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細胞間素活化轉錄因子 NF-KB 引起眼球內炎性反應的研究  
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## **Expression of Fractalkine and CX3CR1 in Experimental Autoimmune Anterior Uveitis**

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**Abstract.**

**Purpose.** To demonstrate the expression and location of fractalkine and receptor, CX3CR1, in iris/ciliary body and thus establish their roles in experimental autoimmune anterior uveitis, an animal model of human acute anterior uveitis.

**Methods.** Uveitis was induced with the injection of melanin-associated antigen intraperitoneally and into the left footpad simultaneously. Some rats were treated with a putative NF- $\kappa$ B inhibitor, pyrrolidine dithiocarbamate (PDTC; 40 mg/kg/day) every day after immunization. At defined time points, fractalkine and CX3CR1 mRNA expression were semiquantified by using RT-PCR. Fractalkine in aqueous humor was determined by ELISA. The cellular sources of fractalkine were determined by immunohistochemical staining.

**Results.** Fractalkine mRNA was found to be upregulated in iris/ciliary body 9 days after immunization, preceding clinical disease onset. CX3CR1 mRNA exhibited peak levels at day 14, coincident with disease onset. Fractalkine in aqueous humor showed a similar expression profile to mRNA expression. PDTC markedly inhibited the expression of fractalkine mRNA in iris/ciliary body and fractalkine protein in aqueous humor. Immunohistochemical staining revealed fractalkine was prominently expressed on vascular endothelial cells.

**Conclusions.** This study was the first to provide evidences of fractalkine –CX3CR1

interactions in the genesis of experimental autoimmune anterior uveitis. The activation of fractalkine gene is, at least in part, via a NF- $\kappa$ B-dependent pathway. Therefore, selective anti-fractalkine or anti- NF- $\kappa$ B therapy may become a therapeutic potential for acute anterior uveitis treatment.

## **Introduction:**

Experimental autoimmune anterior uveitis (EAAU) is a T-cell-mediated autoimmune disease that serves as an animal model for human acute anterior uveitis.<sup>1,2</sup>

Immunization of Lewis rat with bovine melanin-associated antigen (MAA) and appropriate adjuvants resulted in a disease with many of the clinical and pathologic features of the human disease<sup>3</sup>. EAAU differed particularly from other models because the inflammation remained exclusively anterior without retina and choroid involvement<sup>4,5</sup>.

Chemokines are small chemoattractant cytokines that induce leukocyte accumulation at inflammatory sites and modulate inflammatory activities via the recruited cells<sup>6,7</sup>.

Chemokines are most likely implicated in the pathogenesis of autoimmune diseases.

According to NH<sub>2</sub>-terminal cysteine motifs, chemokines can be grouped into four families: C, CC, CXC, and CX<sub>3</sub>C<sup>8</sup>. Fractalkine, a member of CX<sub>3</sub>C chemokine, differs greatly from other chemokines for it can exist either as a soluble form or a membrane-bound form<sup>9</sup>. The soluble form acts as a potent chemoattractant for monocytes/macrophages, T-cells and natural killer cells whereas the membrane-bound form functions as an adhesion molecule<sup>10,11</sup>. Fractalkine exerts its biological activity by binding to a G-protein-coupled receptor known as CX<sub>3</sub>C receptor 1 (CX<sub>3</sub>CR1)<sup>12,13</sup>.

CX3CR1 expressed mainly on specific types of leukocyte, which enable these cells to migrate in response to the concentration gradients of fractalkine<sup>14,15</sup>

Because of its important biological activities, fractalkine has been previously reported to play a role in the pathogenesis of diverse inflammatory diseases<sup>16</sup>. These include cardiac allograft rejection, arthritis, and psoriasis, and toxic neuronal injury<sup>17-20</sup>. In the eye, previous investigators had demonstrated the presence of fractalkine in normal cultured ocular endothelial cells *in vitro*<sup>21</sup>. However, to our knowledge, no investigations to date have been conducted to evaluate fractalkine expression in ocular inflammatory disease.

The nuclear factor kappa B (NF- $\kappa$ B) is a transcriptional factor that can regulate the expression of pro-inflammatory genes, including cytokines, adhesion molecules and chemokines<sup>22,23</sup>. Previous studies had demonstrated the central role of NF- $\kappa$ B activation in the regulation of fractalkine gene induction in aortic smooth muscle cells *in vitro*<sup>24</sup>. However, there were no data available about the role of NF- $\kappa$ B *in vivo* on fractalkine activation.

The purpose of this study was to investigate the time course of the expression of

fractalkine and its receptors, CX3CR1, in the iris/ciliary body in EAAU. In addition, we examined the effect of NF- $\kappa$ B inhibitor, Pyrrolidine Dithiocarbamate (PDTC), on fractalkine gene and protein expression in vivo to determine whether fractalkine activation is NF- $\kappa$ B-dependent.

## **Materials and Methods**

### **Animals**

Lewis rats, 6–8 weeks old and weighing 125-160 g, were used for the experiments.

All animals were treated in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

### **Antigen and induction of EAAU**

MAA was prepared as previously described by Borekhuyse and Kuhlmann with a modification<sup>18</sup>. The iris and ciliary body were carefully obtained from fresh pigmented bovine eyes. The tissue was gently homogenized and filtered through a wire mesh to remove cellular debris and connective tissue. The homogenate was centrifuged at  $1.2 \times 10^5 g$  at 4°C for 15 min and washed once with phosphate-buffered saline (PBS) at pH 7.4. The resulting pellet was resuspended in 2% sodium dodecyl sulfate (SDS) (Bio-Rad, Richmond, CA) and incubated at 70°C for 10 min. After centrifugation, the pellet was washed three times with water. The insoluble antigen was dried and stored at –20°C.

To induce EAAU, Lewis rats received two separated injections on the same time: 1).

MAA was suspended in PBS, emulsified (1:1) in complete Freund's adjuvant (Sigma,



St.Louis, MO) and injected into left hind footpad in a volume of 0.05 ml. 2). MAA was emulsified with 1µg purified Bordetella pertussis toxin (List, Campbell, CA) and injected intraperitoneally in a volume of 0.05 ml.

### **Clinical examination**

The rats were clinically observed on a daily basis with slitlamp biomicroscopy for clinical signs of ocular inflammation. Disease severity was clinically assessed with a scale ranging from 0 to 4: 0 = normal; 1 = slight iris-vessel dilatation and some anterior chamber cells; 2 = iris hyperemia, with some limitation in pupil dilation, anterior chamber cells, and a slight flare; 3 = a miotic, irregular, hyperaemic, and (sometimes) slightly damaged iris, with a considerable flare and cells (especially with accumulation near the iris); and 4 = a seriously damaged and hyperaemic iris, a miotic pupil often filled with protein, and cloudy gel-like aqueous humor (AqH).

### **Tissue preparation**

Rats were sacrificed on days 3 ( $n = 5$ ), 9 ( $n = 5$ ), 11 ( $n = 5$ ), 14 ( $n = 5$ ), 18 ( $n = 5$ ), and 25 ( $n = 5$ ) after immunization. The eyes were harvested. Both eyes of the experimental animals were used, e.g. 10 eyes from 5 rats were examined at each time point. The eyes were quickly dissected, and the iris and ciliary body were isolated from the remaining ocular tissue by using an operating microscope.

### **Preparation of RNA and cDNA**

Total RNA was extracted from the iris/ciliary body with Trizol reagent (Life, Gaithersburg, MD). One microgram of total RNA from each sample was annealed for 5 min at 65°C with 300-ng oligo(dT)(Promega, Madison, WI) and reverse transcribed to cDNA by using 80 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT)(Gibco, Grand Island, NY) per 50 µg reaction for 1 h at 37°C. The reaction was stopped by heating for 5 min at 90°C.

### **Polymerase chain reaction**

The amplification was performed with a thermocycler. (MJ Research, Waltham, MA)

The 50-µl reaction mixture consisted of 5 µl cDNA, 1 µl of sense and antisense primer, 200 µM of each deoxynucleotide, 5 µl 10× Taq polymerase buffer, and 1.25 U Taq polymerase (Promega, Madison, WI). Fractalkine primers were:

5'-GAATTCCTGGCGGGTCAGCACCTCGGCATA-3',

5'-AAGCTTTTACAGGGCAGCGGTCTGGTGGT-3' (DNAFax, Taipei, Taiwan);

CX3CR1 primers were: 5'-AGCTGCTCAGGACCTCACCAT-3',

5'-GTTGTGGAGGCCCTCATGGCTGAT-3' (DNAFax, Taipei, Taiwan); β-actin

primers were: 5'-CTGGAGAAGAGCTATGAGCTG-3',

5'-AATCTCCTTCTGCATCCTGTC-3' (DNAFax, Taipei, Taiwan). Conditions for amplifying fractalkine and CX3CR1 were as follows: denaturation, 1 min at 94°C, and elongation, 3 min at 72°C. For the annealing temperature, 62°C was designed for fractalkine and CX3CR1. At the end of amplification, the reaction mixture was heated for 10 min at 72°C and then cooled to 4°C. A 10- $\mu$ l sample of each polymerase chain reaction (PCR) product was separated by performing gel electrophoresis on 2% agarose containing ethidium bromide (Sigma, St. Louis, MO) and then analyzed under ultraviolet light against the DNA molecular length markers. The intensity of the products was analyzed by using an image analyzer (Digital 1D Science; Eastman Kodak, Rochester, NY), and the amount of PCR-amplifiable material in each reverse-transcribed sample was standardized against the amount of a housekeeping gene rat  $\beta$ -actin.

### **Quantification of leukocytes in AqH**

Aqueous humor (AqH) was collected from the eyes by using a 30-gauge needle immediately after the animal was sacrificed. The AqH was pooled in silicon-treated microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA). A volume of 2  $\mu$ L AqH from one rat was stained with 0.4% trypan-blue solution, and the number of leukocytes was

counted under phase-contrast microscopy.

### **Quantification of fractalkine in AqH**

The levels of fractalkine in the AqH obtained from rats with EAAU were quantitated on days 3 ( $n = 5$ ), 9 ( $n = 5$ ), 11 ( $n = 5$ ), 14 ( $n = 5$ ), 18 ( $n = 5$ ), and 25 ( $n = 5$ ) after immunization by using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The ELISA assay was repeated twice. The sample was diluted up to 50<sup>1</sup> and used for the tests. Optical density was determined at  $A_{450}$  with a microplate reader (Bio-Rad, Richmond, CA). The chemokine concentration was determined from standard curves by using recombinant standards supplied by the manufacturer.

### **Immunohistochemistry**

Formalin-fixed, paraffin-embedded tissue sections were placed on slides,

deparaffinized in xylenes, and rehydrated through graded ethanol into PBS.

Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol, then

the sections were treated with 5% normal rat serum and incubated overnight with

anti-fractalkine immunoglobulin G (IgG) (150 nm/ml)(Santa Cruz Biotechnology,

Santa Cruz, CA) at 4°C. Thereafter, a biotinylated secondary antibody against rat IgG

and an avidin-biotinylated peroxidase complex (Santa Cruz Biotechnology, Santa Cruz, CA) were used with 3-3'diaminobenzidine as a peroxidase substrate. Sections were counterstained with hematoxylin, dehydrated, and mounted.

### **NF- $\kappa$ B inhibitor, PDTC treatment**

To examine whether NF- $\kappa$ B is involved in regulating fractalkine gene, ten rats were randomly selective and received intraperitoneal injection of PDTC (40mg/kg) every day after MAA induction. On 14<sup>th</sup> day of immunization, the day with high levels of fractalkine mRNA and protein expression, the rats were sacrificed. Fractalkine mRNA in iris/ciliary body and fractalkine concentration in aqueous humor after PDTC treatment was determined.

### **Statistical analysis**

Values in the figures and the text are expressed as mean  $\pm$  SEM. Difference among the amounts of the chemokine at the different time points were evaluated by one-way analysis of variance followed by Bonferroni multiple comparison test, as appropriate.

Values of  $p < 0.05$  were considered as significant.

### **Results:**

### **Clinical scores of EAAU**

Clinical signs for EAAU appeared on day 14 post-immunization. The disease reached its peak on day 19, with a clinical score of  $2.60 \pm 0.35$ . Then, recovery started. The disease totally recovered by around day 30.

### **Iris and ciliary-body fractalkine and CX3CR1 mRNA expression**

Fractalkine mRNA was upregulated on day 9 and reached its peak on day 11, preceding onset of clinical disease. Thereafter, fractalkine mRNA declined gradually but stayed at higher than control level during the course of disease. (Fig. 1A)

CX3CR mRNA, the receptor for fractalkine, was upregulated on day 11. The maximal expression of CX3CR1 mRNA was on day 14, concurrent with disease onset, then, returned abruptly to control level on day 18. (Fig. 1B)

### **Fractalkine levels in aqueous humor**

In aqueous humor, fractalkine protein rise significantly on day 9, prior to clinical disease onset. It reached peak on day 14, concurrent with disease onset. Thereafter it declined in parallel with the severity of clinical disease. (Fig. 3)

### **Quantification of leukocytes in Aqueous humor**

Lymphocytes and monocytes were the dominant leukocyte subsets in aqueous humor, in parallel with the upregulation of fractalkine protein. (Fig.2)

### **Effects of NF- $\kappa$ B inhibitor, PDTC, on fractalkine mRNA in iris/ciliary body and fractalkine protein in aqueous humor**

On day 14, PDTC treatment resulted in a reduction in fractalkine mRNA in iris/ciliary (P=0.001). Furthermore, treatment with PDTC significantly reduced the levels of fractalkine protein in aqueous humor ( $720 \pm 110$  pg/ml vs  $280$  pg/ml  $\pm 70$ , P= 0.001).

### **Immunohistochemical staining of fractalkine**

Immunohistochemical analysis on day 14 revealed that fractalkine was prominently expressed on endothelial cells of vessel wall in iris/ciliary body (Fig. 4A). Control sections after omission of primary antibodies showed only background staining (Fig. 4B).

### **Discussion**

Local expression of fractalkine may represent an important level control in directing

the trafficking of monocytes and T cells into sites of inflammation<sup>25</sup>. In this study, we demonstrated a strong expression of fractalkine before and during the evolution of EAAU. Furthermore, upregulation of fractalkine was associated with expression of cognate receptor; CX3CR1, suggesting that fractalkine and CX3CR1 may participate in the pathogenesis of rat model of acute anterior uveitis. To our knowledge, this is the first study to identify the expression of fractalkine in ocular inflammatory disease.

Large leukocytes infiltration, especially monocytes and T lymphocytes, in iris/ciliary body is the pathological hallmarks of EAAU<sup>26</sup>. To recruit certain type of leukocytes to the site of inflammation, multi-step processes that include, establishment of chemattractant gradient, firm attachment of leukocytes to and then migration across the vascular endothelial cells should be properly regulated<sup>27-29</sup>. Previous studies had demonstrated that fractalkine could act as a chemoattractive cytokine to attract monocytes and lymphocytes, and an adhesion molecules to promote transendothelial migration of leukocytes in a variety of inflammatory diseases<sup>30,31</sup>. In this study, we found that fractalkine was upregulated prior to the infiltration of monocytes and lymphocytes, and onset of clinical disease. Given the mechanistic parallels between various inflammations elsewhere in the body, we could reasonably postulate that the early expression of fractalkine may contribute to providing a mechanism for the later



influx of monocytes and T cells.

Our observations that CX3CR1 mRNA was upregulated during the evolution of EAAU provided further evidences of fractalkine –CX3CR1 interactions in selective recruitment of lymphocytes and monocytes into iris/ciliary body. Furthermore, regulation of CX3CR1 expression represented another level of control in the clinical course and severity of diseases. To be an adhesion molecule, fractalkine is capable of binding to leukocytes expressing CX3CR1<sup>32</sup>. CX3CR1 is reported to express on the surface of monocytes, T lymphocytes, and NK cells, the main infiltrating cells in EAAU<sup>33,34</sup>. Therefore, the increases in CXCR3 expression facilitates these leukocytes to be firmly entrapped by fractalkine-secreting cells and then subsequent to promote leukocyte accumulation and inflammatory reactions. At the peak of clinical disease, however, the abrupt decreases in CX3CR1 expression make these CX3CR1-bearing cells unresponsive to fractalkine and then less attract to the inflammatory sites, which marked the initiation of disease resolution.

By immunohistochemistry, we observed that vascular endothelial cells of iris/ ciliary body are the cellular sources of fractalkine in rat model of acute anterior uveitis.

Previous reports of other inflammatory diseases also demonstrated that vascular

endothelial cells are capable of producing fractalkine<sup>35</sup>. We postulate that the localization of fractalkine in vascular endothelium bears strategic meanings in the leukocyte recruitment from circulation into iris/ ciliary body. It is only induced in vascular endothelial cells that fractalkine could accessibly contact with leukocytes in systemic circulation to act as an adhesion molecule in the membrane-bound form, and could build concentration gradient between local tissue and systemic circulation to act as a chemoattractant cytokine in soluble form.

The present study revealed that NF- $\kappa$ B inhibitor effectively suppressed fractalkine gene expression, indicating activation of fractalkine gene is, at least in part, by means of a NF- $\kappa$ B- dependent mechanism in experimental autoimmune anterior uveitis.

Previous in vitro study demonstrated that fractalkine can be induced by proinflammatory cytokines TNF- $\alpha$  and IL-1 in aortic endothelial cells<sup>36</sup>. However, TNF- $\alpha$  and IL-1 themselves are activated through NF- $\kappa$ B. Therefore, in vivo during EAAU, it is possible that NF- $\kappa$ B influences fractalkine expression by direct activation of fractalkine or indirect alterations of other mediators.

In summary, the study was the first to demonstrate expression of fractalkine during ocular inflammatory disease of experimental autoimmune anterior uveitis. This

expression may direct CX3CR1 receptor-expressing mononuclear cell subsets to inflammatory sites. Furthermore, this study has also identified that the activation of fractalkine was by a NF- $\kappa$ B-dependent pathway. These findings may not only provide a new sight to clarify the molcecular mechanisms of acute anterior uveitis, but also indicate that fractalkine or NF- $\kappa$ B might be a drug target for therapeutic applications.

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## **Legends:**

### **Fig. 1**

Expression of fractalkine (A) and CX3CR1(B) mRNA in the iris/ciliary body from Lewis rats at different time points during the course of EAAU. The control represents the normal rats not been immunized. Bar charts represent the relative intensity of mRNA of fractalkine. Line charts represent the clinical scores of the disease. The intensity of mRNA was analyzed using an image analyzer and the relative intensity was determined by comparison of its intensity with that of  $\beta$ -actin. Data are presented as the mean  $\pm$ SEM in five rats. \*  $p < 0.05$ , when compared to normal control.

### **Fig. 2**

The concentration of fractalkine in aqueous humor from Lewis rats at different time points during the course of EAAU were determined by ELISA. The control represents the normal rats not been immunized. Bar charts represent the concentrations of fractalkine in aqueous humor. Line charts represent the clinical scores of the disease. Data are presented as the mean  $\pm$  SEM in five rats. \*  $p < 0.05$ , when compared to normal control.

### **Fig. 3**

Correlations of fractalkine concentration with numbers of different subsets of leukocytes in aqueous humor from Lewis rats at different time point during the course of EAAU. The control represents the normal rats not been immunized. Bar charts

represent number of different types of leukocyte in aqueous humor. Line charts represent the concentration of fractalkine. Data are presented as the mean $\pm$  SEM in five rats. \*  $p < 0.05$ , when compared to normal control.

#### **Fig. 4**

Effect of NF- $\kappa$ B inhibitor, Pyrrolidine Dithiocarbamate (PDTC), on fractalkine mRNA expression in iris/ciliary body (A) and fractalkine protein concentration in aqueous humor (B) of Lewis rats immunized with melanin-associated antigen. The rats were treated with PDTC after immunization. They were sacrificed on day 14.

Total RNA and aqueous humor was extracted. Columns marked as “Normal” represent rats without immunization with MAA, while “MAA” represent rats immunization with MAA. “PDTC-treated” represent rats immunization with MAA and treated with PDTC (40mg/kg) everyday. The intensity of mRNA was analyzed using an image analyzer and the relative intensity was determined by comparison of its intensity with that of  $\beta$ -actin. Data are presented as the mean  $\pm$  SD in five rats.

Significant differences: \* $P < 0.05$

#### **Fig. 5**

Immunohistochemical staining for fractalkine in iris/ ciliary body from Lewis rats

with experimental autoimmune anterior uveitis on days 14 post-immunization.

Positive staining was found in vascular endothelial cells (arrow) in ciliary body

(X1000)

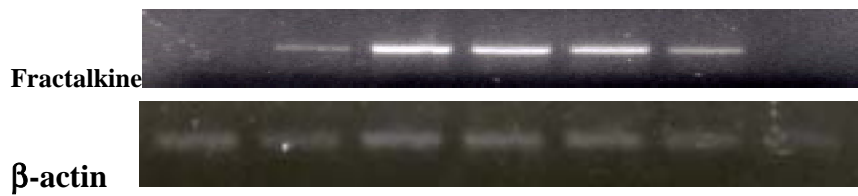
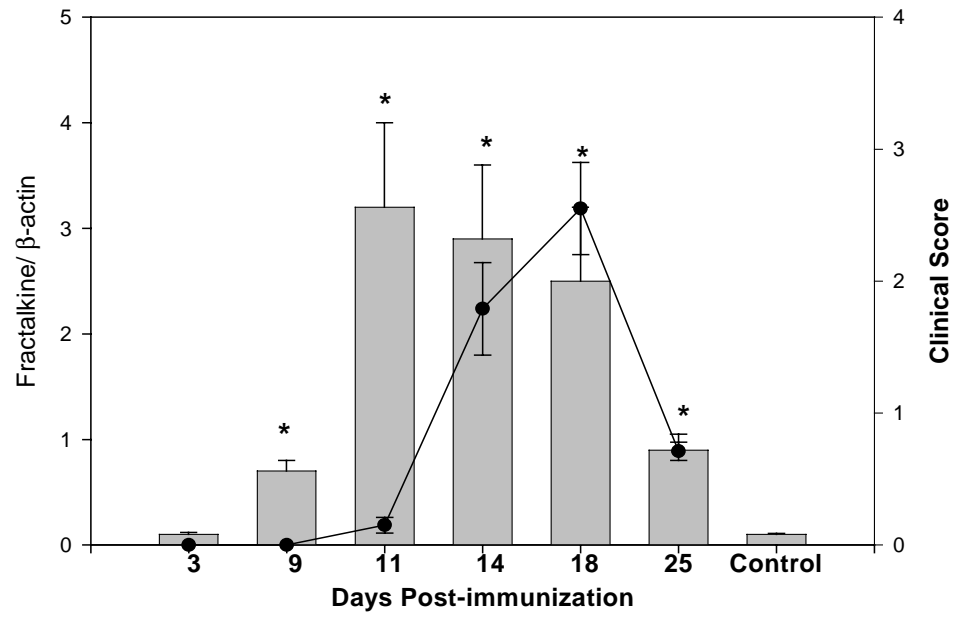


Fig. 1A

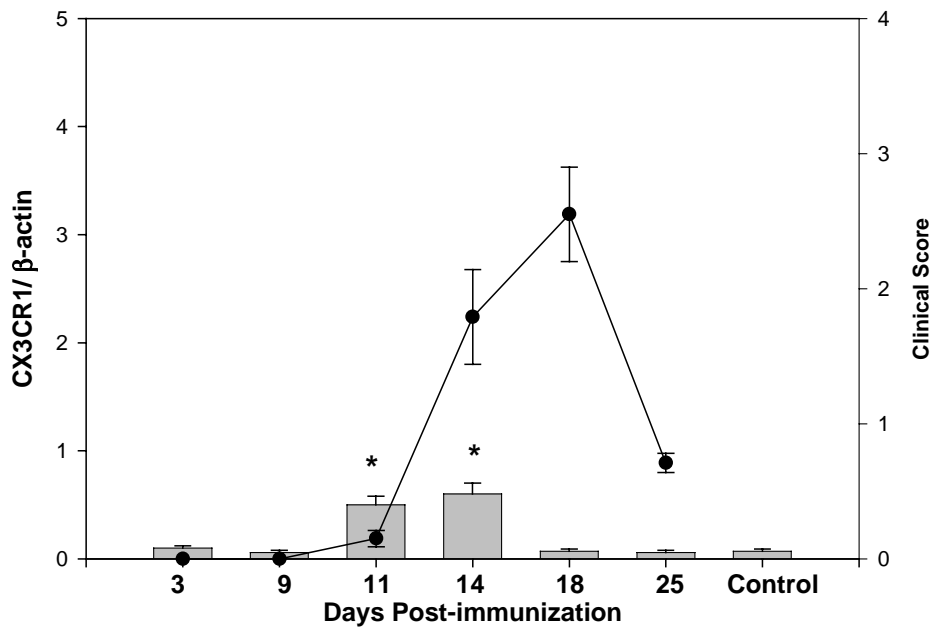


Fig. 1B

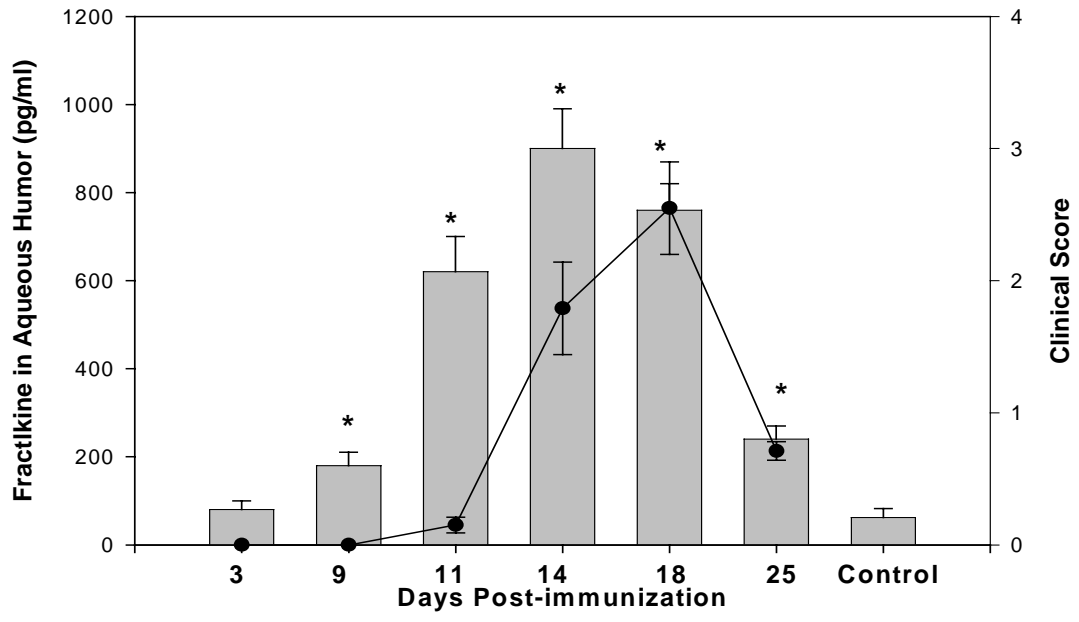
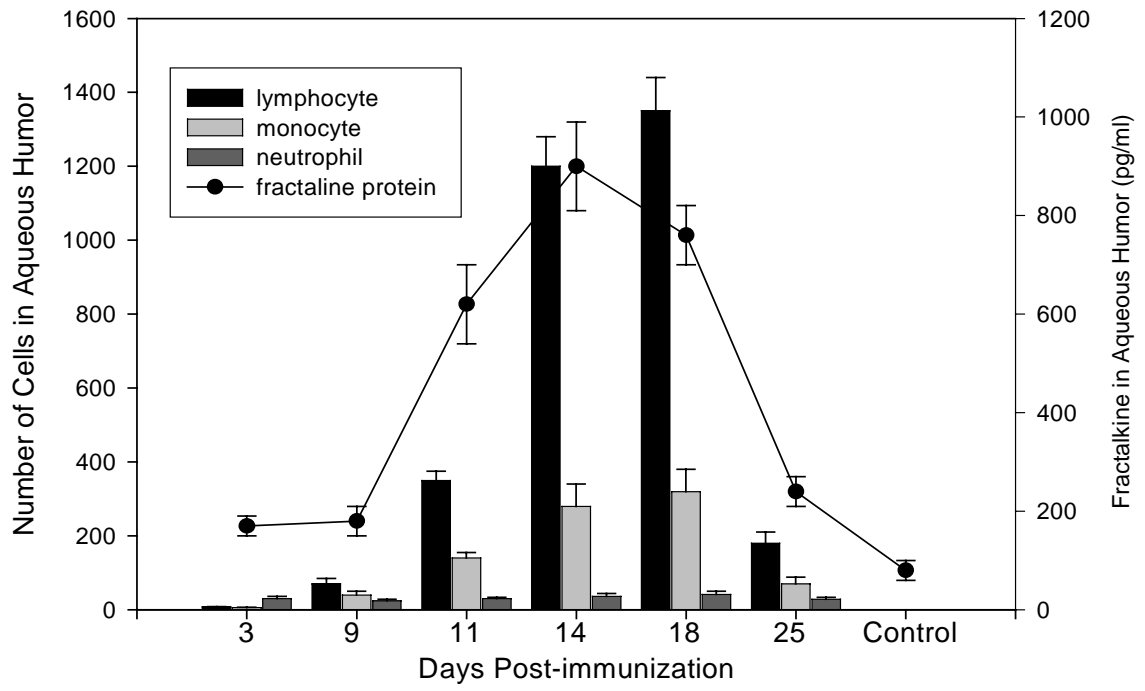
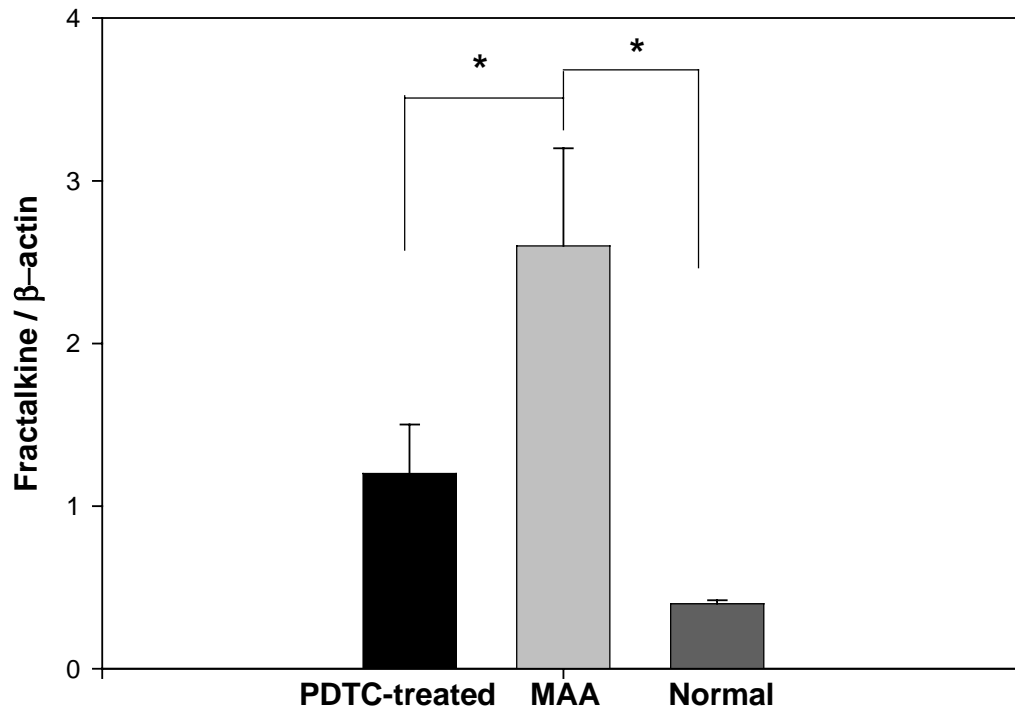


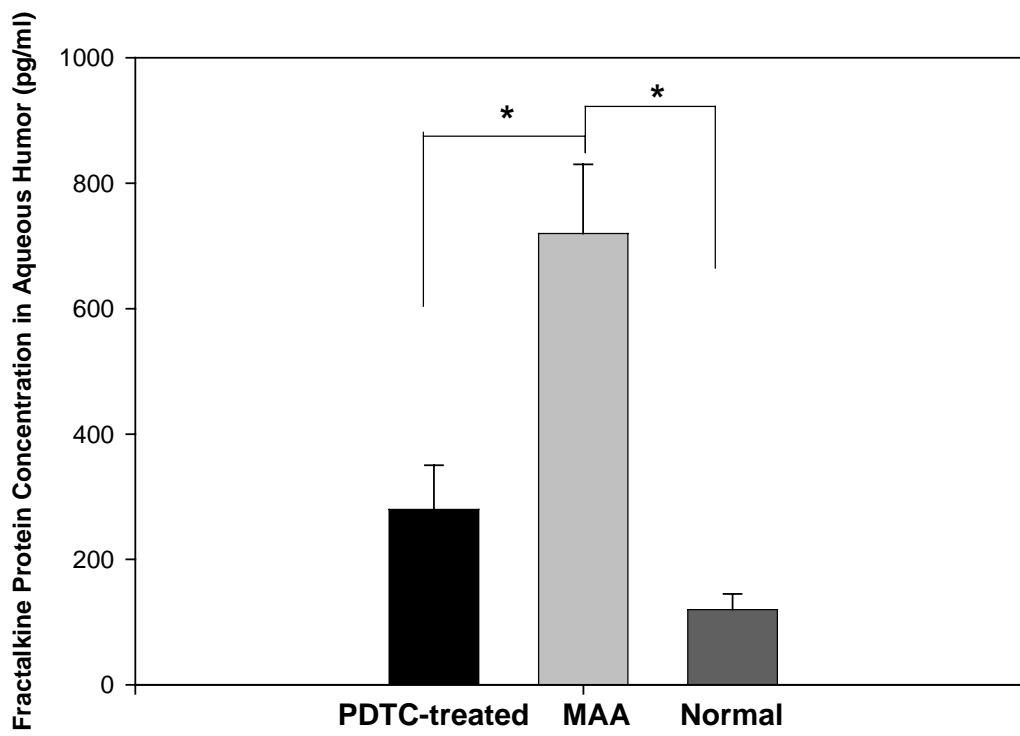
Fig. 2



**Fig 3**



**Fig. 4A**



**Fig. 4B**



