Temporal Dynamic Changes of Phenotypic Expression of Peripheral CD4 Cells during Environmental Allergen Challenge in an Experimental Model of Canine Atopic Dermatitis: A Pilot Study

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ABSTRACT. The immunopathology behind atopic dermatitis (AD) involves a myriad of inflammatory cells and their mediators. Thymus and activation-regulated chemokine (TARC) plays a significant role in the inflammatory phase by recruiting CCR4+ TH2 cells. In addition, CD25+ activated T cells further propagate the allergic response after sensitization by producing cytokines. The purpose of this pilot study was to evaluate how exposure to a common allergen (house dust mite, HDM) would affect the proportions of circulating CD4+CCR4+ TH2 cells and CD4+CD25+activated T cells in an experimental model of AD using high-IgE Beagles. In this experimental model, previously sensitized Beagles develop lesions and pruritus upon allergen challenge consisting of 3-day environmental exposure, 3 hours/day. Blood samples were obtained before, during, and after the end of challenge (days 0, 2, 4, and 17). Clinical signs were evaluated and scored at the same time points. Peripheral blood mononuclear cells (PBMCs) were isolated and used for flow cytometry to identify proportions of CD4+cells positive for either CCR4 or CD25 receptors. Both CD4+cell types (CD25+ and CCR4+) peaked at day 17, when clinical signs had resolved. It is proposed that the increase of circulating CD4+CCR4+ and CD4+CD25+cells most likely demonstrates the sensitization status of environmental allergen challenge. Understanding of these two cell types could prompt additional research concerning therapeutic drugs for AD.

KEY WORDS: activated T cells, atopic dermatitis, canine, CD4, CD25.

Atopic Dermatitis (AD) is a chronic disease affecting 10% of the canine population, sharing many similarities between humans and dogs in both clinical signs and pathogenesis [8,16]. Extensive research has been done in efforts to explain the pathogenesis of AD, including the role of TH2 cells, activated and regulatory T cells, and their cytokines. Despite these attempted measures, the exact mechanisms behind AD are still incompletely understood. Therefore, it is essential to continue identifying the key players involved in the production of AD lesions, including activated T cells, and ultimately the resolution of clinical signs. In order to design more effective methods of treatment, specifically immunomodulation, emphasis on a stronger understanding of cellular kinetics in atopic individuals is crucial.

The current understanding of AD involves the percutaneous absorption of the allergen and an encounter with the allergen-specific IgE on Langerhans’ cells [22]. The antigen is processed and presented to allergen-specific T-lymphocytes, leading to an expansion of TH2 lymphocytes. According to previous studies, chemokine receptor 4 (CCR4) in AD dogs plays a significant role in mobilizing T cells to the lesion after induction by Thymus and Activation-Regulated Chemokine (TARC/CCL17) ligand [14]. Infiltrating T cells then release cytokines responsible for allergen-specific IgE production and eosinophilic inflammation [29]. After additional allergen exposure, the antigen binds to IgE attached to mast cells, causes cross-linking of IgE, and perpetuates a release of various inflammatory mediators such as histamine, prostaglandins, and leukotrienes [23]. It has also been found that mRNA of CCL17 and its receptor CCR4 are primarily expressed in lesional skin of atopic dogs and are not detected in non-lesional canine skin [15]. Although the CCR4 receptor is preferentially expressed on TH2 cells, other subsets including activated TH1 cells have displayed expression of the same receptor [6].

Another major component of the atopic allergic response involves activated CD4+ T lymphocytes, which perpetuate inflammation by producing cytokines such as IL-4, IL-5, or IL-13 [1]. Circulating memory T cells in AD individuals have been shown to express increased markers of activation including CD40L, class II histocompatibility antigen (HLA-DR), and CD25+ (IL-2R) [2]. In addition to measuring cell surface markers, the products of these activated T cells can also be assayed including interferon-γ and soluble interleukin-2 receptor (sIL-2R) [4]. Increased proportions of soluble IL-2 receptor have been reported in human patients with AD correlating with disease severity [7]. CD4+ T cells expressing CD25 and HLA-DR were also found to be associated with severity of disease in a study involving human AD patients [28].

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Another study evaluated CCR4 expression in both dogs with naturally occurring disease and in dogs experimentally sensitized to Japanese cedar pollen [14]. The experimental sensitization was carried out subcutaneously thus not mimicking real life sensitization. These dogs did not develop clinical lesions despite the development of allergen specific IgE. Thus, it is unknown whether those results could apply to an experimental model in which clinical lesions develop after epicutaneous allergen challenge. An additional study by Maeda et al. [15] assessed lesional expression of CCR4, using dogs with naturally occurring AD and no challenge with allergens was carried out. Neither of these studies evaluated changes over time and how these cells may correlate with the development and/or severity of clinical lesions. In order to answer these questions an experimental model in which clinical lesions can be induced and followed over time would be necessary.

In the past, researchers interested in studying the pathology of AD have used murine models and have even selected colonies of dogs with some of the immunological features of AD such as high IgE. However, after antigenic challenge with house dust mites (HDM), previous canine models were not successful in eliciting an accurate representation of clinical signs [5, 26]. The canine model used in this study develops a dermatitis that is consistent immunologically, histologically, and clinically with canine AD after allergen exposure [17]. The dogs in this colony had been previously sensitized by repetitive epicutaneous exposure to HDM (Dermatophagoides farinae). The conditions in which they are housed is controlled, with indoor concrete runs, and no access to HDM-harvesting objects such as stuffed toys, soft bedding, and carpets. The benefits of a controlled canine model for AD allow proper timing of antigen provocation and lesion development, in addition to consistently reproducing clinical signs after epicutaneous challenge with HDM antigen.

Therefore, the aim of this pilot study was to investigate the kinetics of peripheral blood mononuclear cells (PBMCs), specifically CD4+CCR4+ and CD4+CD25+ T cells, after HDM challenge using a canine model for AD. It was hypothesized that the proportion of peripheral CD4+CD25+ and CD4+CCR4+ T cells should increase after HDM provocation. In addition, it was hypothesized that, since CCR4+ cells are recruited in the skin at the time of development of lesions, a decrease in peripheral CCR4+ would be noticed at the peak of clinical signs, and that an increase would be detected at the time of resolution of dermatitis.

MATERIALS AND METHODS

Animals and evaluation of clinical signs: All procedures were approved by the Institutional Animal Care and Use Committee of the University of Florida. A colony of six high-IgE Beagles previously sensitized to HDM epicutaneously was used (five intact females and one intact male). All of the Beagles were 6 years of age and housed in a research facility where exposure to HDM antigen was strictly controlled. All dogs had positive atopy patch test reactions to HDM and positive intradermal skin test reactions in previous studies [20]. In order to recreate the natural exposure to HDM, the antigen (50 mg/dog/day of HDM culture, Greer, Lenoir, NC, U.S.A.) was placed on the floor of a plastic air kennel allowing the dogs to be exposed for 3 hr/day on 3 consecutive days. A previously validated scoring system [Canine Atopic Dermatitis Extent and Severity Index (CADESI) version 3] was used to evaluate clinical signs on days 0, 2, 4, and 17 (before, during, and after conclusion of the challenges) [21]. The purpose of the final blood sampling was to choose a time at which all dogs were most likely to be clear of lesions, considering variation between individuals. Based on previous studies (data not shown) it was known that all of the dogs in this particular colony had shown resolution of clinical signs up to 14 days post-antigenic stimulation, therefore day 17 was selected as a common time for blood sampling.

Blood sampling: Whole blood samples (5 ml) were obtained on days 0, 2, 4 and 17 after the provocation of HDM antigen. Samples were mixed with heparin and used for PBMC separation.

Separation of PBMCs: Heparinized whole blood samples were diluted with an equal volume of Dulbbecco’s PBS (Sigma) and then layered on Histopaque 1077 (Sigma, St. Louis, MO, U.S.A.). Following centrifugation at 350 × g at room temperature for 45 min, a layer of PBMCs was collected and washed twice with washing buffer.

Flowcytometric analysis: Freshly isolated PBMCs were incubated with anti-canine CD4 (Serotec, Kidlington, Oxford, UK), anti-human CCR4 (BD PharMingen, San Jose, CA, U.S.A.), and anti-human CD25 (DakoCytomation, Carpinteria, CA, U.S.A.) monoclonal antibodies in washing buffer at 4°C for 30 min. Antibodies with the appropriate isotypes (purified rat IgG2a and mouse IgG1, BD PharMingen) were used as negative controls. The PBMCs were then stained with FITC-conjugated anti-rat IgG2a and PE-conjugated anti-mouse IgG1 monoclonal antibodies (BD PharMingen) in washing buffer at 4°C for 30 min. After gating the fraction of lymphocytes, proportions of CCR4/CD4 and CD25/CD4 were determined by the flow cytometer (FACS calibur, BD PharMingen).

Statistics: Clinical scores along with proportions of CD4+CCR4+ and CD4+CD25+ PBMCs were statistically analyzed among the days using Dunnett. All analyses were performed using a statistical software package (JMP version 5.1.2, SAS Institute, Cary, NC, U.S.A.). A P<0.05 was considered significant.

RESULTS

Clinical Scores (see Fig. 1): After the environmental allergen challenge, all dogs developed clinical signs consistent with AD. Lesions included erythema and a macular/papular eruption on the lateral abdomen, ventral abdomen, and axillae. The peak of clinical scores using the CADESI
Locating scoring system was on day 4 and was significantly different from day 0 ($P<.0001$). No dermatological signs were observed on days 0 or 17.

**Flow cytometric analysis**

$CD4^{+}CCR4^{+}$ PBMCs (see Fig. 2): No statistically significant change in $CD4^{+}CCR4^{+}$ cells was seen over time or on day 17 compared to baseline. The overall proportion of $CCR4^{+}$ cells throughout the challenge was greater than $CD25^{+}$ cells. Levels of $CD4^{+}CCR4^{+}$ cells in three out of the 6 dogs followed a similar pattern, reaching a nadir soon after the peak of clinical signs. There was variation in $CD4^{+}CCR4^{+}$ cell levels among the other three dogs over time, one gradually declining, one gradually increasing, and the other declining after a steep slope of increase. Two of the dogs that showed a $CD4^{+}CCR4^{+}$ nadir at day 2 (rather than day 4) also had an earlier peak of clinical signs on the same day.

$CD4^{+}CD25^{+}$ PBMCs (see Fig. 3): Overall there was marked individual variation among the dogs; however, day 17 was statistically different from day 0 ($P=0.008$). All but one dog had $CD4^{+}CD25^{+}$ levels highest at day 17. This particular outlier also had the highest percentage of regulatory cells ($CD25^{+}$) cells at day 4 and subsequently had the lowest CADESI score that same day. The clinical signs (CADESI) followed an inverse pattern compared to the percentage of $CD4^{+}CD25^{+}$ cells. At the peak of clinical signs, the dog with the highest CADESI score also had one of the lowest percentages among the other dogs of $CD25^{+}$ cells T-regulatory cells measured in peripheral blood.

**DISCUSSION**

In this pilot study, clinical signs representative of canine AD were noted after 3 days of epicutaneous provocation with HDM. Resolution of clinical signs was evident in all dogs by day 17. The proportion of $CD4^{+}CD25^{+}$ cells on day 17 was significantly different than day 0. There was more variation among subjects for this particular cell type although the individual percentages of $CD4^{+}CD25^{+}$ cells were inversely proportional to the degree of clinical signs. Due to the large individual variation, no significant changes were found for $CD4^{+}CCR4^{+}$ cells over time in this pilot study.

Chemokine receptor 4 is primarily expressed on $T_{h}2$ cells of AD dogs and plays a significant role in mobilizing T cells to the lesion [12]. Previous studies in humans have suggested that peripheral blood levels of CCR4 ligand (TARC) as well as CCR4 chemoattractant macrophage-derived chemokine (MDC) have significantly correlated with AD clinical scoring indices [10, 13, 30, 27]. To the best of the authors’ knowledge, there have been no such studies evaluating similar trends of $CCR4^{+}$ cells themselves as they pertain to clinical severity in AD dogs. Although no significant changes in $CCR4^{+}$ cells over time were noted, there was value in investigating this particular association. Future studies using a larger population of atopic dogs could poten-
tially produce a more significant correlation of CCR4+ cells with the progression of CADES1 scores and subsequent resolution of clinical signs.

A recent study demonstrated that the same anti-human CD25 antibody used in this study recognizes activated T-lymphocytes, for particular use in allergic diseases [18]. The pattern seen in this study with respect to CD4+CD25+ cells was overall variable even though there was a significant difference in the number of these cells measured before HDM provocation and at resolution of clinical signs. The elevation in peripheral levels of CD25+ lymphocytes is most likely a result of environmental sensitization. This correlates with previous reports in human patients with AD in which higher levels of CD25+ cells were seen compared to healthy controls [19]. Two separate studies evaluating the therapeutic affects of IFN-γ in human AD patients have shown a decrease in the number of peripheral or dermal infiltrates of CD25 expression, in parallel with clinical improvement [11, 19]. As a means of indirectly assessing activated T cell dynamics, levels of soluble IL-2R, a protein released by activated T cells, have also been found to become significantly higher in AD than in normal controls in addition to being significantly correlated with clinical severity [7, 28]. The subsequent increase in peripheral CD4+CD25+ T cells after HDM provocation specifically seen in this study has also been seen in an animal model of allergic asthma involving Rhesus monkeys experimentally sensitized to D. farinae [25]. Although both studies share similar outcomes at the end of sensitization, it is difficult to make a direct comparison as continuous levels of peripheral CD4+CD25+ cells were not assayed throughout the trial detailed in Schegle et al as seen in this study involving the Beagle model for AD.

The pattern of CD4+CD25+ kinetics was similar to that of related human studies, reflecting the effects of allergen sensitization. Several different subpopulations of CD4+CD25+ T cells including CD4+CD25high have been evaluated in the past [12, 24]. A positive relationship between this cell type and allergen-specific IgE has been seen, as well as suggestion of Th2-promoting effects [12, 24]. In the study involving CD4+CD25high cells there was evidence of a spring seasonal peak of these cells that correlated positively with peaks in local pollen seasonality [12]. Similarly, after exposure of epicutaneous antigen, the Beagles in this study also showed a significant increase in peripheral CD4+CD25+ cells supporting the idea that allergen sensitization results in an increase in peripheral activated T cells.

The major strength in this study is the fact that a canine model for AD was used, showing clinical signs that are identical to those of naturally occurring atopic dogs in a controlled environment. The use of the canine model allowed for better control of HDM provocation, initiation of clinical signs, and monitoring of its development with limited variables. Unfortunately, due to the small sample size (n=6), there was a higher degree of variation with a limited number of discrete trends. A larger sample size for this particular canine model would be ideal for use in future studies to generate a greater power of data and to show more statistically significant trends of T cell proportions. The availability of a negative control would have made the experimental design of this study stronger. In contrast, the objective of this study was not to compare this particular atopic colony with normal dogs but to evaluate kinetic changes of CD4+ cellular phenotypes in high-IgE beagles during allergen challenge over time and in relationship to either the presence or absence of cutaneous lesions. For the purposes of this pilot study, kinetic changes in PBMCs over time within the same individual were evaluated by using peripheral blood levels of CD4+CCR4+ and CD4+CD25+ cells at day 0 as a control. Future studies involving a larger population comparing the atopic Beagles to normal controls will be necessary to further investigate this topic.

A deeper immunopathologic understanding of AD could be explored through flow cytometric analysis of these same T cell populations from lesional biopsies. In humans it has been shown that there is a large pool of memory T cells in normal skin that can initiate and perpetuate immune reactions in the absence of T cell recruitment from the blood [3]. It would be interesting to evaluate whether this may be an important pathway for the development of cutaneous lesions in canine AD. Activated T cells may be considered as possible future targets for treatment as they have been found to decrease with successful therapy. A study in human medicine showed a decrease in levels of CD25+ cells after immunomodulating therapy using IFN-γ [11]. Additionally, one clinical study involving recombinant IFN-γ in client-owned dogs with AD reported significant improvement in clinical signs after two months [9]. This form of therapy could be an area of interest for future studies involving this particular canine model of AD.

In summary, this pilot study suggests that, in a canine model for AD, provocation with HDM eventually results in a significant increase of circulating CD4+CD25+ cells most likely demonstrating the sensitization status of atopic individuals after environmental allergen challenge. In this pilot study, changes in CD4+CCR4+ cells were not statistically significant due to the large individual variation. Individual dogs showed a decrease at the peak of the lesions and a subsequent increase at the time of resolution of lesions. Larger studies with more dogs, both allergic and normal controls, are necessary before drawing conclusions on the changes of this population of T cells in this experimental model of canine AD.

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