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Novel Tools for Elucidating the Immunology of Allergy

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ABSTRACT

Background: Anaphylactic shock causes an estimated 1,500 deaths every year in the United States alone and millions suffer from allergic rhinitis. Despite the documentation and investigation of atopic (IgE-mediated) diseases for over a century, much is unknown about several aspects of this area of immunology. Type I allergies occur when allergen-specific Immunoglobulin E (IgE) antibodies are formed against an otherwise harmless protein. This can lead to symptoms ranging from a runny nose to serious inflammation and, in severe cases, anaphylaxis. Furthermore, the “blocking” effect of IgG4 provides the basis for specific allergen immunotherapy (SIT), where injections of whole allergen extracts are used to reduce and “block” the IgE response. Because only trace amounts of IgE can be found in the blood, discovering details about its origin and relationship to IgG, as well as its behavior in reaction to SIT has proven difficult.

Objective: This study attempts to further elucidate the characteristics of humoral response with regard to memory cells as well as serum allergen-specific IgG.

Methods: The ELISpot procedure was utilized for the enumeration of IgE memory cells circulating in allergic and non-allergic patients, while IgG affinity for those patients was tested on a western blot using the same standardized panel of allergens.

Results: The former procedure indicated that allergic individuals do possess IgE memory cells in the blood which are specific to allergens. Also shown, in the latter experiment, is a unique IgG binding pattern for each participant, even those claiming to be non-allergic.

Conclusions: Overall, IgE memory B cells are present in the peripheral blood of allergic individuals and each individual displays a unique pattern of serum IgG antibodies to specific allergens, whether

or not they are on SIT. In the future, these results may be utilized to predict which allergens atopic individuals will become allergic to, as well as develop new, directed, forms of immunotherapy.

INTRODUCTION

Atopic diseases, such as allergic rhinitis and allergic asthma, have been increasing alarmingly across industrialized nations, with over half of Americans afflicted by some allergic manifestation [7]. This increase in symptoms has occurred in the last few decades and has not been observed in the developing world, suggesting a tie to industrialization. However, current therapeutic methods lack the foundation of modern immunology vital to confronting the causative agent, IgE. Rather than decrease the IgE response in patients, SIT treatments focus on increasing IgG levels in the blood [1, 8]. IgG4 has been shown to inhibit IgE levels in allergic patients and also to be present in high levels when commonly exposed to an antigen, such as beekeepers with insect venom [10]. The hygiene hypothesis asserts that higher standards of living prevent children in industrialized nations from encountering as many infectious agents, leaving them more vulnerable to allergies [4]. It has been shown that infection with certain types of parasites in childhood leads to significantly lower chances for atopic disease; though the cause for this is unknown, high levels of IgG4 antibodies can be detected in patients infected with those parasites [10].

Though no research has isolated a single cause, nor a single cure for allergic disease, there are many studies which suggest a clear distinction between the “infectious microcosm” of atopic individuals presenting symptoms, and those who are non-allergic [4]. Being exposed to the dust and microbes present on a farm, as well as higher diversity of bacterial or fungal taxa in home-dust, have both been linked to lower instances of allergies. The gut microbiome also seems to be relevant to atopic diseases, as an altered microbial flora is correlated with food allergy and children who have been on antibiotics show an increased risk for asthma [4]. All of these infectious agents have been linked to atopic disease, however the current atmosphere of treatment is unable to incorporate these findings into directed treatment. Allergic disease combines many genetic and environmental factors in a way that has still yet to be understood fully.

Current forms of therapy work to “desensitize” patients by exposing them to the allergens they are producing IgE against; however, the mode of doing so involves crude mixtures of allergens which may fail to effect a response, or may even worsen the reaction and cause development of new allergies [1]. There are currently few methods for determining the specific protein(s) in the

extract which are stimulating the immune response. The lack of information about IgE hinders forward progress in advancing treatment methods. IgE antibodies are present in such small quantities that they represent a “bottleneck” for the characterization of a complete library of allergen epitopes [6]. The novel techniques our lab has developed to explore vaccine responses will be applied to allergy with the hope that more efficient forms of immunotherapy can be pursued.

In this study, selected participants' peripheral blood was tested with memory B cell ELISpot procedure to determine the number of allergen-specific cells. The blood samples were processed, and then stimulated in culture for six days so that the memory cells differentiated into antibody secreting cells (ASCs). The ASCs were then tested on a plate coated with our panel of standardized allergens so that the allergen-specific cells could be counted. These results indicate that while no circulating allergen-specific ASCs were detected, IgE

memory cells were present. The same set of standardized allergen extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) then transferred to a PVDF membrane and probed with a serum sample from selected participants. IgG was then detected with anti-human IgG-HRP in order to determine binding of each patient's IgG to the allergens in question. Each individual possesses a unique pattern of bands whether on SIT or not. An individual who does not have allergic rhinitis showed bands to 'Type I' grass pollen allergens (i.e. Phl p 1), but few others. This indicates that non-allergic individuals do not progress into 'molecular spreading' [5].

METHODS

Memory ELISpot

The stimulation of PBMCs and determination of ASCs by ELISpot has been previously described in detail [2]. To detect IgE rather than IgG, plates were coated with

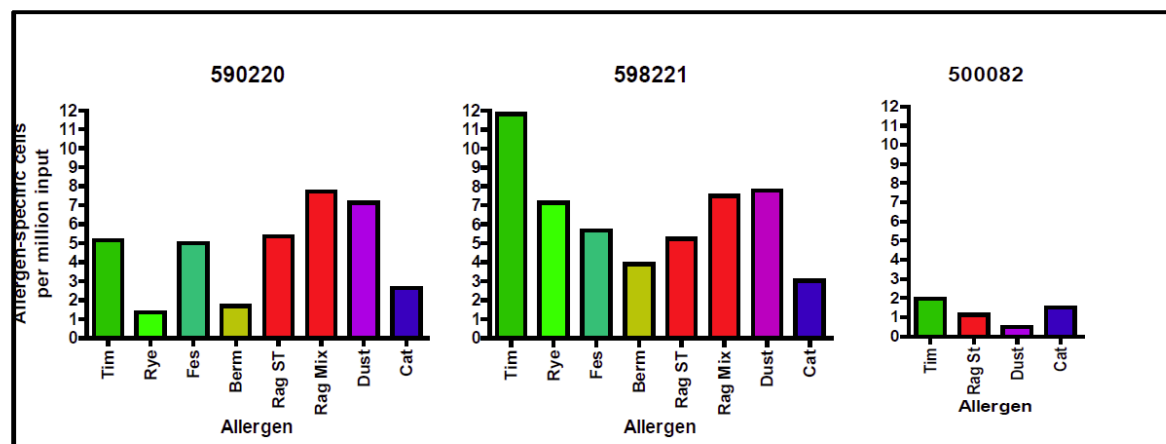


Figure 1: Numbers of allergen-specific memory B cells were determined by ELISpot.

10µg/ml of goat polyclonal anti-IgE (1µg/well) (Bethyl, Montgomery, TX) and detected with the same polyclonal antibody conjugated to HRP (Bethyl, Montgomery, TX). Standard allergens were also coated at (1µg/well) (AllerMed, San Diego, CA).

SDS-PAGE and Western Blot

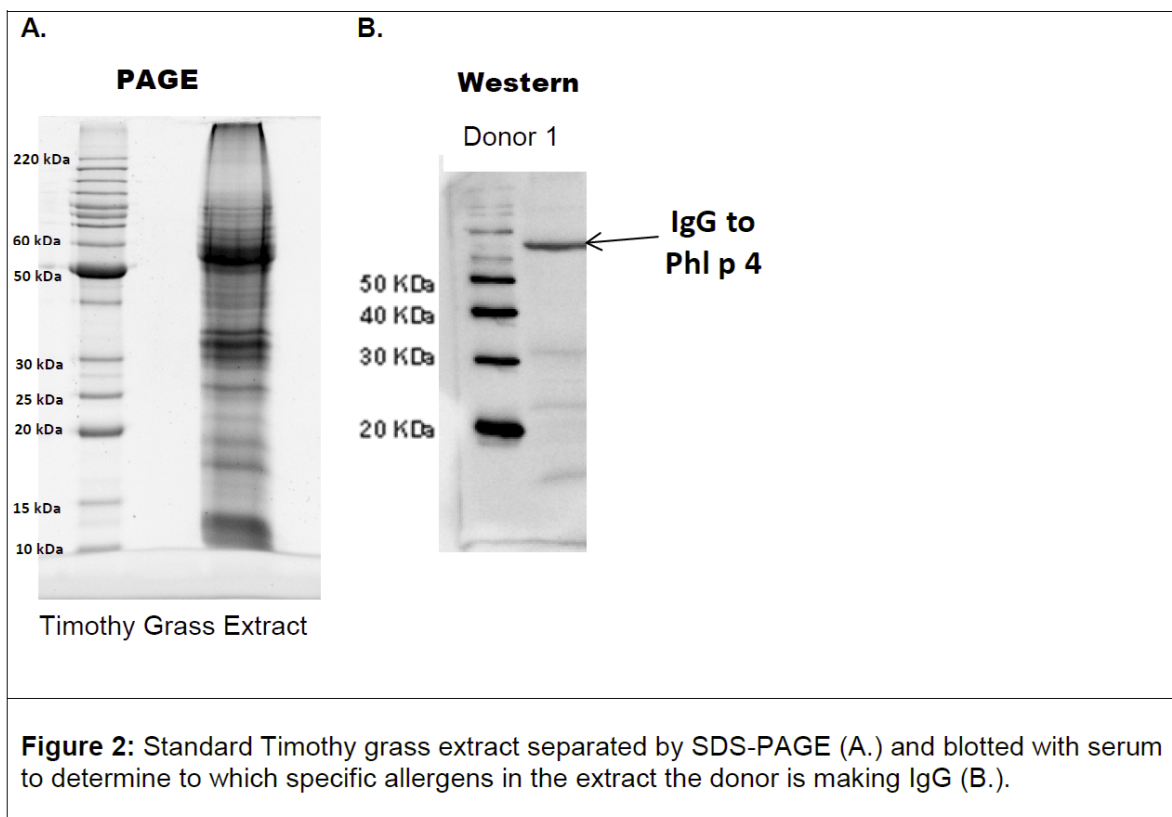
The standard allergens of interest (Timothy grass, meadow Fescue, perennial Rye, Bermuda grass, ragweed, dust mite, and cat hair) (AllerMed, San Diego, CA) were separated by SDS-PAGE using a 12.5% gel. The gels were then transferred to PVDF membranes using a semi-dry transfer protocol. Membranes were blocked with 3% dry milk powder for 1 hour. Blots were then slowly shaken with the 'primary antibody' for the blots,

plasma from allergic individuals diluted 1:100 in 3% milk, for 1 hour. Blots were developed using goat anti-human IgG Fc conjugated to HRP (Jackson ImmunoResearch, West Grove, PA) at 1:5000 in 3% milk followed by Luminata ECL reagent (Millipore, Billerica, MA) and imaged.

RESULTS AND DISCUSSION

Memory ELISpot

The presence or absence of IgE positive, allergen-specific memory cells is still controversial in mouse models [3, 9] and has not been closely examined in humans. In order to determine whether such cells are present in the peripheral blood of allergic individuals, we adapted the IgG memory ELISpot [2] to detect cells which upon pan



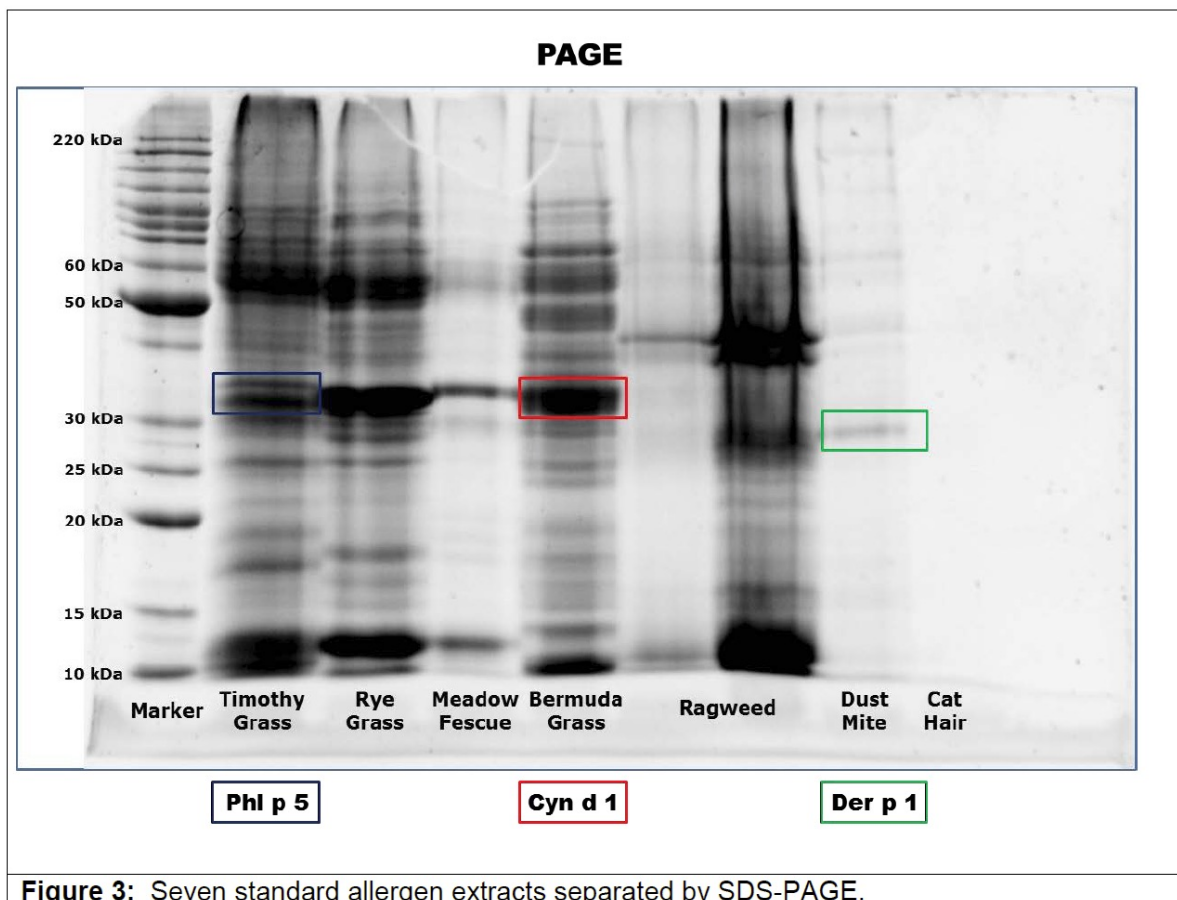
stimulation produce IgE and are likely IgE positive cells with a memory phenotype. We were able to detect such cells to seven allergens in three allergic individuals, as shown in Figure 1.

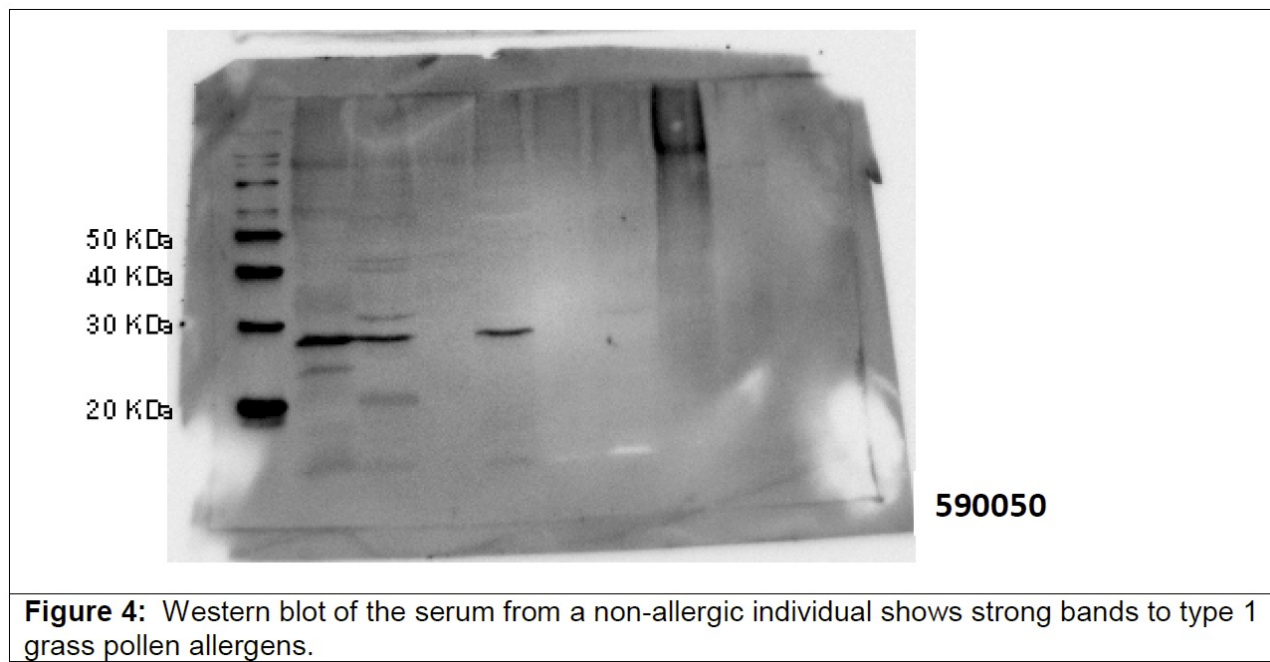
Allergen-specific IgG by Western blot

It is well known that 'blocking' IgG4 is induced by SIT via vaccine-like mechanisms [1]. To our knowledge, however, the total IgG response has not been examined to several allergens among many individuals. To this end, we developed an assay to explore to which specific allergen proteins in crude extracts allergic and non-allergic individuals made total IgG. Whole standardized allergen extracts were first run on 12.5% SDS-PAGE

gels, transferred to PVDF membranes and blotted with the serum of several individuals. Total IgG was then detected and visualized. Figure 2a shows an example of Timothy grass extract separated by PAGE and then blotted with serum from an individual with IgG to Phl p 4 (Figure 2b). Figure 3 shows all seven allergen extracts separated by SDS-PAGE, highlighting Phl p 5, Cyn d 1, and Der p 1.

The first individual analyzed by this method is 'non-allergic'. This donor has no symptoms of allergic rhinitis. By this western blot analysis, this individual has intense IgG bands to group 1 grass pollen allergens (Phl p 1, Lol p 1, Fes p 1) as well as Bermuda grass (Cyn d 1) (see Figure 4). Recent work has shown that children who develop allergic





rhinitis via Timothy grass pollen do so in certain progressions, typically starting with Phl p 1, but progressing to the other Timothy allergens via 'molecular spreading' [5]. The fact that this non-allergic individual has IgG only to the type 1 allergens may indicate an important mechanism in the development of allergic rhinitis, which has stopped with type 1, rather than progressing further. Whether the IgG is the cause (perhaps due to natural IgG₄) or is simply an indicator that this person has only made an immune response to type 1 is still under investigation.

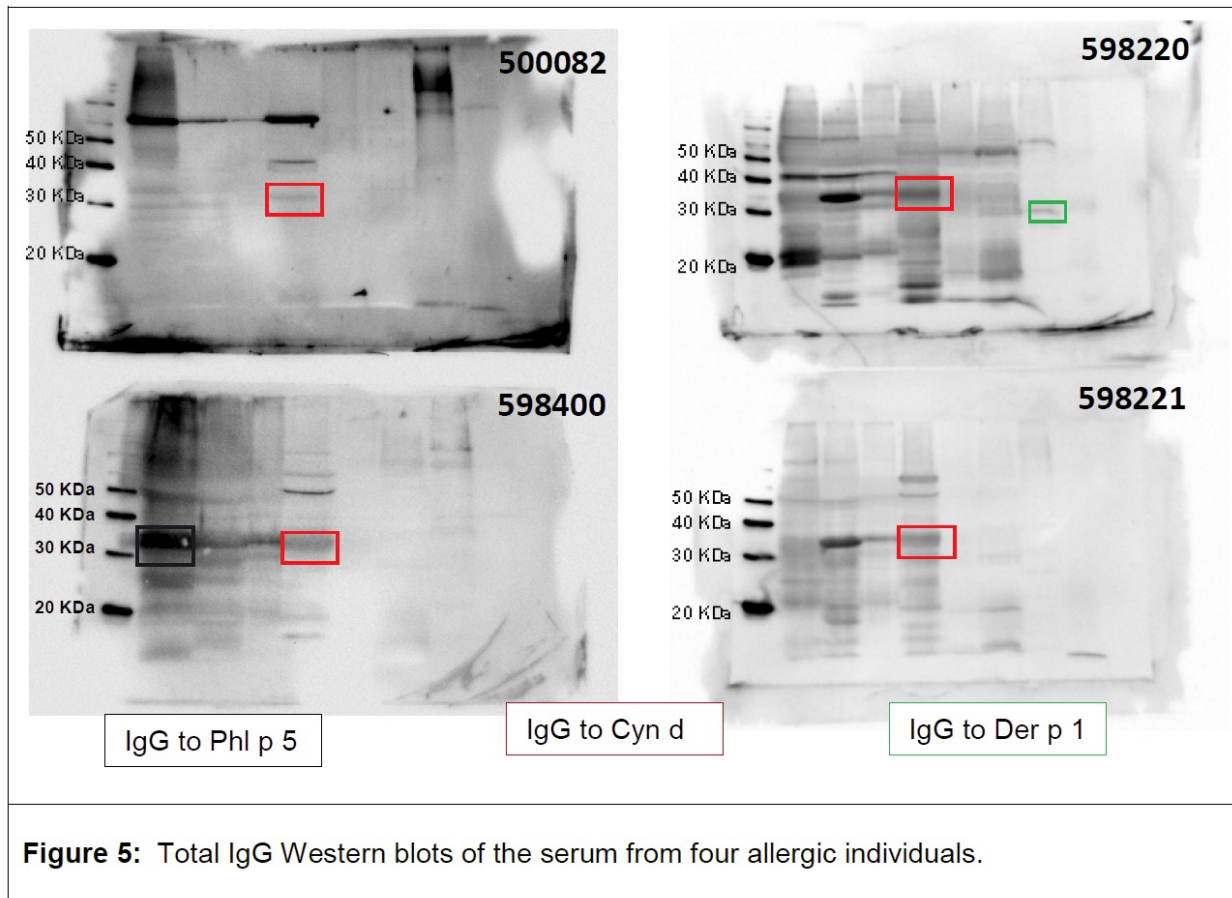
Unlike the non-allergic donor, allergic individuals show intense bands to many of the allergens present. Representative blots from four individuals are shown in Figure 5 and a summary of bands to known allergens is shown in Table 1. Donor 500082 has strong bands to Phl p 4 and dust mite allergens. Donor 598400 has been on SIT for many years and has intense bands to grass pollens,

including type 5 grass allergens. 598220 and 598221 are both allergic individuals that are not on SIT and they both show many strong bands. Many of these bands are to uncommon or uncharacterized allergen proteins, indicating that much work remains in understanding these allergens.

Work remains to be done to determine what the presence of detectable amounts of allergen-specific total IgG indicates, as well. IgG₁ and IgG₂ are not capable of blocking the formation of IgE/allergen complexes and may have no effect on basophil and mast cell activation, or may even enhance it. Conversely, the presence of IgG₁ may simply be a more easily detectable surrogate for IgG₄, whereas the bands we are detecting may actually indicate the ability to block these responses. As this work continues, we will focus on this relationship, the mechanism of stopping 'molecular spreading' in non-allergic

individuals and the origin and function of IgE

memory cells in humans.



	Timothy Grass	Perennial Rye	Meadow Fescue	Bermuda Grass	Rag Weed	Dust Mite	Cat Hair
* 590050	Phl p 1	Lol p 1		Cyn d 1			
510111	Phl p 4	Lol p 4	Fes p 4	Cyn d 1	Amb a 1 Amb a 2		
598221		Lol p 5	Fes p 5				Fel d 1/3/4
598220		Lol p 2/3 Lol p 5		Cyn d 1 Cyn d 7	Amb a 1 Amb a 3/5/7	Der p 1 Der p 4	
* 598400	Phl p 2/7 Phl p 5	Lol p 5	Fes p 5	Cyn d 1 50 kDa unknown			
500082	Phl p 4	Lol p 4	Fes p 4	Cyn d 1 40 kDa unknown 60 kDa unknown		Der p 2/5	Fel d 1/3/4 Fel d 2
* 598401	Phl p 4 Phl p 5	Lol p 4				Der p/f 2/5 Der p 4	
* 598402	Phl p 5	Lol p 5	Fes p 5	60 kDa unknown		Der f 1 Der p/f 2/5 Der p 4	

Table 1: Summary of total IgG bands from western blots.

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