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The studies presented in this thesis focus on components involved in allergy development, the design of novel allergy therapies, and the off-label use of current allergy therapies.

**ALLERGY DEVELOPMENT**

**Route of sensitization**

In Chapter 2 a case study of food anaphylaxis induced through cutaneous sensitization is presented. Although this route of sensitization had previously been indicated in murine studies and observational studies, this was the first study to demonstrate both clinical and immunological evidence for sensitization to a foodstuff in a human, through cutaneous use of a moisturizer for the treatment of eczema. From the data presented in this study clinicians are urged to emphasize to their patients the importance of using a bland topical treatment for eczema. Indeed, this report was the subject of a news release by the American Academy of Allergy, Asthma and Immunology, as well as several Australia-wide media outlets (Newspapers: Canberra Times, Herald Sun; Television: “Insight” on SBS). The broader clinical relevance of this finding is highlighted by a recent report from our research group, describing an individual developing anaphylaxis to oats, following the use of oatmeal-containing skin products to treat atopic dermatitis. Furthermore, atopic dermatitis and early onset eczema have recently been identified as strong risk factors for the development of peanut allergy in infants.

Recently published mouse studies reveal the potential mechanism behind the development of oral food allergy through cutaneous sensitization. They have shown a pivotal role for TSLP-activated basophils in inducing IgE-mediated intestinal food allergy following epicutaneous sensitization through a disrupted skin barrier. Upon skin barrier disruption, basophils infiltrate the skin in response to epithelial cell-derived TSLP and release Th2 type cytokines, including IL-4. It is thought that the IL-4 released by basophils into the periphery induces the Th2 cell polarization, resulting in allergen-specific IgE production and an increase in intestinal mast cells, allowing for intestinal inflammation upon oral allergen encounter. Early oral exposure may prevent this process by inducing tolerance prior to cutaneous sensitization. With this in mind the LEAP clinical trial was initiated, randomizing 640 infants at high risk for peanut allergy (defined by severe eczema or egg allergy) to consume or avoid peanuts until 5 years of age. The results showed that earlier consumption of peanuts correlates with decreased frequency of peanut allergy development in both sensitized and non-sensitized infants. The role of eczema/atopic dermatitis and timing of food introduction in the development of food allergy are now considered highly relevant. However, using this information for the prevention of food allergy may not be as simple as it would seem, as is exemplified by a randomized controlled trial on the use of moisturizer to prevent atopic dermatitis/eczema and allergic sensitization in infants. Although atopic dermatitis/eczema frequency was lower in the treatment group, the frequency of allergic sensitization was not affected.

**Antigen presentation**

Although a role for basophils in the cutaneous sensitization route of food allergy development started to emerge, their role in direct antigen presentation was still a matter of debate. A heightened interest into this function of human basophils was sparked by several high impact publications claiming murine basophil antigen presentation was both necessary and sufficient for the initiation of Th2 dominant responses. The methods used to conclude this consisted of both OVA-allergic and helminth-infected mouse models, whereby depletion of basophils prevented the development of Th2 immunity, while depletion of CD11c DC did not. Subsequent human basophil studies revealed a lack of, or minimal, MHC Class II and costimulatory molecule expression in stimulated basophils, and an inability to induce allergen-specific T cell activation through direct antigen presentation. The basophils were stimulated for 16-24 hours with allergens, TLR2 ligands and cytokines IL-3 and IFN-γ. Considering the restricted panel of stimulants used and the fact that stimulated human eosinophils, of the same lineage as basophils, show maximal MHC Class II expression at day 2-4 of culture, it was conceivable that the optimal conditions for in vitro induced MHC Class II expression in basophils had not yet been achieved. Furthermore, evidence of increased ex vivo HLA-DR expression in human basophils had emerged from patients with systemic lupus erythematosus (SLE) compared to healthy controls, emphasizing that under specific circumstances HLA-DR up-regulation on basophils does occur. Basophils were detected in the lymph nodes and spleen of SLE patients, providing the opportunity for these cells to interact directly with antigen-specific T cells. The study conducted in Chapter 3 aimed to further investigate the potential of human basophils to present antigen to T cells. The data showed that basophils could be induced to express MHC Class II (up to 17%) and demonstrated the components required for peptide/MHC Class II complex assembly. This was also evident at gene expression levels, through detection of relevant mRNA transcripts. Furthermore, varying levels of these transcripts were also detected in MHC Class II-negative basophils after stimulation, indicating the potential for a larger proportion of cells to become MHC Class II-positive under appropriate conditions. Confirming previous human basophil studies, minimal costimulatory molecule expression was detected and basophils were found to be incapable of antigen presentation to CD4+ T cells with whole protein. Furthermore, the ability of basophils to present peptide to T cells, which does not require antigen uptake or processing, was also assessed. Again, basophils were incapable of inducing peptide-specific T cell activation, indicating that a lack of antigen uptake and processing may not be the only issue at play, and that antigen presentation may also be hampered by a lack of costimulatory molecule expression. The question remains: if the expressed MHC Class II is insufficient to induce T cell proliferation, what is its function and does it influence other cells? In this context, it would be of interest to assess the possible effects of peptide presentation in the absence of costimulatory molecule expression by these basophils, as this may induce T cell anergy.

In addition to the inability of human basophils to act as APC, the original findings of this feature in murine basophils were also disputed, with concerns that the depletion methods employed for both DC and basophils were flawed. The method of DC depletion involved the...
that inflammatory CD11c+CD49b+FcεRI+ cells producing IL-4 to initiate a Th2 response. In a model of helminth infection, it was discovered RI+ DC from the same lymph node, suggesting that basophils are poor antigen presenting cells despite detectable MHC Class II expression. Further investigation revealed low levels of HLA-DM and invariant chain expression, components required for successful loading of peptides into the MHC Class II grooves. A recent study provides some insights into the discrepancies between murine basophil phenotype (MHC Class II and costimulatory expression) and their apparent lack of antigen presenting abilities. The findings show that basophils are capable of inducing a Th2 response to haptens and peptides, which may bind directly to membrane MHC Class II, but not to proteins. This was confirmed to be due to impaired antigen uptake and processing. These findings further emphasize the differences between murine and human basophils. While murine basophils are capable of MHC Class II and costimulatory expression, and exogenous peptide presentation, our data and that of others has shown human basophils to be limited to low levels of MHC Class II expression. In addition to these findings, the first genetically engineered basophil-deficient mouse models revealed that DC, not basophils, were necessary for the initiation of Th2 immunity to helminth infection.

Although basophils may not fulfill the criteria of functional APC, they can produce substantial amounts of IL-4 and have been shown to be involved in Th2 immunity development as an accessory cell, while professional APC interact directly with naïve T cells. The extent of their contribution to Th2 polarization may depend on the biological setting, route of delivery (cutaneous) and the particular antigens involved. Recent murine studies also point to other cells producing IL-4 to initiate a Th2 response. In a model of helminth infection, it was discovered that inflammatory CD11c+CD49b+FcεRI+ DC were capable of initiating a Th2 response through the production of substantial amounts of IL-4. Further, the initiation of a Th2 response in a mouse model of peanut allergy showed the IL-4 required for Th2 skewing to originate from naïve CD4+ T cells, inducing cell differentiation and amplification in an autocrine/paracrine manner. The production of IL-4 from the naïve CD4+ T cells was dependent on DC OX40L expression, and was independent of innate IL-4 producing cells. Combined, these studies suggest that Th2 polarization may occur through multiple non-exclusive pathways.

Since the first reports of murine basophil antigen presentation in 2009, it has become clear that basophil identification is more complex than originally appreciated, and that basophil properties may differ significantly between mice and men. So far, studies of human basophils have found no evidence for basophils acting as APC. Our data have confirmed this, and provided additional evidence of their limitations in this role. Although it remains possible that blood-derived basophils do not represent the abilities of basophils present in tissue in a pro-inflammatory environment not yet replicated in vitro, the evidence to date does not support a role for human basophils in antigen presentation.

**ALLERGY THERAPEUTICS**

**Peanut allergy PIT**

The studies performed in Chapters 4 and 5 provided the information needed to design a safe and effective peptide based immunotherapy for peanut allergy. Although the data regarding the dominance of particular T cell epitopes involved in peanut allergy are of great value in characterizing mechanism of disease, the most important aspect of these studies has been to further the development of a novel therapeutic. The choice of peptides for the further development of a therapeutic is based on their ability to induce a proliferative and cytokine-producing response by CD4+ T cells from peanut allergic individuals. The strength of the induced response, as well as the responder frequency to the peptide among allergic cohorts, was taken into account during this selection. Indeed, previous murine studies have shown dominant T cell epitope-derived peptides to be more efficient at inducing tolerance than those of minor epitopes. In addition, different peptide-based therapeutics currently in clinical trial with Circassia Ltd (UK) have been designed on the same principles and are showing highly promising clinical efficacy. Further considerations include diverse HLA binding of the peptides and the inability of the peptide to be recognized by IgE or activate basophils in vitro. Diverse HLA binding is necessary to design a PIT that will be effective across ethnically diverse populations. The results show diverse binding of the selected peptides to HLA-DR, HLA-DP and HLA-DQ molecules. Although antigens are most commonly presented on HLA-DR molecules, identification of dominant peptides presented on HLA-DP or DQ molecules is highly beneficial to the design of a widely applicable PIT, as these molecules are more conserved across different populations. Finally, the precise core T cell epitopes within both the Ara h 1 and Ara h 2 proteins were identified, allowing for the selection of the shortest possible peptides, thereby minimizing the risk of IgE binding or effector cell activation.

The three short peptides containing the five dominant T cell epitopes of the major peanut allergen Ara h 2 identified in Chapter 4 were further investigated in Chapter 6, with regard to their resistance to gastrointestinal enzymatic digestion for potential oral delivery. Though not yet implemented for any existing peptide-based allergen immunotherapy, there is increasing interest in this route of delivery, due to the ease of administration and the tolerogenic profile of APC present in the oral and intestinal mucosa. Several murine studies have shown the potential for this route of peptide delivery, one of which reported the induction of tolerance.
in a mouse model of food (egg) allergy. Indeed, during the last decade the benefits of this route of delivery have been realised in other areas of peptide-based therapeutics, evident by the emergence of commercial oral peptide delivery platforms such as Peptelligence and the release of Linaclotide, a 14-mer peptide delivered orally to treat chronic constipation and irritable bowel syndrome. For successful oral peptide delivery, several hurdles must be overcome. The first is enzymatic and acidic degradation in the stomach and intestine. Although coating products exist to protect a protein or peptide from degradation in the stomach, the induction of oral tolerance is highly desirable in AIT and would require immediate exposure to the peptide. The findings presented in Chapter 6 indicate a reasonable level of peptide resistance to gastrointestinal enzymatic degradation, and show how this resistance can be influenced through residue substitution in positions removed from the predicted enzyme digest sites. The results of Chapter 6 concern only three of the five T cell epitopes identified in Ara h 2, and further studies are required to assess the digestive resistance of the two remaining epitopes of Ara h 2, and the recently identified T cell epitopes of Ara h 1. Other hurdles would also need to be overcome for these peptides to maintain bioavailability following oral ingestion, however the recent development of new technologies to improve peptide stability may assist in this process, should this eventually be the preferred route of delivery.

Of the peptides described in Chapters 4 and 5, a selection has now been chosen to form a novel peanut allergy PIT, named PeanutVax. Peptide stability and toxicity testing of this selection, in preparation for a phase I/IIa clinical trial, is currently underway. This will be the first peptide-based immunotherapy for a food allergy, and therefore it is difficult to make a prediction of efficacy. Clinical trials of the Cat-PAD peptide immunotherapy have shown promising results, with sustained unresponsiveness for up to two years after a three-month immunotherapy regimen of only four intradermal injections and the induction of oral tolerance is highly desirable in AIT and would require immediate exposure to the peptide. The findings presented in Chapter 6 indicate a reasonable level of peptide resistance to gastrointestinal enzymatic degradation, and show how this resistance can be influenced through residue substitution in positions removed from the predicted enzyme digest sites. The results of Chapter 6 concern only three of the five T cell epitopes identified in Ara h 2, and further studies are required to assess the digestive resistance of the two remaining epitopes of Ara h 2, and the recently identified T cell epitopes of Ara h 1. Other hurdles would also need to be overcome for these peptides to maintain bioavailability following oral ingestion, however the recent development of new technologies to improve peptide stability may assist in this process, should this eventually be the preferred route of delivery.

Though originally intended for severe allergic asthma, omalizumab can now be administered to patients with ABPA. This is based on numerous studies, including our placebo-controlled
trial, confirming its safety and efficacy in this patient group. The off-label use of omalizumab has recently been assessed in similar controlled trials for other disorders, including non-atopic asthma, atopic dermatitis and chronic idiopathic urticaria, highlighting the broad applicability of this drug. In addition to these applications, omalizumab has potentially found a place in food allergy treatment, where it is used to prevent immediate hypersensitivity reactions during the course of oral allergen immunotherapy. With the broad application of this therapeutic, the challenge now lies in identifying biomarkers that may predict the efficacy of omalizumab in patients prior to administration. Considering the high cost of omalizumab and the duration required to evaluate efficacy, these markers would be of great value, not only on a socio-economic level but to the individual patients as well.

SUMMARY OF FINDINGS

In the studies presented in this thesis, components involved in allergy development were investigated, targets for the design of novel allergy therapies were identified, and the off-label use of current allergy therapies were assessed. The main findings are:

- Food allergy can be induced through repeated exposure of barrier-disrupted skin to the allergen, even in an adult (Chapter 2).
- Isolated human basophils express low levels of MHC Class II upon in vitro stimulation with IL-3, GM-CSF and IFN-γ, which is not accompanied by expression of costimulatory molecules CD40, CD80 or CD86 (Chapter 3).
- Human MHC Class II positive basophils are incapable of inducing allergen-specific T cell activation, through presentation of either whole allergen or allergen-derived peptide in vitro (Chapter 3).
- There are five dominant CD4+ T cell epitopes within the major peanut protein Ara h 2, which can be combined into three short HLA-degenerate peptides. These peptides are not recognized by peanut-specific IgE (Chapter 4).
- There are ten dominant CD4+ T cell epitopes within the major peanut protein Ara h 1, which can be combined into seven short HLA-degenerate peptides. These peptides do not induce IgE-mediated basophil activation (Chapter 5).
- The substitution of cysteine residues in T cell epitope peptides with serine residues, a standard procedure in therapeutic peptide preparation for clinical use, may alter peptide resistance to enzymatic digestion, with implications for the potential of oral route of delivery (Chapter 6).
- Omalizumab is safe and effective in reducing basophil sensitivity to Aspergillus fumigatus and exacerbation rates in patients diagnosed with ABPA even when serum IgE levels are in excess of the dosing guidelines (Chapter 7).

CONCLUDING REMARKS

The mechanisms involved in the development of allergic disease are not yet fully understood. Further identification of risk factors and the cellular mechanisms underlying allergic disease will assist in establishing what is required to reverse the recent increase in disease prevalence. In the meantime, allergen immunotherapy (AIT) remains the most promising therapeutic, due to its ability to modify the specific immune response with long-lasting effects. Recent advances in this field, such as allergen modification (e.g. hypoallergenic forms or T cell epitope peptides) and route of delivery (intradermal and intralymphatic), show promise in expanding the applicability of AIT to additional allergy types and disease severities, as well as reducing adverse effects and treatment duration. Further improvement of AIT may be achieved upon the identification of the requirements for achieving the ultimate goal of sustained unresponsiveness. In addition to immunotherapy, a vast array of biological therapeutics is currently available, and several existing therapeutics may be applicable for certain allergic diseases through off-label use. Placebo-controlled trials with carefully defined patient groups are necessary for the validation of these off-label treatments. Upon validation, such therapeutics may be best applied through a personalized approach, where specific treatment protocols are established for each individual. This is becoming feasible with the emergence of affordable cutting edge techniques e.g. genome sequencing, and will ultimately prevent unnecessary treatment and associated costs.
REFERENCES


