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Original Research Article

Quantitative variations of $CD4^+CD25^+$ cells in Peking duck-white leghorn chimeras based on bone marrow mesenchymal stem cells

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Abstract

Purpose: *To develop a chimera via microinjection of poultry xenogeneic bone marrow mesenchymal stem cells (BMMSCs), and to assess its immune tolerance based on variations in proportion of $CD4^+CD25^+$ cells in $CD4^+$ cells (specific $CD4^+CD25^+$ cells).*
Methods: *BMMSCs were flush out from femurs and tibias of Peking ducks with phosphate-buffered saline and cultured. Their morphology was determined with a microscope. Several surface markers (i.e., CD44, CD45, CD71, CD73 and CD34) were used to identify the cells.*
Results: *The results indicate successful chimera development. $CD4^+CD25^+$ cells derived from the thymus of chimeras were migrated to the spleen and cecal tonsils. This migration was more obvious in chimeras than in the control group, suggesting a more robust immune system in the chimeras. The migration tendency gradually decreased with time. There were significant increases in specific $CD4^+CD25^+$ cells, TGF-β and IL-10 in cecal tonsils throughout the experimental period (30 days). However, in thymus and spleen, variations in specific $CD4^+CD25^+$ cells were observed only on the 1st day post-hatching.*
Conclusion: *The results suggest a relatively pure BMMSC population without contaminating hematopoietic stem cells. Differentiation of the BMMSCs into osteoblasts and adipocytes was inducible, indicating typical MSC character.*

Keywords: *Bone marrow mesenchymal stem cells, Immune tolerance, Chimera, Specific $CD4^+CD25^+$ cells, Cell migration*

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INTRODUCTION

Mesenchymal stem cells (MSCs) are widely focused on for their differentiation capacity and potential therapeutic effects on many diseases, e.g., autoimmune disease and graft rejection. MSCs can be obtained from several sources, e.g., bone marrow, fetal blood, fetal liver, and adipose tissue [1,2]. MSCs can be extensively expanded and differentiated *in vitro* into a variety of other cell types, e.g., osteogenic, chondrogenic, and adipogenic cells, depending on the stimulus and cultural conditions [3-5]. MSCs can regulate immune responses invoked in tissue injury, transplantation, and autoimmunity. In addition, MSCs also participate in immune regulation by suppressing T cell proliferation and increasing the amount of regulatory T cells (T_{reg}s) [6].

T_{reg}s are specialized in immune suppression [7]. These cells suppress effector T cells proliferation

and subsequently inhibit cytokines production via effector T cells, maintaining self-tolerance of the host [8]. In 1995, $CD4^+CD25^+$ T cells were identified with suppressive properties of T_{reg}s in mice [9]. Subsequently, FoxP3, a specific marker for T_{reg}s, was identified in several species, and $CD4^+CD25^+FoxP3^+$ cells were described as T_{reg}s [10,11]. FoxP3 was not identified via expressed sequence tag [12] or polymerase chain reaction (PCR) in chickens [13]. $CD4^+CD25^+$ cells in poultry exerted suppressive effects similar to those of T_{reg}s in mammals. Thus, $CD4^+CD25^+$ cells in chickens were selected as target T_{reg}s in the present study. A vital role was played by these cells in the induction and maintenance of self- and non-self tolerance, and they have also been implicated in chimera generation [14]. However, studies on $CD4^+CD25^+$ cells have typically focused on mammals, while studies on changes in $CD4^+CD25^+$ cells in chimeras formed via xenogenic MSCs in poultry are rare. Thus, the role of MSCs in changes of $CD4^+CD25^+$ in chimeras remains unknown.

In this paper, a chimera model was developed via microinjection of donor cells, i.e., bone marrow MSCs (BMMSCs) from Peking ducks, into the blood vessels of White Leghorns at Hamburger Hamilton Stage 13 (HH13) [15]. Then, variations of $CD4^+CD25^+$ cells in the thymus, spleen, cecal tonsils, and Bursa of Fabricius of the chimeras were studied. TGF-β and IL-10 are positively correlated with changes in $CD4^+CD25^+$ cells [16,17]. Moreover, MSCs affect T cell proliferation via TGF-β and IL-10 [5]. Thus, the mRNA expressions of TGF-β and IL-10 were also examined. Therefore, this paper was to explore the immune tolerance of chimeras and lay a foundation for studies on MSC therapy.

EXPERIMENTAL

Experimental animals

Newly-hatched Peking ducks were purchased from the Chinese Academy of Agricultural Sciences (Beijing, China), and hatching White Leghorn eggs were obtained from the China Agricultural University (CAU) (Beijing, China). Animal care and experimental procedures were conducted according to international guidelines [18] and the ethical committee of CAU (approval no. SKLAB-2014-07-01).

Isolation and culture of BMMSCs

Femurs and tibias from day-old Peking ducks were harvested and separated from the surrounding muscles and connective tissue using surgical scissors and forceps. Subsequently, they were disinfected with medicinal alcohol (75 %) for 3 min, and then washed with phosphate-buffered saline (PBS). Thereafter, both ends of the femurs and tibias were cut off, and the marrow was flushed out with PBS into a clean bench. The marrow was centrifuged at 1000 rpm/min for 5 min. The pellets were re-suspended in a complete medium, containing fetal bovine serum (FBS, 10 % (v/v)) (Gibco, Carlsbad, CA, USA), L-glutamine (2 mM) (Sigma, St. Louis, MO, USA), basic fibroblast growth factor (bFGF, 10 ng/mL) (PeproTech, Rocky Hill, USA), epithelial growth factor (EGF, 10 ng/mL) (Sigma) and penicillin/streptomycin (10^4 IU/mL) (Sigma) in a Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Gibco). The re-suspended pellets containing 1×10^6 cells were subsequently seeded onto a 25-cm^2 flask (Corning, NY, USA) and incubated under an atmosphere of 5 % CO_2 with the constant temperature of 37 °C. PBS was applied twice to wash off the non-adherent cells 24 h later. The morphology of the primary cells was observed on the 2nd and 5th days after seeding. The primary cells were cultured until they reached a confluence of 80 %, and subsequently the cells were trypsinized into dissociated cells, passaged, and marked as passage one (P1). In general, the cells were homogeneous after three passages (P3). Thus, P3 cells were used in subsequent experiments.

Characterization of BMMSCs using surface markers

A fluorescence-activated cell sorting (FACS) apparatus (Becton Dickinson, Franklin Lakes, NJ, USA) was used for the detection of CD44-FITC (fluorescein isothiocyanate; Southern Biotech, Birmingham, AL USA) and CD45-PE (phycoerythrin; Southern Biotech). Results of the FACS were analyzed using FlowJo software (Stanford University, Stamford, CA, USA). Reverse transcription n-PCR technology was used for the detection of CD71, CD73 and CD34 markers. Trizol reagent (GenStar, Beijing, China) was applied to isolate the total RNA from P3 cells. A reverse transcription system (Promega, Madison, WI, USA) was utilized to synthesized and amplified cDNA. The specific primers and cycle conditions were described in Supplementary Data Table S1 and S2.The PCR products were visualized using agarose gel (2 %) electrophoresis.

Induction of osteogenic and adipogenic differentiation of BMMSC

The P3 cells were adherent-cultured until 60 %-confluence was achieved. Subsequently, they

were incubated in osteoblast- or adipocyte-inducing conditional media containing FBS(10 %), dexamethasone (0.5 mM) (Sigma), β-glycerophosphate (10 mM) (PeproTech), vitamin C (50 µg/mL) and penicillin/streptomycin (10^4 IU/mL) (Sigma). The adipogenic-inducing conditional media contained FBS (10 %), dexamethasone (1mM), isobutyl-methylxanthine (IBMX, 0.5 mM) (PeproTech), insulin (10 µg/mL) (PeproTech), indomethacin (200µM) (PeproTech) and penicillin/streptomycin (10^4 IU/mL). A control group was also cultured in complete medium. Cell media from each group was replaced every 2 or 3 days. After incubation for 10 days, expression of alkaline phosphatase (ALP) was detected using an ALP stain kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). After incubation for two weeks, Alizarin Red staining was applied to detect the calcium nodes, and Oil Red-O staining was applied to assess the accumulation of intracellular lipid.

Development and identification of chimeras

P3 cells were used as donor cells (5×10^6 cellsmL^{-1}). After washing twice with PBS, 2 µL of the donor cells was microinjected into the blood vessel of a White Leghorn at HH13 using a microneedle. The microinjected embryos subsequently hatched into chimeras. To verify the chimeras, a secondary injection and PCR were performed after hatching. The cycling parameters and primers were set as described in Supplementary Data Table S2 and S3, respectively.

Quantification of $CD4^+CD25^+$ cells

Lymphocytes in the target organs of the hosts on the 1st, 7th, 14th, and 30th days post-hatching (D1, D7, D14, and D30, respectively) were stained using anti-chicken CD4-FITC (Southern Biotech) and CD25-PE as previously described [19]. FACS was applied to analyze the variations in $CD4^+CD25^+$ cells. The proportion of $CD4^+CD25^+$ cells in $CD4^+$ cells (specific $CD4^+CD25^+$ cells) was used to characterize immune suppression. Fold-changes were expressed in terms of number of specific $CD4^+CD25^+$ cells relative to control group.

Detection of TGF-β and IL-10

Quantitative real-time PCR (qRT-PCR) was applied using a LightCycler system (Roche, Rotkreuz, Switzerland) described previously [20]. The primers and cycling parameters were set as described in Supplementary Data Table S4 and S5, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was applied as a reference gene to obtain the expression levels of TGF-β and IL-10. Fold-changes relative to the control group in expression levels were calculated to access the variation.

Statistical analysis

Student's *t*-test was applied for statistical analysis using a Statistical Package for the Social Sciences (SPSS, SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Morphology of BMMSCs

Primary cells isolated from bone marrow were cultured for 5 days to achieve 80 % confluence prior to passage. The morphologies of the 2-day cells and the 5-day cells ares hown in Figure 1-a and 1-b, respectively. After culturing for 2 days, most of the suspended target cells adhered to the plate, showing triangular, polygonal and long fusiform shapes with clear boundaries. With prolonged culture, 80 % confluence was gradually achieved on the 5th day, and protrusions with a shuttle shapes were observed for most of the 5-day cells. The confluent cells were subsequently digested and passaged. Figure 1-c shows the morphology of the P1 cells. During passage, the impurities were gradually eliminated, and the relatively pure P3 cells (Figure 1-d) were used in subsequent experiments.

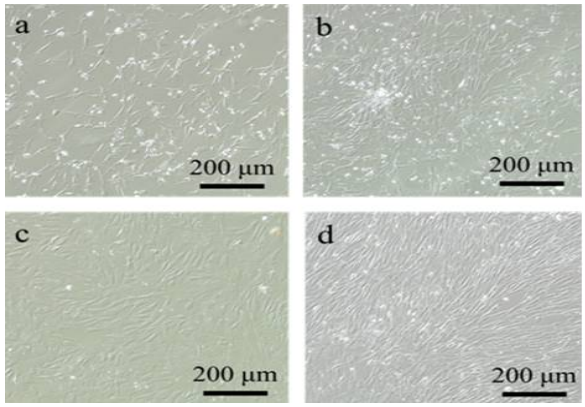

Figure 1: Morphology of the primary BMMSCs. ***Note:*** *(a) After primary culture for 48 h, BMMSCs exhibited triangular, polygonal and long fusform shapes with clear boundaries. (b) After primary culture for 5 days, BMMSCs gradually reached confluence, and most cells showed protrusions. (c) The P1 cells of BMMSCs were relatively homogeneous. (d) The P3 cells of BMMSCs were relatively pure and showed fibroblast-like properties*

Surface markers of BMMSCs

To characterize the P3 cells, several surface markers were applied and detected using FACS or reverse transcription-PCR. The results of the FACS analysis (Figure 2a and 2b) revealed that most of the P3 cells (93.2 %) expressed CD44, while the number of cells expressing CD45 was negligible (only 0.6 %). The results of the reverse transcription-PCR analysis (Figure 2c) showed that the P3 cells were CD71- and CD73-positive but CD34-negative. CD44, CD71 and CD73 are markers of BMMSCs, and CD45 and CD34 are markers of hematopoietic stem cells [21], which are common impurities in BMMSCs, indicating that the P3 cells were relatively pure BMMSC population without contaminating hematopoietic stem cells.

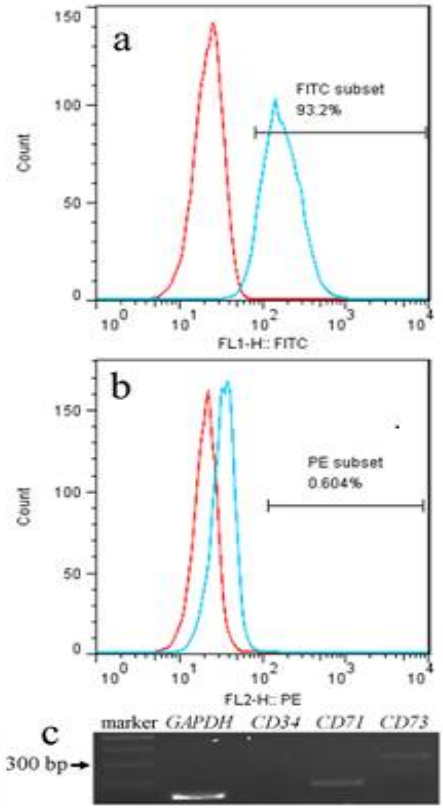

Figure 2: Expression of selected surface markers of BMMSCs. ***Note:*** *(a) FACS analysis of CD44-FITC. Red: control; blue: CD44-FITC. (b) FACS analysis of CD45-PE. Red: control; blue: CD45-PE. (c) Reverse transcription-PCR analysis the positive expression of CD71 and CD73, and the negative expression of CD34*

BMMSC differentiation *in vitro*

BMMSCs can be differentiated into osteoblasts and adipocytes [22]. In this paper, the differentiation of the P3 cells was induced *in vitro*. Figure 3 shows the progress of osteoblast differentiation. The cells cultured with complete medium (control group) remained unchanged throughout the 14-day culture experiment, showing a fusiform shape (Figure 3a). On the 7th day, the induced P3 cells aggregated together as nodules with multiple stratifications and obvious crystallization (Figure 3b). On the 10th day, the induced P3 cells stained brownish after treatment with ALP (Figure 3c), which is a characteristic of osteoblasts. Thus the induced P3 cells preliminarily differentiated into osteoblasts. On the 14th day, the morphology of the induced P3 cells significantly changed into a larger polygonal shape. Simultaneously, the induced P3 cells were stained with Alizarin Red (Figure 3d), suggesting that osteoblast differentiation was successful.

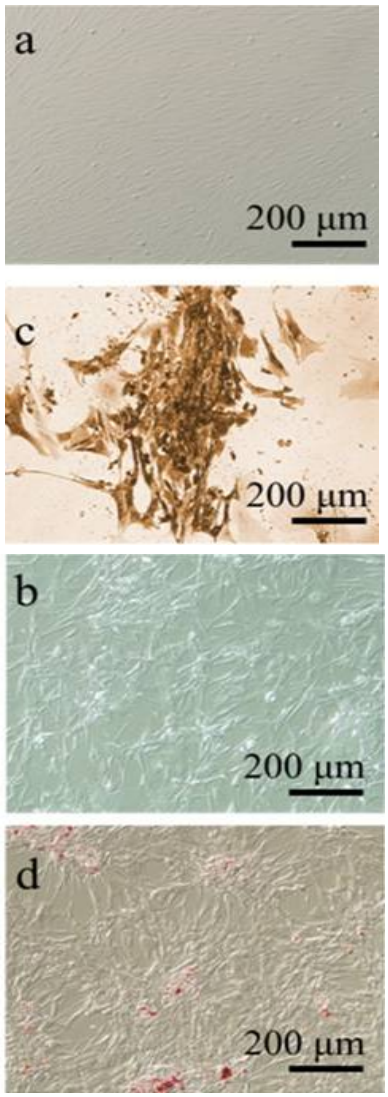

Figure 3: Osteogenic differentiation of BMMSCs. ***Note:*** *(a) In the control group, the cell morphology showed no changes during cultured with complete medium. (b) After a 7-day-induction, the cells assembled and formed nodules. (c) After a 10-day-induction, the cells were stained using the ALP staining kit. (d) After a 14-day-induction, the cells showed nodules via alizarin red staining*

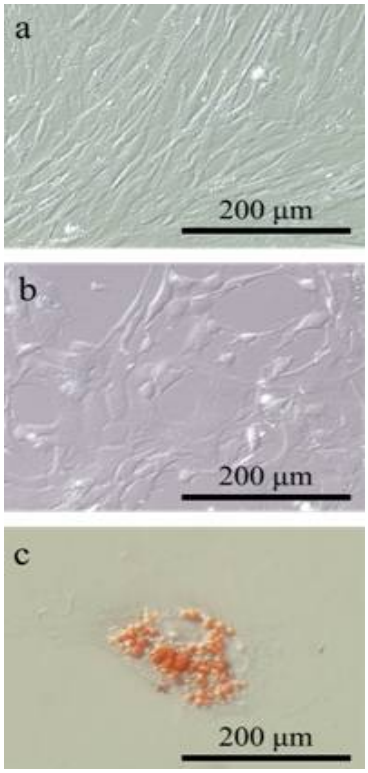

Figure 4: A dipogenic differentiation of BMMSCs. ***Note:*** *(a) In the control group, the cell morphology showed no changes during cultured in complete medium. (b) After induction for 4 days, the cells increased in size and showed a round morphology. (c) After induction for two weeks, the cells were detected via oil red O staining*

Figure 4 shows the progress of adipocyte differentiation. The cells of the control group remained constant during cultured for 14 days, as shown in Figure 4a. On the 4th day, the morphology of the induced P3 cells was altered to an oblate shape (Figure 4-b). On the 14th day, the larger oblate cells were dominant on the plate. In addition, lipid droplets were clearly visible inside the cells, and these cells showed positive staining with Oil-Red-O (i.e., the orange sections in Figure 4-c), suggesting that adipocyte differentiation was achieved. The osteoblast and adipocyte differentiation further confirmed the BMMSCs.

Identification of chimeras

The P3 cells identified as BMMSCs were microinjected into the embryos of White Leghorns to generate Peking duck-White Leghorn chimeras. To identify the chimeras, a secondary injection on D7 was performed, and PCR verification was also carried out. The performance of the chimera after a secondary injection at the wing vein using the same P3 cells (i.e., experimental group) was examined. Chimeras subjected to a secondary injection with PBS were used as a negative control group, and White Leghorns microinjected with PBS at embryo period and injected secondly with P3 cells on D7 were used as a positive control group. On D14, variations in specific $CD4^+CD25^+$ cells in the experimental and control groups were studied using FACS (Figure 5a). Comparison of the positive control group with the negative one showed an obvious decrease in population of specific $CD4^+CD25^+$ cells in the thymus and spleen, and a sharp increase in the cecal tonsils (about 3-fold). This result suggests that the injection of P3 cells induced an immune reaction in the positive control group. In the experimental group, the specific $CD4^+CD25^+$ cells in all the target organs remained approximateto the negative control group. The microinjected P3 cells were tolerated in the experimental group. In addition, the DNA from the chimeras on D1 was detected using PCR.

The results (Figure 5b) showed that a specific gene from Peking duck was detected in all test organs. These results illustrate that a chimera could be produced through microinjection.

Specific $CD4^+CD25^+$ cell variations

The immune tolerance of the chimeras was examined based on variations of the specific $CD4^+CD25^+$ cells. Avian $CD4^+CD25^+$ cells derived from the thymus can migrate to the cecal tonsils and spleen [17]. To examine the migration of these cells in chimeras, different organs of the target chimeras were collected, and the specific $CD4^+CD25^+$ cell variations were assessed.

Figure 6 shows the specific $CD4^+CD25^+$ cell variations in target immune organs of D1 chimeras. The specific $CD4^+CD25^+$ cells in the thymus were significantly reduced (a loss of nearly 50 %) compared with the control group microinjected with only PBS. In the spleen and cecal tonsils, the specific $CD4^+CD25^+$ cells were significantly increased (about 2-fold and 3.5-fold, respectively). In both experimental and control groups, $CD4^+CD25^+$ cell expression was negligible in the Bursa of Fabricius. These results indicate a more obvious migration of $CD4^+CD25^+$ cells in the D1chimeras than in the control group.

Figure 7 shows the variations of specific $CD4^+CD25^+$ cells and the accompanying TGF-β and IL-10. On both D7 and D30, no significant variations in specific $CD4^+CD25^+$ cells and target cytokines were observed in the thymus and spleen. However, in the cecal tonsils, specific $CD4^+CD25^+$ cells, TGF-β and IL-10 were markedly increased on D7 and moderately on D30.

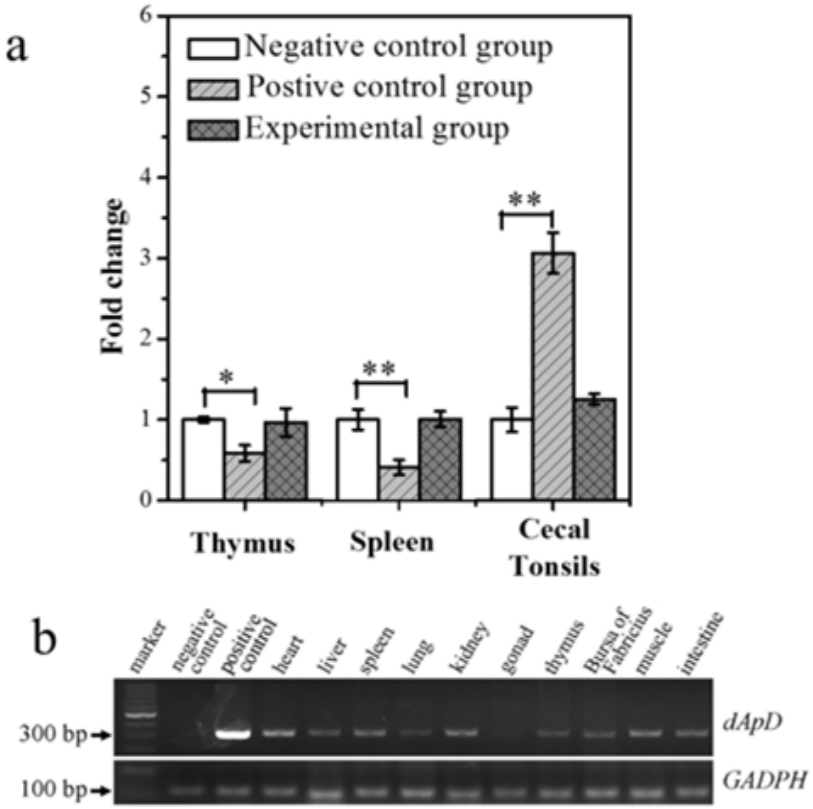

Figure 5: Identification of chimeras through secondary injection and PCR. ***Note:*** *(a) variations of specific $CD4^+CD25^+$ cells in target organs at D14.* ***Note:*** *the data represent the means ± SEM of 3-5 individuals; *p < 0.05, **p < 0.01; (b) expression of the duck gene in different organs*

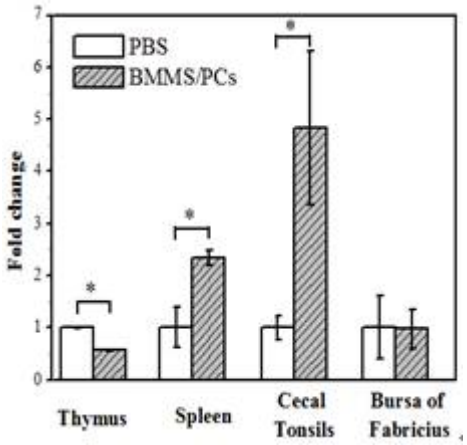

Figure 6: Variations in specific $CD4^+CD25^+$ cells in target organs on D1. ***Note:*** data represent the mean ± SEM (n = 3 – 5); *$p < 0.05$, **$p < 0.01$

DISCUSSION

In the present study, variations in specific $CD4^+CD25^+$ cells in chimeras generated via heterogeneous MSCs were investigated. MSCs possess several advantages, e.g., multi-lineage differentiation, potential tissue reconstruction, high immunomodulatory ability, and less immunogenicity [23]. Previous studies primarily focused on MSCs in humans and mice [1-4]. Not much research has been conducted on poultry. Such studies are of great significance for understanding of immune tolerance in poultry.

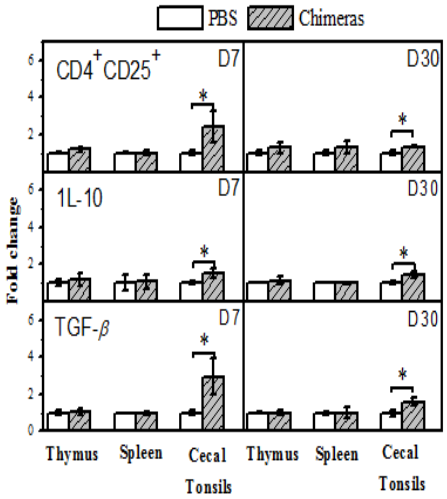

Figure 7: Variations of specific $CD4^+CD25^+$ cells, TGF-β and IL-10 in different organs on D7 and D30. ***Note:*** *The data represent the means ± SEM of 3-5 individuals; *p < 0.05, **p < 0.01*

The selected BMMSCs were isolated from Peking ducks, cultured and passaged for 3 generations to ensure a relatively pure BMMSC population, and the expressions of several surface markers (i.e., CD44, CD45, CD34, CD71

and CD73) in the selected BMMSCs were examined. As a cell surface glycoprotein, CD44 functions as a growth-anchoring site for MSCs. CD45 is a well-known marker of leukocyte common antigen expressed in virtually all white blood cells and hematopoietic stem cells [22].

The fact that CD44 expressed positively and CD45 expressed negatively illustrate the MSC character of the cultured P3 cells. The hematopoietic stem/progenitor cell marker CD34 [22] has been implicated in the transportation of hematopoietic stem cells and in inflammatory responses and lymphocyte homing. CD71, a transferrin receptor, is necessary for iron transport and regulation of the intracellular iron concentration [24]. CD73 is an important signaling molecule for lymphocyte differentiation [25]. The fact that CD71 and CD73 express positively and CD34 expressed negatively could be further evidence of MSCs in the present study.

Multilineage differentiation capacity is a characteristic of stem cells. MSCs have been differentiated into cells in mesodermal lineages *in vitro* in several studies [1-2,25]. In the present study, the target P3 cells were also differentiated into osteoblasts and adipocytes using different inducing agents (i.e., dexamethasone, vitamin C, and β-glycerophosphate to induce osteoblast differentiation; IBMX, insulin, dexamethasone, and indomethacin to induce adipocyte differentiation). Inducing factors stimulate or inhibit the expression of target genes via their effects on cell signaling pathways. The selected inducing agents stimulated the expressions of osteoblast and adipocyte genes and ultimately differentiated the target P3 cells into osteoblasts and adipocytes, confirming the successful isolation and culture of BMMSCs in the present study.

The relatively pure BMMSCs (i.e., the P3 cells) were microinjected into the embryos of White Leghorns to produce chimeras. To identify the chimeras, a secondary injection and PCR verification was used. In the positive control group, the injected P3 cells from Peking ducks were regarded as exogenous invasion inducing inflammation in the recipient. The recipient subsequently responded to the P3 cells, inducing variations of the specific $CD4^+CD25^+$ cells. $CD4^+CD25^+$ cells in avian can acquire super-suppressive properties once inflammation subsides [13] (D14 in this paper). Hence, specific $CD4^+CD25^+$ cells sharply increased in the cecal tonsils which were sensitive to inflammation. $CD4^+CD25^+$ cells originate in the thymus and migrate to the peripheral organs [17], inducing a decrease in specific $CD4^+CD25^+$ cells in the thymus.

Spleens are the storage organs of $CD4^+CD25^+$ cells, and the migration of $CD4^+CD25^+$ would subsequently decrease the specific $CD4^+CD25^+$ cells in the spleen. In the experimental groups, the first microinjection successfully generated chimeras, and the P3 cells in the secondary injection were considered autologous cells and had little effect. The fact that specific Peking duck gene could be detected using PCR in all tested organs of the D1 chimeras also indicates successful generation of a Peking duck-White Leghorn chimera.

Variations in specific $CD4^+CD25^+$ cells reflected immune tolerance of the chimeras. Shanmugasundaram *et al* [17] reported the migration of $CD4^+CD25^+$ cells from the thymus to the peripheral organs in avian organisms. This migration was also observed in chimeras in the current study, except in Bursa of Fabricius. Since Bursa of Fabricius is a source of B-lymphocytes, the presence of T lymphocytes in this organ may be negligible under normal conditions (without inflammation). There was a more obvious migration in the chimeras than in the control group. Ducks have a more robust immune system than chickens, so the chimeras might be superior to the wild chicken with respect to $CD4^+CD25^+$ cell migration.

In the cecal tonsils of chimeras, a significant increase in specific $CD4^+CD25^+$ cells could be observed when compared with the control group, while in other organs, specific $CD4^+CD25^+$ cells remained approximately the same. This may be attributed to the functions of the target organs. The intestine, as an organ which handles food residue, presents an anaerobic bioreactor programmed with enormous population of bacteria, and is sensitive to inflammation. Since MSCs can be utilized to treat Crohn's disease [26], the intestine would be one site of action for MSCs. In addition, MSCs are involved in immune regulation response and so they might directly increase the population of specific $CD4^+CD25^+$ cells in the cecal tonsils.

IL-10 is an anti-inflammatory cytokine that inhibits macrophage and dendritic cell functions, while TGF-β is an immunosuppressive cytokine [5]. Both cytokines can suppress the activated $CD4^+CD25^-$ T cells proliferation. Moreover, TGF-β has been implicated in mediation of MSC responses [27]. Thus, in the present study, variations in TGF-β and IL-10 accompanied changes in specific $CD4^+CD25^+$ cells.

CONCLUSION

The findings of the present study reveal that $CD4^+CD25^+$ cells showed more obvious migration on D1 from the thymus to the spleen and cecal tonsils in chimeras than that in White Leghorns microinjected with PBS, indicating a relatively robust immune system in the chimeras. The migration tendency gradually decreases with time. BMMSCs induces significant increases in specific $CD4^+CD25^+$ cells, and in TGF-β and IL-10 in cecal tonsils, although the increases decline with time. However, in the thymus and spleen, variation in specific $CD4^+CD25^+$ cells is only obvious on D1, while on D7 and D30, no obvious variation is observed in the chimeras compared with the controls. The immune tolerance was studied in chimeras in this study should benefit foundation studies on MSC therapy.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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REFERENCES

1. *Campagnoli C, Roberts IAG, Kumar S, Bennett PR, Bellantuono I, Fisk NM. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 2001; 98: 2396-2402.*
2. *Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 2006; 24: 1294-1301.*
3. *Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.*
4. *Wakitani S, Saito T, Caplan AI. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 1995; 18: 1417-1426.*
5. *Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 2000; 61: 364-370.*
6. *Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M. Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells. Arthritis Rheum-US 2009; 60: 1006-1019.*
7. *Shanmugasundaram R, Selvaraj RK. Effects of in vivo injection of anti-chicken CD25 monoclonal antibody on regulatory T cell depletion and CD4+ CD25− T cell properties in chickens. Dev Comp Immunol 2012; 36: 578-583.*
8. *Workman CJ, Szymczak-Workman AL, Collison LW, Pillai MR, Vignali DA. The development and function of regulatory T cells. Cell Mol Life Sci2009; 66: 2603-2622.*
9. *Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains.CD25. Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995; 155: 1151-1164.*
10. *Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; 299: 1057-1061.*
11. *Adeegbe D, Bayer AL, Levy RB, Malek TR. Cutting edge: allogeneic CD4+ CD25+ Foxp3+ T regulatory cells suppress autoimmunity while establishing transplantation tolerance. J Immunol 2006; 176: 7149-7153.*
12. *Shack LA, Buza JJ, Burgess SC. The neoplastically transformed.CD30hi Marek's disease lymphoma cell phenotype most closely resembles T-regulatory cells. Cancer ImmunolImmunother 2008; 57: 1253-1262.*
13. *Selvaraj RK. Avian CD4+CD25+ regulatory T cells: Properties and therapeutic applications. Dev Comp Immunol 2013; 41: 397-402.*
14. *Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, Mak TW, Sakaguchi S. Immunologic self-tolerance maintained by CD25+ CD4+ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med 2000; 192: 303-310.*

15. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. J Morphol1951; 88: 49-92.
16. Shanmugasundaram R, Selvaraj RK. Regulatory T cell properties of chicken CD4+CD25+ cells. J Immunol 2011; 186: 1997-2002.
17. Shanmugasundaram R, Selvaraj RK. CD4+CD25+ regulatory T cell ontogeny and preferential migration to the cecal tonsils in chickens. PloS One 2012; 7: e33970.
18. World Health Organization. Principles of laboratory animal care. WHO Chron 1985; 39: 51-56.
19. Yu XX, Rui L, Shao Q, Liu HW, Lu YN, Zhang YC, Li ZD. Changes of CD4+ CD25+ cells ratio in immune organs from chickens challenged with infectious bursal disease virus strains with varying virulences. Viruses 2015; 7: 1357-1372.
20. Guo Q, Shao Q, Xu WP, Rui L, Sumi R, EguchiF., Li, ZD. Immunomodulatory and Anti-IBDV Activities of the Polysaccharide AEX from Coccomyxa gloeobotrydiformis. Marine Drugs 2017: 15: 36.
21. Ohishi M, Schipani E. Bone marrow mesenchymal stem cells. J Cell Biochem 2010; 109: 277-282.
22. Bai CY, Hou LL, Ma YH, Chen LN, Zhang MH, Guan WJ. Isolation and characterization of mesenchymal stem cells from chicken bone marrow. Cell Tissue Bank 2013; 14: 437-451.
23. Nagaishi K, Arimura Y, Fujimiya M. Stem cell therapy for inflammatory bowel disease. J Gastroenterol2015; 50: 280-286.
24. Marsee DK, Pinkus GS, Yu H. CD71 transferrin receptor: an effective marker for erythroid precursors in bone marrow biopsy specimens. Am J Clin Pathol 2000; 134: 429-435.
25. Bai CY, Li XC, Hou LL, Zhang MH, Guan WJ, Ma YH. Biological characterization of chicken mesenchymal stem/progenitor cells from umbilical cord Wharton's jelly. Mol Cell Biochem 2013; 376: 95-102.
26. Adams S C, Xing Z, Li J. Immune-related gene expression in response to H11N9 low pathogenic avian influenza virus infection in chicken and Pekin duck peripheral blood mononuclear cells. Molecular immunology2009; 46(8): 1744-1749.
27. Ciccocioppo R, Bernardo ME, Sgarella A, Maccario R, Avanzini MA, Ubezio, C, Minelli A, Alvisi C, Vanoli A, Calliada F, et al. Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease. Gut 2011; 60: 788-798.