Investigating the Mechanism of Dendritic Cell Allergen Response to Ara h2 Peanut Protein in a Canine *In Vitro* Model

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June 7, 2016

Background

Atopic dermatitis, more commonly known as eczema, is a common skin condition that affects a substantial proportion of the United States population. Specifically, 31.6 million individuals currently have atopic dermatitis, with at least 17.8 million having a moderate to severe case [18]. With a prevalence of almost ten percent in the United States, atopic dermatitis is more pervasive in America than diseases such as cancer and HIV [24]. The disease is even more common in children, with ninety percent of children getting mild to severe atopic dermatitis before the age of 5 [2]. Half of these individuals do not grow out of the disease, and can be faced with the eczema into their teenage years and adult life.

Atopic dermatitis is found throughout the United States, although some geographic variation is present. For example, incidence rates range from 18.1% in states such as Maryland, to 8.6% in South Dakota [21]. Interestingly, the incidence of atopic dermatitis is highest in East Coast states, although there is no accepted reason

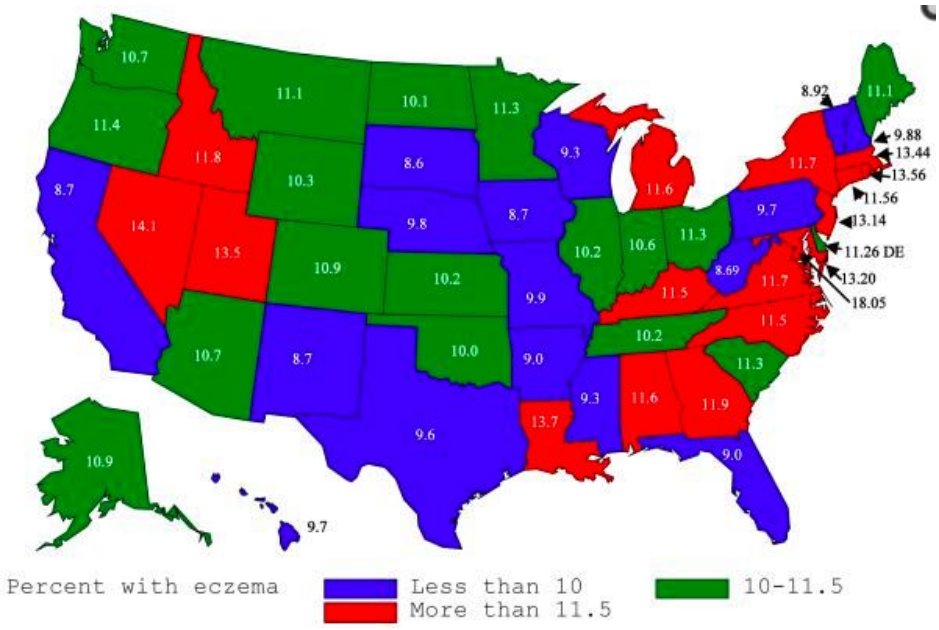

Figure 1: Incidence of atopic dermatitis in the United States. (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3130508/)

for this trend. Thus, it is evident that atopic dermatitis is a widespread disease affecting a large population of the United States.

The symptoms of atopic dermatitis vary depending on the age of the subject. Atopic dermatitis can be found in children as young as two to three months of age, where it is usually present in a mild to moderate form [2]. Still, chronic atopic dermatitis can affect individuals for their whole lives, and can quickly take a toll on the individual's quality of life. Symptoms usually manifest in the form of rashes and dry skin, with bumps and discoloration in more severe cases. The constant irritation can lead to scratching, which can expose the individual to harmful and possibly life threatening infections. Along with the physical symptoms, affected individuals must often cope with a significant psychosocial burden [2]. Individuals and family members are burdened with time-consuming treatment regimens for the disease, as well as dietary and household changes.

Atopic dermatitis also has a significant financial impact on families with affected individuals. Estimates show that treatment can range from 100 dollars to over 2000 dollars per patient per year [7]. This takes a large toll on the healthcare industry, with a direct cost of almost one billion dollars per year. Despite the existence of treatment regimens, such as various moisturizers, cleansing products, steroids, and abstaining from bath additives, the actual cause of atopic dermatitis is still unknown [10][16]. Therefore, these treatments do not target the cause of atopic dermatitis, but rather attempt to alleviate the symptoms, making for an overall ineffective strategy at combatting the disease.

Given the discrepancy between the high prevalence of the disease and the lack of effective treatments, it is it is evident that atopic dermatitis is a disease that requires more

attention and research. Thus, we aim to investigate a possible mechanism of atopic dermatitis development. If successful, this research has the potential to lead to a better understanding of the pathways involved in atopic dermatitis, which can ultimately lead to more effective medicines and treatments.

Although there is no currently known cause of atopic dermatitis, researchers have suggested a possible role of allergenic response in its development [8]. Specifically, food hypersensitivity can contribute to the development of atopic dermatitis, as well as asthma and hay fever. Many dermatologists embrace this suggested link, and have found that the elimination of specific foods that comprise most food allergies, such as soy, peanuts, and milk, can lead to significant improvement in eczematous symptoms [4]. Allergy is an increasing phenomenon across the world. In the U.S. alone, roughly 15 million people suffer from food allergies [11]. Among these, the most common food allergy is peanut, with 3.1 million Americans allergic. These numbers parallel the widespread (more than 3 million US cases per year) incidence of atopic dermatitis [2].

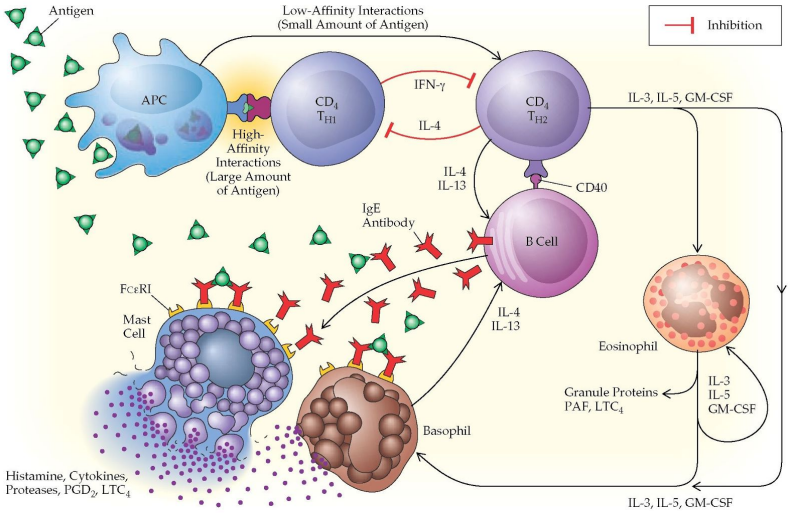

Figure 2. IgE pathway. (http://what-when-how.com/wp-content/uploads/2012/04/tmp4C45.jpg)

The currently accepted allergenic response takes place through the Immunoglobulin E (IgE) pathway of mast cell activation to a foreign, non-pathogenic epitope, as summarized in

figure 2 [12]. However, there is evidence to suggest an alternate pathway of allergen response, through dendritic cells. Dendritic cells are essential in the immune system, linking the adaptive and innate immune systems. Their dendrites ensnare bacteria, virus, and other antigens. Once activated, the dendritic cells migrate through the blood to lymph nodes to activate naive helper T cells [20]. Dendritic cells are largely responsible for kickstarting the first step in the adaptive immune system.

The role of dendritic cells in atopic dermatitis has been touched upon, where researchers showed that the inflammatory microenvironment of atopic dermatitis has a significant impact on dendritic cell activation, as shown in figure 3 [5]. Furthermore, inflammatory diseases of the skin, such as atopic dermatitis, have been associated with higher dendritic cell count, suggesting a role of dendritic cells in the pathways of these diseases [6]. In addition, the role of dendritic cells in atopic dermatitis is plausible due to the high volume of dendritic cells in the skin, known as Langerhans cells. Widespread errant dendritic cell activation could manifest as atopic dermatitis, with symptoms of skin rashes and irritability. The migration of dendritic cells from the skin through tissue could cause localized inflammation via activation of the inflammatory response with basophil and mast cell activation [20].

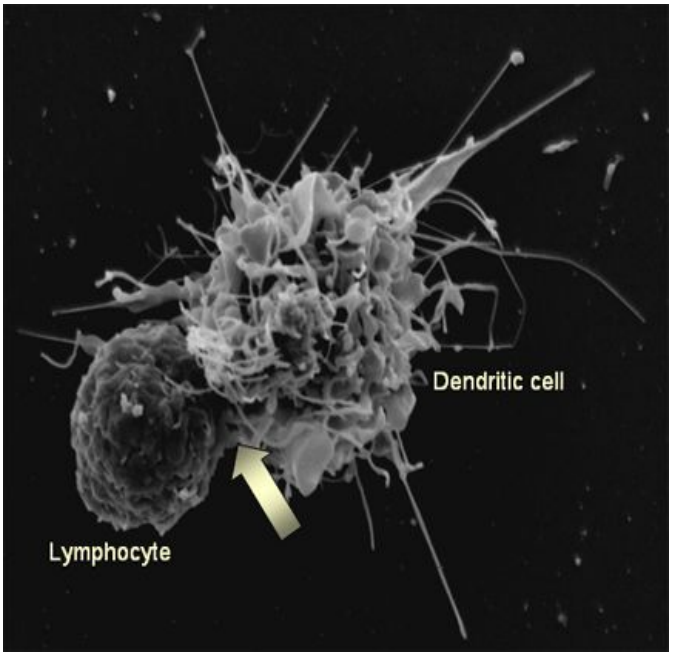

Figure 3: A Dendritic Cell Activating a Lymphocyte. http://www.hemacare.com/blog/wp-content/uploads/2014/04/dendritic-cell.gif

Generally, dendritic cells are activated by PAMPs, Pathogen Associated Molecular Patterns. Epitopes from food allergy can be mistaken for antigen from pathogenic molecules, creating harmful inflammation. Thus, given the possible relationship of food allergens and atopic dermatitis, as well as a correlation between dendritic cell activation and atopic dermatitis, the proposed investigation aims to determine whether food allergens can cause dendritic cell activation.

In order to create a model for experimentation, peanut allergens were chosen as they are the most common type of food allergy [11]. Specifically, the Ara h2 protein, a trypsin inhibitor [15], was used to model peanut allergenic proteins. Ara h2 has been shown to be most directly responsible for the allergenic properties of peanuts, and elicits the highest reaction with individuals with peanut hypersensitivity [14].

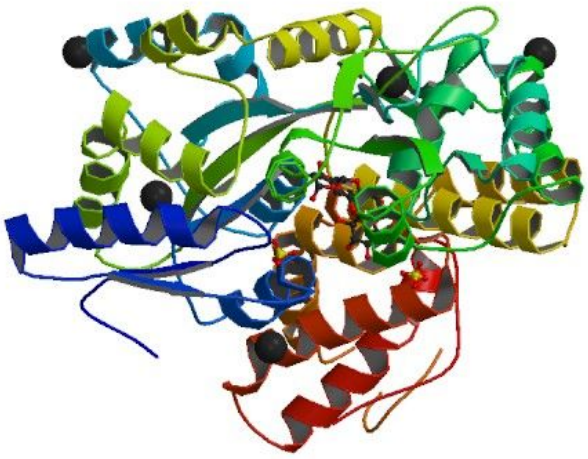

Figure 4: Ara h2. (http://www.rcsb.org/pdb/explore.do?structureId=3ob4)

Dendritic cell activation was measured with CD83, a known marker of mature dendritic cells [14]. CD83 is expressed on mature dendritic cells, and promotes MHC class II and CD86 expression [1]. Beyond this, the role CD83 plays in dendritic cells is currently unknown. Still, CD83 qualifies as an effective marker for dendritic cell activation because CD83 mRNA is significantly upregulated during dendritic cell maturation, and CD83 has been used in previous studies to separate and quantify mature dendritic cells [19].

For monetary reasons and cell yield, canine cells were used in experimentation. A canine model is viable because dendritic cells grown in vivo from a canine source have shown functional and morphological characteristics similar to human dendritic cells [23]. Furthermore, the CD83 marker chosen to measure dendritic cell activation is shown to have similar increased expression in mature human dendritic cells, providing evidence that canine cells and the chosen marker are feasible for experimentation [17].

As with any study, there were several limitations that prevented the results of this experiment from being directly applicable in a clinical setting. First and foremost is the incomparability of an *in vitro* experiment to actual *in vivo* responses. However, as this experiment is the first step in exploring the possible dendritic cell pathway in allergen response and atopic dermatitis, an *in vitro* experiment is better suited. Another important limitation is the focus of a single allergen. Due to time constraints, it was not possible to analyze many food allergens. Instead, Ara h2 was chosen to model peanut allergies, as it is the most common form of food allergies. Lastly, the investigation did not include any technical replicates, due to a limitation on the number of cells that can be obtained. This is not desirable as it causes the data to be less reliable due to possible outliers and experimental errors.

Overall, the experiment aimed to discover a possible secondary pathway leading to atopic dermatitis, driven through a dendritic cell allergenic response. The knowledge gained from this study helped contribute a deeper understanding of dendritic cell allergenic response to the scientific community, as well as make progress towards the eventual understanding of what causes atopic dermatitis. This information could eventually be used to help the millions of people around the world who suffer from this disease [18].

The hypothesis of this investigation was that Ara h2 administration will cause an increase in the CD83 expression of PHA induced dendritic cells. There are two independent variables in the study: phytohaemagglutinin (PHA), and Ara h2. We used PHA to cause differentiation of the canine peripheral mononuclear cells (PBMC) into canine dendritic cells, while we used Ara h2 to study its effect on the CD83 expression of these cells. We used PHA at a concentration of 8 ug/mL due to the same concentration being used in the only known previous study implementing this method [25]. We selected a 10 ug/mL concentration of Ara h2 because this concentration has been shown to elicit the most noticeable effect in previous studies that stimulated cells with purified Ara h2 [9].

The use of PHA to induce this differentiation was implemented due to its prior success in inducing canine PBMC to differentiate into naive dendritic cells [25]. However, because this method varies drastically from the more established use of granulocyte macrophage colony-stimulating factor (GM-CSF) to induce differentiation, this study is also investigating the efficacy and reproducibility of the use of PHA in inducing dendritic cell differentiation.

The dependent variable of the experiment is the measured CD83 expression of the dendritic cells, measured through FITC-conjugated anti-CD83 antibodies. CD83 was chosen as a marker for dendritic cell activation due to the extensive literature linking it to a sign of dendritic cell maturation [14][19].

To put the results in context, we chose *E. Coli* Lipopolysaccharide (LPS) as the positive control in the experiment. LPS has been shown to activate naive dendritic cells, and has been used in the capacity of a positive control in similar experiments [23]. We used a concentration of 10 ug/mL to replicate the effects of LPS in previous experimentation. Due to time constraints,

we were unable to run multiple experimental replicates. However, we were able to conduct two trials.

The dependent variable is twofold, measuring the CD83-expressing dendritic cell count with flow cytometry as described in the methods section. These cell surface receptors measure dendritic cell activation [23]. Because of the relatively novel nature of the differentiation procedure we used, we also examined the efficacy of PHA in causing the differentiation of PBMC into dendritic cells.

Methods and Materials

We obtained canine peripheral blood through generous donations from the Blue Ridge Veterinary Blood Bank. We used the Sigma Aldrich Accuspin System - Histopaque 1077 to spin down the peripheral blood and isolate the PBMC [22]. We followed the manufacturer's standard centrifugation procedure, spinning the 50 mL tube at 800 x g for 15 minutes at room temperature, and then transferring the mononuclear band with a sterilized Pasteur pipet into a sterile centrifuge tube. We washed this isolated band twice with PBS in order to remove any remaining erythrocytes.

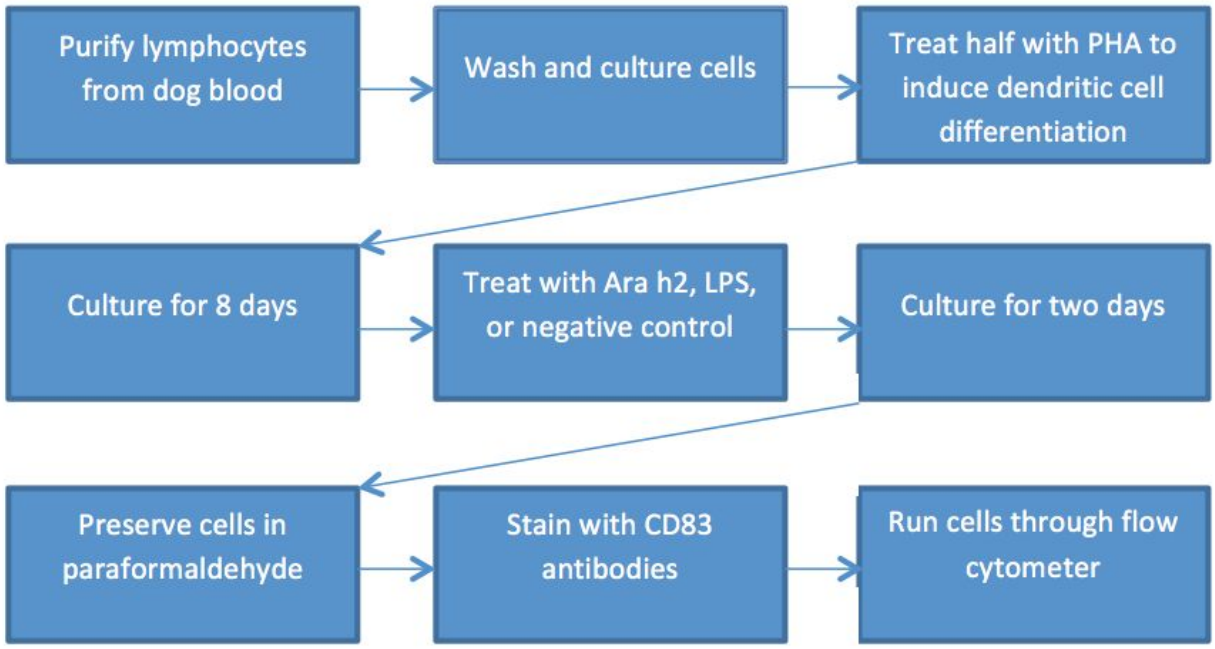

Figure 5. Flow Diagram for Methods.

After isolation, we cultured the PBMC for 2 days with RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO2 humidified atmosphere [25]. The cells grew in two T-25 flasks at an initial concentration of 0.5 x 10^6 cells/mL, separated due to the high cell concentration seen after initial isolation. After these 2 days, 50 uL of PHA was added

to one of the T-25 flasks at a concentration of 8 ug/mL to induce differentiation. We incubated the cells with PHA for 8 days, following the optimal time period for PHA incubation [25]. During the incubation period the cell media was changed as needed.

Due to the dual suspended and attached nature of dendritic cells, the cells had to be trypsinized as well as spun down when replacing media. Cells were incubated in 1 mL of trypsin for 5 minutes at 37°C in 5% CO_2, and then incubated for periods of 2 minutes until the cells were completely detached. For cell washes, we spun down the suspended cells at 1315g for 10 minutes and washed them with PBS. Following the 8 day incubation with PHA and analysis between +PHA and -PHA, we split the cells into a 6 well plate, with wells containing +PHA, +PHA+LPS, and +PHA+Ara h2/peanut powder.

We obtained purified Ara h2 from Protein labs, which was originally delivered in a 20 mM Tris and 0.15 M NaCl buffer (pH = 7.7). The Ara h2 solution was diluted to 500 uL with PBS containing 10% glycerol, and stored at -80°C. Upon thawing, we diluted it to a concentration of 100 ug/mL and added 15 uL of this solution to the well for a final concentration of 10 ug/mL with the cells.

After culturing the cells in PHA, LPS, or no treatment for two more days, we fixed the cells in a 2% paraformaldehyde (PFA) solution. Following fixation, we washed the cells with an incubation buffer comprised of 0.5 grams of BSA in 100 mL 1X PBS, spinning the cells at 2000g for 5 minutes. After resuspension in 100 uL of incubation buffer, we blocked the fixed cells with this same incubation buffer for 10 minutes. At the end of the 10 minute window, we set aside half of the cell suspension to remain unstained (for downstream analysis of background fluorescence), and brought the remaining half of the suspension up to 100 uL again. We then

incubated the second half of the cell suspension with the FITC-conjugated CD83 antibody for 1 hour in the dark. After this 1 hour period, we washed both the unstained and stained cells and resuspended in 100 uL of PBS to prepare for flow cytometry analysis. We ran the cells through the flow cytometer according to standard procedure, and analyzed the data using an ANOVA test in order to compare multiple sets of data.

Results

Figures 6-8 were taken at various points during the experimental process. Figure 6 shows the PBMC prior to the incubation with PHA, Figure 7 shows the cells on day 7 of incubation with PHA, and figure 8 shows an image of cells without PHA. It is visible in these images that the cells that received PHA have a larger and more branched morphology and noticeable clumping This is much different from the cells that did not receive PHA, which are smaller and don't go clump. This suggests that the cells have adopted some dendritic cell morphology. Dendritic cells have sticky dendrites that latch onto antigens in the bloodstream. In culture, this can result in a clumping effect that we saw [23]. This suggests that the incubation with PHA was successful in differentiating the canine PBMC into canine dendritic cells, although further testing is necessary before this can be conclusively asserted. It's possible that it didn't create functional cells.

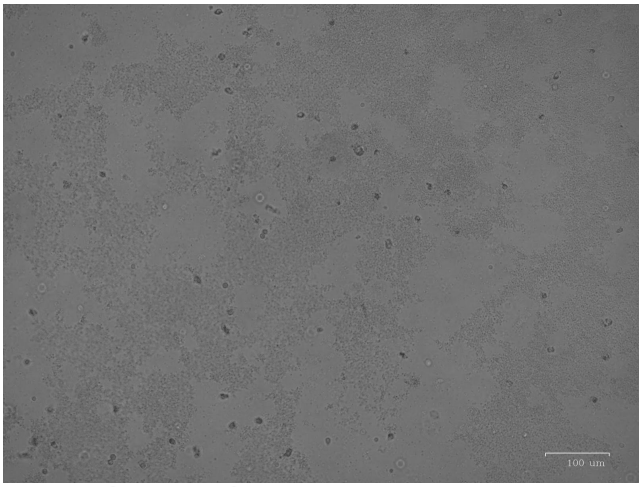

Figure 6. Canine PBMC pre-PHA treatment.

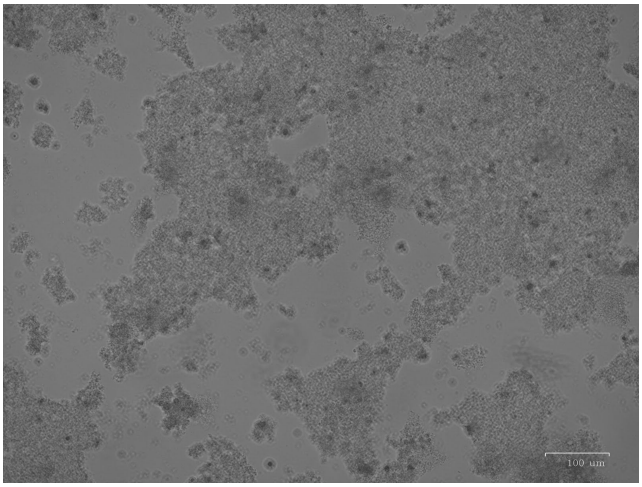

Figure 7. Cells on day 7 following PHA treatment.

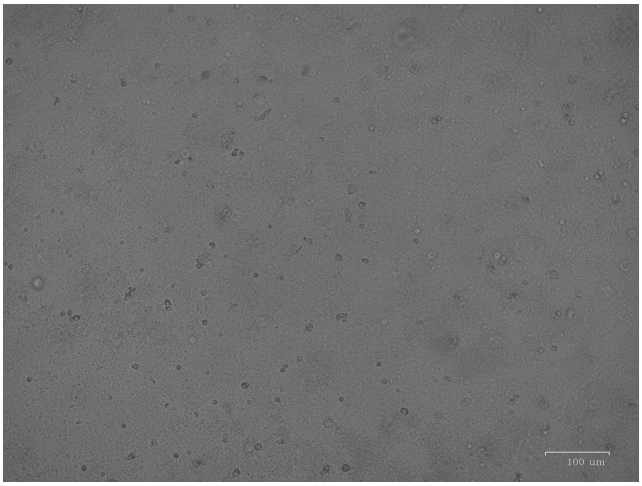

Figure 8. Day 7 cells without PHA treatment.

Following incubation with PHA, the cells were subjected to Ara h2, LPS, or left to culture without any additives (negative control). The flow cytometry results of the antibody staining for these three populations is shown in table 1.

Table 1. The Effect of Peanut Powder on Fluorescence-Measured Cell Activation.

Cell Type	Unstained Mean Fluorescence	Stained Mean Fluorescence
+LPS	149.160	147.820
-LPS	149.165	148.625
+Peanut	149.140	148.275

This information is also displayed in a graphical representation in Figure 9. Because we ran more than two levels of the independent variable, we used an ANOVA test to determine the statistical due significance of the difference between the data. The results of this test can be seen in table 2, which show the data were not statistically significantly different, with a p value of .31988.

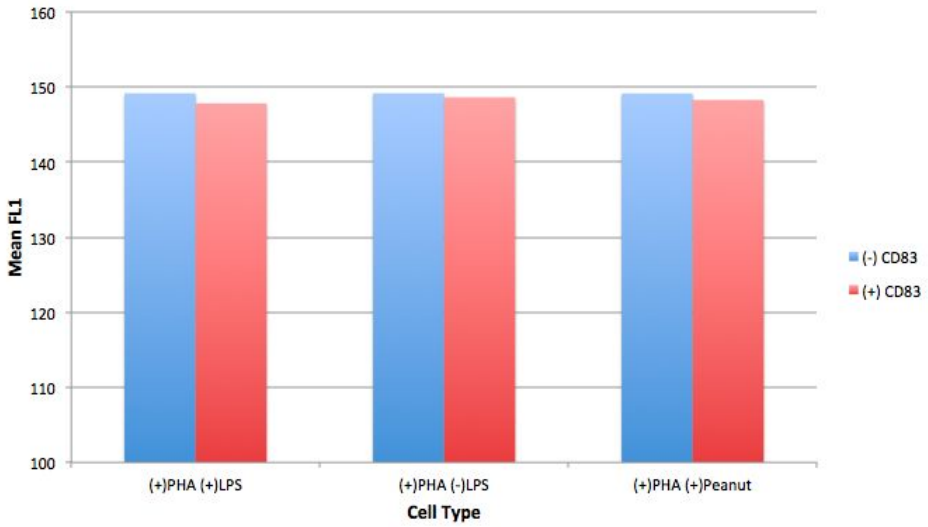

Figure 9. The Effect of Peanut Powder on Mean Fl-1 Fluorescence.

Table 2. ANOVA Statistical Results for Mean FL-1 Fluorescence.

Sample type	Mean	Variance	Std. Dev.	Std. Err.
No antibody	149.155	0.3715	0.6095	0.2488
Antibody +LPS	147.82	0.1922	0.4384	0.31
Antibody -LPS	148.625	3.7813	1.9445	1.375
Antibody +Peanut	148.275	0.3121	0.5586	0.395

Overall, there was no statistically significant difference between any of the IV levels, thus showing that there was no meaningful difference in FL1 Fluorescence (and presumably CD83 presence and dendritic cell activation) between samples treated with LPS (intended to induce dendritic cell activation), samples without LPS, and samples treated with the peanut powder (variable under investigation).

Furthermore, because there was no statistically significant difference between groups stained with the antibody and groups not stained with the antibody, the detected fluorescence could likely be due to background fluorescence from the cells, rather than due to the antibody. It's also possible that fluorescence caused by the PBMC that was not differentiated by the PHA overwhelmed any difference between the groups. This is corroborated by the consistent downward trend seen in data. However, as this was not statistically significant more trials and perhaps a clearer activation detection method is required for further analysis.

Another trial is being conducted in order to eliminate possible sources of error. Additionally, this secondary trial will use purified Ara h2 rather than store bought peanut powder, which offers a more promising outcome. Due to our inability to use the flow cytometer (machine error), data will be collected through the spectrophotometer.

Conclusion

The purpose of the experiment was twofold: to test if phytohemagglutinin (PHA) could induce canine peripheral blood mononuclear cells (PBMCs) to become naive dendritic cells, and to test if ara h2 activated these canine dendritic cells. Our research found evidence that PHA induced morphological changes in canine PBMC to create structures that resembled those of dendritic cells. However, results for the ara h2 were inconclusive. When run under the flow cytometry, only background fluorescence was measured for both the ara h2 cultures and the LPS positive control. This suggests that either some experimental error occurred while staining or processing the cells, or that the PHA induction simply produced cells that had some characteristics of dendritic cells but didn't function as them. Further trials have to be conducted to determine which possible explanation is correct, or if there's another explanation altogether. As such, neither hypothesis proposed can be supported by the evidence at this time. One possible follow up experiment could be to explore the effect of ara h2 on human dendritic cells rather than induced canine cells, which would increase viability for clinical application. This separation, while more financially costly, would be a marked improvement upon the current one. Other allergenic molecules could also be tested in lieu of ara h2, creating a more diverse base of chemicals to test the alternative allergenic pathway theory. If that data is promising, then in vivo trials could experiment with adaptive immune system targeted treatment for atopic dermatitis.

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