Research Square

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Bone Marrow Mesenchymal Stem Cells Regulate IL-4 and INF-γ Expression in AR Mouse Model

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Abstract

Background:

Bone marrow mesenchymal stem cells can promote the recovery of immune balance and regulate the balance of Th1/2 cells. Allergic rhinitis is a disease with Th1/2 imbalance mediated by IgE. It's unclear whether BMSCs could regulate AR disease. In this study, the possible role of BMSCs was explored.

Methods :

AR mouse model was established by ovalbumin (OVA). 18 models were randomly divided into three groups: AR-sensitized, Stem-cell-returned, Medium-returned; six unsensitized mouses named normal-control. IgE, IL-4 and INF-γ levels were measured by Elisa. Observing migration of BMSCs by immunofluorescence. Flow cytometry used to detect changes of Th1/2. STAT 4/6 protein level was detected by Western-blot.

Results :

After OVA-sensitization, IgE, IL-4 and STAT6 levels were higher, INF-γ and STAT4 level was lower. Flow cytometry revealed a decrease in Th1 cell and an increase in Th2 cell in AR group. After BMSCs treatment, t IgE, IL-4 and STAT6 levels in SCRg and MRg were lower than that in AR group, and tINF-γ and STAT4 level were higher than hat inAR group. Flow cytometry showed that the content of Th1 cell increased while Th2 cell decreased.

Conclusions:

BMSCs return treatment could decrease the expression of IL-4, promote the expression of INF-γ and regulate the balance of Th cell, and the mechanism was closely related to STAT4/6 signaling pathway. However there was no statistical difference between SCRg and MRg, so the role of BMSCs maybe achieved through paracrine function rather than multi-directional differentiation potential.

Background

Allergic rhinitis (AR), is an common chronic inflammatory disorder of the nasal airways with Th1/Th2 imbalance[1]. Many signal pathways are involved in the regulation, and large numbers of cytokines are secreted. Signal transduction and activator of transcription (STAT) is one type of transcription factor discovered in recent years which is closed related to immune disease[2]. STAT 4 and STAT 6 (STAT 4/6) are two subtypes of STAT and are the important factors mediating Th1/Th2 imbalance. Numerous studies have shown that STAT 4/6 plays an important role in the pathogenesis of AR. STAT 4/6 regulates

the differentiation of Th cells into Th1/Th2 subpopulations by regulating the expression of cytokines such as IgE, INF-γ and IL-4. STAT 4 can regulate the differentiation of immature Th cells into Th1, and promote the production of INF-γ. STAT 6 can regulate the differentiation of immature Th cells into Th2, induces secretion of IL-4 and promote inflammatory response[3-4].

IL-4 is an immunoregulatory cytokine secreted primarily by activated Th2 cells in allergic inflammations, such as AR and asthma. It's also an important cellular cytokine in the jak-stat protein pathway. IL-4 can enhance the antigen-forming ability of B cells, increase the expression of low-affinity receptors in the IgE segment and induce the differentiation of IgE-positive cells. However, the IL-4 receptor have no endogenous kinase activity and require receptor-associated kinases as a driving factor for signal transduction. The JAK kinase family played this role. The JAK /STAT signaling pathway was composed of three major structural proteins: one JAK protein and two stat proteins. IL-4 was phosphorylated by binding to the JAK family receptor, and then the stat phosphorylation agent dimerizes. The stat protein then nucleates to regulate target gene transcription to activate the cascade.

Bone marrow mesenchymal stem cells (BMSCs) are multipoint progenitor cells derived from bone marrow, which can be evolved into osteoblasts, chondrocytes, nerve cells and hepatocytes, etc, have become the most widely used cell in tissue engineering research and clinical laboratory research. Recent studies have shown that BMSCs have weak immunogenicity and potent immunomodulatory effects, and have therapeutic effects on a variety of immune diseases[5]. Many studies have entered clinical trials.

By reading the literature, we found that BMSCs can promote the recovery of immune balance. It inhibits T cell, B cell, nature killer(NKC) and dendritic cell(DC) activities, and regulate Th1/2 cell balance, then achieve immune regulation function through a variety of mechanisms[6]. Smail Ogulur reported that intranasal application of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can inhibit airway inflammation in a mouse model of acute asthma[7]. Stem cells regulate the immune environment by inhibiting the over activation of Th 2 cells and the production of Tregs, alleviated the symptoms of immune disease and reduced the secretion of Th 2 cells secreting cytokines (including IL-4, IL-5 and IL-13), as well as decreased IgE levels and mucus production8. Its regulation mechanism has a highly relevance with the pathogensis of AR, but there are few studies related to AR.

We hypothesized that BMSCs can alleviate AR symptoms by regulating the STAT 4/6 signaling pathway, and designed this experimental program: AR mouse model, was established, BMSCs were isolated and cultured, then BMSCs or BMSCs medium was returned through mouse tail vein, to study its action in AR. At the same time, we verified that whether the mechanism of BMSCs is through differentiation or paracrine.

Methods

Material and animal models

Mature healthy male mouse purchased from National Rodent Laboratory Animal Resources (Shanghai, China). All animal care and experimental procedures were ethical and approved by the Tongji University Institution Animal Care and Use Committee.

The AR mouse model was prepared as follows. A total of 25 mature healthy male mice were used to establish AR mouse model. Each mouse was first sensitized intraperitoneally with 0.3 mg of OVA and 30 mg of AL(OH)3 every other day for a total of seven times. From day 15, the mouses were treated with 0.5% OVA aerosol to stimulate AR symptoms for five times. The symptoms were observed and the frequencies of scratching and sneezing were assessed using the procedure previously described by Al Suleimani M but with modifications. Finally, 22 AR mouse model were obtained. We randomly excluded 4 of them and divided the remaining into 3 groups (6 in each group), named Stem cell returned group (SCRg), Medium returned group (MRg), AR-sensitized group (ARg). Meanwhile, the other 6 mature healthy male mouse were named Normal control group (NCg).

BMSCs preparation and intervention therapy

Four weeks old mice were selected, sacrificed by cervical dislocation, then soaked in 75% ethanol for 15 min. Quickly cut the skin and muscles of the hind limbs under aseptic conditions, and the bone marrow is taken. The obtained BMSCs were cultured in a low-sugar DMEM culture medium (containing 10% FBS, 100 u/ml penicillin, and 100 mg/ml streptomycin), and changed every 3 days until the cells were up to about 90% , and the cells were digested and passaged. After digesting with 0.25% trypsin, it was inoculated into a new medium, and the inoculation density was 0.5-1.0*10^4 pieces/cm2. A total of 2 passages were taken, p2 cells were taken and counted, and the supernatant was taken. The collected cells were resuspended again with PBS and controlled to contain 1.0*10^6 cells per 0.2 ml.

On the 21st day, the SCRg group was transfused with BMSCs via the tail vein, which were labeled with CM-Dil and a total of 1.0×10^6 of p2 generation, once daily for 5 days; MRg group was transfused with the same dose of medium supernatant which were taken during BMSCs culture. AR g and NC groups returned the same dose of PBS.

Determination of serum IgE and cytokines IL-4 and INF-γ

The mouses were anesthetized by intraperitoneal administration of pentobarbital (40 mg/kg). These animals were sacrificed by rapid decapitation, and then blood and nasal mucosa were collected. Part of the nasal mucosa was taken from the front of the inferior turbinate and the anterior segment of the nasal septum, and immediately placed in liquid nitrogen.The levels of IgE, IL-4 and INF-γ in guinea pigs were determined by ELISA (RB Inc., Maryland, USA). The latter part of the mouse head was retained and examined for decalcification staining.

Flow cytometry for detecting Th1/2 cell

Th1 cells were labeled with CD4 + IFN-γ + antibody, and Th2 cells were labeled with CD4 + IL4 + antibody. Part of fresh nasal mucosa tissue was dissociated, resuspended in cell wash solution(PBS with 2% BSA),

and adjusted the cell concentration to 1*106 /ml. Then added fluorescently labeled CD4 antibody （eBioscience，85-11-0041-81) (concentration: 0.125ug per tube) and reacted at 4 ° C for 30 minutes in the dark. Next, washed with pre-cold PBS to remove unbound antibody, and then resuspended in PBS. Fixd and punched 1ml Fix & Perm (Caltag, GAS-003), and incubated at 4 ° C in the dark for 45min. After washing once in PBS and twice in buffer, added fluorescently labeled IFN-γ (eBioscience, 85-12-7311-82) and IL 4 (eBioscience, 85-12-7041-81) antibodies, 5ul each, and incubated at 4 ° C for 30min in the dark, washed with pre-cold PBS to remove unbound antibody. Finally, it was detected by flow cytometry.

Western blot analyses of STAT 4/6

The nasal mucosa, which was frozen in liquid nitrogen, was homogenized in 1 mL of protein lysis buffer (PBS containing 0.1% Triton X-100) and centrifuged at 14,000 g for 10 min at 4 °C. Nasal mucosa lysates from each group were analyzed by Western blot.The blot was blocked with PBS-T containing 1% skim milk and then incubated with a 1:1000 diluted STAT 4 antibody (AbCAM, ab68153) or a 1:1000 diluted STAT 6 antibody (AbCAM, ab32520) at room temperature. After three additional washes, the blots were incubated with anti-mouse secondary antibody (1:5000) then conjugated to horseradish peroxidase for 1h at room temperature. The bands were visualized using EZ-ECL detection reagents.The second batch of images was quantified using Quantity One software. B-actin was employed as an endogenous control for protein normalization. The experiments were performed in duplicate.

Results

Immunofluorescence of BMSCs in nasal mucosa and Nasal sinus decalcification staining

Immunofluorescence of BMSCs in nasal mucosa Tissue from the specimens were fixed in 10% buffered formalin. Immunohistochemical stain were performed on formalin-fixed and paraffin-embedded 4um sections. Fluorescence microscope was applied to observe and photograph the migration of CM-Dil-labeled BMSCs in nasal mucosa. It can be seen that there was red fluorescence of BMSCs in the nasal mucosa, and the nasal mucosa cells were stained with DAPI and displayed in blue（Fig. 1). At the same time, we decalcified the cross section of the nasal sinus of the animal model. It can be observed that the AR mouse model group is thicker than the nasal mucosa basement membrane and the submucosal lamina propria inflammatory cell infiltration is significantly increased. After intervention with BMSCs or the medium of BMSCs, the inflamematory cell infiltration of the lamina propria was not as severe as in the AR group, and there was no statistical difference between the SCRg and MRg（Fig.2）.

Reduction of IgE levels in serum

The IgE level in the serum of the AR-sensitized group was higher than those in the control group ($p<0.05$). Compared with the AR group, IgE levels were significantly lower after treatment with BMSCs or the medium of BMSCs ($p < 0.01$) (Fig. 3), but there was no statistical difference between the SCRg and MRg groups.

Reduction of IL-4 and INF-γ levels in serum

The level of IL-4 in the AR-sensitized group was higher than that in the control group ($p<0.05$). Compared with the ARg, IL-4 levels were significantly decreased after after treatment with BMSCs or the medium of BMSCs ($p < 0.01$) (Fig. 4), but there was no statistical difference between the SCRg and MRg groups. After sensitization with OVA, the level of INF-γ is lowered, after treatment with BMSCs or the medium of BMSCs, INF-γ levels were elevated in SCRg and MRg. Also there was no statistical difference between the SCRg and MRg. (Fig. 5)

Flow cytometry to detect changes in Th1 / 2 cell content

In the NCg, the content of Th1 cells was significantly higher than that of Th2 cells. After OVA sensitization, the content of Th2 cells increased (including the group of ARg, SCRg, MRg). And after treatment with BMSCs or the medium of BMSCs, the content of Th1 cells were significantly more than that in ARg, and the content of Th2 cells were decreased. But there was no statistical difference between the two groups.(Fig.6)

Western blot results of STAT 4/6 expression pathway protein

As shown in Fig 7 Western blot analysis showed that the expression level of STAT 6 in the AR-sensitized group were higher than that in the control groups ($p<0.05$), and the expression level of STAT 6 in the SCRg and MRg group were lower, but there was no statistical difference between the SCRg and MRg. The expression level of STAT 4 in the AR-sensitized group were lower than that in the control groups ($p<0.05$), while the expression of STAT 4 in the SCRg and MRg was higher than those in ARg and NC group, also there was no statistical difference between the SCRg and MRg . (Fig. 7)

Discussion

The pathophysiology of AR is chronic airway inflammation, with infiltration of eosinophils, mast cells, and CD4+ T lymphocytes, expressing many cytokines, such as IgE, IL-4, and INF-γ. Lymphocytes develop into Th1 or Th2 cells. They can be classified based on their cytokine production in the immune system. Th2 cells produce IL-4, IL-10, and other cytokines that are primarily involved in IgE-mediated delayed type-1 hypersensitivity and contribute to the recruitment of eosinophils[9,10].

BMSCs are adult stem cells derived from mesoderm. Which have the potential for self-renewal and multi-directional differentiation. BMSCs can differentiate into various cells, such as osteoblasts, chondrocytes, nerve cells and hepatocytes, etc[11,12]. Due to its easy separation and culture, low risk of conversion to cancer and low immunogenicity[7,13-15], BMSCs have become a hotspots of tissue engineering research and clinical application experimental research. At present, it has been extensively reported that BMSCs have been used for cell replacement therapy, inhibiting inflammation and promoting tissue repair. In addition, they can also replicate the target gene to the host as a cell carrier, which provides much of evidence for diseases treatment [16,17].

Currently, BMSCs have been shown to have weak immunogenicity and unique immuno-negative properties, which can inhibit the activity of T cells, B cells, natural killer cells (NKC) and dendritic cells (DC) at different levels[18,19]. In vivo, BMSCs have a complex regulatory effects on the imbalance of Th1/Th2 immune response. Stem cells can modulate the immune environment by inhibiting excessive activation of Th2 cells and production of Tregs. Reversal of disease symptoms and reduction of Th2 secreting cytokines (including IL-4, IL-5 and IL-13) and reduction of IgE levels and mucus production can be observed. Additionally, it has been reported that BMSCs can exert immunosuppressive effects by reducing the activation and differentiation of B and T cells[20,21].

It was found that BMSCs can regulate the immune response by reducing IL-4, IL-6, and then increase the levels of IL-10 and INF-r, thereby regulating the differentiation of various T cell subtypes. This is consistent with our findings. After stem cell re-infusion and culture medium re-infusion22, IL-4 levels in the blood of AR mice decreased, while INF-r levels increased, indicating that the immune response was down-regulated. However, there was no difference of IL-4 and INF-r between the SCRg and MRg. This suggests that both approaches can perform the similar effect on immune system. Furthermore, it was found that the content of IgE was decreased; and the symptoms of AR mouse was alleviated, evaluated by behavior. This confirmed the result again. However, there was still no statistically significant difference between the two groups and not clear which aspect played a role.

After stem cell re-infusion and medium-return intervention, we used proteomics to detect the stat protein levels in the nasal mucosa. We found that the stat4/6 protein content was significantly higher in AR-sensitized group, indicating that the stat pathway was activated, a, and participated in the AR immune response. After stem cell reinfusion and medium-return intervention, the stat4/6 protein activation was reduced. It suggested that those interventions can regulate STAT 4 pathway in AR immune response. However, there was no statistically significant difference between the two groups and the mechanism was not clear.

We investigated the effect of BMSCs on AR immune response and its possible mechanism, using Elisa, flow cytometry, and proteomics analysis to detect expression changes of related cytokines and possible signaling pathways in AR mouse model after stem cell reinfusion and medium-return intervention. We found that these interventions can reduce the IgE level and IL-4 cytokines in AR mouse and increase INF-r level to balance Th1/Th2, and inhibit the AR immune response. In addition, stem cell re-infusion and medium re-infusion interventions activate the STAT4/6 signaling pathway. It was also found that the two interventions achieved similar results with no statistical difference between them. therefore, it can be concluded that BMSCs may perform effects on AR immune response through a large amount of mediators produced by direct secretion or the paracrine pathway, which regulated the cytokines and further regulated the immune response. However, the mechanism is complex and further in-depth experimental research is still needed.

Conclusions

Treatment of BMSCs and the medium of BMSCs can reduce the expression of IL-4, promote the expression of INF-γ and regulate the expression of Th cell in AR mouse model, and its mechanism is closely related to the STAT 4/6 signaling pathway. Moreover, the mechanism of action of BMSCs in regulating immune balance may be achieved by paracrine function rather than multi-directional differentiation potential.

Abbreviations

BMSCs	Bone marrow mesenchymal stem cells
OVA	ovalbumin
SCRg	Stem cell returned group
MRg	Medium returned group
ARg	AR-sensitized group
NCg	Normal control group
AR	Allergic rhinitis
STAT	Signal transduction and activator of transcription

Declarations

Ethics approval and consent to participate

All animal care and experimental procedures were ethical and approved by the Tongji University Institution Animal Care and Use Committee.

Consent for publication

Not applicable

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Authors' contributions

All the authors conceived and designed the study. Chuanliang Zhao and Wentao Zou participated and carried out the experiment. Chuanliang Zhao wrote the first draft of the manuscript, interpreted the data and Jiaxiong Zhang modified and confirmed the final version. Jingwen Sun and Xiaojing Cai also participated and carried out the experiment.

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Conflicts of Interest

The authors declare that they have no competing interests

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

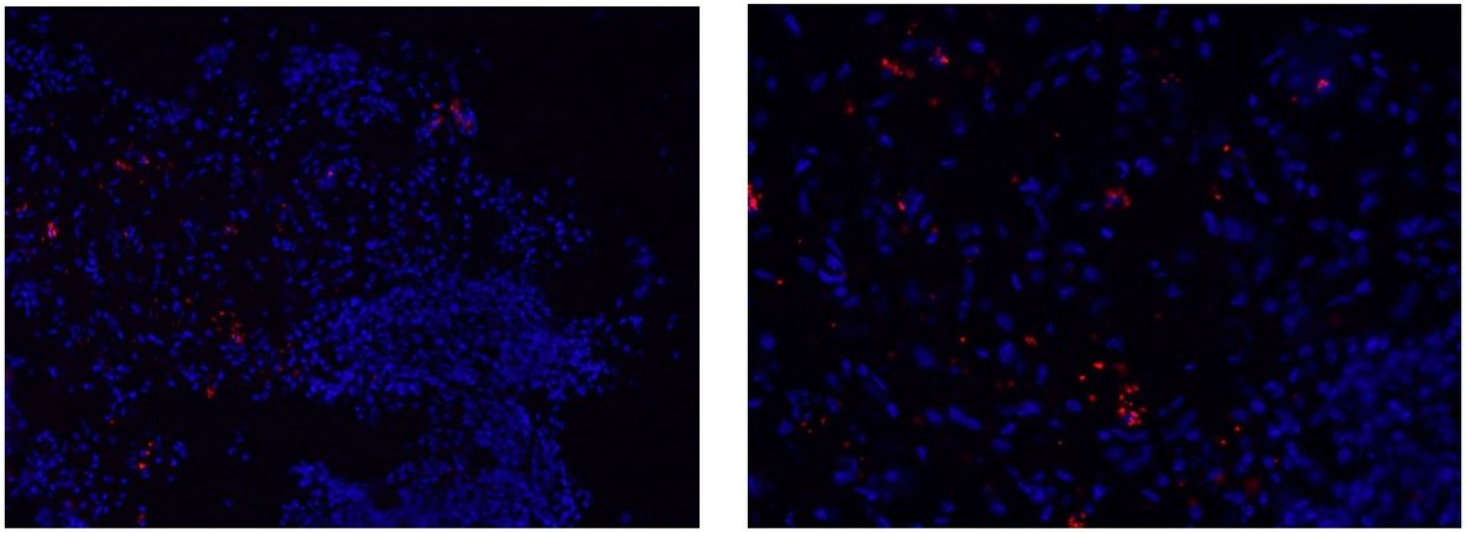


Figure 1

The migration of BMSCs was observed under a fluorescence microscope. BMSCs were visualized in the nasal mucosa (red light), and the nasal mucosa cells were stained with DAPI and displayed in blue.

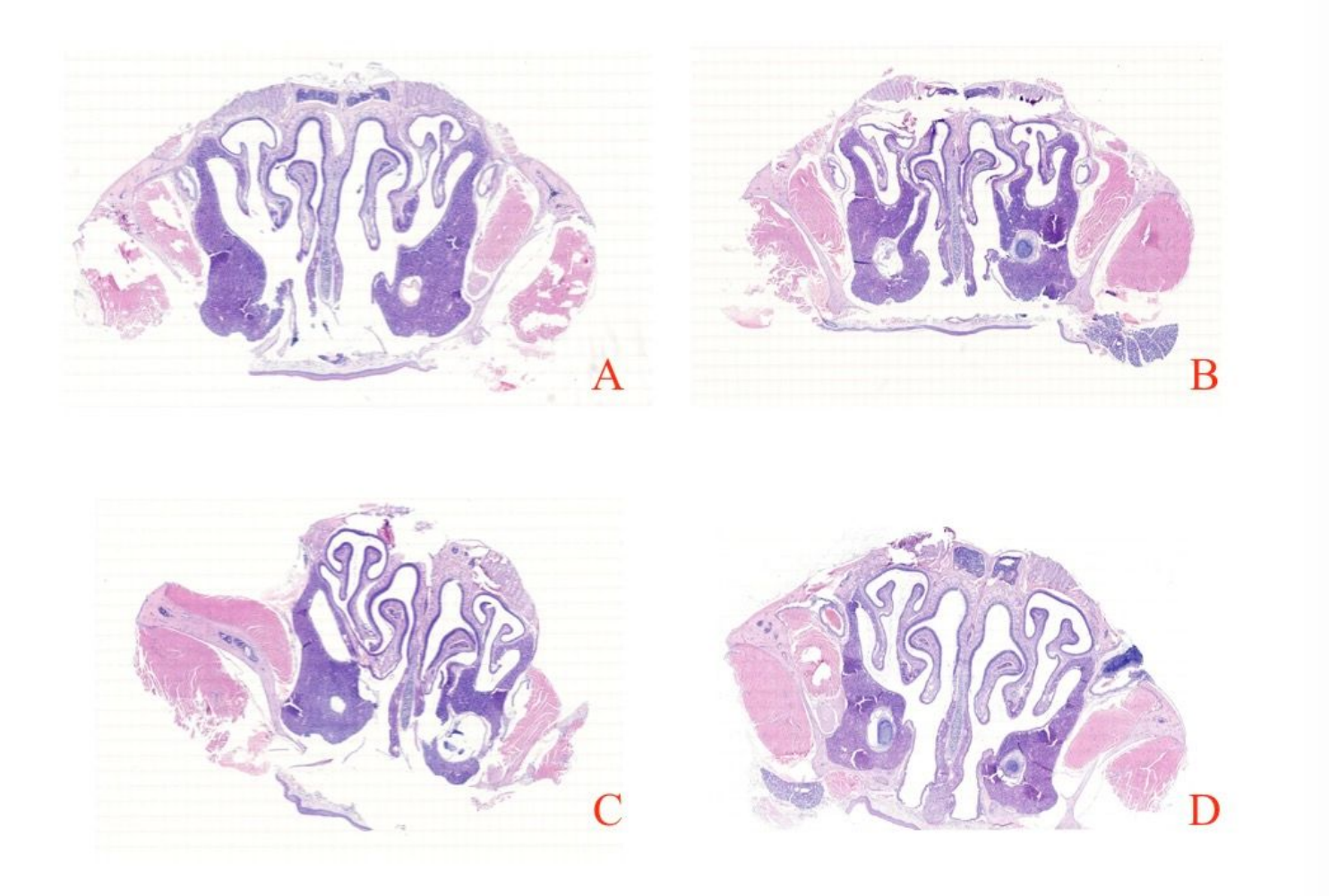


Figure 2

Through the decalcification staining of the sinus cross section, the nasal mucosa basement membrane thickening and the submucosal lamina propria inflammatory cell infiltration were significantly increased in the AR mouse model group(including the group of ARg, SCRg, MRg). This phenomenon was significantly alleviated after intervention with BMSCs or BMSCs medium. (A:ARg. B:SCRg. C:MRg. D:NCg)

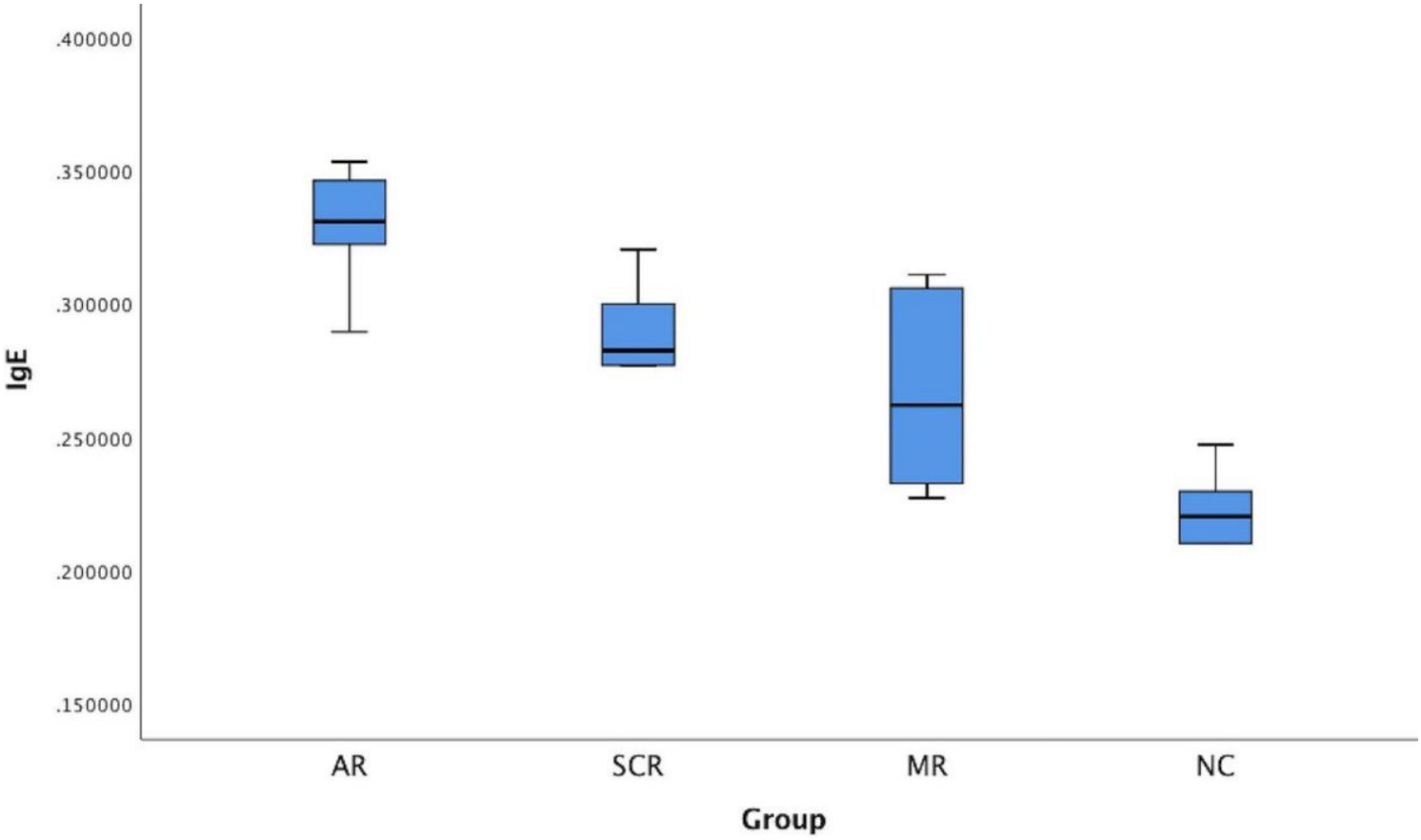


Figure 3

After OVA sensitization, IgE level in the serum increased significantly(including the group of ARg, SCRg, MRg) compared with NCg. After treatment with BMSCs or the medium of BMSCs, the IgE level in the SCRg and MRg groups decreased, but there was no statistical difference between the two groups.

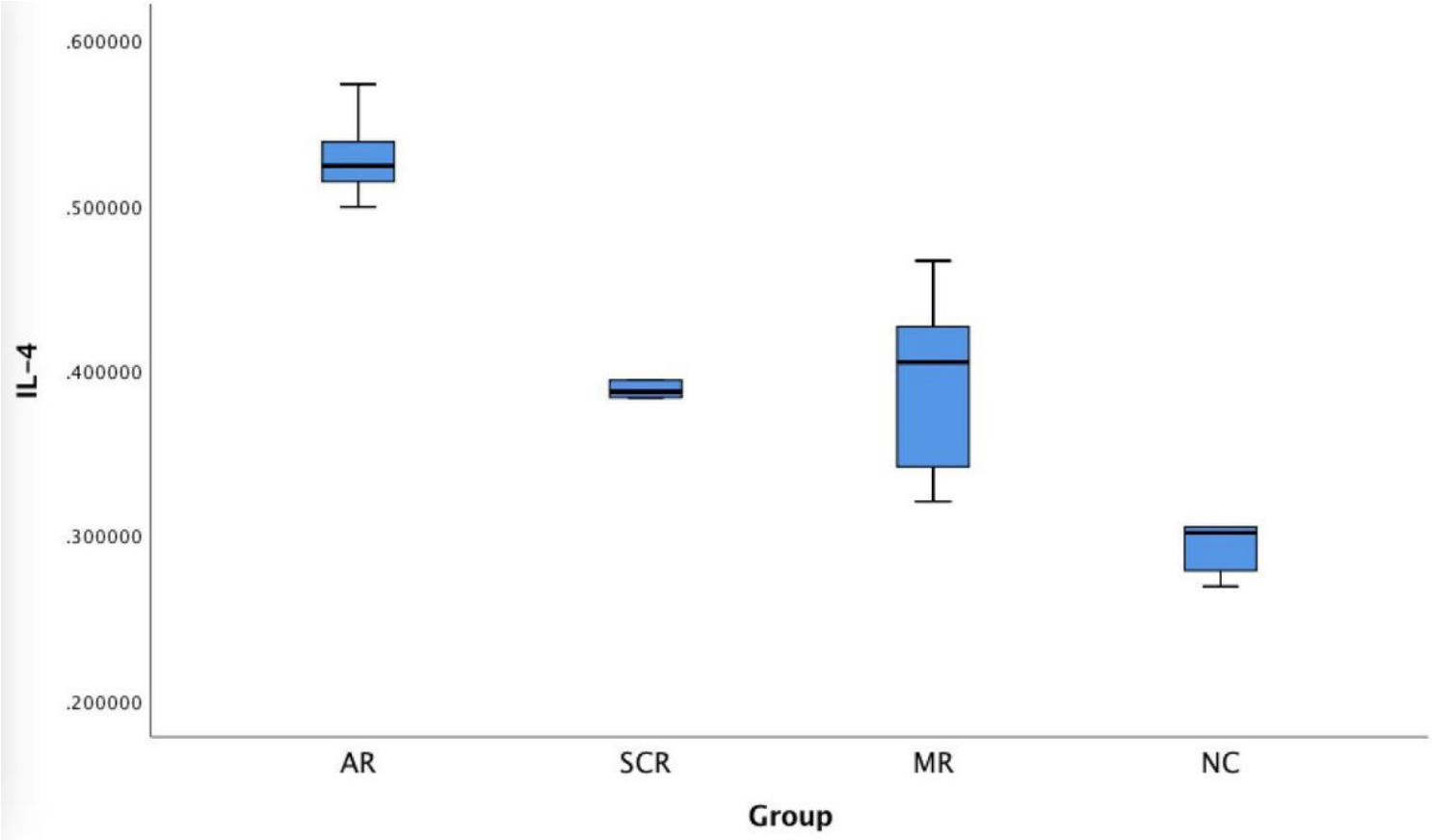


Figure 4

The IL-4 level in the AR-sensitized group (including the group of ARg, SCRg, MRg) was higher than that in the control group. After treatment with BMSCs or the medium of BMSCs, the IL-4 level in the SCRg and MRg groups decreased, but there was no statistical difference between the two groups.

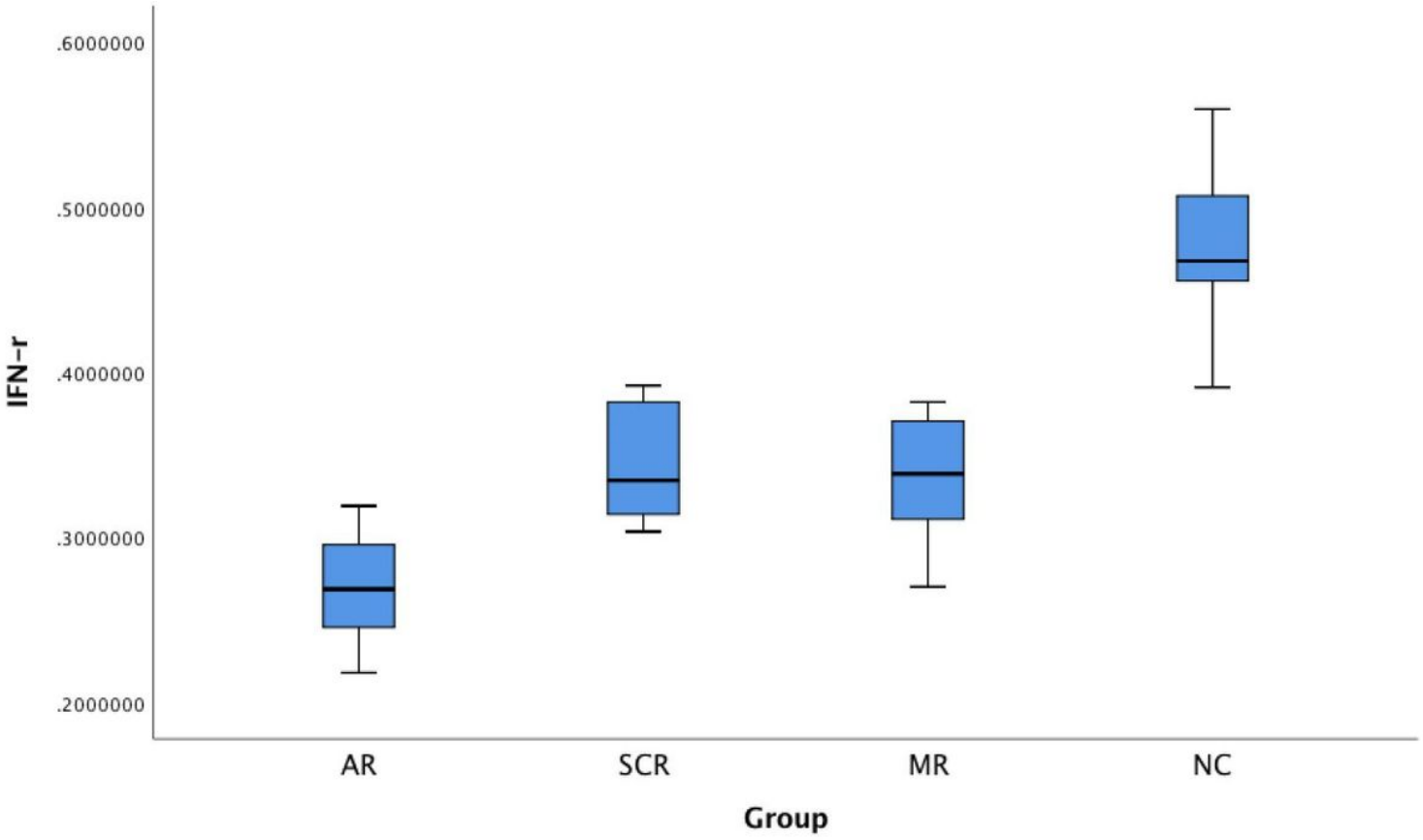


Figure 5

The level of INF-γ in the AR-sensitized group (including the group of ARg, SCRg, MRg) is lower than that in NCg, but the levels of INF-γ in SCRg and MRg are higher than in ARg.

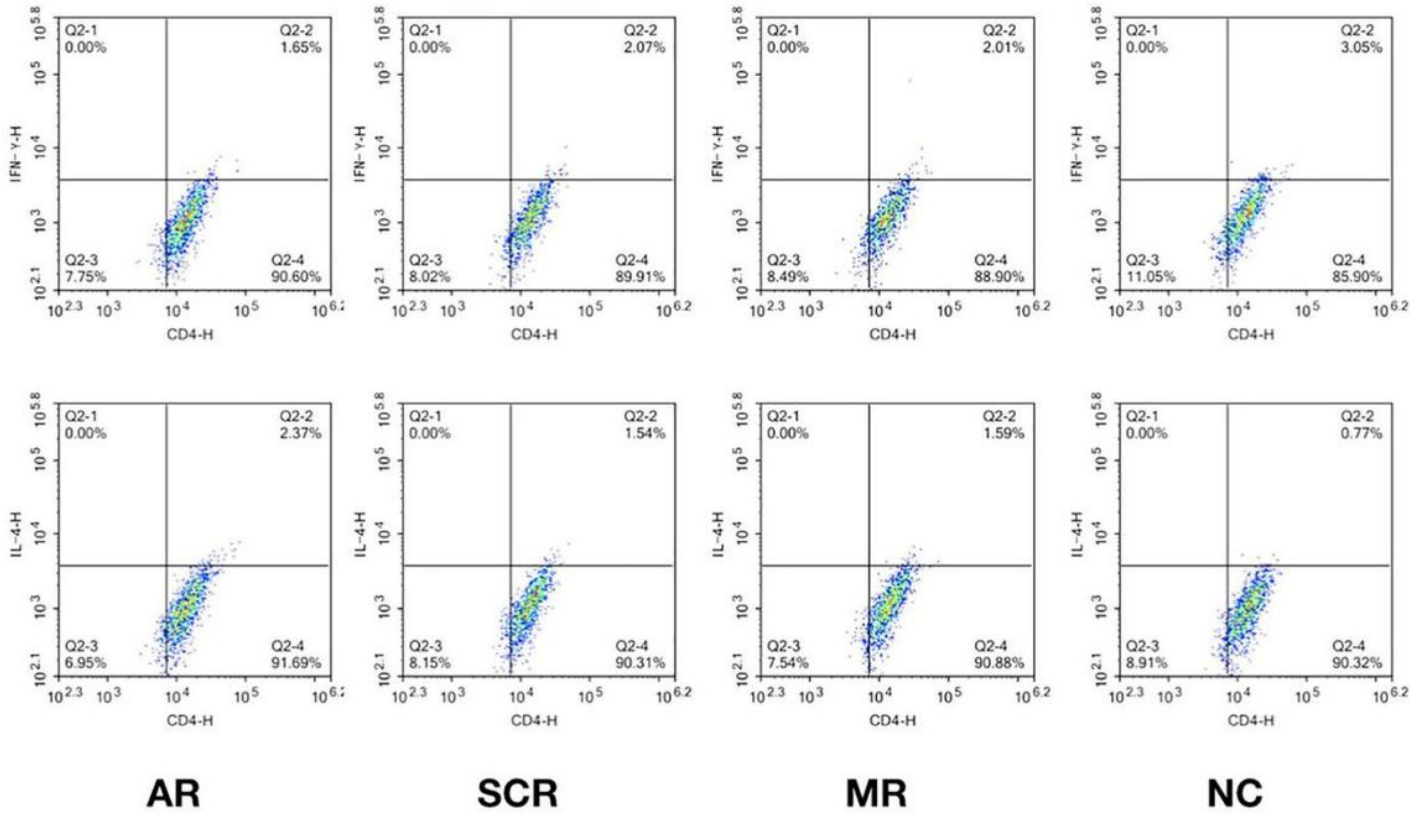


Figure 6

As showen in the figure, the ratio of Th1/2 was reduced in AR, and the Th2 cells were largely differentied. In SCR and MR, Th1 cells were increased and the ratio of Th1/2 was increased.

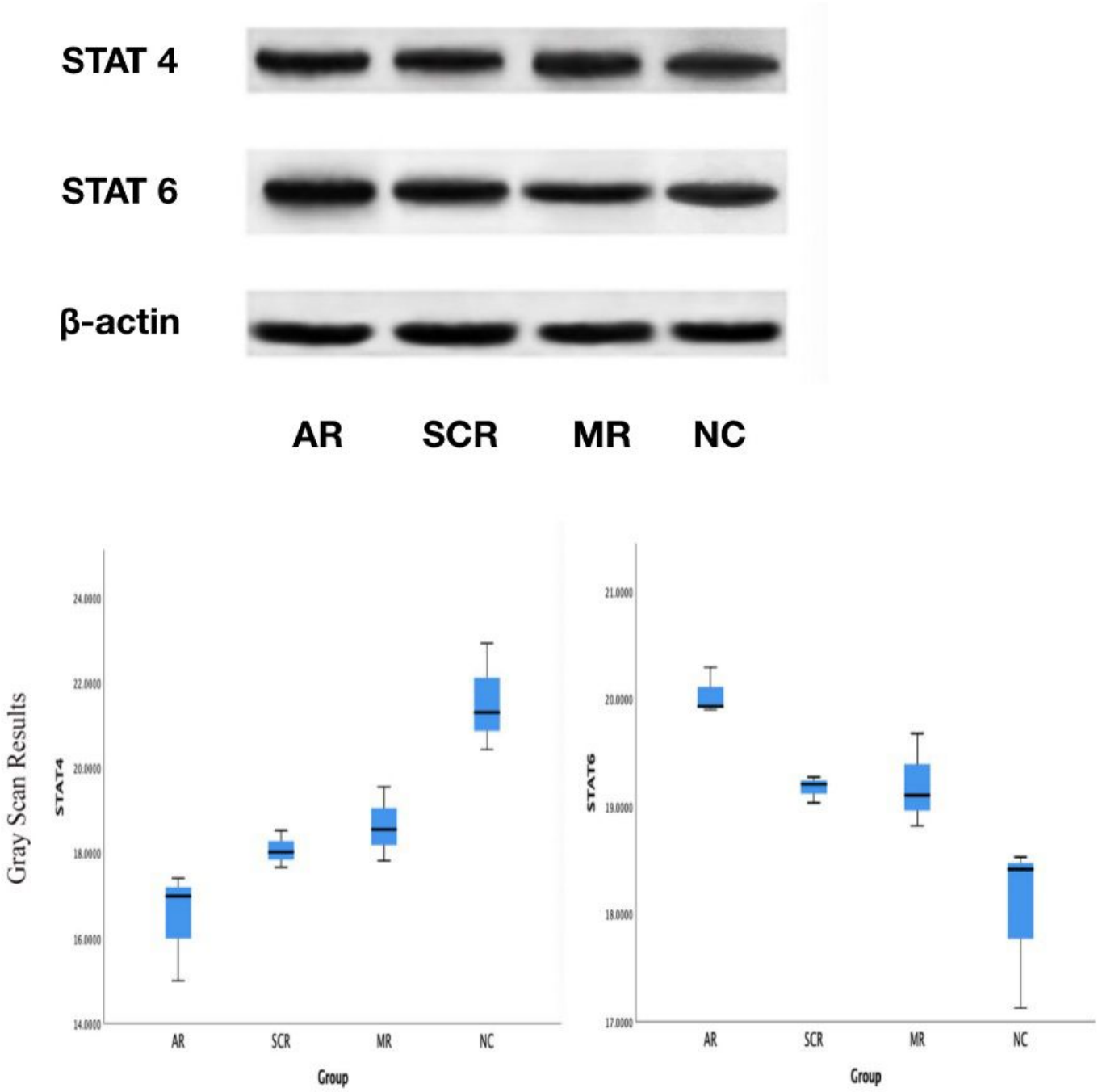


Figure 7

As shown in Fig, WB showed that the level of STAT 6 in the AR-sensitized group (including the group of ARg, SCRg, MRg) were higher than that in NCg, and the expression level of STAT 6 in the SCRg and MRg were lower than that in ARg, but there was no statistical difference between the SCRg and MRg. the level of STAT 4 in the AR-sensitized group (including the group of ARg, SCRg, MRg) were lower than that in NCg.