The Use of CpG Oligodeoxynucleotides as an Adjuvant for Immunotherapy to Mediate the T-Helper Cell Response Against Dog Allergies

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Abstract - Dog allergies are a nationwide concern, with 15 million Americans suffering from this allergy. Currently, the treatment against allergies, allergen immunotherapy (AIT), lacks efficacy in treating most allergies and has yet to focus on improving AIT for dog allergies. This study is the first to determine whether or not adding a bacterial adjuvant, CpG ODNs, to AIT will prevent an allergic response to dog allergens. *In vitro* observation of cell responses to dog allergens, combined with and without CpG ODNs, was assessed. When evaluating the effectiveness of adding the CpG ODN, the type of T-helper cell response was determined through flow cytometry. Results indicated a decrease in Th2 cells, with no increase in Th1 cells. Overall, this demonstrates that the use of CpG ODNs has the potential to amend the immune response. Ultimately, the goal of this research extends beyond treating dog allergies. Ideally, researchers hope to fully understand the method of using bacterial adjuvants against allergies so the treatment can be widespread across different types of allergies.

Key Words – T-helper 2 cells, CpG ODNS, AIT

INTRODUCTION

More than 50 million Americans suffer from allergies; three in ten are allergic to dogs (Grayson, 2018). There are currently seven known dog allergens, classified as Can f 1-7, that are considered a major risk factor for developing allergic rhinitis and asthma (Chan & Leung, 2018) As allergies are growing at a rapid rate, at 6.4% increase per year (cdc.gov, 2017), the need for an effective treatment is critical.

Allergies present themselves when a foreign antigen is displayed to immune cells. Allergens are recognized by CD4 T helper (Th) cells, specifically, CD4 Th2 cells that stimulate the production of immunoglobulin E (IgE) antibodies from B cells through chemical signals, such as interleukins (IL) (Reece et al., 2018). The type of interleukin secreted determines the nature of the T cell response. Interleukin associated with the Th2 response encourages the production of IgE, ultimately leading to an allergic response. Allergic responses cause symptoms that can range from watery eyes, sneezing, rashes, to anaphylaxis (Grayson, 2018). To cure these allergic responses there is currently only one treatment method, allergen immunotherapy (AIT) (Cox, 2018). AIT works to counteract the Th2 allergic response by promoting the production of immunoglobulin antibodies and interleukins associated with the Th1 response, which normally occurs in response to intracellular parasites (Janeway, 1999). AIT involves the gradually increasing introduction of allergenic substance that an individual is allergic to. The goal is to modify the immune system away from an allergic Th2 response upon exposure. However, AIT faces barriers, such as a lack of effectiveness when using the crude allergen extract; therefore, future research must focus on new solutions to overcome this obstacle (Chan & Leung, 2018). One possible solution is the addition of an adjuvant, a substance that modulates immune responses to an antigen.

Adjuvants have been shown to amplify the effect of immunotherapy by modulating the immune response (Shi, 2019). By recruiting specific populations of immune cells, adjuvants play a role in stimulating a Th1 response (Shi, 2019). One promising adjuvant is the cytosine-guanine oligodeoxynucleotide (CpG ODN). CpG ODN is a short single-stranded segment of bacterial DNA that contains unmethylated CpG dinucleotides (invivogen.com). CpG ODNs are agonists of Toll-like receptor (TLR) 9, which is important because TLRs play a key role in the induction of Th1 immune responses (Farrokhi et al., 2017). The use of CpG ODNs in immunotherapy has the potential to be curative in the treatment of allergic diseases (Farrokhi et al., 2017). Thus, the focus of this study is to improve immunotherapy for dog allergies with the use of the CpG ODN adjuvant. This study is the first of its kind to use dog allergens and the CpG ODN adjuvant together. In the end, this treatment may have the potential to alter the immune pathway, ultimately stopping allergic reactions and reducing the risk of anaphylaxis, a potentially deadly condition.

I. The Immune System and Th1/Th2 Cell Response

Th2 cells play an important role in the humoral immune response by releasing interleukins to signal the production of antibodies, specifically the allergic antibody, IgE (Umetsu & DeKruyff, 1997). When produced, IgE binds to mast cells, producing an allergic response (Galli & Tsai, 2012). Th2 cells represent a harmful adaptive response to antigens by amplifying and prolonging allergic inflammation, as well as

late-phase reactions, which are associated with airway hyperreactivity that can lead to asthma and allergic rhinitis (Umetsu & DeKruyff, 1997). By contrast, Th1 cells inhibit the development of allergic reactions because the chemicals secreted, IFN-γ and IL-12, do not promote IgE synthesis (Umetsu & DeKruyff, 1997).

It has been found that the Th1 cell response cascade inhibits the proliferation of Th2 cytokines, thus inhibiting allergic responses (reviewed in D'Elios et al., 2011). The Th1 cell response includes the secretion of IL-12 and IL-18, which signal for IFN-γ and inhibit the secretion of IgE (Yoshimoto et al., 1997). However, Th2 cells cannot signal for the synthesis of IgE when Th1 cytokines are proliferating because these chemicals secrete signals for different antibody production (Oriss et al., 1997). Therefore, the ideal immune response is the Th1 cell response. It is possible to induce a Th1 reactions over a Th2 reaction with the use of adjuvants, one being CpG ODNs. CpG ODNs have been shown to suppress the Th2 response and induce a Th1 response; therefore, CpG ODNs are currently studied as a tool to induce Th1 cell responses.

II. CpG ODNs Help Induce Th1 Cell Response

Preclinical models of asthma have demonstrated that CpG-ODNs are potent inhibitors of atopic (allergic) responses, suppressing Th2 cytokines and reducing airway eosinophilia and levels of IgE (reviewed in Fonseca & Kline, 2009). Airway eosinophilia was significantly reduced ($p < 0.01$) in mice treated with CpG ODN immunotherapy compared to mice treated with regular AIT (Jain et al., 2003). CpG ODNs are currently being developed as a vaccine adjuvant to prevent or treat allergies (reviewed in Hanagata, 2017). Thus, the introduction of an adjuvant provides a promising alternative to improve immunotherapy.

CpG ODNs bind to cells that express toll-like receptor 9 (TLR9) inducing a response that is biased toward Th1 immunity (Shirota & Klinman, 2014). In 2018, Lehto et al. (2018) found that mice who were allergic to timothy grass allergen and received CpG ODN immunotherapy reacted with a Th1 cell type response, resulting in a significant decrease in allergen-specific IgE levels ($p < 0.05$). This finding indicates that the CpG ODN sequences worked to decrease and weaken the allergic symptoms. The results are promising, suggesting that this technique, AIT with CpG ODNs, could be applied with different allergens.

Another study by Srivastava et al. (2015) analyzed the use of CpG ODNs with AIT against peanut allergies in a murine mouse model. Only 30% of the mice that received the CpG ODN with the peanut allergen had an allergic response, whereas 100% of the mice receiving the allergen alone had an allergic reaction. Additionally, a significant decrease in IgE was observed ($p < 0.001$), indicating a shift to a Th1 response. Further, Marshall et al. (2001) conducted a study with similar results, examining the properties of CpG ODNs linked with Amb a 1, a ragweed allergen, *in vitro*. This study found a marked reduction of allergen-induced IL-4 ($P < 0.05$) and IL-5 ($P < 0.05$) production while also showing an increase of IFN-γ ($P < 0.005$). Together, the data indicates a shift away from the Th2 response, since IFN-γ is connected to the Th1 response. Thus, the linkage of Amb a 1 to CpG ODNs appears to alter the immune response through the downregulation of the Th2 cytokine response by promoting the secretion of IFN-γ. Other studies, such as Kitagaki et al. 2002, have analyzed the relationship between interleukins and the Th2/Th1 response.

Since interleukins are the signaling molecules that determine whether a response is Th1 or Th2, they are an important factor in the immune pathway. Kitagaki et al. (2002) found CpG ODN-induced suppression of established Th2 responses in mice through the inhibition of antigen-induced IL-5 and the production of IL-12. When CpG ODNs were administered with immunotherapy, the production of IL-5 was significantly inhibited ($p = 0.01$). In other words, the therapy stimulated the production of interleukins associated with the Th1 response resulting in Th2 response being inhibited.

Overall, the results from multiple studies provide promising results for the use of CpG ODN sequences in immunotherapy to prevent allergies. The use of CpG ODNs has been shown to decrease the Th2 allergic response with ragweed (Lehto et al., 2018) and peanut allergens (Srivastava et al., 2015), but has never been tested with dog allergens. Therefore, this study will be the first of its kind to focus on how CpG ODNs are able to affect the immune pathway of cell cultures from patients sensitized to dog allergens. The goal of this study is to inhibit allergic response by inducing a Th1 cell response.

1. Obtain lymphocytes and culture the cells with commercially bought dog allergens
2. Break the cells cultures into four treatment groups, one being the experimental, which is IL-2 with an active form of CpG ODNs
3. Analyze cell proliferation of Th2 and Th1 cells, using in the different cell cultures by flow cytometry and FlowJo software

H_0: Lymphocytes that receive the CpG oligodeoxynucleotide adjuvant immunotherapy will not experience a decrease in the Th2 immune reaction.

H_1: CpG oligodeoxynucleotide adjuvant immunotherapy will significantly decrease the Th2 immune reaction within the dog sensitized lymphocyte cultures.

METHODS

I. Samples

Twelve human blood samples were obtained as cryopreserved buffy coat with peripheral blood mononuclear cells (PBMC's) from the biobank under IRB approval, six were sensitized to dogs and six were non-allergic. The blood samples were separated and organized in 96 well plates. For each sample there two negative controls, a positive control, and an experimental group. One negative control contained

the sample and dog allergen only. The other negative control contained the cells, Can f 1-7, IL-2, and inactive CpG ODNs. The experimental group contained the sample with the IL-2, active CpG ODN, and allergen. Finally, the positive control group contained the sample and IL-2 only.

II. CpG Oligodeoxynucleotide

The CpG ODN were purchased from InvivoGen. The purchased adjuvant was ODN-2395 - TLR ligand; this was a class C synthetic CpG ODN.

III. PBMC Separation

Diluted blood with PBS in equal amounts and layered on top of Ficoll-Paque PLUS density gradient media (#GE17-1440-03, Millipore Sigma) and centrifuge. Obtained the buffy coat layer by using a transfer pipet, mixed with PBS to rinse, centrifuge. Freezing media containing 10% DMSO with 90% fetal bovine serum (FBS) and frozen to -80°C.

IV. Preparation of Cell Cultures

Rinse frozen buffy coats PBS and resuspended in X-vivo 20 (Lonza) + 20% FBS at 1x10^6 per ml. Spin 10' at 400g full brake. Aspirate off liquid and resuspend pellet in 10 ml PBS, take out 10ul for counting. Calculate cells/well for 96 well plate 3x10^5 cells per well needed. Separate out depending on conditions. Pellet cells and add to 200ul of X-vivo with a final 4ul of Acetone Precipitate Dog Extract (Stallergenes Greer) with CpG ODN 2395 or CpG ODN 2395 Control (InvivoGen) as indicated and incubated at 37°C for 1h. Add 0.3ul IL-2 (100U/ml) to ensure T cell surivial. For 96 well plate there will be ~300ul of media/cells. CpG at 0.6ul (2uM final concentration InvivoGen ODN 2395). Re-suspend cells to 1x10^6/ml in X-vivo 20+20% FBS media and plate at 5x10^5 per well and add PBS in empty wells around cells and leave for 5-7 days. During incubation media was not replenished and viability was assessed during flow cytometry using LIVE/DEAD fixable aqua.

V. Intracellular Staining of Cells

BD biosciences Fix and Perm Fixation and Permeabilization kit GAS003. LIVE/DEAD Fixable Dead Fixable Stain Dye BD biosciences L34957. Take ~1x10^6 cells out per FACs tube and spin down 12000 RPM for 5min. Add 1ml PBS(or in dye free (D10 or R10) media). Decant off and keep at room temp 20 min. Spin 12000 rpm for 5 min, leave the residual ~300ul. Add antibody as recommended by manufacturer in dark hood and mix. Incubate Room temp 20' in dark. Wash with 1ml PBS and spin 12000 rpm for 5 min. Add 210 ul of Cytofix/cytoperm for 20 min. Wash x2 1ml each with Perm buffer and spin 12000 rpm for 5min, leave residual fluid. Add intracellular antibody as recommended and incubate 30min at room temp in dark. Spin and resuspend in 1%PFA. Prepare Comp tubes.

VI. Flow Cytometry

For evaluation of surface receptors, LIVE/DEAD Fixable Aqua stain (#L34957, ThermoFisher) was used to determine living cells followed by antibodies directed against surface markers CD4 (#317417), CD8 (#344741), CD19 (#302205), and CD21 (#354911, BioLegend). Cells were incubated with antibodies at room temperature for 20 min and then washed with PBS. For intracellular staining cells were permeabilized with True-Nuclear™ Transcription Factor Buffer Set (BioLegend) per protocol followed by incubation with antibodies against T-bet and Fox-P3 for 1h at 37°C and fixed. Figure 1 shows a detailed list of all antibodies. Samples were acquired using a Fortessa flow cytometer (BD Bioscience) and analyzed using FlowJo software.

Laser	EM Filter	Marker	Color / Format	Host / Target	Isotype	Catalog *	Amout used per 1E6 cells (ul)	Voltages	
FSS								400	A,H,W
SSC								250	A,H,W
637	670/30	GATA-3	APC	Mouse anti-Human	IgG2b κ	653805	5	610	
637	780/60	CD4	APC-Cy7	Mouse anti-Human	IgG2b κ	317417	5	450	
488	530/30	CD19	FITC	Mouse anti-Human	IgG1 κ	302205	5	450	
405	525/50	Live/ Dead Aqua	BV510	All Species		#L34957	1	500	
405	610/20	CD8	BV 605	Mouse anti-Human	IgG1 κ	344741	5	400	
561	586/15	FoxP3	PE	Mouse anti-Human	IgG1 κ	320007	5	550	
561	610/20	T-bet	PE-Tx Red (PE-Dazzle 594)	Mouse anti-Human	IgG1 κ	644827	5	485	
561	670/30	CD25	PE-Cy5	Mouse anti-Human	IgG1 κ	302607	5	560	
561	780/60	CD21	PE-Cy7	Mouse anti-Human	IgG1 κ	354911	5	600	

Figure 1. ***Antibody list for flow cytometry.*** These are all the antibodies used for flow cytometry including the EM filter, marker, color, heat/target, isotope, catalog, amount used, and voltage.

VII. Statistics

Data analysis was performed in Excel (Microsoft) to define the mean and standard deviation. A t-test was used to find significance between groups. Alpha was set at 0.05.

RESULTS

I. Cell Cultures

Before evaluating the types of cells that proliferated in each sample, pictures of each cell culture were taken (Figure 2). The pictures depicted high cell proliferation, however, the pictures did not reveal what type of T cells were present.

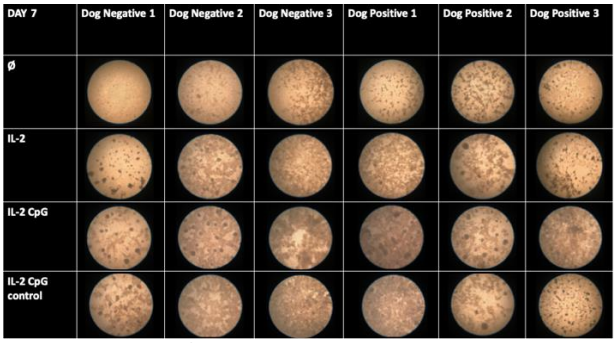

Figure 2. ***Pictures of cell cultures.*** This picture shows the cell cultures of half the dog allergic and non-allergic samples. These pictures were taken seven days after incubation. The dark spots indicate cell proliferation.

II. T Helper Cell Characterization

T helper cells were stained and run through flow cytometry in order to identify the type of T cells that proliferated. The family of T helper cells was derived based upon the transcription factors present; cells that are GATA-3+ are Th2 cells, and cells that are T-Bet + are Th1 cells. The percent of Th1 and Th2 cells were compared between the dog allergic treatments and non-allergic treatments. Using flow cytometry percent of cells that were GATA-3+ or T-Bet + were determined (Figure 3).

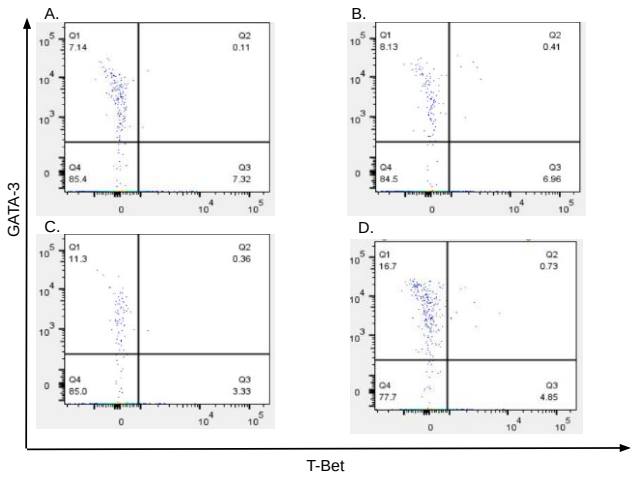

Figure 3. ***Flow cytometry results of an dog allergic sample.*** A. The graph shows the T-cells for the control group. B. The T-cell results for the group that only received IL-2 and the allergen. C. The T-cells that resulted from IL-2 CpG treatment. D. The T-cells that resulted from the CpG control group. Note that this data represents only one sample and demonstrates where the following data originates from.

III. Observing the T Helper Cell Responses to Major Dog Allergens Can f 1-7

After flow cytometry, the percentage of cells that were GATA-3+ (indicating the presence of Th2 cells) was averaged. Standard deviation was then calculated for each average. Results showed a decrease in Th2 cells with the allergic group that received the IL-2 CpG treatment compared to the non-allergic IL-2 CpG group at a significant value ($p = 0.04954$). In the dog negative control group, 26.99% of the cells were GATA-3+, in the IL-2 CpG dog negative group 29.72% of the cells were GATA-3+, compared to 25.91% of dog positive control cells. These values were compared to the dog positive IL-2 CpG group that had 5.90% of their cells GATA-3 positive (Figure 4).

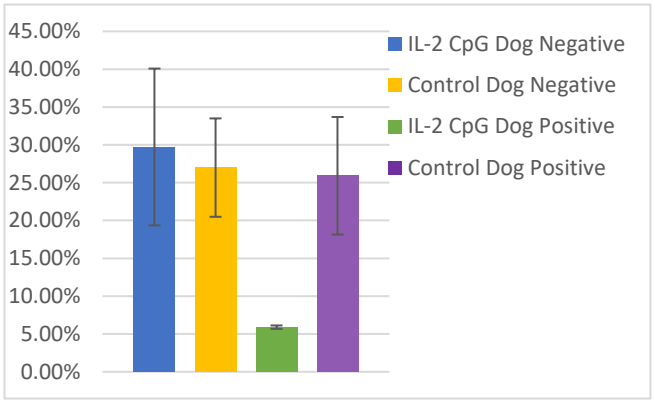

Figure 4. ***The percent of cells that are GATA-3+ and T-Bet-.*** This graph shows the averages of GATA-3+ or Th2 cells for each treatment group and also includes standard deviation error bars. The averages were taken from the six samples in each treatment group.

The percent of T helper cells that are T-Bet+ represent the Th1 cell population. Averages were taken from each allergic and non-allergic treatment group. Standard deviation was derived from these averages. The overall level of T-Bet + cells were higher in the allergic and non-allergic control, but there was not a significant increase ($p = 0.211$, $p = 0.391$) among any of these values (Figure 5).

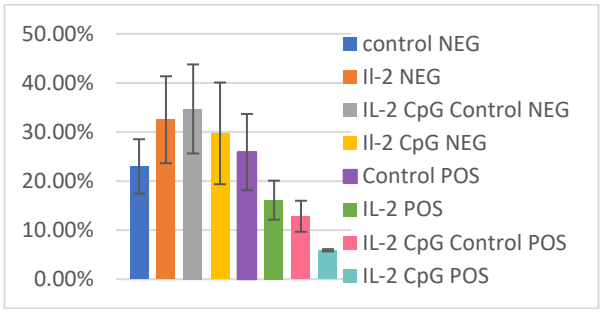

Figure 5. ***The Percent of Cells that are GATA-3 - and T-Bet+.*** These cells were identified by flow cytometry based on if the T-Bet transcription factor was present. Each sample average and standard deviation are depicted in the graph above. Significance was calculated, however, no significance was observed.

DISCUSSION

This study aimed to determine whether or not adding CpG oligodeoxynucleotides to AIT would alter the T helper cell response from a Th2 response to a Th1 response of a dog allergic individual. Results revealed there was a decrease of Th2 cells produced by the group that was dog allergic and received the active CpG treatment and this finding was statistically significant ($p = 0.04954$). This is important since Th2 cells are associated with an allergic response. However, to fully evaluate the effectiveness of the use of CpG ODNs,

the Th1 cell population was also observed. Using transcription factor T-Bet and flow cytometry, the percent of proliferated cells that were Th1 type was found. The important group to observe was the dog allergic experimental treatment group, which was compared against all the control groups. Ultimately, we did not detect a noticeable increase in Th1 cells. This is an interesting finding. Due to the properties of the CpG ODN, as the population of Th2 cells decreased, the Th1 cell population should have increased. This was not the case. Future research should repeat this study with the goal of advancing the field of immunology.

This study has the following limitations. Ideally, our goal was to have fresh lymphocytes, however, the cells were obtained from the biobank were frozen. Once samples were thawed and cells were counted, there was a limited number of living cells due to poor cryopreservation. This low cell count potentially affected the results. Additionally, this study used a commercial mixture of dog allergenic proteins, which included Can f 1-7. Ideally, future studies should look to make their own protein so that they are using one specific allergen and the exact amount of the allergen they are using is known. Going forward, this study should be replicated with samples that have higher cell counts by using a fresh sample compared to a frozen one and using fresh lab made recombinant allergens.

In the future, immunotherapy research must continue. If our method can be used successfully with dog allergens, it is likely that it can become more widespread and be utilized with even more allergies or other diseases, such as cancer (Hanagata, 2017), and even in making normal childhood vaccinations more effective with the addition of CpG adjuvants. Therefore, it is important to repeat this study for more reliable results and to use this study's methods as a model for future studies that work at targeting the body's T helper cells.

Much research has shown the benefits of living and working with dogs, from the joys of companionship to the life-saving attributes of service dogs. However, millions of Americans are faced with the obstacle of debilitating dog allergies, potentially preventing them from interacting with various breeds of dogs. Currently, allergy treatments are limited, and allergen immunotherapy has yet to show clear effectiveness and efficacy against dog allergens. The use of a bacteria adjuvant with immunotherapy has shown promising results in altering the immune response against other allergens. Therefore, this study was the first of its kind to evaluate the use of CpG ODNs against dog allergies. Results of this study, although not statistically significant, showed a decrease in Th2 cells, with no increase in Th1 cells. Be that as it may, this study suggests a trend that promotes the use of CpG ODNs as a method to alter the immune response to dog allergens. In the future, there are several factors that should be addressed. Research should aim to use a single recombinant allergen and further investigate dosing of CpG. In addition, the amount of allergen used in each treatment should also be evaluated, with the ultimate overall goal to complete the study *in vivo*. Finally, researchers should turn to other emerging methods such as the use of nano-allergens. Nano-allergens are modified nanoparticles that are able to detect the number of epitopes on an allergen to recognize the severity of that allergen. This could be an important method to explore, as it has the potential to bring the field of treating allergies to a new level of personalized medicine.

In the end, results from this paper, along with past findings concerning the use of CpG ODNs as an adjuvant for immunotherapy (Lehto et al., 2018; Srivastava et al., 2015; Marshall et al., 2001) lay the groundwork for future research to uncover a more efficient method for treating allergies. As allergies are on the rise, it is important that a reliable treatment is developed, as the threat of dog allergies is ever present. Even when a dog leaves a public area, evidence of their allergens remain for several hours and even days. In addition, AIT can cost over a thousand dollars a year. Our study suggests further exploration into effective adjuvant therapy, with the hope of developing methods that could spread across more than one allergy. Ultimately, the goal of all allergy researchers is to provide a successful treatment for the millions of people who are limited in the interactions they can have with man's best friend.

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