Induction of Immunological Tolerance in Chicken (*Gallus domesticus*) to DT40 Cells Stably Transfected with GFP

Wenjing Jin, Chen Zhao*, Peng Sun, Xiaoping Wang, Wenxin Zhang, Jihong Yuan,
Fang Yuan, Yan Li and Zandong Li

State Key Laboratories for AgroBiotechnology, Department of Biochemistry and Molecular Biology,
College of Biological Sciences, China Agricultural University,
No. 2, Yuanmingyuan West Road, 100193, Beijing, China

Genetically modified cells expressing xenogeneic proteins may trigger strong immunological responses when introduced into animals; however, these responses may be prevented by inducing immunological tolerance. In this study, we have introduced allogeneic cells into chicken embryos via embryonic blood vessel microinjection and induced immunological tolerance to them. Chicken DT40 cells stably transfected with the *GFP* gene (DT40-GFP cells) were produced and used as antigens to induce immunological tolerance by this method at 65–70 h of incubation for preimmunization. Enzyme-linked immunosorbent assays and lymphocyte proliferation assays were performed to determine the serum antibody and lymphocyte responses in hatched chickens after reinjection of the cells at 6 weeks post hatching. Our findings indicated that humoral and cellular immunological responses to allogeneic DT40 cells and DT40-GFP cells expressing xenogeneic proteins can be suppressed by preimmunization during embryogenesis; moreover, an effect on lymphocyte proliferation was observed.

Key words: chickens, stably transfected DT40, tolerance induction

J. Poult. Sci., 46: 356–362, 2009

Introduction

For many applications, long-term transgene expression and the survival of genetically modified cells are essential in transgenic animals; however, it is becoming increasingly clear that many gene products and transplanted cells carrying foreign genes have the potential to elicit significant immune response (Tang *et al.*, 1992; Mohammed *et al.*, 1998), because the host immune system recognizes their protein products as being foreign. Many scientists have explored the mechanism of tolerance and have attempted to induce immunological tolerance to transplanted cells since the early 1950s. Owen (Owen, 1945) reported long-term chimerism in erythroid cells in twin cattle and proposed that the effect was due to vascular anastomoses between the embryos, which permitted the exchange of primordial hematopoietic tissue. This led Burnet (Burnet, 1959) to theorize that contact with an

antigen very early in life would induce specific tolerance, and this theory was subsequently verified by Billingham *et al.* (Billingham *et al.*, 1953), who induced tolerance to skin grafts from black-coated mice in two adult white mice by inoculating neonatal white mice with lymphoid cells from the donor. Owen's experiments were repeated in chicken embryos by Hasek (Hasek, 1953). The finding of Miller in 1961 (Miller, 1961) that transplantation reactions and other forms of cellular immunity are controlled by the thymus revealed the importance of T cells. These findings suggest that the most reliable way to establish tolerance to transplantation antigens is to induce lymphohematopoietic chimerism during early embryogenesis.

An efficient means of introducing antigens into early embryos is required. Immunological tolerance to xenogeneic proteins produced by retrovirally transduced cells has successfully been induced (Bagley *et al.*, 2002), and it has been shown that it is easier to induce tolerance by intravenous inoculation of the antigen (Cremer *et al.*, 1983; Scherer *et al.*, 1989). We have previously demonstrated that tolerance could be induced by inoculating chicken embryos with protein antigen (Zhao *et al.*, 2006). Here, we investigated whether specific immune tolerance to cell-surface proteins and foreign gene products could be induced by introducing stable transfectants into chicken embryos at an early stage of development. DT40 is a

Received: February 26, 2009, Accepted: March 31, 2009

Correspondence: Prof. Z. Li and Dr. C. Zhao, College of Biological Sciences, China Agricultural University, No. 2, Yuanmingyuan West Road, 100193, Beijing, China. (E-mail: lzdws@cau.edu.cn)

* Present address: Biological Resource Laboratory, Laboratory for Intellectual Fundamentals for Environmental Studies, National Institute for Environmental Studies, 16-2 Onogawa, Tuskuba, Ibaraki 305-8506, Japan

widely used avian leukosis virus-induced B cell line, derived from Hyline SC chickens (Chang *et al.*, 2004; Terasaki *et al.*, 2008). DT40 cells carrying the GFP gene (DT40-GFP cells) were produced by electroporation and then introduced into chicken embryos via embryonic blood vessel microinjection. The chickens treated during embryogenesis were injected with the relevant cells after hatching, and serum samples were collected and analyzed by enzyme-linked immunosorbent assays (ELISAs) to detect the presence of anti-DT40 and anti-DT40-GFP antibodies. In addition, lymphocyte proliferation assays (LPAs) were used to examine the lymphocyte response to DT40 and DT40-GFP. Our results indicated that both humoral and cellular immunological responses to allogeneic DT40 and DT40-GFP were suppressed. These data may help to establish a new method of preventing immune responses to foreign gene products and transplanted cells in transgenic animals.

Materials and Methods

Cell Culture and Transfection

DT40 cells (kindly provided by Dr. JY Han, National University of Seoul, Seoul, Korea) were cultured at 38°C under 5% CO₂ in DMEM/F-12 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone), 1% non-essential amino acids (HyClone), 1% pyruvic acid sodium salt (HyClone) and 1% penicillin/streptomycin (HyClone). After 3 days in culture, the cells, at a density of 5×10^6 cells/800 μ l, were transfected with 20 μ g of linearized plasmid pEGFP-N1 using a Gene Pulser™ electroporator (Bio-Rad, Hercules, CA, USA) set at an optimized condition of 550 V and 50 μ F. The transfected cells were maintained for 24 h in the above culture medium and then cultured in selection medium containing 1.5 mg/ml G418 (Sigma, St. Louis, MO, USA) for 2 weeks. All surviving colonies were selected and screened by fluorescence microscopy (Olympus, Tokyo, Japan). Cells were harvested, washed twice with phosphate-buffered saline (PBS, pH 7.6), and then suspended in PBS to a final concentration of 2×10^4 to 3×10^4 cells/ μ l (for microinjection) or 5×10^5 cells/100 μ l (for post-hatch injection).

Embryonic Preimmunization by Blood Vessel Microinjection

Fertilized Jingbai 938 (White Leghorn, Gallus domesticus) eggs were washed with 0.1% benzalkonium bromide, and incubated. At 65–70 h of incubation eggs were swabbed with 70% ethanol, and a roller bit was used to open a small window near the position of the embryo. DT40-GFP cells (4×10^4 to 6×10^4 cells) were directly injected through the window into embryonic blood vessels, using a micropipette with an outer tip diameter of 30–40 μ m under a stereomicroscope (SMZ-10, Nikon). The window was then sealed with Parafilm, and the eggs were incubated, with the blunt end up, until hatching. The newly hatched chickens were wing-banded and housed in a sterilized animal room to avoid possible infection

(Pieskus *et al.*, 2008) at 30°C for 1 week after hatching and thereafter at 20°C, with free access to water and food.

Post-hatch Challenge and Serum Sample Preparation

At 6 weeks post-hatching, the chickens were injected via wing vein with DT40-GFP cells (5×10^5). The positive group was composed of age-matched chicks that were not inoculated with antigenic cells during embryogenesis but were immunized at 6 weeks post-hatching. The chickens in the negative control group did not receive treatment at embryogenesis or post-hatching. Blood samples were collected from each chicken and were stored overnight at 4°C. The serum was separated from the clotted blood by centrifugation at $10,000 \times g$ for 5 min; all serum samples were stored at -20°C until analysis.

Anti-DT40 and Anti-DT40-GFP Antibody Detection

ELISAs were conducted to detect specific antibodies. The whole DT40 or DT40-GFP cells were coated onto ELISA plates directly (Epstein *et al.*, 1985). Briefly, flat-bottom 96-well ELISA plates (EIA/RIA, Costar, Corning Inc., Corning, NY, USA) were coated overnight at 4°C with 1×10^4 cells/well in carbonate-bicarbonate buffer (pH 9.6), followed by a 1.5 to 2 h blocking period with 1% (w/v) gelatin dissolved in the coating buffer. After the plates were washed three times with washing buffer (PBS containing 0.05% [v/v] Tween 20), the serum samples (diluted 1: 400) were added to the wells and incubated at 37°C for 1 h. The wells were washed, and horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG (1: 400 dilution) (kindly provided by Prof. JX Zhao, China Agricultural University, Beijing, China) was added, followed by incubation at 37°C for 1 h. The wells were washed again, and substrate (TMB, Amresco, Solon, OH, USA) was added. The plates were incubated for 15 min at 37°C, and then the reaction was stopped by adding 2 M H₂SO₄. The optical density (OD) of each well was determined using an automated ELISA plate reader (model 550, Bio-Rad) equipped with a 492 nm filter. Serum samples with an OD value more than 2.1 times that of the negative control were considered positive for antibodies.

Lymphocyte Preparation and the LPAs

Peripheral anticoagulated blood samples, obtained in a heparin tube, were used for the LPAs. An amount of chicken lymphocyte separation medium (TBD, Tianjin, China) equal to the original volume of the blood sample was added to the blood collection tube, followed by centrifugation at 1,500 rpm for 30 min. The peripheral blood mononuclear cells were harvested from the interface, immediately washed twice in D-Hanks solution, and centrifuged at 2,000 rpm for 10 min at room temperature. After washing again, the cells were resuspended in 2 ml of complete RPMI 1640 medium (HyClone) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cell resuspension was then passed slowly through a fiberglass mesh at 37°C for 40 min–1 h, to remove most of the dead cells and B lymphocytes. The filtered cells were counted, diluted to a concentration of 4×10^6 cells/ml, and cultured at 100 μ l/well in 96-well

microtiter plates. Triplicate lymphocyte cultures (4×10^5 cells/well) were stimulated in complete RPMI 1640 medium with or without 500 DT40 cells and 500 DT40-GFP cells that were each used as a stimulus to induce lymphocyte proliferation, for 60 h at 37°C under 5% CO₂. During the last 4 h, the wells were pulsed with 20 μ l of MTS (Promega Corp., Madison, WI, USA) to assess cellular proliferation. The proliferative response was measured as the OD at 450 nm of the suspension in the well compared with that of the cells and media alone, using an automated ELISA plate reader.

The results are expressed as the OD (mean $\pm$ SE) of triplicate cultures. T cell tolerance, as assessed by LPA, was described by the stimulating index (SI). The amount of proliferation was calculated as follows:

Stimulating index (SI) = $[\text{OD}_{\text{stimulated cells}} - \text{OD}_{\text{stimulus control}}] / [\text{OD}_{\text{non-stimulated cells}} - \text{OD}_{\text{medium control}}]$. Herein, stimulated cells group and Non-stimulated cells group mean lymphocytes with or without DT40 or DT40-GFP cells to be stimulus respectively; Stimulus control is DT40 or DT40-GFP cells only in culture medium and medium control is just medium in wells. Samples with a mean SI value below 1.0 were considered to be completely tolerant.

Statistical Analysis

All data are expressed as the means $\pm$ SE. Comparisons within groups were made using SPSS 13.0 (SPSS Corp., Chicago, IL, USA). A p -value < 0.05 (t -test) was considered significant.

Results

Expression of Plasmid-transfected EGFP in DT40 Cells and Distribution of Cells Introduced into Chicken Embryos

The vector pEGFP-N1, which contains a highly effective enhancer to improve the expression of GFP, was transfected into DT40 cells by electroporation. Stable transfectants were identified by selection using G418; GFP gene was detected by PCR method, and GFP expression was monitored by fluorescence microscopy (Fig. 1A). Our results indicate that the transgene was integrated into the genome of the cells and expressed successfully.

To determine if DT40-GFP cells can survive and give rise to a chimera after introduction into chicken embryos, injected embryos from embryonation day (ED) 4 to ED 19 were isolated, and the GFP fluorescence was examined. Fluorescent cells were observed continuously before hatching (day 19). Most of the DT40-GFP cells were present in the head of the embryo, the allantois and the yolk sac; a few cells were detected in the liver, spleen, and other organs. However, no cells were found in the thymus. The number of fluorescent cells decreased as development progressed, and the cells were stellate or fibrotic in shape (Fig. 1B). A number of non-cellular fluorescent particles were detected; this may represent lysed cells that released their GFP, which might have acted as an effective inducer of tolerance.

Immunogenicity of DT40 and DT40-GFP in Specific-pathogen Free (SPF) Chickens

To directly examine the immunogenicity of the DT40-GFP cells, 20 SPF chickens, 1 month old, were intravenously injected with 2.5×10^6 DT40-GFP cells. Serum samples were collected from day 4 to day 35 after injection, and specific antibody responses against the allogeneic cells were detected by ELISA. Ten age-matched SPF chickens were used as negative controls.

Anti-DT40 and anti-DT40-GFP antibodies were produced, with peak production on day 10 post-injection. Interestingly, a significant difference in the antibody level was observed between the plates coated with DT40 versus DT40-GFP cells during the assay. As shown in Fig. 1C, the mean OD (0.65 ± 0.09) on day 10 post-injection was significantly higher with DT40-GFP cells than with DT40 cells (0.40 ± 0.09 , $p < 0.05$), and the OD values for both cell types were significantly higher than the OD for the negative control (0.09 ± 0.01). These data indicate that heterogeneous cells and transgene expression can trigger immune responses.

Induction of DT40-GFP Cell-induced Tolerance by Preimmunization during Embryogenesis

Twenty one chickens that hatched from 42 fertilized eggs were microinjected with DT40-GFP cells at 65–70 h of embryogenesis and injected with the same cells at 6 weeks post-hatching. Fifteen (Six chicks died after hatching) blood samples were collected 10 days later. Serum and lymphocytes were isolated for ELISA and LPA, respectively. The results were classified into three subgroups based on the OD or SI: tolerance, partial tolerance, and non-tolerance. A significant difference in the mean OD or SI was observed between any two of these subgroups.

For ELISAs, the average antibody responses to DT40 (0.34 ± 0.06) and DT40-GFP (0.38 ± 0.11) before reinjection were significantly higher than those for the negative control (DT40: 0.23 ± 0.03 , DT40-GFP: 0.24 ± 0.05) and the SPF control (DT40: 0.21 ± 0.05 , DT40-GFP: 0.22 ± 0.06), demonstrating a weak immunological response in the chicks prior to their post-hatch immunization. After reinjection, there was no significant increase in the antibody response to DT40 cells (see Fig. 2A) in three samples (0.42 ± 0.02 , $p = 0.401$) compared with the response before reinjection, although the average OD values were higher than those for the negative control. This suggests that the samples were tolerant to antigens from the DT40 cells. Six samples (0.55 ± 0.03) showed a weak response to DT40, but the antibody level was significantly lower than that for the positive controls (0.90 ± 0.24 , $p = 0.029$). The other samples were non-tolerant (1.07 ± 0.50), with OD values similar to those of the positive controls. In contrast, the response to DT40-GFP cells (Fig. 2B) after reinjection (1.28 ± 0.29) was significantly greater than the response to DT40 cells, even though a few samples were slightly lower. Thus, it appears that DT40-GFP cells induced an improved immunogenicity com-

Fig. 1. **A:** The fluorescent DT40-GFP cells were detected by dark-field and fluorescence microscopy ($\times 200$) ; **B:** Distribution and appearance of DT40-GFP cells following their introduction into chicken embryos. (a-c) Cells in the head ($\times 40$), brain ($\times 200$), and allantois ($\times 40$), respectively, of a day-4 embryo. (d-f) Cells in the head ($\times 100$), brain ($\times 200$), and allantois ($\times 100$), respectively, of a day-9 embryo; **C:** ELISA of specific antibody responses to allogeneic cells in SPF chickens. Significant differences were observed between the two tests ($p < 0.01$) and between the test sets and the negative control ($p < 0.001$).

Fig. 2. **15 chickens were injected with DT40-GFP during embryogenesis and post-hatching.** Serum anti-DT40 and anti-DT40-GFP antibodies detected by ELISA. The average OD at 492 nm is shown by the bars; lowercase letters a, b, c and d, above the bars indicate significant differences ($p < 0.05$, t -test); and letter n represents numbers of animals in each subgroup.

Fig. 3. 15 chickens were injected with DT40-GFP during embryogenesis and post-hatching. Analysis of the T cell proliferative response to DT40 cells and DT40-GFP cells determined by LPA. The average SI is shown by the bars; lowercase letters a, b and c, above the bars indicate significant differences ($p < 0.05$, t -test); and letter n represents numbers of animals in each subgroup.

pared with DT40 cells.

On lymphocyte proliferation, DT40 tended to have a greater effect than DT40-GFP (2.14 ± 1.53 vs. 1.45 ± 0.94 , respectively, Fig. 3. A-B), but the difference was not significant ($p = 0.144$). Seven samples (3.35 ± 1.45) showed a strong proliferative response to DT40, similar to that of the positive control (3.08 ± 0.84). Two of these samples (1.19 ± 0.05) demonstrated a significant difference compared with the positive control (2.02 ± 0.50 , $p = 0.044$) when stimulated with DT40-GFP cells, and thus these samples were considered non-responsive. Five samples (1.31 ± 0.23) were weakly responsive to DT40 stimulation, and three of them were also non-responsive to DT40-GFP (0.72 ± 0.12). Cellular proliferation could not be triggered by either stimulus in three samples (0.71 ± 0.08 , with DT40; 0.75 ± 0.14 , with DT40-GFP).

These results suggested that immune responses to allogeneic cells could be suppressed by preimmunization with the cells during embryogenesis.

Discussion

The long-term survival of genetically modified cells is critical for acquiring foreign pharmaceutical proteins in transgenic animals. However, the direct transplantation of genetically modified cells into animals usually induces a strong immunological response. Thus, it is necessary to prevent host immune responses to gene products as well as the cells used for gene delivery. In this study, we investigated whether allogeneic cells carrying foreign genes could induce specific tolerance by preimmunization via embryonic blood vessel microinjection at 65–70 h of incubation.

To our knowledge, this is the first report showing the induction of immunological tolerance to transgenic cells via the embryonic microinjection in chickens. The cells could survive in the embryo for a long time and induce a state of chimerism, even after hatching; therefore, continuous antigen stimulation might have existed in the chicks

before they were immunized at 6 weeks post-hatching, and this probably would have resulted in a greater immune response. On the other hand, this implies a sufficient chance for the developing immune system to encounter and process antigens, and may be favorable for the induction of tolerance, though there is no direct evidence for this (Starzl *et al.*, 1992).

Both ELISA and LPA suggested that immune responses to specific allogeneic cells could be suppressed by preimmunization with the cells during embryogenesis, and double-negative results were observed in a few chicks. A significantly greater response to DT40-GFP than to DT40 was observed ($p < 0.01$) in ELISA, indicating that the DT 40 cells that had incorporated the *GFP* gene had greater humoral immunogenicity than their wild-type counterparts. Insertion of the *GFP* gene into the genome, which may cause variations in self gene expression in the DT40 cells; the altered proteins may display non-self antigens on the cell surface. It is confirmed by the conclusion that the small differences in the three-dimensional structure between an introduced molecule and native proteins can be detected by the immune system (Schellekens, 2002). The LPA is used as an *in vitro* surrogate to assess the overall quality and character of the cellular immune response (Haddad *et al.*, 1991; Sitz and Birx, 1999; Sottong *et al.*, 2000). Although our ELISA results are less than ideal, our LPA results demonstrate that a cellular immunological response could be suppressed. Furthermore, we showed that the mean SI following stimulation by the cells with the same type as those injected during embryogenesis was lower than that by another cell type without pretreatment of the embryos.

In addition, chickens that were microinjected with DT 40-GFP cells via embryonic blood vessels and injected with DT40 cells during week 6, and microinjected with DT40 cells via embryonic blood vessels and injected with DT40-GFP cells post hatching, were also detected with the same method. These results indicate that tolerance to

antigens from DT40-GFP cells did not automatically result in tolerance to DT40 cells, and vice versa. GFP was also used as a stimulus, suggesting that tolerance to allogeneic cells does not necessarily produce tolerance to the cytoplasmic protein.

Our results indicate that a T cell non-response may be responsible for the induction of tolerance in chickens. It is similar to conclusion made by Husby (Husby *et al.*, 1994) that antigen feeding induced T cell tolerance but not B cell in humans. Although both B and T cells can be made tolerant, it is more important to tolerize T cells than B cells, because B cells cannot make antibodies to most antigens without the help of T cells. In birds, T cells develop in the thymus, and B cells develop in the bursa of Fabricius (Glick, 1956; Cooper *et al.*, 1965; Toivanen *et al.*, 1972; Hengartner *et al.*, 1988). The maturing T cells are positively and negatively selected during intrathymic development before their migration to the thymic medulla and then to the peripheral lymphoid tissues (Scollay *et al.*, 1984; Hengartner *et al.*, 1988; Kisielow *et al.*, 1988; Penit *et al.*, 1988; Sha *et al.*, 1988). However, clonal selection may miss some T cells and give rise to double-negative cells with a limited capacity for mounting an immune response in the peripheral blood (Caveno *et al.*, 1999; Van Meerwijk *et al.*, 1999). The same situation may occur in other lymphocytes such as B or NK cells. Our previous attempts at inducing immunological tolerance to soluble proteins demonstrated that the antibody response in hatched chickens challenged with the same protein post-hatching could be effectively suppressed in 53.8% of chickens exposed to casein and in 62.5% (inoculating at 65–67 h of incubation) and 33.33% (inoculating at 67–70 h of incubation) of chickens exposed to BSA. Cells were comparatively harder to be induced tolerance than soluble proteins, considering the multiple antigens and major histocompatibility complex (MHC). Thus, not all the chickens were tolerant. Besides, after microinjection, the DT40-GFP cells injected into chicken embryos may die or leak out of the embryos. The immune system would not meet the cells as antigens during embryogenesis, that will result in non-tolerance to the cells. The differences between individual chickens may be also a reason.

In summary, we have developed a method for inducing immunological tolerance to allogeneic cells in chickens. Both serum antibody responses and lymphocyte proliferation could be suppressed. Further research will focus on developing an effective tolerance induction protocol for other cells that can secrete foreign proteins. New attempts will be made to produce transgenic chimeric chickens which can deposit pharmaceutical proteins that may be collected in their egg yolk (Kitaguchi *et al.*, 2008) after immunological tolerance to genetically modified cells and their products can be successfully induced.

Acknowledgments

This work was supported by the grants from 2007AA100504 from the State 863 High-technology R&D Project

of China.

References

- Bagley J, Tian C, Sachs DH and Iacomini J. Induction of T-cell tolerance to a MHC class I alloantigen by gene therapy. *Blood*, 99: 4394–4399. 2002.
- Billingham RE, Brent L and Medawar PB. Actively acquired tolerance of foreign cells. *Nature*, 172: 603–606. 1953.
- Burnet FM. The clonal selection theory of acquired immunity. Cambridge: The University Press. 1959.
- Caveno J, Zhang Y, Motyka B, Teh SJ and Teh HS. Functional similarity and differences between selection-independent CD4⁺ CD8⁺ alphabeta T cells and positively selected CD8 T cells expressing the same TCR and the induction of anergy in CD4⁺ CD8⁺ alphabeta T cells in antigen-expressing mice. *Journal of Immunology*, 163: 1222–1229. 1999.
- Chang H and Delany ME. Karyotype stability of the DT40 chicken B cell line: macrochromosome variation and cytogenetic mosaicism. *Chromosome Research*, 12: 299–307. 2004.
- Cooper MD, Peterson RD and Good RA. Delineation of the Thymic and Bursal Lymphoid Systems in the Chicken. *Nature*, 205: 143–146. 1965.
- Cremer MA, Hernandez AD, Townes AS, Stuart JM and Kang AH. Collagen-induced arthritis in rats: antigen-specific suppression of arthritis and immunity by intravenously injected native type II collagen. *Journal of Immunology*, 131: 2995–3000. 1983.
- Epstein SL and Lunney JK. A cell surface ELISA in the mouse using only poly-L-lysine as cell fixative. *Journal of Immunological Methods*, 76: 63–72. 1985.
- Glick B, Chang TS and Jaap RG. The bursa of Fabricius and antibody production on the domestic fowl. *Poultry Science*, 35: 224–226. 1956.
- Haddad EE and Mashaly MM. In vivo effects of TRH, T3 and cGH on antibody production and T- and B-lymphocytes proliferation in immature male chickens. *Immunological Invest*, 20: 557–568. 1991.
- Hasek M. Parabiosis of birds during embryonic development. *Cesk Biology*, 2: 265–270. 1953.
- Hengartner H, Odermatt B, Schneider R, Schreyer M, Walle G, MacDonald HR and Zinkernagel RM. Deletion of self-reactive T cells before entry into the thymus medulla. *Nature*, 336: 388–390. 1988.
- Husby S, Mestecky J, Moldoveanu Z, Holland S and Elson CO. Oral tolerance in humans. T cell but not B cell tolerance after antigen feeding. *Journal of Immunology*, 152: 4663–4670. 1994.
- Kisielow P, Bluthmann H, Staerz UD, Steinmetz M and Von BH. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4⁺ CD8⁺ thymocytes. *Nature*, 333: 742–746. 1988.
- Kitaguchi K, Minoura M, Noritake M, Mizutani M, Kinoshita K, Horio F and Murai A. Determination of Immunoglobulin Y Concentration in Yolk Extract Prepared by Water Dilution Method: Comparisons among Three Strains of Chickens. *The Journal of Poultry Science*, 45: 82–87. 2008.
- Miller JFAP. Immunological function of the thymus. *Lancet*, 2: 748–749. 1961.
- Mohammed SM, Morrison S, Wims L, Trinh KR, Wildeman AG, Bonselaar J and Etches RJ. Deposition of genetically

- engineered human antibodies into the egg yolk of hens. *Immunotechnology*, 4: 115–125. 1998.
- Owen RD. Immunogenetic consequences of vascular anastomoses between bovine twins. *Science*, 102: 400–401. 1945.
- Penit C and Vasseur F. Sequential events in thymocyte differentiation and thymus regeneration revealed by a combination of bromodeoxyuridine DNA labeling and antimitotic drug treatment. *Journal of Immunology*, 140: 3315–3323. 1988.
- Pieskus J, Kazeniauskas E, Butrimaite-Ambrozeviciene C, Stanevicius Z and Mauricas M. Salmonella Incidence in Broiler and Laying Hens with the Different Housing Systems. *The Journal of Poultry Science*, 45: 227–231. 2008.
- Schellekens H. Immunogenicity of therapeutic proteins: clinical implications and future prospects. *Clinical Therapy*, 24: 1720–1740; discussion 1719. 2002.
- Scherer MT, Chan BM, Ria F, Smith JA, Perkins DL and Geffer ML. Control of cellular and humoral immune responses by peptides containing T-cell epitopes. *Cold Spring Harb Symp Quant Biology*, 54 Pt 1: 497–504. 1989.
- Scolley R, Wilson A and Shortman K. Thymus cell migration: analysis of thymus emigrants with markers that distinguish medullary thymocytes from peripheral T cells. *Journal of Immunology*, 132: 1089–1094. 1984.
- Sha WC, Nelson CA, Newberry RD, Kranz DM, Russell JH and Loh DY. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature*, 336: 73–76. 1988.
- Sitz KV and Birx DL. *Lymphocyte Proliferation Assay*. HIV protocols. Humana Press, 17: 343–353. 1999.
- Sottong PR, Rosebrock JA, Britz JA and Kramer TR. Measurement of T-lymphocyte responses in whole-blood cultures using newly synthesized DNA and ATP. *Clinical Diagn Lab Immunology*, 7: 307–311. 2000.
- Starzl TE, Demetris AJ, Murase N, Ildstad S, Ricordi C and Trucco M. Cell migration, chimerism, and graft acceptance. *Lancet*, 339: 1579–1582. 1992.
- Tang DC, DeVit M and Johnston SA. Genetic immunization is a simple method for eliciting an immune response. *Nature*, 356: 152–154. 1992.
- Terasaki K, Hirayama H, Kasanga CJ, Maw MT, Ohya K, Yamaguchi T and Fukushi H. Chicken B Lymphoma DT40 Cells as a Useful Tool for in vitro Analysis of Pathogenic Infectious Bursal Disease Virus. *Journal of Veterinary Medical Science*, 70: 407–410. 2008.
- Toivanen P, Toivanen A and Good RA. Ontogeny of bursal function in chicken. I. Embryonic stem cell for humoral immunity. *Journal of Immunology*, 109: 1058–1070. 1972.
- Van Meerwijk JP and MacDonald HR. In vivo T-lymphocyte tolerance in the absence of thymic clonal deletion mediated by hematopoietic cells. *Blood*, 93: 3856–3862. 1999.
- Zhao C, Song C, Wang X, Li Z, Sha J, Han H, Zhang Y and Li Z. Induction of immunological tolerance in chickens inoculated with xenogeneic antigens at an early stage of embryonic development. *Developmental & Comparative Immunology*, 30: 431–440. 2006.