International Immunopharmacology 70 (2019) 295–301

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

$CD24^{hi}CD38^{hi}$ B regulatory cells from patients with end plate inflammation presented reduced functional potency

Chengping Xu[a,1], Meiyan Zhang[b,1], Kai Li[a], Ming Ni[a], Yunpeng Bai[a], Jun Zhang[a], Xin Song[a], Jian Wang[a,*]

[a] Department of Orthopedics, Pudong New Area People's Hospital affiliated to Shanghai University of Medicine & Health Sciences, Shanghai, China
[b] Department of Emergency Medicine and Critical Care, Shanghai East Hospital, Tongji University School of Medicine, Shanghai, China

ARTICLE INFO

Keywords:
$CD24^{hi}CD38^{hi}$ Breg
End plate inflammation
IL-10
PD-L1

ABSTRACT

Problem due to disc degeneration is frequently found in the aging population. However, severe pain and accompanying end plate inflammation is only found in a small subset of patients, who can be of a younger age than most people with severe disc degeneration, with no apparent cause. We hypothesized that deficiencies in B regulatory (Breg) cells might contribute to the aberrant inflammation in these patients. However, we found that the frequency of $CD24^{hi}CD38^{hi}$ Breg cells was significantly higher in patients than in controls. To investigate Breg function, $CD24^{hi}CD38^{hi}$ Breg cells were stimulated via CD40L/αIg and via *Staphylococcus aureus* Cowan. Interestingly, the expression of IL-10 and TGF-β1 was significantly lower in patients than in controls. The expression of PD-L1 was comparable between patient $CD24^{hi}CD38^{hi}$ Bregs and control $CD24^{hi}CD38^{hi}$ Bregs. Control $CD24^{hi}CD38^{hi}$ Bregs, but not patient $CD24^{hi}CD38^{hi}$ Bregs, could suppress the expression of TBX21 and RORC2 in stimulated $CD4^+$ T cells, in a manner that was dependent on IL-10 and PD-L1. The expression of FOXP3, on the other hand, was dependent on TGF-β. In addition, PD-L1 reduced the viability of $CD4^+$ T cells. Together, we demonstrated that the patients with end plate inflammation did not present a reduction in $CD19^+CD24^{hi}CD38^{hi}$ Breg frequency, but presented a reduction in $CD19^+CD24^{hi}CD38^{hi}$ Breg function.

1. Introduction

The vertebral end plate is the cartilage structure located between the intervertebral disc and the vertebra [1,2]. Mechanically, the end plate can distribute physical stress between the discs and the adjacent vertebrae and prevent the disc nucleus from bulging [3]. The end plate also serves as a nutrient transport pathway to the cells inside the disc nucleus. Small solutes, such as glucose, oxygen, and lactate, may pass through to end plate to and from the intervertebral disc via passive infusion, and the efficiency of the transport is directly correlated with the area of contact [4,5].

Chronic low back pain is a debilitating disease with increasing prevalence worldwide [6]. Degenerative changes in the innervated end plate can contribute to both painful symptoms and disc atrophy due to altered stress distribution and nutrient delivery. Age-related changes in proteoglycan and collagen, such as gradual thinning and calcification, play a part in end plate degeneration [7]. Inflammation around the end plate may also contribute to end plate degeneration [8]. Infiltration of immune cells, including T cells, macrophages, and neutrophils, are found in the disc tissue of patients with degenerated and herniated discs, as well as in experimental models of disc degeneration [9–12]. These immune cells release a multitude of cytokines, including tumor necrosis factor α (TNFα), interferon γ (IFNγ), interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-10, IL-17, and IL-23 [13–19]. Spontaneous release of inflammatory mediators by herniated disc tissues is also observed [20,21]. Both other groups and ours have demonstrated that patients with end plate degeneration and disc diseases display aberrant Th17 inflammation, characterized by IL-17 upregulation and associated changes in antigen-presenting cells [22–25]. Hence, it should be investigated whether regulatory immune cells could suppress the inflammatory responses in end plate inflammation.

B regulatory (Breg) cells are a collection of B lymphocytes with the potential to suppress inflammatory responses and maintain tolerance [26]. The $CD24^{hi}CD38^{hi}$ transitional B cell subset is among the best-characterized Breg cells, and was shown to suppress T cell responses via IL-10 release and the PD-L1 and CD80/86 pathways. Diseases characterized by chronic pathological inflammation, such as systemic lupus erythematosus, multiple sclerosis, inflammatory bowel diseases, and

* Corresponding author at: Department of Orthopedics, Pudong New Area People's Hospital, 490 Chuanhuan South Road, Shanghai, China.
E-mail address: jianwangmd@aliyun.com (J. Wang).
[1] These authors contributed equally to the work.

https://doi.org/10.1016/j.intimp.2019.02.034
Received 18 December 2018; Received in revised form 9 February 2019; Accepted 20 February 2019
Available online 07 March 2019

allergies, have been associated with dysfunctions in the Breg compartment [27–31].

The function of Breg cells in patients with disc degeneration and end plate inflammation has not been extensively investigated. We hypothesized that Breg cells might suffer from dysregulation and dysfunction in these patients, resulting in exacerbated inflammation that could contribute to further disc and end plate degeneration. With this goal, we compared the circulating Breg cells in controls and patients with idiopathic end plate inflammation.

2. Methods

2.1. Sample collection

The diagnosis, recruitment, sample collection, and experimentation were conducted at Pudong New Area People's Hospital. The patient group included 35 subjects, including 20 females and 15 males at 38.5 ± 6.1 (mean ± standard deviation) years of age at a body-mass index (BMI) at 24.1 ± 3.1 kg/m^2. The control group included 35 healthy individuals, including 22 females and 13 males at 40.1 ± 5.8 years of age at a BMI of 23.8 ± 2.7 kg/m^2. Exclusion criteria included, past or present injuries at the low back or hip, inflammatory arthritis, ankylosing spondylitis, malignancies, autoimmunity, and chronic virus infections. Six out of 35 patients and 7 out of 35 controls were smokers, and no participant were alcohol dependent, though 22 out of 35 patients and 28 out of 35 controls were casual consumers of alcohol. Patients and controls were matched in gender, age, BMI, smoking status, and alcohol consumption status. The patients reported constant, motion-restricting low back pain from the end plate that worsened with movement, without previous traumatic events or radicular symptoms. Additionally, 8 patients experienced dual hip or dual thigh pain above the knee, and 3 patients experiences unilateral hip pain. Lesions at the end plate were diagnosed and confirmed via a combination of CT scanning, MRI examination, and X-ray radiography.

After receiving written informed consent from each participant, peripheral blood was collected via venipuncture. PBMCs were collected using Ficoll gradient centrifugation (Sigma).

2.2. Flow cytometry

To evaluate the frequency of $CD24^{hi}CD38^{hi}$ Breg cells, PBMCs were incubated under 4 °C in dark with Aqua dead cell stain (Life Technologies), anti-CD19, anti-CD24, and anti-CD38 mAbs (BioLegend). To examine the expression of PD-L1 by Breg cells, an additional anti-PD-L1 mAb (BioLegend) was added. After 30 min, the cells were washed twice to remove excess antibodies and processed in FACSCanto II system (BD).

2.3. Breg stimulation

$CD19^+CD24^{hi}CD38^{hi}$ Breg cells (5×10^5 per experiment) were stained with surface antibodies and sorted in FACSAria system (BD). The cells were then incubated with were incubated in unstimulated culture media, with 1 μg/mL recombinant human CD40L carrier-free (BioLegend) + 1 μg/mL goat anti-human IgG + IgM $F(ab')_2$ (Jackson Laboratories), or with 1 μg/mL SAC (Sigma), for 72 h. The cells and the supernatant were separated by centrifugation. IL-10 and TGF-β1 ELISAs were performed using Human IL-10 Quantikine ELISA kit and Human TGF-beta 1 Quantikine ELISA kit (R&D Systems), respectively.

2.4. Breg/$CD4^+$ T cell coculture

$CD4^+$ T cells were purified from whole PBMCs via negative selection of non-$CD4^+$ T cells (Miltenyi Biotec). $CD4^+$ T cells (5×10^5 per experiment) were incubated in the presence or absence of an equal number of $CD19^+CD24^{hi}CD38^{hi}$ Breg cells, with or without 1 μg/mL SAC, 1 μg/mL anti-CD3, and 2 μg/mL anti-CD28 mAbs (BioLegend). When indicated, human IL-10 neutralizing JES3-19F1, human/mouse TGF-β1 neutralizing 19D8 (BioLegend), and PD-L1 blocking MIH1 (Thermo Fisher Scientific) were added at 10 μg/mL. Isotype control antibodies, including Rat IgG2a κ and Mouse IgG1 κ were added in separate experiments. Supernatant was replaced every 72 h. To evaluate the expression of $CD4^+$ T cell transcription factors, at the end of co-culture, $CD4^+$ T cells were re-isolated via negative selection. Viability of $CD4^+$ T cells was determined using anti-CD4 mAb (BioLegend) staining and Aqua dead cell stain.

2.5. RT-PCR

Following cell harvest, the RNA was extracted using RNeasy Mini kit (Qiagen) combined with RNase-free DNase I (Qiagen) to remove contaminating DNA. Reverse transcription was performed using iScript cDNA Synthesis kit (Bio-Rad). PCR was performed using SYBR Green Master Mix (Applied Biosystems) in a 7500 Fast Real-Time PCR system (Applied Biosystems). Initiation was performed for 10 min at 95 °C, followed by 40 cycles of amplification (20 s at 95 °C plus 60 s at 60 °C). Each gene was examined in triplicates and the result was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression level. The TaqMan primers, including Hs00894392_m1 for TBX21, Hs01076112_m1 for RORC, Hs01085834_m1 for Foxp3, Hs00961622_m1 for IL-10, Hs00204257_m1 for PD-L1, Hs00998133_m1 for TGF-β1, and Hs02786624_g1 for GAPDH (Thermo Fisher), were used for each gene.

2.6. Statistics

Individual data or mean ± standard deviation were presented. Two-tailed tests were performed for all comparisons. Unpaired *t*-test was used to compare Breg frequency between control and patient. Two-way ANOVA followed by Sidak's test was used to compare the characteristics of Breg cells and $CD4^+$ T cells from control and patient under various forms of stimulation. One-way ANOVA followed by Dunnett's test was used to compare the effects of IL-10, TGF-β, and PD-L1 inhibition. A p value smaller than 0.05 was considered significant.

3. Results

3.1. Frequency of $CD24^{hi}CD38^{hi}$ B cells in patients with end plate inflammation

To evaluate Breg cells, the peripheral blood mononuclear cells (PBMCs) were collected from 35 patients with idiopathic end plate inflammation and 35 healthy controls with matching age and gender. Using previously published gating strategy [29], we examined the frequency of the $CD24^{hi}CD38^{hi}$ population in total circulating B cells (Fig. 1A). As a group, the patients presented significantly higher frequencies of $CD24^{hi}CD38^{hi}$ B cells than controls (Fig. 1B), but the difference was small, and high variability was observed for both the patient group and the healthy group.

3.2. Expression of inhibitory molecules by $CD24^{hi}CD38^{hi}$ Breg cells in patients and controls

To assess the functional capacity of $CD24^{hi}CD38^{hi}$ Breg cells in patients and controls, the expression of inhibitory molecules was examined with or without stimulation. The IL-10 transcription was low under unstimulated condition but significantly increased in the presence of CD40L and anti-Ig (αIg), and was further increased in the presence *Staphylococcus aureus* Cowan (SAC) (Fig. 2A). Additionally, stimulated $CD24^{hi}CD38^{hi}$ Breg cells in patients presented lower IL-10 transcription than that in controls. The transcription of PD-L1 was higher in stimulated Bregs than unstimulated Bregs, but no significant

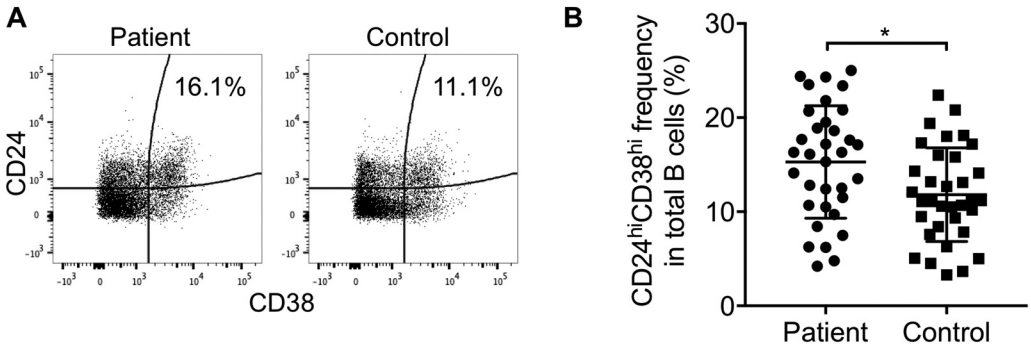

Fig. 1. Frequency of $CD24^{hi}CD38^{hi}$ cells in $CD19^+$ B cells in patients and controls.
PBMCs isolated from patients with end plate inflammation and healthy controls were stained for CD19, CD24, and CD38 expression directly ex vivo. (A) Representative gating of $CD24^{hi}CD38^{hi}$ cells (upper right quadrant) in pregated $CD19^+$ B cells from one patient and one control. (B) Frequency of $CD24^{hi}CD38^{hi}$ cells in $CD19^+$ B cells from 35 patients and 35 controls. $^*p < 0.05$.

difference was observed between patients and controls (Fig. 2B). The TGF-β1 expression was also significantly elevated following CD40L/αIg stimulation and SAC stimulation, and was significantly lower in patients than in controls (Fig. 2C).

To confirm protein expression, we stained $CD24^{hi}CD38^{hi}$ Breg cells for PD-L1 expression on the surface (Fig. 3A). The frequency of PD-$L1^+$ $CD24^{hi}CD38^{hi}$ Breg cells elevated from under 5% in unstimulated condition to approximately 10% after stimulation (Fig. 3B). No significant differences between patients and controls were observed. IL-10 and TGF-β1 secretion by $CD24^{hi}CD38^{hi}$ Breg cells was below the detection limit under unstimulated condition. After stimulation, both could be detected and the levels of expression were significantly lower in patients than in controls (Fig. 3C and D).

3.3. *$CD24^{hi}CD38^{hi}$ Breg cells significantly modulated the expression of $CD4^+$ T cell transcription factors*

Murine IL-10-producing B cells suppressed the differentiation of Th1 and Th17 cells [32]. Murine Breg cells could also elevate the expression of FOXP3 and promote Treg differentiation via TGF-β expression. To examine whether these capacities exist in $CD24^{hi}CD38^{hi}$ Breg cells, we co-incubated $CD24^{hi}CD38^{hi}$ Breg cells with autologous $CD4^+$ T cells, without stimulation and with SAC and anti-human CD3 and CD28 (3/28) for stimulation. The level of transcription factor expression in $CD4^+$ T cells was then examined. Due to the limitation that the frequency of $CD24^{hi}CD38^{hi}$ Breg cells in PBMCs was low, we only performed this experiment in patients and controls with higher than average Breg frequencies. The expression of TBX21, encoding the Th1-lineage transcription factor T-bet, was significantly elevated by SAC + 3/28 stimulation in both patients and controls, and was significantly lower in patients than in controls (Fig. 4A). In controls, the addition of Breg cells resulted in lower TBX21 expression in stimulated $CD4^+$ T cells, while such effect was not observed in patients (Fig. 4A). RORC2, which encodes the Th17-lineage transcription factor RORγt, was also upregulated by stimulation (Fig. 4B). Interestingly, in controls but not patients, the presence of Breg cells significantly reduced the RORC2 expression in stimulated $CD4^+$ T cells, resulting in lower RORC2 expression in stimulated $CD4^+$ T cells in controls than in patients (Fig. 4B). The expression of FOXP3, encoding the Treg signature transcription factor, was higher in stimulated $CD4^+$ T cells in controls than in patients (Fig. 4C). The addition of $CD24^{hi}CD38^{hi}$ Breg cells did not change the transcription of FOXP3 in either patients or controls.

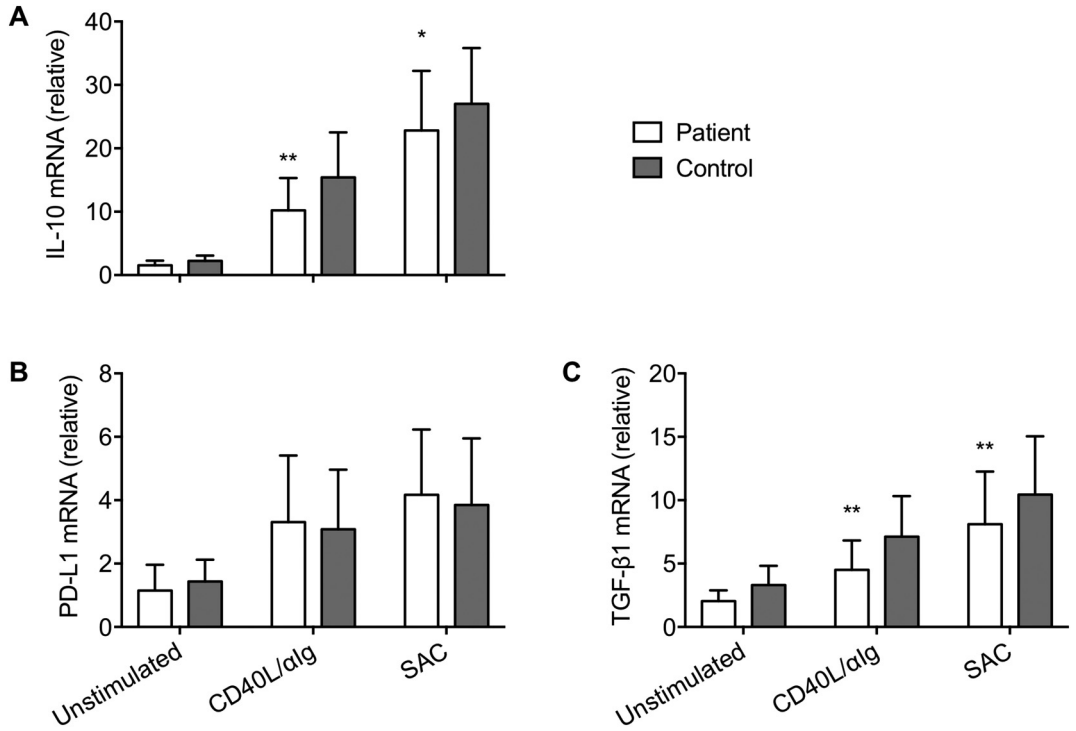

Fig. 2. Transcription of inhibitory molecules by $CD24^{hi}CD38^{hi}$ Breg cells.
$CD24^{hi}CD38^{hi}$ Bregs were flow cytometry-sorted and incubated for 72 h in blank unstimulated media, with CD40L/αIg stimulation, or with SAC stimulation. The mRNA transcript levels of (A) IL-10, (B) PD-L1, and (C) TGF-β1 were shown. $N = 35$ for both groups in (B), (C), and (D). $^*p < 0.05$. $^{**}p < 0.01$.

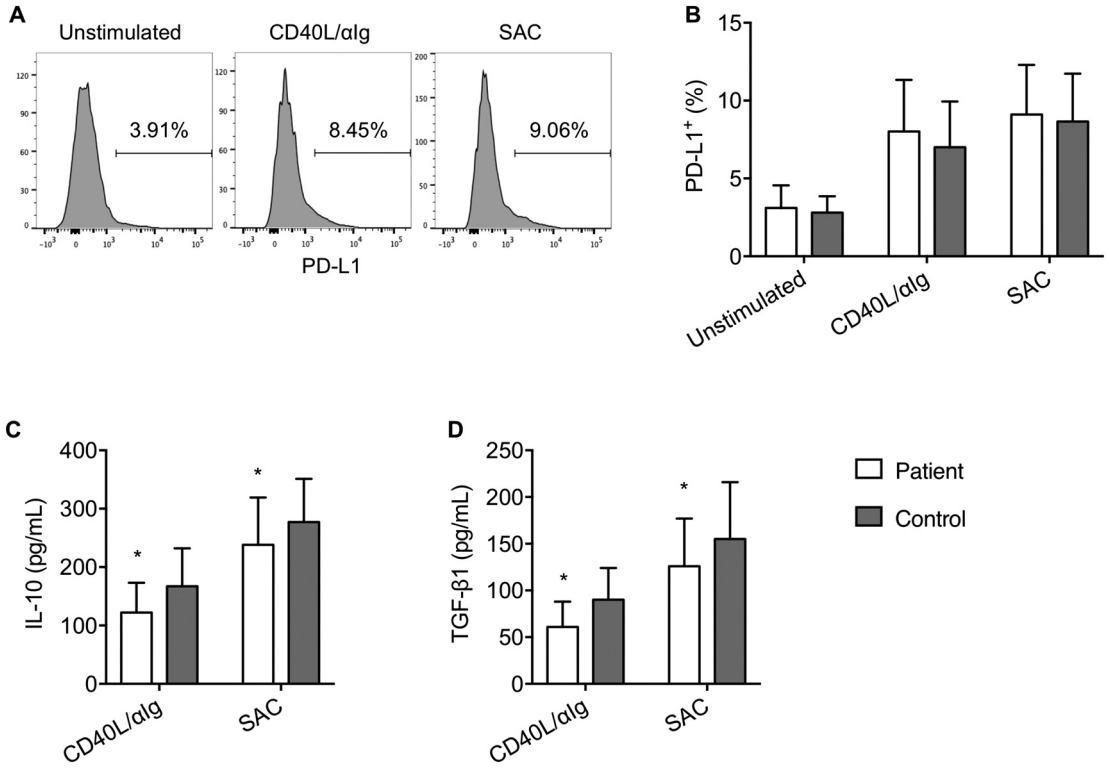

Fig. 3. Protein expression of inhibitory molecules by $CD24^{hi}CD38^{hi}$ Breg cells.
$CD24^{hi}CD38^{hi}$ Bregs were flow cytometry-sorted and incubated for 72 h in blank unstimulated media, with CD40L/αIg stimulation, or with SAC stimulation. (A) Representative gating of surface PD-L1 expression in one patient. (B) The frequency of PD-$L1^+$ $CD24^{hi}CD38^{hi}$ Breg cells in patients and controls under unstimulated condition or with CD40L/αIg or SAC stimulation. (C) IL-10 release in the supernatant following stimulation. (D) TGF-β1 release in the supernatant following stimulation. N = 35 for both groups in (B), (C), and (D). $*p < 0.05$.

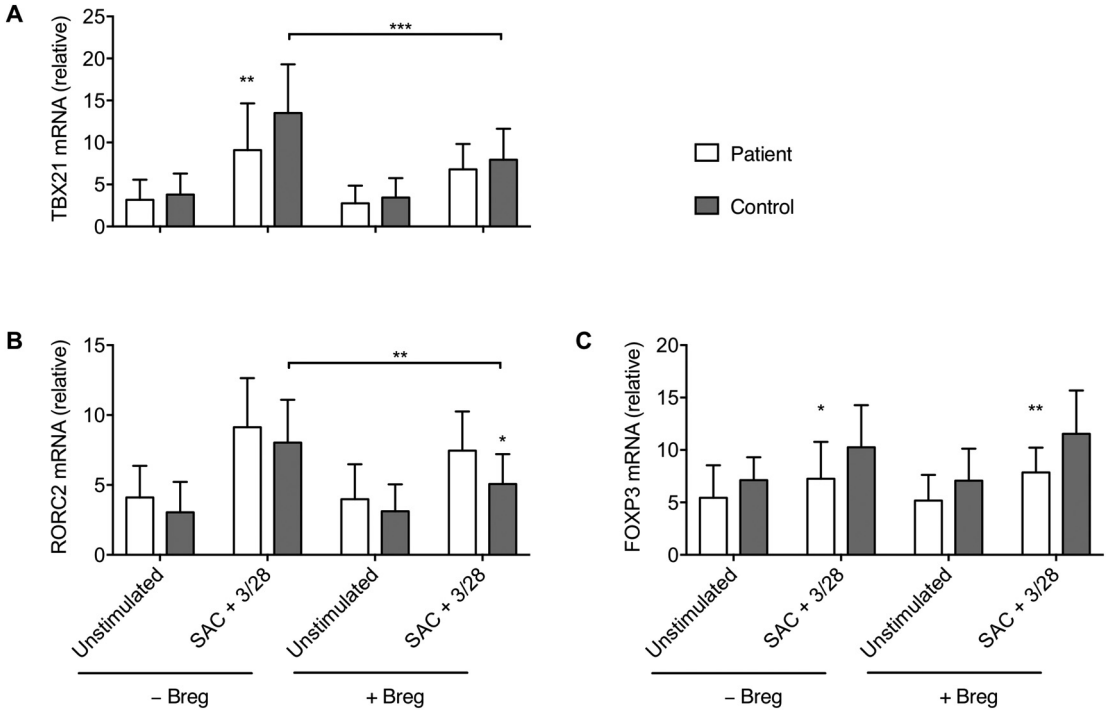

Fig. 4. Transcription factor expression by $CD4^+$ T cells with or without autologous Breg.
$CD24^{hi}CD38^{hi}$ Bregs were co-incubated with autologous $CD4^+$ T cells at 1/1 ratio, in the absence or presence of SAC and anti-human CD3 and CD28 (SAC + 3/28) stimulation. As control, Breg was absent in select cultures. Following 6-day co-incubation, $CD4^+$ T cells were negatively selected and lysed. The transcription of (A) TBX21, (B) RORC2, and (C) FOXP3 was evaluated. $N = 17$ for each group. $*p < 0.05$. $**p < 0.01$. $***p < 0.001$.

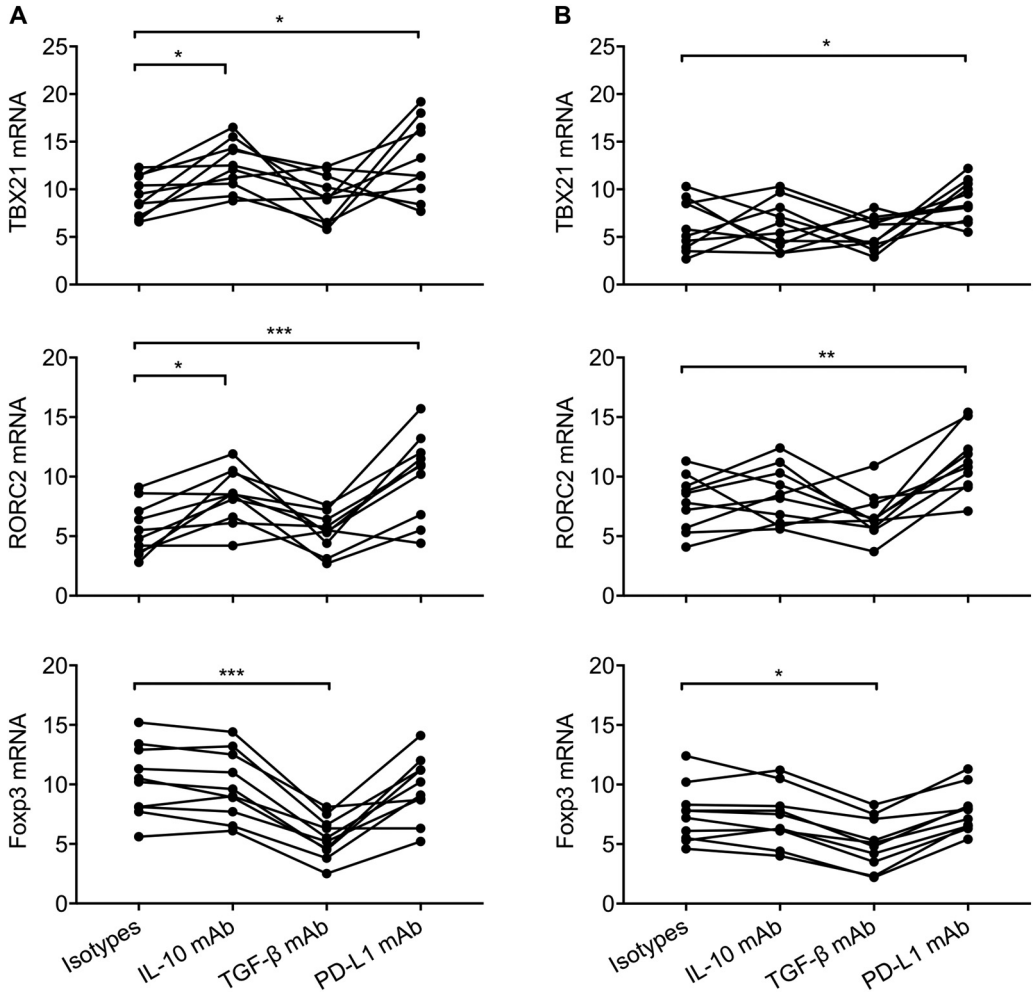

Fig. 5. Effect of IL-10, TGF-β, and PD-L1 inhibition in Breg/CD4$^+$ T cell co-culture.
CD24^{hi}CD38^{hi} Bregs were co-incubated with autologous CD4$^+$ T cells at 1/1 ratio, in the presence of SAC + 3/28 stimulation. Anti-human IL-10, TGF-β, and PD-L1 mAbs and corresponding isotype controls were added in separate experiments. The TBX21, RORC2, and Foxp3 expression in (A) control CD4$^+$ T cells and (B) patient CD4$^+$ T cells were examined following 6-day co-incubation. *p < 0.05. **p < 0.01. ***p < 0.001.

3.4. CD24^{hi}CD38^{hi} Breg-mediated suppression of TBX21 and RORC2 was dependent on IL-10 and PD-L1, and Foxp3 on TGF-β

To investigate the mechanism of action of CD24^{hi}CD38^{hi} Breg cells, we neutralized IL-10, TGF-β, and PD-L1 using corresponding blocking antibodies. Compared to isotype controls, anti-IL-10 and anti-PD-L1 monoclonal antibodies (mAbs) significantly elevated the expression of TBX21 and RORC2, in control subjects (Fig. 5A), while anti-TGF-β mAb did not produce a significant result. In patients, anti-PD-L1 mAb, but not anti-IL-10 mAb or anti-TGFβ mAb, significantly elevated the expression of TBX21 and RORC2 (Fig. 5B). Foxp3 expression, on the other hand, was significantly suppressed by anti-TGF-β mAb in both controls and patients, and was unaffected by anti-IL-10 mAb or anti-TGFβ mAb.

3.5. PD-L1, but not IL-10 or TGF-β, reduced the viability of CD4$^+$ T cells

Subsequently, we examined the effect of IL-10, TGF-β, and PD-L1 blocking on the viability of CD4$^+$ T cells, measured using Aqua dead cell stain in flow cytometry. Compared to isotype controls, anti-PD-L1 mAb significantly increased the viability of patient CD4$^+$ T cells and control CD4$^+$ T cells (Fig. 6A and B). The anti-IL-10 mAb or anti-TGF-β mAb, on the other hand, did not produce a significant difference.

4. Discussion

Disc degeneration due to aging is found in most individuals, yet, only a small subset experience significant pain accompanied with end plate lesion and inflammation, with no apparent cause and occasionally at younger ages. Other groups and we have found that low back pain is frequently accompanied by aberrant proinflammatory immune responses at the degenerated disc, the end plate, and systemically in circulating T cells [3,23–25,33]. These observations might be associated with a defective regulatory compartment of the immune system.

Since Breg cells play an important role in the maintenance of tolerance and suppression of autoimmune responses, we hypothesized that a lack of Breg cells might be found in these patients. However, the frequency of CD19$^+$CD24^{hi}CD38^{hi} Breg cells was higher in patients than in controls, though large variations were found in both groups. Subsequently, we examined the expression of inhibitory molecules expressed by CD19$^+$CD24^{hi}CD38^{hi} Breg cells from patients and controls. Interestingly, patient Breg cells exhibited lower IL-10 expression and TGF-β1 expression upon antigen-dependent (CD40L/αIg) stimulation and antigen-independent (SAC) stimulation. The expression of PD-L1, on the other hand, was comparable between patient Breg cells and control Breg cells. While control Breg cells suppressed the expression of TBX21 and RORC2 in CD4$^+$ T cells, patient Breg cells was incapable of altering the expression of these transcription factors. The mechanism of action by CD19$^+$CD24^{hi}CD38^{hi} Breg cells was most commonly

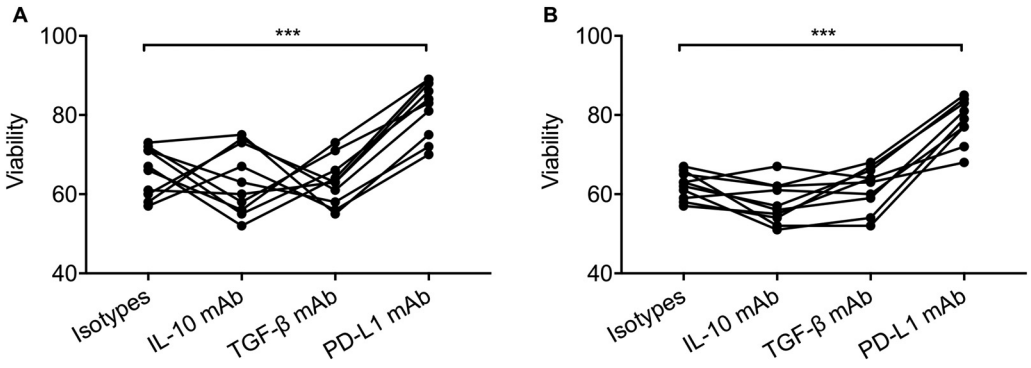

Fig. 6. Effect of IL-10, TGF-β, and PD-L1 inhibition on the viability of $CD4^+$ T cells.
$CD24^{hi}CD38^{hi}$ Bregs were co-incubated with autologous $CD4^+$ T cells at 1/1 ratio, in the presence of SAC + 3/28 stimulation. Anti-human IL-10, TGF-β, and PD-L1 mAbs and corresponding isotype controls were added in separate experiments. The viability of control $CD4^+$ T cells, and the viability of (B) patient $CD4^+$ T cells, were examined following 6-day co-incubation. ***$p < 0.001$.

attributed to the production of IL-10, PD-L1, and other surface molecules such as CD80/86 and CD1d [26]. In this study, we observed that the $CD19^+CD24^{hi}CD38^{hi}$ Breg cells possessed the capacity of producing TGF-β1 in both patients and controls. TGF-β1 is instrumental to the development of Treg cells, and TGF-β-expressing Breg cells promoted Treg differentiation [34–36]. Here, we showed that TGF-β inhibition reduced the expression of Foxp3 but did not affect the expression of TBX21 or RORC2, or the viability, of the $CD4^+$ T cells in the coculture with $CD19^+CD24^{hi}CD38^{hi}$ Bregs. In controls, IL-10 and PD-L1, but not TGF-β, contributed to the suppression of TBX21 and RORC2, while in patients, PD-L1 contributed to the suppression of TBX21 and RORC2. In both controls and patients, PD-L1, but not IL-10 or TGF-β, reduced the viability of stimulated $CD4^+$ T cells. Together, we demonstrated that the patients with end plate inflammation did not present a reduction in $CD19^+CD24^{hi}CD38^{hi}$ Breg frequency, but presented a reduction in $CD19^+CD24^{hi}CD38^{hi}$ Breg function.

Due to the above discoveries, strategies that enhance Breg function might provide new opportunities for the treatment of disc degeneration and end plate inflammation. However, several challenges are present. First, the experiments were performed entirely using peripheral blood Breg cells, and the high frequency of blood Breg cells did not translate to high functional potency. Hence, the frequency and function of Breg cells at the disc and infiltrating blood from the end plate should be investigated, upon receiving relevant samples. Also, in the laboratory setting, activation of Breg cells is usually performed using anti-Ig, CD40L, PMA, ionomycin, and TLR agonists such as SAC [26]. The suitability of these stimulants in the setting of disc degeneration may appear questionable at first, since these agents were rarely present in blood. However, several studies have identified that low-virulence bacterial and viral infection may be associated with disc degeneration and end plate inflammation [37,38]. Whether these foreign antigens could stimulate Breg cells in vivo should be further investigated. Also, the pros and cons of using Breg cells versus immunosuppressive drugs should also be considered. It remains unclear whether improvement in Breg function could actually reduce the rate of disc degeneration and ameliorate low back pain. For this reason, the association between Breg function and clinical parameters of the disease should be further examined. The patients should be followed for an extended period of time to investigate the association between Breg function and frequency with clinical progression of the disease. In addition, animal models have been developed to simulate human disc disorders and degeneration, including spontaneous models with collagen mutations or accelerated aging, and vertebral damage models induced by chemical or mechanical insults [39]. The role of Breg cells should be further confirmed in these animal models.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the Academic Leader Program of Pudong New Area Commission of Health and Family Planning (PWRd2017-08); the Science and Technology Development Funding of Pudong New Area (PKJ2017-Y33); the Pudong New Area Health System Key Speciality Project (PWZzk2017-015).

References

[1] R.J. Moore, The vertebral endplate: disc degeneration, disc regeneration, Eur Spine J. Springer 15 (Suppl. 3) (2006) S333–S337, https://doi.org/10.1007/s00586-006-0170-4.
[2] R.J. Moore, The vertebral end-plate: what do we know? Eur. Spine J. (2000), https://doi.org/10.1007/s005860050217.
[3] J.C. Lotz, A.J. Fields, E.C. Liebenberg, The role of the vertebral end plate in low back pain, Glob spine J. SAGE Publications 3 (2013) 153–164, https://doi.org/10.1055/s-0033-1347298.
[4] A. Nachemson, T. Lewin, A. Maroudas, M.A.R. Freeman, In vitro diffusion of DYE through the end-plates and the annulus fibrosus of human lumbar inter-vertebral discs, Acta Orthop. (1970), https://doi.org/10.3109/17453677008991550.
[5] S. Roberts, H. Evans, J. Trivedi, J. Menage, et al., Histology and pathology of the human intervertebral disc, J. Bone Joint Surg. Am. 88 (Suppl. 2) (2006) 10–14, https://doi.org/10.2106/JBJS.F.00019.
[6] J.K. Freburger, G.M. Holmes, R.P. Agans, A.M. Jackman, J.D. Darter, A.S. Wallace, et al., The rising prevalence of chronic low back pain, Arch Intern Med. NIH Public Access 169 (2009) 251–258, https://doi.org/10.1001/archinternmed.2008.543.
[7] Antoniou J, Goudsouzian NM, Heathfield TF, Winterbottom N, Steffen T, Poole AR, et al. The human lumbar endplate: evidence of changes in biosynthesis and denaturation of the extracellular matrix with growth, maturation, aging, and degeneration. Spine. 1996. doi:https://doi.org/10.1097/00007632-199605150-00006.
[8] C. Xiong, Y. Huang, H. Kang, T. Zhang, F. Xu, X. Cai, Macrophage inhibition factor–mediated CD74 signal modulate inflammation and matrix metabolism in the degenerated cartilage endplate chondrocytes by activating extracellular signal regulated kinase 1/2, Spine (Phila Pa 1976) 42 (2017) E61–E70, https://doi.org/10.1097/BRS.0000000000001726.
[9] M.F. Shamji, L.A. Setton, W. Jarvis, S. So, J. Chen, L. Jing, et al., Proinflammatory cytokine expression profile in degenerated and herniated human intervertebral disc tissues, Arthritis Rheum. NIH Public Access 62 (2010) 1974–1982, https://doi.org/10.1002/art.27444.
[10] A. Geiss, K. Larsson, K. Junevik, B. Rydevik, K. Olmarker, Autologous nucleus pulposus primes T cells to develop into interleukin-4-producing effector cells: an experimental study on the autoimmune properties of nucleus pulposus, J. Orthop. Res. 27 (2009) 97–103, https://doi.org/10.1002/jor.20691.
[11] A. Kanerva, B. Kommonen, M. Gronblad, J. Tolonen, A. Habtemariam, J. Virri, et al., Inflammatory cells in experimental intervertebral disc injury, Spine (Phila Pa 1976) 22 (1997) 2711–2715, https://doi.org/10.1097/00007632-199712010-00002.
[12] S. Dudli, A.J. Fields, D. Samartzis, J. Karppinen, J.C. Lotz, Pathobiology of modic changes, Eur. Spine J. 25 (2016) 3723–3734, https://doi.org/10.1007/s00586-016-4459-7.
[13] C.K. Kepler, D.Z. Markova, A.S. Hilibrand, A.R. Vaccaro, M.V. Risbud, T.J. Albert, et al., Substance p stimulates production of inflammatory cytokines in human disc cells, Spine (Phila Pa 1976) 38 (2013) E1291–E1299, https://doi.org/10.1097/BRS.0b013e3182a42bc2.
[14] J.M. Cuéllar, P.M. Borges, V.G. Cuéllar, A. Yoo, G.J. Scuderi, D.C. Yeomans, Cytokine expression in the epidural space: a model of noncompressive disc herniation-induced inflammation, Spine (Phila Pa 1976) 38 (2013) 17–23, https://doi.org/10.1097/BRS.0b013e3182604baa.
[15] M.V. Risbud, I.M. Shapiro, Role of cytokines in intervertebral disc degeneration: pain and disc content, Nat Rev Rheumatol. NIH Public Access 10 (2014) 44–56, https://doi.org/10.1038/nrrheum.2013.160.
[16] D. Purmessur, B.A. Walter, P.J. Roughley, D.M. Laudier, A.C. Hecht, J. Iatridis, A role for TNFa in intervertebral disc degeneration: a non-recoverable catabolic shift,

Biochem. Biophys. Res. Commun. 433 (2013) 151–156, https://doi.org/10.1016/j.bbrc.2013.02.034.
[17] C. Shen, J. Yan, L.-S. Jiang, L.-Y. Dai, Autophagy in rat annulus fibrosus cells: evidence and possible implications, Arthritis Res Ther. 13 (2011) R132, https://doi.org/10.1186/ar3443.
[18] S. Kawaguchi, T. Yamashita, K. Yokogushi, T. Murakami, O. Ohwada, N. Sato, Immunophenotypic analysis of the inflammatory infiltrates in herniated intervertebral discs, Spine (Phila Pa 1976) 26 (2001) 1209–1214. Available: http://www.ncbi.nlm.nih.gov/pubmed/11389385.
[19] C. Weiler, A.G. Nerlich, B.E. Bachmeier, N. Boos, Expression and distribution of tumor necrosis factor alpha in human lumbar intervertebral discs: a study in surgical specimen and autopsy controls, Spine (Phila Pa 1976) 30 (2005) 44–53 discussion 54. Available: http://www.ncbi.nlm.nih.gov/pubmed/15626980.
[20] C.L. Le Maitre, J.A. Hoyland, A.J. Freemont, Catabolic cytokine expression in degenerate and herniated human intervertebral discs: IL-1beta and TNFalpha expression profile, Arthritis Res Ther 9 (2007) R77, https://doi.org/10.1186/ar2275.
[21] J.G. Burke, R.W.G. Watson, D. McCormack, F.E. Dowling, M.G. Walsh, J.M. Fitzpatrick, Spontaneous production of monocyte chemoattractant protein-1 and interleukin-8 by the human lumbar intervertebral disc, Spine (Phila Pa 1976) 27 (2002) 1402–1407. Available: http://www.ncbi.nlm.nih.gov/pubmed/12131736.
[22] P. Tian, Z.-J. Li, X. Fu, X.-L. Ma, Role of interleukin-17 in chondrocytes of herniated intervertebral lumbar discs, Exp Ther Med. Spandidos Publications 10 (2015) 81–87, https://doi.org/10.3892/etm.2015.2449.
[23] J. Wang, W. Sun, A. Bond, C. Xu, K. Li, D. Ren, et al., Patients with idiopathic endplate inflammation displayed aberrant Th17 responses resulting in proinflammatory monocyte activation, Exp. Cell Res. (1 Feb 2018) 33–38, https://doi.org/10.1016/j.yexcr.2018.01.006.
[24] H. Jiang, Y. Deng, T. Wang, J. Ma, P. Li, P. Tian, et al., Interleukin-23 may contribute to the pathogenesis of lumbar disc herniation through the IL-23/IL-17 pathway, J Orthop Surg Res. BioMed Central 11 (2016) 12, https://doi.org/10.1186/s13018-016-0343-8.
[25] J. Wang, W. Sun, A. Bond, C. Xu, K. Li, D. Ren, et al., A positive feedback loop between Th17 cells and dendritic cells in patients with endplate inflammation, Immunol. Investig. (2018) 1–13, https://doi.org/10.1080/08820139.2018.1496097.
[26] C. Mauri, M. Menon, Human regulatory B cells in health and disease: therapeutic potential, J. Clin. Investig. (2017), https://doi.org/10.1172/JCI85113.
[27] S. Sattler, G.-S. Ling, D. Xu, L. Hussaarts, A. Romaine, H. Zhao, et al., IL-10-producing regulatory B cells induced by IL-33 (Breg(IL-33)) effectively attenuate mucosal inflammatory responses in the gut, J Autoimmun. Elsevier 50 (2014) 107–122, https://doi.org/10.1016/j.jaut.2014.01.032.
[28] Wang X, Zhu Y, Zhang M, Wang H, Jiang Y, Gao P, et al. Ulcerative colitis is characterized by a decrease in regulatory B cells. J Crohns Colitis. The Oxford University Press; 2016;51: 127–145. doi:10.1093/ecco-jcc/jjw074.
[29] Blair PA, Noreña LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic lupus erythematosus patients. Immunity. 2010;32: 129–140. doi:https://doi.org/10.1016/j.immuni.2009.11.009.
[30] S. Knippenberg, E. Peelen, J. Smolders, M. Thewissen, P. Menheere, J.W. Cohen Tervaert, et al., Reduction in IL-10 producing B cells (Breg) in multiple sclerosis is accompanied by a reduced naïve/memory Breg ratio during a relapse but not in remission, J. Neuroimmunol. (2011) 80–86, https://doi.org/10.1016/j.jneuroim.2011.08.019.
[31] R. Kamekura, K. Shigehara, S. Miyajima, S. Jitsukawa, K. Kawata, K. Yamashita, et al., Alteration of circulating type 2 follicular helper T cells and regulatory B cells underlies the comorbid association of allergic rhinitis with bronchial asthma, Clin. Immunol. (2015), https://doi.org/10.1016/j.clim.2015.02.016.
[32] N. Carter, E. Rosser, C. Mauri, Interleukin-10 produced by B cells is crucial for the suppression of Th17/Th1 responses, induction of T regulatory type 1 cells and reduction of collagen-induced arthritis, Arthritis Res Ther 14 (2012) R32, https://doi.org/10.1186/ar3736.
[33] R. Rahme, R. Moussa, The modic vertebral endplate and marrow changes: pathologic significance and relation to low back pain and segmental instability of the lumbar spine, Am. J. Neuroradiol. (2008) 838–842, https://doi.org/10.3174/ajnr.A0925.
[34] W. Chen, J.E. Konkel, Development of thymic Foxp3 $^{+}$ regulatory T cells: TGF-β matters, Eur. J. Immunol. 45 (2015) 958–965, https://doi.org/10.1002/eji.201444999.
[35] S.G. Zheng, Regulatory T cells vs Th17: differentiation of Th17 versus Treg, are the mutually exclusive? Am J Clin Exp Immunol. e-Century Publishing Corporation 2 (2013) 94–106. Available: http://www.ncbi.nlm.nih.gov/pubmed/23885327.
[36] K.M. Lee, R.T. Stott, G. Zhao, J. SooHoo, W. Xiong, M.M. Lian, et al., TGF-β-producing regulatory B cells induce regulatory T cells and promote transplantation tolerance, Eur. J. Immunol. 44 (2014) 1728–1736, https://doi.org/10.1002/eji.201344062.
[37] R. Ganko, P.J. Rao, K. Phan, R.J. Mobbs, Can bacterial infection by low virulent organisms be a plausible cause for symptomatic disc degeneration? A systematic review, Spine (2015), https://doi.org/10.1097/BRS.0000000000000832.
[38] K. Alpantaki, P. Katonis, A.G. Hadjipavlou, D.A. Spandidos, G. Sourvinos, Herpes virus infection can cause intervertebral disc degeneration: a causal relationship? Bone Joint J (2011), https://doi.org/10.1302/0301-620X.93B9.27002.
[39] M. Alini, S.M. Eisenstein, K. Ito, C. Little, A.A. Kettler, K. Masuda, et al., Are animal models useful for studying human disc disorders/degeneration? Eur. Spine J. (2008), https://doi.org/10.1007/s00586-007-0414-y.