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Accelerated Central Nervous System Autoimmunity in BAFF-Receptor-Deficient Mice

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Abstract

B cell activating factor (BAFF) is critical for B cell survival, a function that is mediated by BAFF receptor, (BAFF-R). The role of BAFF (or BAFF-R) in the multiple sclerosis model, experimental autoimmune encephalomyelitis (EAE), was examined using BAFF-R-deficient mice. BAFF-R deficiency resulted in paradoxically increased severity of EAE induced by myelin-oligodendrocyte glycoprotein (MOG) peptide 35-55. Inflammatory foci in BAFF-R-deficient mice comprised increased numbers of activated macrophages expressing BAFF and correlated with increased BAFF secretion. Thus, BAFF-R may be important in regulating EAE, possibly by influencing macrophage function through a mechanism that involves modulation of BAFF expression.

Keywords

B-cell activating factor; BAFF-Receptor; Experimental autoimmune encephalomyelitis; Multiple sclerosis; Central nervous system inflammation; Macrophages; T-Cells

1. Introduction

Multiple sclerosis (1) is an immune-mediated disease of the central nervous system (CNS) (2), in which inflammation is thought to be regulated by both T cells and B cells (3). The significance of B cells in the pathogenesis of MS is highlighted by the benefit of B cell depletion therapy with intravenous rituximab in MS patients. However, a recent phase II clinical trial of B cell-activating factor (BAFF) blockade with Atacicept, a recombinant TACI Ig fusion protein, led to worsening disease activity in MS patients (4). It is possible that the deleterious results of systemic anti-BAFF therapy may have derived from effects not just on the survival of B cells, which is mediated through B cell activating factor receptor (BAFF-R) (5), but also on regulation of T cell, B cell and macrophage function, which may

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be mediated by other BAFF receptors including transmembrane activator calcium-modulator and cyclophilin ligand interactor (TACI), and B cell maturation antigen (BCMA) (6). Whereas BAFF is primarily expressed by myeloid cells and radioresistant stromal cells in the lymphoid organs and spleen (7-8), BAFF receptors are expressed in B cells, subsets of T cells and macrophages (9-12). In particular, macrophages, which are a major source of inducible BAFF, may become activated upon stimulation with BAFF via BAFF-R, TACI or BCMA (11-12).

The current investigation addresses whether BAFF plays a role in regulating B cells, T cells and macrophages in the development of the T cell-dependent model of EAE in which Th17 activity and IL23 are required (13). BAFF-R-deficient mice, which express no BAFF-R resulting in significantly reduced numbers of mature B cells (9), were used to examine the effects of BAFF-R deficiency on susceptibility to EAE. The results demonstrate that, paradoxically, BAFF-R-deficient mice developed an earlier onset and increased severity of p35-55-induced EAE, with more widespread inflammation in the spinal cord. The inflammatory foci in the CNS consisted of increased numbers of activated macrophages, many of which expressed BAFF, which correlated with increased systemic BAFF secretion. In addition, there was a similar increase in peripheral pro-inflammatory cytokines in BAFF-R-deficient mice with EAE. These findings suggest that BAFF-R-deficient mice respond to the lack of the receptor BAFF-R and relative B cell deficiency by generating increased levels of BAFF, which may act through TACI or BCMA to promote pro-inflammatory T cell and macrophage responses that facilitate the development of CNS autoimmunity.

2. Materials and Methods

2.1. Peptide

Rodent myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 (MEVGWYRSPFSRVVHLYRNGK) was synthesized and reverse-phase chromatographically purified by New England Peptide (Gardner, MA).

2.2 Mice

Wild-type C57BL/6 mice were purchased from The Jackson Laboratory (JaxWest). Homozygous BAFF-R^{-/-} (knockout) mice on a B6 background, which are deficient in BAFF-R, created using a targeting vector to produce a floxed BAFF-R allele by inserting lox P sites flanking exon 3 and 4 and a neomycin resistance cassette flanked by two FRT sites (9), were obtained from Yoshiteru Sasaki, (CBR Institute, Harvard University, Boston, MA). BAFF-R deficient mice used in the present study are distinct from A/WySnJ mice which have a mutated BAFF-R but are still able to bind BAFF, and are characterized by B cell maturational defects (14-15).

2.3. EAE induction and clinical analysis

Eight to 12 week old female C57BL/6, and BAFF-R KO mice were administered 300 µg of MOG peptide in 0.1 mL PBS emulsified in 0.1 mL complete Freund's adjuvant (CFA) containing 4mg/mL heat-inactivated Mycobacterium tuberculosis H37RA (Difco, Detroit, MI) via subcutaneous flank injection on day 0, followed by 200 ng of Bordetella pertussis

toxin (List Biological Laboratories) in 0.2 mL of PBS administered intraperitoneally on day 0 and 48 hours post immunization. The mice were weighed and observed daily. Clinical scoring of motor weakness was graded on a 0-5 point scale as follows (16): 0 = no clinical disease, 1 = decreased tail tone, 2 = mild monoparesis or paraparesis, 3 = severe paraparesis, 4 = paraplegia and or quadraparesis, and 5 = moribund or death. All experiments were performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines at the University of California, Davis.

2.4. Histology

At 21 or 43 days post-immunization with either MOG p35-55, BAFF-R KO and wild-type B6 mice were anesthetized by intraperitoneal injection of ketamine/xylazine prior to intracardiac perfusion with saline followed by phosphate buffered 4% (w/v) paraformaldehyde. Spinal cords and brains were removed and post-fixed at room temperature with phosphate buffered 4% paraformaldehyde for one hour. Spinal cord sections were transected in the mid-lumbar and cervico-thoracic regions, and brain sections were transected mid-sagittally. Tissue sections were processed for paraffin embedding and cryostat sectioning. Fixed brain and spinal cord tissues were embedded in paraffin, sectioned into 6 μm sections, and stained with H&E to identify inflammatory cells. Inflammatory foci (containing 10 or more inflammatory cells per focus) were counted and areas measured (μm^2), in the parenchyma and meninges of brain, lumbar and cervical spinal cord sections from each mouse by an examiner in a blinded manner. For cryostat sectioning, fixed CNS tissues were cryoprotected by immersing tissues in 30% sucrose at 4° C overnight, embedded in OCT cryostat mounting medium (Sakura Finetek), frozen in liquid nitrogen cooled 2-methylbutane, sectioned (transversely for spinal cord, and sagittally for brain) into 10 μm sections, and stained for immunofluorescence and confocal microscopy.

2.5. Immunohistology

Primary antibodies used in this study include BAFF (rat anti-mouse mAb, Abcam), activated macrophage/microglia marker IBA-1 (goat anti mouse polyclonal Ab, Abcam), CD3 (rabbit anti-mouse polyclonal Ab, Abcam), and nuclear dye Hoechst 33258 (Invitrogen). Cryostat sections were immunostained with monoclonal and polyclonal primary antibodies. Biotinylated secondary antibodies binding to antibodies against BAFF were detected with rhodamine-conjugated streptavidin (Jackson ImmunoResearch Laboratories). FITC-labeled secondary antibodies were used to detect IBA-1 and CD3 expression. Cryostat sections mounted on poly-L-lysine coated slides were allowed to dry, and then pre-fixed with 0.5 % paraformaldehyde, washed with phosphate buffered saline (PBS) pH 7.2, blocked with a donkey block solution containing 10% donkey serum (Jackson ImmunoResearch Laboratories), MEM/Hepes (Invitrogen), 1 % (v/v) Penicillin (PCN)-Strep (Invitrogen), and 0.05% (v/v) sodium azide for 15 minutes at room temperature, and then washed with PBS, blocked with strep-avidin and biotin (Vector Labs) blocking solutions, rinsed with PBS and incubated overnight with primary antibodies in donkey block solution at 4° C. Cryostat sections were subsequently washed in PBS, incubated with secondary antibodies in donkey block solution containing 10 % donkey serum for 30 minutes at room temperature, then washed with PBS, incubated with strep-avidin rhodamine in donkey block solution for 15 minutes at room temperature, washed with PBS, and then were post-fixed with methanol at

-20° C, counterstained with Hoechst nuclear stain (2 µg/mL in PBS) and mounted in Vectashield.

2.6. Imaging and image analysis

H&E stained spinal cord sections were viewed with a Nikon E600 (Tokyo, Japan) microscope and a 40× objective lens (Plan Apochromat, NA 0.95), and detected with a color CCD camera (LE-470, Optronics, Goleta, CA). Quantification of inflammatory foci was performed by counting the number of inflammatory foci and measuring areas of each focus using Stereoinvestigator (version 8, Micro Bright Field, Inc., Williston, VT). Fluorescent images were captured by confocal microscopy with a Zeiss LSM 510 META (Carl Zeiss, Germany) laser scanning confocal microscope and an oil DIC 63× objective lens (Plan Apochromat, NA 1.4). Three dimensional images of EAE spinal cord sections were generated by collecting a z-series of confocal images. To evaluate the proportion of macrophages among Hoechst positive cells in inflammatory foci, Volocity (version 4.0, Improvision Inc.) software was used to measure and analyze objects in 3-dimensional images.

2.7. Splenocyte activation and cytokine analysis

Day 12 MOG peptide-immunized BAFF-R KO and wild-type controls were euthanized by CO₂ inhalation. Spleens were removed from each group of mice, homogenized using a Dounce homogenizer, and filtered using a 100 µm mesh filter. Cell suspensions were centrifuged for 10 minutes at 1000 g, washed in RPMI-5 containing RPMI 1640, 5% heat inactivated FBS, 10 mM HEPES, 1% (v/v) Penicillin (PCN)/Streptomycin (strep), 50 µM 2-mercaptoethanol. Cell pellets were resuspended in RPMI-5, Ficoll Hypaque (Amersham Pharmacia Biotech) solution was layered under the media, and the suspension was centrifuged at 800 × g for 15 minutes at room temperature. Cells were collected from the Ficoll Hypaque interface, transferred to 50 mL conical tubes and washed in RPMI-5 media. Cell pellets were resuspended in RPMI-10 media containing RPMI-1640, 10% heat inactivated FBS, 10 mM HEPES, 1% (v/v) PCN/Strep, 50 µM 2-mercaptoethanol, and counted using a hemocytometer. Splenocytes (5×10^5 cells/well) were cultured in flat-bottomed, 96-well plates in media (RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U mL⁻¹ penicillin, 0.1 mg mL⁻¹ streptomycin, 0.5 µM 2-mercaptoethanol and 10% FBS) in the presence of MOG p35-55 peptide (0, 10, 20, 50 µg mL⁻¹), human MOG 1-125 (0, 20, 33 µg mL⁻¹) or media control. Cytokines were measured in the supernatants (at 48 h: IL-2, IL-6; 72h: IFN-γ, TNF-α, BAFF; 96h: IL-17; 120 h: IL-10) of cultured splenocytes using OPTeia ELISA sets (BD Pharmingen) or Quantikine ELISA KIT for BAFF (R&D Systems) as per protocol. Splenocytes were pooled from seven animals per group and wells were plated in triplicate. Data were analyzed using a SOFTmax ELISA software and were expressed as means ± SEM of triplicate wells.

2.8. Statistical Analysis

Data are shown as means ± SEM. A t-test was used for parametric data (for 2 groups) and Mann-Whitney U statistics were used for non-parametric data (for 2 groups). Fisher's exact test was used to determine whether differences in proportions were significant. P-values <0.05 were considered statistically significant.

3. Results

3.1. BAFF-R-deficient mice develop an earlier onset and increased severity of p35-55-induced EAE

Similar to BAFF-deficient mice, BAFF-R-deficient mice have reduced numbers of mature B cells (9). Following immunization with p35-55, BAFF-R-deficient mice had an earlier onset of clinical disease and increased severity of acute EAE compared to wild-type (10) (Fig. 1A), (Table 1). Thus, BAFF-R expression is not required for p35-55-induced EAE and its absence results in more severe disease.

3.2. CNS inflammatory foci are increased in BAFF-R-deficient mice and have an increased proportion of activated macrophages

The inflammatory foci were five-fold greater in area in the spinal cords of p35-55 immunized BAFF-R-deficient mice compared to p35-55 immunized wild-type controls during the chronic phase of EAE (43 days) (Fig. 1D-G, Table 2). In contrast, at 21 days, levels of inflammation appeared similar (Figure 1B-C). A possible explanation for this may be that the histological measures used to gauge inflammation, cross sectional area of and numbers of foci are not sufficiently sensitive to detect statistically significant differences early on in the course of EAE. To determine the cellular composition of the lesions, we examined histological sections with markers IBA-1 for activated macrophages and CD3 for T cells. Compared with p35-55 immunized wild-type controls, BAFF-R-deficient mice immunized with p35-55 had increased levels of IBA-1, which co-localized with cells expressing increased levels of BAFF in spinal cord infiltrates at both 21 and 43 days. (Fig 2A-D). In contrast, while there was some increased expression of the pan T-cell marker, (CD3) at 43 days in p35-55 immunized BAFF-R-deficient mice compared with p35-55 immunized wild-type controls, it did not significantly colocalize with increased BAFF expression. Furthermore, the increase in CD3 expression was not as prominent as the elevation in IBA-1 expression in p35-55 immunized BAFF-R-deficient mice. (Data not shown). Thus, the inflammatory foci in BAFF-R-deficient mice comprised larger numbers of activated macrophages many of which expressed BAFF. Moreover, BAFF-R deficiency resulted in a higher proportion of activated macrophages within inflammatory infiltrates compared with wild-type controls following p35-55 immunization (Fig 2E), possibly via upregulation of other BAFF receptors in macrophages.

3.3. p35-55 immunization promotes increased pro-inflammatory T cell activation in BAFF-R-deficient mice

Increased susceptibility to p35-55-induced EAE could also reflect changes in T cell activation. Therefore, splenocyte culture supernatants were assessed for Th1 cytokines (IL-2, IFN- γ and TNF- α), Th17 cytokines (IL-17, IL-6), and Th2 cytokine (IL-10). BAFF-R-deficient mice immunized with p35-55 produced increased levels of IL-2, and IL-6 (Fig. 3A-B), as well as IFN- γ and TNF- α (Fig. 3C-D) compared with similarly immunized wild-type controls. At the same time, secretion of IL-10 was markedly reduced in BAFF-R-deficient mice (Fig. 3F). IL-17 production was similar for both groups (Fig. 3E). To address whether susceptibility to p35-55-induced EAE in BAFF-R-deficient mice could also reflect alterations in systemic BAFF regulation, splenocyte culture supernatants were examined for

BAFF expression. Splenocytes from BAFF-R-deficient mice produced two to four-fold greater levels of secreted BAFF compared to wild-type animals, even when not stimulated by antigen (Fig. 3G).

4. Discussion

In a recent MS clinical trial of Atacicept, a recombinant TACI-Ig fusion protein which binds BAFF and its associated ligand APRIL and functions to prevent BAFF ligand activation of its receptors, MS patients developed worsening disease activity (4). Our present study of BAFF-R deficiency in which the absence of BAFF-R led to worsening CNS autoimmunity and inflammation may provide some insight into the results of the Atacicept trial. BAFF is critical for the survival of B cells (17). It has been shown that BAFF-deficient mice have decreased numbers of mature B cells and have impaired antibody production (17). BAFF-R-deficient mice have a phenotype similar to BAFF-deficient mice, showing reduced numbers of mature B cells and displaying impaired antigen dependent antibody responses (9). In particular, BAFF-R-deficient mice have a significant decrease in the number of mature B2 cells and marginal zone B cells but have no significant reduction in the number of B1 cells, similar to BAFF-deficient mice, suggesting that the effect of BAFF on B cell survival is mediated through BAFF-R (9).

While it is evident that BAFF-R mediates the B cell survival effects of BAFF, the results of the present study suggest that the increased severity of CNS inflammation observed in BAFF-R-deficient mice are more likely to be attributable to the effects of BAFF on T cell regulation and changes in macrophage function than through the promotion of B cell survival. In addition to their expression on B cells, BAFF receptors are also expressed in T cells and macrophages, suggesting that BAFF may play a role in T cell regulation and macrophage function (9-12). In the model presented here, BAFF may exert its pro-inflammatory effects through the NF-Kappa B pathways (18-19) via other BAFF receptors including TACI and BCMA. Increases in IL-6 and TNF- α in the setting of BAFF-R deficiency suggest increased macrophage activation possibly associated with elevated BAFF expression.

Macrophages, which are a major producer of inducible BAFF, express all three BAFF receptors, BAFF-R, TACI and BCMA, in certain contexts (11-12). Furthermore, these receptors may be upregulated in the presence of BAFF stimulation (11). In the current study, BAFF-R-deficient mice were used to examine the effects of BAFF-R deficiency on susceptibility to a T cell model of EAE. We have demonstrated that BAFF-R is not required for EAE induced by immunization with p35-55. The observation that T cells from BAFF-R-deficient EAE mice, cultured with p35-55, secrete increased levels of pro-inflammatory cytokines implies that priming is not impaired in BAFF-R-deficient mice, and is possibly even enhanced. Consistent with the latter possibility is our observation that BAFF-R-deficient mice exhibit an earlier onset and increased severity of p35-55-induced EAE, along with increased levels of IBA-1 staining and increased proportions of IBA-1 positive macrophages within inflammatory lesions at day 21, which may result from increased macrophage activation via upregulation of other BAFF receptors.

The observation that EAE is more severe in BAFF-R-deficient animals suggests that BAFF-R may be important in modulating EAE, possibly by influencing macrophage function. Increased levels of BAFF in the CNS and expression of BAFF in-vitro in BAFF-R-deficient mice compared with p35-55 immunized wild-type controls suggest that BAFF may contribute to chronic CNS inflammation and increased CNS autoimmunity possibly through the activation of other BAFF receptors in the absence of BAFF-R. A caveat is that the association between systemic BAFF levels and EAE susceptibility may depend on the context of disease. For example, in the setting of intact BAFF-R expression, EAE susceptibility in SJL/J mice immunized with PLP p139-151 was associated with alterations in B cell homeostasis as well as decreased serum BAFF levels at the peak of disease (14 days) while B10.S mice resistant to EAE induction were shown to have an early increase in serum BAFF levels at 7 days following immunization with PLP p139-151 but not at later time points, compared with PBS and adjuvant controls (20).

It is possible that BAFF-R can mediate its effects on macrophage function and T cell regulation in EAE independently of mature B cells. This may result in a bias toward pro-inflammatory cytokine responses in p35-55 immunized BAFF-R-deficient mice compared with similarly immunized wild-type controls. Alternatively, since B cells are critical for recovery in EAE, through an IL-10 dependent mechanism (21), the reduction in the number of B cells and the markedly reduced IL-10 production may contribute to the impaired recovery in EAE in BAFF-R-deficient mice. In addition, the lack of mature B cells may also promote EAE induction by decreasing the proportion of CD4⁺CD25⁺FoxP3⁺ regulatory T cells, and by regulating macrophage and other antigen presenting cell (APC) function that enhance encephalitogenic pro-inflammatory cytokine responses (22). Generating BAFF-R/B cell deficient (uMT) mice to determine the effects of combined BAFF-R and B cell deficiencies on susceptibility to EAE may help to assess directly whether BAFF-R may affect T cell regulation and macrophage function independently of B cells.

Our observations appear to be relevant in explaining the unanticipated worsening of MS in patients treated with Atacicept. Systemically directed anti-BAFF therapy in MS such as in the Atacicept study (4) may result in worsening disease activity through various mechanisms. Treatment with Atacicept may have led to a paradoxically increased expression of BAFF systemically and in the CNS, as well as an increase in systemic T-cell activation and enhanced pro-inflammatory macrophage function, as is seen in the setting of BAFF-R deficiency in our study, resulting in the unexpected worsening of CNS inflammation observed in those patients. In contrast, anti-BAFF therapies specifically targeting the CNS may play an important role in preventing the development of chronic CNS inflammation and progression of disease in MS.

In summary, our findings indicate that BAFF-R is important in EAE regulation. BAFF-R deficiency was associated with more severe EAE, elevated levels of BAFF, macrophage activation and greater myelin-reactive pro-inflammatory immune responses. The possibility that some of these effects may occur independently of B cells will require further investigation. Paradoxical worsening of EAE through selective inhibition of one member of BAFF receptors, suggests that activity of elevated BAFF on other BAFF family receptors,

may promote pro-inflammatory myeloid and T cell responses in MS or other autoimmune diseases.

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Figure 1. p35-55 immunized BAFF-R-deficient mice have accelerated EAE

Mean $\pm$ SEM. clinical scores of wild-type (WT) and BAFF-R-deficient mice in p35-55 induced EAE are shown (* $P < 0.05$ Mann-Whitney; $n = 7$ per group for days 0 through 31, and $n = 4$ per group for days 32 through 43) **A**) Data pooled from two independent EAE experiments and representative of two independent EAE experiments. Day 21 lumbar spinal cord sections from wild-type (**B**) and BAFF-R-deficient (**C**) and day 43 p35-55-induced EAE lumbar spinal cord sections from representative wild-type (**D, F**) and BAFF-R-deficient mice (**E, G**) were stained with H&E. Images acquired with a 40 $\times$ objective lens. Scale bar 100 μ m.

Figure 2. BAFF-R-deficient mice have increased levels of activated macrophages in CNS spinal cord inflammatory foci at 21 and 43 days following immunization

Three color immunofluorescence for IBA-1 (FITC-labeled), BAFF (rhodamine-labeled), and nuclei (Hoechst) shows elevated levels of activated macrophage marker, IBA-1, which colocalized with BAFF expression in inflammatory foci in day 21 p35-55-induced EAE lumbar spinal cord in BAFF-R-deficient (A) compared to p35-55 immunized wild-type controls (B), and which colocalized with increased BAFF expression in inflammatory foci of 43 day p35-55-induced EAE spinal cord in BAFF-R-deficient (C) compared to p35-55 immunized wild-type controls (D). Images acquired with a 63× objective lens. Scale bar 50 μm (A, B) and 100 μm (C, D). Mean ± s.e.m. Proportion of FITC-labeled IBA-1 cells among Hoechst positive cells in inflammatory foci of wild-type and BAFF-R-deficient EAE lumbar spinal cord sections are shown (E) (**P < 0.01, t-test, n = 3 foci per group).

Figure 3. p35-55 immunization promotes increased splenocyte activation in BAFF-R-deficient mice

Secretion of IL-2, IL-6, IFN- γ , TNF- α , IL-17, IL-10 and BAFF from cultured splenocytes (pg/mL), (A-G) derived from day 12 p35-55 immunized BAFF-R-deficient mice compared to p35-55 immunized wild-type controls. Cultured splenocytes were stimulated with p35-55 antigen (0, 10, 20 and 50 ug/mL). Cytokines were measured in the supernatants (at 48 h: IL-2, IL-6; 72h: IFN- γ , TNF- α , BAFF; 96h: IL-17; 120 h: IL-10) of cultured splenocytes. Splenocytes were pooled from seven animals per group and wells were plated in triplicate. Data are representative of two independent experiments.

Table 1
Clinical and Histological Characteristics of EAE

	Incidence	Mean Day of Onset ^a	Mean Maximum Disease Score ^b	Histological Score ^{c,d}
BAFF-R-deficient	7/7	11.4 ± 0.4 **	3.4 ± 0.43 (p=0.2994)	101 ± 23 × 10 ³ (n = 29) **
C57BL/6	7/7	16.1 ± 1.1	2.7 ± 0.48	19.3 ± 6.7 × 10 ³ (n = 16)

^a Mean day of disease onset of individual animals in each group ± sem.

^b Mean of maximum disease score of individual animals in each group ± sem.

^c Total area of parenchymal foci (um²) in 43 day lumbar spinal cord samples

^d n = number of foci

**
P<0.001

Table 2
Comparison of inflammatory foci in 43 day lumbar spinal cord samples from wild-type and BAFF-R-deficient p35-55-induced EAE mice

	Parenchymal Foci/Section ¹	Meningeal Foci/Section ²	Total Area of Parenchymal Foci ³ (μm ²)	Total Area of Meningeal Foci ⁴ (μm ²)
BAFF-R-deficient	7 ± 2 (n = 5)	7.5 ± 1.1 (n = 5)	101 ± 23 × 10 ³ (n = 29) **	17.8 ± 4.4 × 10 ³ (n = 30) *
Wild-type	5 ± 1 (n = 4)	3.5 ± 2.3 (n = 4)	19.3 ± 6.7 × 10 ³ (n = 16)	3.4 ± 0.9 × 10 ³ (n = 14)

Values are mean ± SEM;

* P<0.05,

** P<0.01;

^{1, 2} n=number of sections;

^{3, 4} n = number of foci