using N1 peptide in a HEK-Blue cell line

The protocol used was taken from the datasheet provided with the HEK-Blue IL-4/IL-13 kit by the manufacturer (*InvivoGen*). A few adjustments were made to the protocol to perform the assay. Cells were grown in a T-75 flask and were used for the detection assay. The cells were detached using 3 ml trypsin, which was neutralised with 7 ml of media (FBS - neutralising agent) to give a total volume of 10 ml. The average cells were counted using a TC10 automated cell counter (*Bio-Rad*) and cultured at a density of 50,000 cells per well of a 96-well plate. Six treatment wells were used in total; 4 wells treated with peptide N1 and 2 wells without peptide as positive and negative controls (shown in results section). Cells in the treatment wells were incubated with 50 μl of N1 peptide (75, 150 and 225 μM, respectively) at 37°C in a rotator shaker for 1 h, whereas the control wells were incubated with filter-sterilized water (DH_2O). Post-incubation, 20 μl of IL-4 cytokine (100 ng/ml) was added to the positive control and three treatment wells. The 96-well plate was sealed using a parafilm and incubated at 37°C with 5% CO_2 for a period of 24 hours. Post-incubation, QUANTI-Blue substrate was prepared using the instructions in the HEK-Blue kit and 180 μl of this solution was added to 6 wells in a fresh 96-well plate. 20 μl of induced HEK-Blue IL-4 cells supernatant from each of the treatment wells was added to the QUANTI-Blue solution. The subsequent results were read using an xMark microplate absorbance spectrophotometer (*Bio-Rad*) at a wavelength of 640 nm.

Statistical analysis

The data were analysed using Microsoft Excel 2013 (Microsoft Inc. Las Vegas, NV, USA). The results were analysed by Student's *t*-test to determine any statistically significant differences between the positive controls and treatment wells in all of the above experiments. The statistical significance was set at $*p<0.05$.

Results

Selected peptides from 12-mer libraries were analyzed by taking 3 random peptides from the 9 individual phage clones isolated from the 5th round of biopanning, demonstrating identical sequence motifs (see next section below). Each sample was diluted, plated and individual clones were isolated, amplified, sequenced and tested for reactivity with the target antigen IL-4Rα using ELISA immunoassays and HEK-Blue inhibition assays.

Phage display biopanning and sequencing

Upon sequencing, 9 of the 10-phage clones showed identical consensus in binding sequence, N1 to N9. Since the peptides from the clones N1 to N9 showed a consensus in sequence, N10 was discarded and was no longer used for further analysis. The sequence of the peptide has not been shown due to intellectual property reasons with Deakin University.

ClustalW2 was used to perform sequence analysis of N1 amino acid with the IL-4 cytokine to observe the similarities between their sequences. This was performed to evaluate the binding capacity of the peptide to IL-4Rα, and as a result, "mimic" the cytokine IL-4 in its amino acid sequence. This also revealed the epitope region of specific amino acid sequence of IL-4Rα that would hybridise with the peptide. Table 2 shows a comparison between IL-4 and the N1 peptide with 6 amino acid residues showing identical residues, and also, three weakly conserved residues.

Table 2. Results obtained from *Clustal*W2 alignments, performed using N1 peptide

Percentage amino acid similarity with 12-mer N1 peptide	With IL-4	With N1 peptide
Identical sequences	6 amino acids (50%)	100%
Strongly conserved	None	100%
Weakly conserved	3 amino acids (25%)	100%

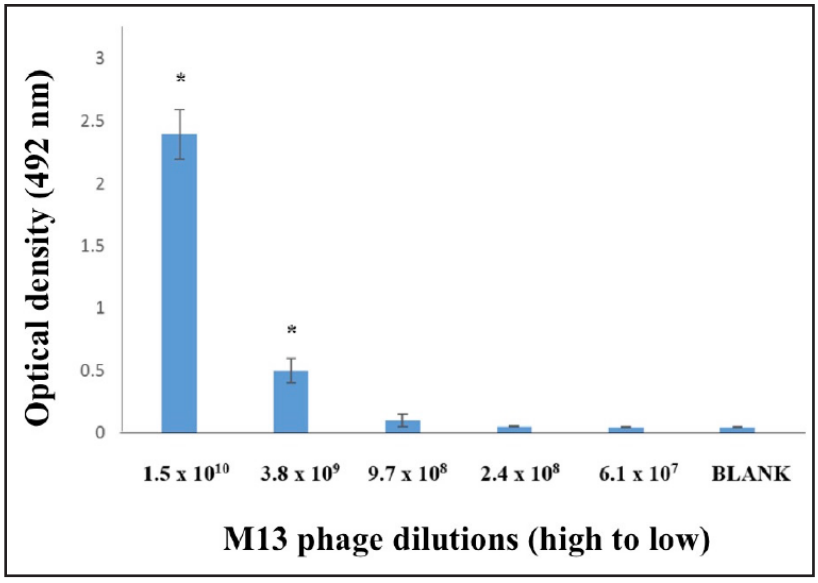

Fig. 2. M13 binding ELISA of phage clone N6. Figure illustrates the absorbance readings for a range of phage dilutions used to show an interaction with IL-4Rα. The results represent the mean of 3 replicates within an experiment and the error bars show standard deviation. A statistical Student's *t*-test revealed a significant difference between the mean absorbance values when compared to the control (*$p < 0.05$).

M13 ELISA with N6 phage clones

An M13 ELISA was performed to further enhance results showing specific binding of the phage peptide to the target IL-4Rα (Fig. 2). The detection was based on a strong affinity between the displayed peptide by the phage clones and the target IL-4Rα. Overall, the selected peptide N6 showed a binding to the IL-4Rα target. The phage clone displaying the peptide gave higher absorbance readings when compared to the control. This is an indication of the accuracy and specificity of the M13 phage binding to its target antigen when used in *in vitro* analysis.

Direct ELISA

In view of the fact that N1 amino acid sequence shared a 50% homology when aligned with cytokine IL-4, it was necessary to conduct further tests on the N1 biopep, to determine its avidity with IL-4Rα. This was performed by using direct ELISA, in which the peptide was allowed to interact with the IL-4Rα in the absence of the cytokines to determine its avidity to the target antigen. Evidently, the differences in absorbance readings obtained showed that N1 biopep had specifically bound IL-4Rα, when compared to the control, which we have recently reported in a previous study [28]. A trend was seen with increasing concentration of the peptide, where higher absorbance readings were recorded on the spectrophotometer [28]. This indicated that the streptavidin-HRP had indeed recognized the biotin on peptide N1, which in turn interacted with the IL-4Rα target antigen [28].

Inhibition ELISA

Shortly after the results were achieved from the direct ELISA, the next step was to verify whether the synthesized N1 biopep was capable of inhibiting the interaction between IL-4 and its receptor IL-4Rα. In this case, the IL-4Rα was immobilized on a 96-well plate, followed by the addition of the IL-4 cytokine. The inhibition was found to be ~73% with respect to the

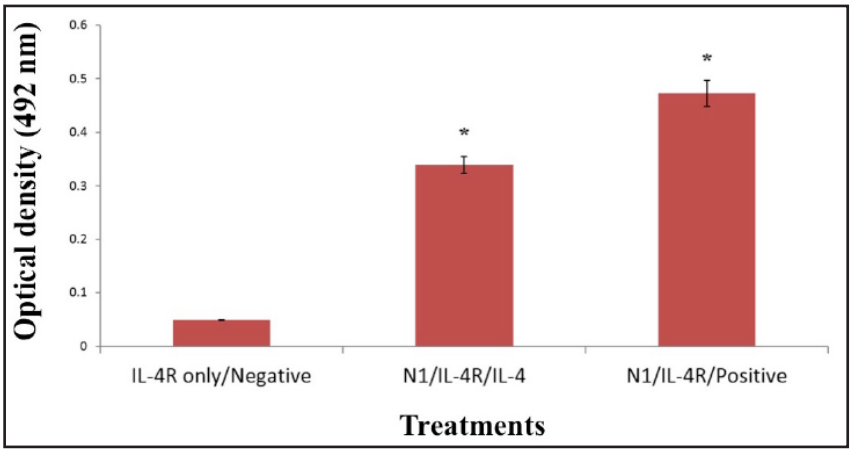

Fig. 3. Inhibition ELISA showed that the biotinylated peptide N1 successfully fastened to the target IL-4Rα in the presence of IL-4, as the inhibitor. The negative control had no biotinylated peptide N1 or cytokine IL-4 (graph bar on far left). The results represent the mean of 3 replicates within an experiment and the error bars show standard deviation. A statistical Student's *t*-test revealed a significant difference between the mean absorbance values when compared to the control (*p < 0.05).

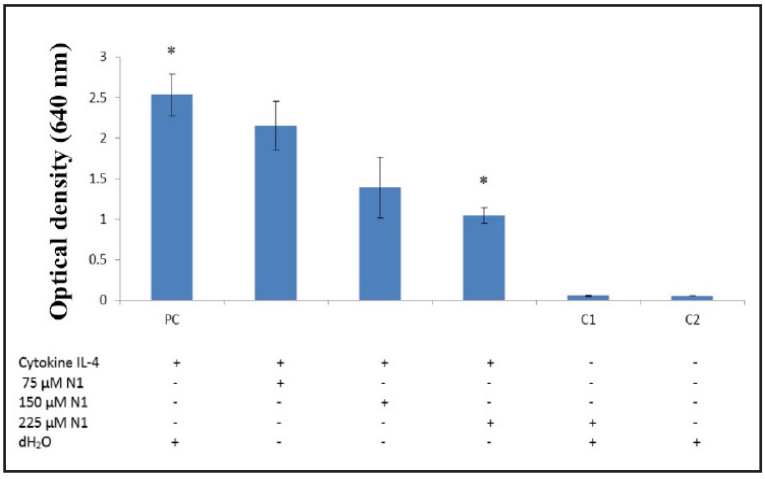

Fig. 4. The peptide was used to antagonize the IL-4Rα to decrease SEAP levels secreted by the HEK-Blue cells. A dose-dependent relationship can be observed with the three treatment wells containing peptide N1. Positive Control (PC) included cells treated with IL-4 cytokine alone. Control 1 (C1) included cells incubated with peptide only and Control 2 (C2) included cells without any treatment. The results represent the mean of 3 replicates within an experiment and the error bars show standard deviation. P<0.05* denotes significance of optical density (OD) readings compared to positive control (PC).

biopep. These results show that the biopep N1 is able to successfully inhibit IL-4 cytokine from binding to its receptor, as shown in Fig. 3.

HEK-Blue cell line inhibition assay with N1 peptide

Once all necessary immunoassays had been carried out to determine the efficacy of the N1 peptide, further analysis was required to confirm the specificity of the peptide *in vitro* in inhibiting the IL-4 signalling pathway. As shown in Fig. 4, with the addition of the peptide N1, a dose-dependent relationship was observed with the increasing concentration of the peptide, which means less SEAP was secreted by the cells, hence less color produced in the presence of the QUANTI-Blue substrate. All positive and negative controls behaved as expected with high and low peaks as suggested by the HEK-Blue IL-4/IL-13 kit. Due to limitation of resources, 225 µM was the highest peptide antagonist dose used. The aim here was not to achieve 100% inhibition of IL-4 signalling but to see if a gradual inhibition of IL-4 signalling can be achieved with increasing N1 peptide antagonist concentration. Indeed, >50% inhibition of the IL-4 signalling pathway was achieved with the highest concentration of peptide at 225 µM (Fig. 5). Consistency was also observed with cell viability when tested for all the treatments and compared to the positive control.

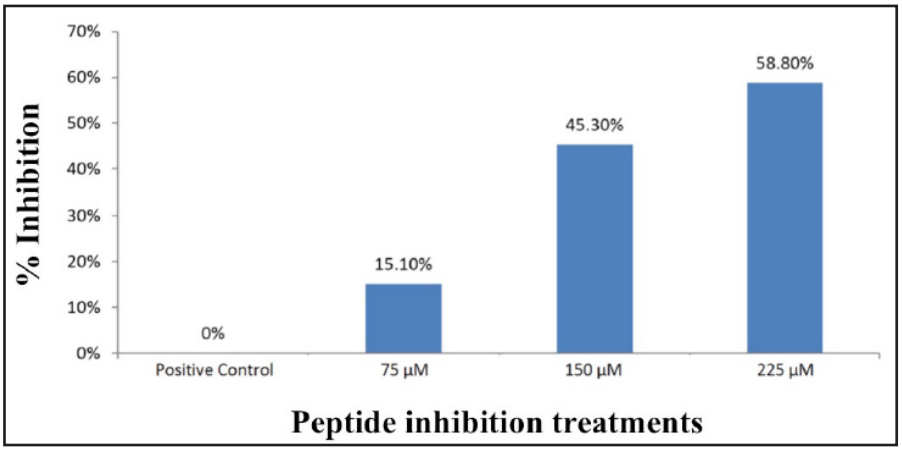

Fig. 5. Percent inhibition of HEK-Blue cells with N1 peptide. The percentage values represented here are a reflection of treatments used in Figure 4 (75 µM, 150 µM and 225 µM). Figure shows incremental inhibition of IL-4Rα signalling from HEK-Blue cells as N1 peptide concentrations are increased. Positive control is denoted at 0%, which is used as a referral point for the standard value at which inhibition begins.

Justifying the results obtained

To validate and substantiate our results, each of the above immunoassay and HEK-Blue analysis was repeated independently 3 times with triplicates taken for each experimental reading. This was done to justify the results and present an unbiased opinion regarding the peptide being used as a novel antagonist.

Discussion

Phage display

IL-4R plays a vital role in allergic diseases since it is able to bind IL-4 and IL-13 cytokines. Activation of Th cells by APC leads to the production of these cytokines. In B cells, these cytokines induce B-cell proliferation, differentiation and class switching of immunoglobulin to produce IgE [30]. In addition, cytokines IL-4 and IL-13 are known to down-regulate the expression of immune mediators by moderately suppressing the DNA transcription regulator nuclear factor κB [30-32]. These cytokines also contribute to IgE priming of mast cells, further influencing increased IgE production [30-32]. Thus, it is necessary to find a novel antagonist to IL-4Rα, to inhibit the interaction of the cytokines with their receptor, hence down-regulating the excess production of IgE and thus the allergic cascade, as a prophylactic treatment.

Current clinical strategies for allergy treatment involve allergen avoidance by patients, pharmacotherapy and specific immunotherapy. Only the third strategy has provided a long-term solution but with little success, since the allergen extracts used are poorly characterized and suffers from batch to batch variation [33]. In addition, natural allergen extracts exhibit high allergenic activity that often cause side effects and it has been reported that SIT with allergen extracts can induce IgE sensitization to new allergens [33, 34]. Other current therapeutics for IL-4 signalling includes Omalizumab, which is a humanised antibody to the Fc region of IgE, and Dupilumab, which is a humanised antibody against IL-4Rα [35, 36].

The display of foreign proteins on the surface of bacteriophage M13 has been used to understand protein-protein interactions at the molecular level. For this reason, phage display technology has been used as a novel approach to identify peptide antagonists [37, 38]. This technology has evolved over the last two decades and has been used for drug discovery and identifying novel synthetic peptides, which can be used as antagonists to many protein-ligand interactions [39-42]. Our findings indicate that a novel synthetic peptide N1 has been identified and characterised as an antagonist to IL-4Rα, which inhibits the interaction between the cytokine IL-4 and its receptor. IL-4, as discussed earlier, plays an important role in the allergic cascade, eventually leading to the release of inflammatory histamines from mast cells and basophils that lead to allergic symptoms. Hence, targeting the receptor was

the main objective of this study.

Phage display has become a method of choice for epitope mapping and has been successfully used in numerous published studies [43-46]. For this reason, it was suggested that sequences N1-N9 that showed consensus in binding represented an epitope that strongly bound with the IL-4Rα target. This was determined from the ClustalW2 alignments that were conducted using the IL-4 and IL-13 amino acid sequences with the N1-N9 amino acid sequence. 6 amino acid residues were exactly identical with the IL-4 (153 amino acids) along with 3 weakly conserved regions. In essence, 50% of the peptide sequence was identical with IL-4. When the same 12-mer sequence was aligned with IL-13 (146 amino acids), only two amino acids were identical while the strongly and weakly conserved amino acids had 3 each. Thus, the peptide sequence mimicked the IL-4 cytokine more than it did IL-13. This is consistent with the study that IL-4Rα subunit binds both IL-4 as well as IL-13. Homologous sequences have blocks of alignments separated by gaps (not shown for intellectual property policies). The gaps usually indicate loop regions with no conserved core secondary structure and hence, explain that the epitope of the peptide is conformational in shape rather than linear [47]. Further, a nucleotide BLAST search was conducted on NCBI using the amino acid sequence of the identified peptide. It was used to compare the peptide sequence with those contained in nucleotide databases by aligning the sequence with previously characterized proteins, therefore assisting to identify homologues that are linked with the IL-4Rα protein. However, an analysis of the homologues revealed no relevant protein that might be linked to the interleukin family of proteins.

M13 binding ELISA with N6 phage clone

This preliminary M13 binding ELISA study carried out with the phage clone N6 laid down the hypothesis that the selected phage clones can indeed bind to the IL-4Rα target, and may therefore, act as a potential inhibitor for interaction with IL-4.

Direct ELISA and Inhibition ELISA

A final aim of this study was to test the ability of the identified antagonist to not only bind with the target but down-regulate or inhibit its interaction with the target IL-4Rα. Hence, an inhibition ELISA was performed using the N1 biopep as the inhibitor. The inhibition ELISA is a highly sensitive and specific immunoassay method that uses an antigen capture method to bind the analytes present in the solution. Results are shown in the graph and a Student's *t*-test analysis of the values compared to the control was highly significant ($*p < 0.05$). This proved that the affinity of N1 biopep for the IL-4Rα was much higher than the cytokine itself. Although the IL-4 cytokine concentration used was lower than the concentration of the biopep N1, it was consistent with the recommendation of the manufacturer. The results were comparable to many other researches where peptide-ligand interactions have proved to be very successful [48, 49]. As a result of the small size of the peptide, they tend to show a higher target-to-background ratios compared to macromolecular compounds.

HEK-Blue cell inhibition assay with N1 peptide

HEK-Blue cells carry simplicity, high sensitivity and low cost to conduct IL-4 signalling experiments. Thus, HEK-Blue detection assay was performed using a HEK-Blue transfected cell line with the N1 peptide as the inhibitor. As the results suggest, an increase in peptide concentration reduced SEAP levels that were detected through a colorimetric analysis. Although some minor changes have been made to the overall protocol of the detection assay, the absorption readings observed were at peak values in the positive control, hence eliminating any doubt about the procedure used. The aim of this experiment was to achieve a >50% inhibition of the IL-4 signalling pathway that would allow the severity of allergic symptoms to be lowered in atopic individuals [50-53]. Although it has been criticized that peptides selected against purified recombinant protein may not be able to access their targets on living cells, it has proved otherwise in our case (explained in section below). This may partly be due to the fact that the peptide binds to a conformational epitope region of the

IL-4Rα. The positive and negative controls were setup to pinpoint any discrepancy in the results. More importantly, the detrimental effects of peptide concentrations were also tested by performing a cell viability test for all the parameters. Consistency in cell count suggested that even at higher doses, the peptide antagonist did not exert any negative impacts on cell growth.

Preferential use of peptides as antagonists

Many pharmacological companies believe that peptides are a menace because of their poor agile properties like poor tissue penetration, serum resistance and quick elimination [54]. However, recent studies suggest that such problems can be tackled with modification of the peptide while other proteins or molecules are more difficult to deal with, due to their larger sizes. There are potential benefits of using peptides as drugs, which come into play when dealing with protein-protein interactions (PPI's). Binding pockets of proteins are sometimes small and unable to fit an antagonist large in size (antibodies or molecules) and hence, using peptides is a more viable option; ranging from two to fifty amino acids in length. Second advantage of using peptides is that it's unlikely to cause any adverse immunological reactions in the subject since they are too small to render any physiological reaction. Thirdly, the chemical diversity found in peptides, as opposed to large molecules, allows more specific interaction with the protein, increasing the antagonist's efficiency to penetrate the conformational structure of the protein [55, 56].

Conclusion

We have sucessfully identified and characterised a novel antagonist to IL-4Rα which down-regulates the interaction of the IL-4Rα with the cytokine IL-4. Using a phage display random peptide library, the biopanning procedure established in identifying and isolating the synthetic peptide antagonist N1. Furthermore, the second aim was to test the identified antagonist using immunoassay techniques and tissue culture assays to justify that the novel peptide was indeed inhibiting the IL-4Rα from interacting with IL-4. Results from ELISA immunoassays suggested that the peptide was proficient in binding to the IL-4Rα with a higher affinity and hence, was able to retard the overall protein-ligand interaction between the cytokines and IL-4Rα. Furthermore, using HEK-Blue IL-4/IL-13 cells, a >50% inhibition was achieved with the peptide with a simple colorimetric analysis. As this peptide targets the two most clinically important cytokines in allergy, it promises to provide for the future treatment for all IgE-mediated allergies.

Acknowledgements

Authors N. Ahmed and P. Dhanapala have been supported by Deakin University Postgraduate Award (DUPA). This study was supported by School of Life and Environmental Sciences and Centre for Molecular and Medical Research (MMR), Deakin University.

Disclosure Statement

The authors have no financial conflict of interest.

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