Hyperthyroid monkeys: a nonhuman primate model of experimental Graves' disease

Y Wang*, L P Wu*, J Fu, H J Lv, X Y Guan, L Xu, P Chen, C Q Gao, P Hou, M J Ji and B Y Shi

Department of Endocrinology, The First Affiliated Hospital of Xi'an Jiaotong University School of Medicine, Xi'an 710061, People's Republic of China

*(Y Wang and L P Wu contributed equally to this work)

Correspondence should be addressed to B Y Shi

Email
shibingy@126.com

Abstract

Graves' disease (GD) is a common organ-specific autoimmune disease with the prevalence between 0.5 and 2% in women. Several lines of evidence indicate that the shed A-subunit rather than the full-length thyrotropin receptor (TSHR) is the autoantigen that triggers autoimmunity and leads to hyperthyroidism. We have for the first time induced GD in female rhesus monkeys, which exhibit greater similarity to patients with GD than previous rodent models. After final immunization, the monkeys injected with adenovirus expressing the A-subunit of TSHR (A-sub-Ad) showed some characteristics of GD. When compared with controls, all the test monkeys had significantly higher TSHR antibody levels, half of them had increased total thyroxine (T_4) and free T_4 , and 50% developed goiter. To better understand the underlying mechanisms, quantitative studies on subpopulations of CD4+T helper cells were carried out. The data indicated that this GD model involved a mixed Th1 and Th2 response. Declined Treg proportions and increased Th17:Treg ratio are also observed. Our rhesus monkey model successfully mimicked GD in humans in many aspects. It would be a useful tool for furthering our understanding of the pathogenesis of GD and would potentially shorten the distance toward the prevention and treatment of this disease in human.

Key Words

- ▶ thyrotropin receptor A-subunit
- ▶ Graves' disease
- ▶ rhesus monkeys

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Introduction

Graves' disease (GD), a common organ-specific autoimmune disease, has its prevalence between 0.5 and 2% in women, and it is ten times more common in women than in men (Lazarus 2012). The thyrotropin receptor (TSHR), as the main autoantigen, plays an important role in the development of GD. It has been shown that the TSHR is expressed as a single chain molecule that undergoes intramolecular cleavage into an extracellular A-subunit and a membrane-spanning B-subunit (Pichurin *et al.* 2006). In addition, the A-subunit alone initiates or

amplifies the immune response, leading to Graves' hyperthyroidism (Chazenbalk 2002, Chen 2003b, Mizutori *et al.* 2006). Over the past decades, various protocols have been put into use in rodents, including inoculation with syngeneic cells expressing TSHR or gene-delivery methods, such as plasmid cDNA or adenovirus vectors encoding the TSHR or its A-subunit, to induce experimental GD (McLachlan *et al.* 2005, Nagayama 2007). However, some characteristics in these rodents model do not mimic accurately those characteristics in typical

Graves' patients (Chen 2003a). In contrast to the rodents' model, these rhesus macaques which exhibit greater similarity to humans with respect to physiology, neurobiology, and metabolism (Disotell & Tosi 2007, Gibbs *et al.* 2007, Pennisi 2007) more closely approximate the individuals that develop GD. None of current therapies can cure GD. Thus, exploring novel treatments for GD has become a priority for all scientific researchers. We have shown that GD could be prevented by the induction of immunotolerance to TSHR in neonatal BALB/c mice and that this is an effective method of prevention for GD (Wu *et al.* 2011). This innovation could potentially be used as a GD vaccine in patients with GD. To expedite the development of a vaccine that could be used in GD patients, a rhesus macaque model that can narrow the gap between rodents and human beings should be established.

CD4+ T helper (Th) lymphocytes have been indicated as a crucial player in GD by secreting various cytokines and providing help for other effector cells (Lichiardopol & Mota 2009). It is well known that adaptive immunity can be differentiated into two distinct subsets: the interferon (IFN)- γ -dominated Th1 cell-mediated immune response and the interleukin 4 (IL4)-dominated Th2 antibody-mediated immune response (Mosmann & Coffman 1989, Singh *et al.* 1999). There is a concern that the Th2 immune response is likely to be pivotal in the Shimojo and M12-TSHR models, while Th1 seems to be important in the DNA-TSHR, Ad-TSHR, and DC-TSHR models (Nagayama *et al.* 2003, Barrett *et al.* 2004, Nagayama 2005). Furthermore, there are contradictory data about Th1/Th2 balance shift in GD patients (Weetman *et al.* 1990, Latrofa *et al.* 2004). These observations suggest that GD cannot be simply identified as a Th1 or Th2 immune-mediated disease. More recently, this simplistic categorization has become more complicated with the definition of the CD4+ subsets into other subpopulations termed regulatory T cells (Tregs) and Th17. Tregs in peripheral tissues play an important role in the maintenance of peripheral immune tolerance and the prevention of autoimmune diseases. Th17 cells have been observed to play a role in numerous autoimmune diseases, and there is more evidence suggesting that they are related to GD (Ganesh *et al.* 2011, Horie *et al.* 2011, Yan *et al.* 2012).

This nonhuman primate model which more closely approximates human GD is the prerequisite for GD vaccine in rhesus monkeys and would potentially shorten the distance toward the prevention and treatment of this disease in human beings in the near future.

Materials and methods

Adenovirus construction

The construction and purification of A-sub-Ad has been described previously (Chen 2003b). A-sub-Ad and control adenoviruses expressing enhanced green fluorescent protein (EGFP-Ad) were propagated in human embryonic kidney 293 cells (HEK293) and purified by ion-exchange chromatographic column. Both the A-sub-Ad and EGFP-Ad used in this study were drawn from the same preparation and stored in aliquots at -80°C .

Animals and immunization

Twelve female rhesus macaques (*Macaca mulatta*), 3 years old and 3–4 kg in weight, were selected from the Kunming Institute of Zoology, Chinese Academy of Sciences (SCXK(dian)2008-0001). All animals were free of known primate pathogens. The monkeys were under the supervision of the The Fourth Military Medical University Laboratory Animal Center. All animal care and treatment were in accordance with the ethics and law in China. Care and treatment were provided to all animals in accordance with the Guide for Care and Use of Laboratory Animals. Adult female rhesus macaques were injected intramuscularly with 6×10^{10} particles A-sub-Ad or EGFP-Ad five times at 3 weeks intervals, and the sera were prepared from blood samples collected at several time points. In all cases, the total vaccine dose was suspended in 1 ml PBS, and the vaccines were delivered intramuscularly in 0.5-ml aliquots into both quadriceps muscles. When necessary, animals were immobilized with 10 mg/kg ketamine hydrochloride (Sigma-Aldrich), injected intramuscularly.

Serum thyroid hormones

Total thyroxine (T_4) and free thyroxine (FT_4) in monkey sera were measured at several time points by RIA, using commercial kits (Beijing Atom High Tech Co., Ltd., Beijing, China).

TSHR antibodies measured by ELISA and TSH-binding inhibition assays

TSHR antibodies (TRAbs) were measured by competitive ELISA, using a commercial kit according to the manufacturer's protocol (Medipan GmbH, Germany, ID, USA). In brief, duplicate serum aliquots were incubated with TSHR immobilized on the ELISA wells. The M22

complex was added. The bound receptor–M22 antibody complex was detected with HRP. The signal was developed with 3,3',5,5'-tetramethylbenzidine, and the OD was read at 450 nm. TRAbs measured by the TSH-binding inhibition (TBI) assays, using a commercial kit according to the manufacturer's protocol (Medipan GmbH). In brief, duplicate serum aliquots (100 µl unless otherwise indicated) were incubated with TSHR immobilized on the coated tubes; ^{125}I -labeled TSH was added and the TRAb complexes were precipitated with polyethylene glycol.

Histological examination of the thyroid gland

Dissected thyroid glands were fixed in 10% buffered formalin (pH 7.4) and then dehydrated and embedded into paraffin. Sections (5 µm thickness) were stained with hematoxylin–eosin. Analysis was performed using the Olympus Cue-2 image analysis system connected to an Olympus compound microscope. The area of each follicle in five random fields was evaluated from each section, and the samples were taken from hyperthyroid and euthyroid.

Viral neutralization assay

A modified protocol based on a previously described assay was used to determine the titer of anti-adenovirus neutralizing antibodies in serum (Rahman 2001, Pilankatta *et al.* 2010). Briefly, all samples were incubated at 56 °C for 30 min to inactivate complement. In advance, HEK293 cells were plated onto flat-bottom 96-well plates (10^4 cells/well in 0.1 ml each). Duplicate serial dilutions of the serum sample were pre-incubated with an equal volume of virus with 50 MOI at 37 °C for 30 min and then transferred to HEK293 cells. The fluorescence was measured at 24 h to assay neutralizing capacity. Background fluorescence was assessed in the cell control, which contained HEK293 cells alone, and virus control fluorescence was determined in wells containing HEK293 cells cultured with virus in the absence of monkey serum. The reciprocal of the mean serum dilution that corresponded to 50% inhibition in EGFP fluorescence was taken as the anti-adenovirus neutralizing antibody titer.

Flow cytometric analysis

Peripheral blood mononuclear cells (PBMC) and spleen lymphocytes were suspended in Flow Cytometry Staining Buffer (eBiosciences, San Diego, CA, USA) at a final concentration of 5×10^6 cells/ml. Foxp3 expression in CD4+CD25+T cells was detected using the

Human/NonHuman Primate Regulatory T cell Staining Kit (eBiosciences) according to the manufacturer's protocol. Briefly, the rhesus macaques' PBMCs and spleen lymphocytes were first surface-labeled with FITC anti-human CD4 and PE anti-human CD25. After fixation and permeabilization, the cells were stained with APC anti-human Foxp3 or a matched isotype control. The CD4+IL17+ cells were detected by stimulation and then cultured for 4 h with phorbol myristate acetate plus ionomycin (at 50 and 1 ng/ml, respectively, Sigma–Aldrich) at 37 °C. These cells were then stained with FITC-CD4 antibody (eBiosciences), fixed, permeabilized, and then cells were incubated with Anti-Human IL17A Alexa Fluor 647 antibody (eBiosciences) for 30 min at 4 °C in dark. Three-color flow cytometric analysis was performed using flow cytometry (FACS Aria, BD Biosciences, San Jose, CA, USA). Data analysis was performed using Cell Quest Software (BD).

Quantitative RT-PCR

mRNA levels of transcription factors forkhead box P3 (FOXP3), RAR-related orphan receptor C (RORC), T-box 21 (TBX21), and GATA binding protein 3 (GATA3) were investigated. The primer sequences were shown in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article. Total RNA was isolated from PBMCs and the spleen lymphocytes using TRI Reagent (Sigma–Aldrich) according to the manufacturer's protocol. RT was performed using the PrimeScript RT reagent Kit (Takara, Syuzou, Shiga, Japan). mRNA expression was determined using an CFX96 Real-Time PCR Detection System (Bio-Rad) and SYBR Premix Taq (Takara). Reactions were initially denatured at 95 °C for 30 s, followed by 40 cycles for 5 s at 95 °C and 30 s at 60 °C. Melting curve analysis was used to control for amplification specificity. A cDNA dilution series of the calibrator was used to set up a standard curve for target genes and the housekeeping gene.

Regulatory T cell isolation and suppression assays

The monkey splenocyte suspensions were added to anti-mCD4-FITC and anti-mCD25-PE antibodies (L200 and M-A251, respectively; BD Biosciences, San Jose, CA, USA) and then incubated at 4 °C for 10 min. We sorted CD4+CD25+ Treg cells with flow cytometry (FACS Aria, BD) and collected CD4+CD25+ Treg cells and CD4+CD25– T cells separately in the two flow tubes.

CD4+CD25– cells (2.5×10^4 /well) were cultured with CD4+CD25+ cells with plate-bound anti-CD3 (SP34; BD Biosciences) onto 96-well round-bottom plates. The

antigen-presenting cells (APC; 5×10^4 /well unseparated total PBMC, mitomycin C for 30 min at 37 °C) were added to all cultures. To evaluate dose-dependent inhibition of cytokine production, titrated numbers of CD4+CD25+ cells were added stepwise in decreasing ratios from 1:1 to 16:1. Culture supernatants were taken after 48 h to measure IFN- γ , IL4, and IL17A by ELISA, using a commercial kit according to the manufacturer's protocol (Mabtech AB, Stockholm, Sweden). Cell-proliferation assays were performed by using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) for the last 4 h of a 3-day culture.

Statistical analyses

Normally distributed data were presented as the mean and S.E.M. Significant differences between the magnitudes of responses between mice in two groups were determined by Mann–Whitney rank sum tests or, when normally distributed, independent samples *t*-test. Serial measurements were compared by repeated measures ANOVA. All statistical analyses were calculated using SPSS for Windows (SPSS 15.0), and a value of 5% was considered significant.

Results

Hyperthyroidism and goiter in rhesus macaque immunized with TSHR A-subunit

An essential feature of GD is elevated thyroid hormone levels (thyrotoxicosis) in association with TRAbs. Sera obtained at several time points from all monkeys were used to measure TT₄ and FT₄ to diagnose hyperthyroidism. The trend of TT₄ and FT₄ in EGFP-Ad-injected monkeys and hyperthyroid monkeys in A-sub-Ad-injected group is shown in Fig. 1a. Two weeks after the third injection, 17% of the monkeys injected with A-sub-Ad showed elevated serum TT₄ and FT₄ levels compared with controls. Two weeks after the fifth injection, the proportion of hyperthyroidism rose to 50% (Fig. 1b). In addition, there was a significant decrease in the weight of hyperthyroid monkeys in the A-sub-Ad animals, from 3.95 ± 0.05 ($\pm$ S.E.M.) to 3.62 ± 0.2 ($\pm$ S.E.M.) ($P=0.05$, Mann–Whitney *U* test, $n=6$; Supplementary Figure 1, see section on supplementary data given at the end of this article). The resting heart rate in hyperthyroid monkeys in the A-sub-Ad-injected monkeys (three monkeys) increased with a mean of 255 ± 14 per minutes ($\pm$ S.E.M.) compared with 188 ± 16 per minutes ($\pm$ S.E.M.) in the control monkeys ($P=0.021$, independent samples *t*-test, $n=6$;

Figure 1

Sera TT₄ and FT₄ in rhesus macaques immunized with A-sub-Ad and EGFP-Ad. (a) General trend of TT₄ and FT₄ in EGFP-Ad-injected monkeys and hyperthyroid monkeys in A-sub-Ad-injected group (three monkeys) throughout the immunization regimen (mean $\pm$ S.E.M.). (b) Individual values of TT₄ and FT₄ for monkeys 2 weeks after the third and fifth injections. The shaded area indicates the mean $\pm$ 2 S.D. of values for the control animals ($n=6$).

Figure 2

Goiter in rhesus macaques immunized with A-sub-Ad. (a) Naked-eye view of the thyroid gland. Normal thyroid was from euthyroid monkey and enlarged gland was from hyperthyroid monkey. (b) Histology of thyroid tissue from euthyroid monkey and hyperthyroid monkey at two magnifications (20 $\times$ and 40 $\times$).

Supplementary Figure 3). The increase in TT₄ and FT₄ in the A-sub-Ad-injected monkeys, combined with their weight loss and increased heart rates, indicated that hyperthyroidism had been successfully induced.

Thyroid overactivity (hyperthyroidism) is usually associated with goiter. In this experiment, 50% (three in six monkeys) of the thyroid glands from A-sub-Ad-injected monkeys were visibly enlarged, unlike the normal-sized glands in the EGFP-Ad group (Fig. 2a). One A-sub-Ad-injected monkey got slight hypertrophy in the thyroid gland. The weight of thyroid gland (g) showed a significant difference in the A-sub-Ad-injected monkeys, with a mean of 0.318 ± 0.04 g ($\pm$ S.E.M.) vs 0.203 ± 0.01 g ($\pm$ S.E.M.) in the EGFP-Ad-injected monkeys ($P=0.018$, independent samples t -test, $n=6$). The thyroid glands of monkeys with high serum TT₄ showed marked hypertrophy and exhibited thyrocyte hypercellularity, with intrusion into the follicular lumen due to hyperactivity (Fig. 2b). Furthermore, the thyroid follicle showed an evident increase in the hyperthyroid monkeys, with a mean value of 51.37 ± 2.31 μm^2 ($\pm$ S.E.M.) vs 26.95 ± 0.6 μm^2 ($\pm$ S.E.M.)

in the euthyroid monkeys ($P=0.01$, independent samples t -test, $n=6$). There was no lymphocytic infiltration in any of the thyroid glands. No ophthalmopathy was observed in A-sub-Ad-injected monkeys.

Figure 3

TSH receptor antibody (TRAb) measured by ELISA and TBI assay. (a) The general trend of TRAb measured by ELISA and TBI assay. (b) The individual values of TRAb, as measured by ELISA and TBI after the third and fifth injections ($P=0.03$, $P=0.04$, respectively, independent samples t -test, $n=6$). The shaded area indicates the mean $\pm$ 2 s.d. of values for the control animals.

Figure 4

Determination of anti-adenovirus neutralizing antibody titer. (a) Anti-adenovirus antibody responses in vaccinated animals. (b) Anti-adenovirus antibodies block entry of Ad-EGFP into HEK293 cells. HEK293 cells were

infected with Ad-EGFP (moi50) after pre-incubation with serial dilutions of the serum. The cell control contained HEK293 cells alone, and the virus control with HEK293 cells cultured with virus in the absence of monkey serum.

Quantitation of TRAb levels

Sera were studied at several time points to detect TRAbs. The TSHR is a major autoantigen in autoimmune hyperthyroidism, and specific autoantibodies (TRAb) acting as TSHR agonists are pathogenic and are a diagnostic hallmark of GD (Smith *et al.* 2007). We examined TRAbs in two ways. First, we measured TRAbs in undiluted serum by competitive ELISA. TRAbs from the EGFP-Ad group were almost undetectable at all time points. In contrast, TRAbs in A-sub-Ad-injected monkeys showed a slight increase following the first injection, fluctuated between the second and fourth injections, and then sharply peaked after the fifth injection (Fig. 3a). A-sub-Ad-injected monkeys showed higher levels after five injections than after three ($P=0.03$, independent samples *t*-test, $n=6$; Fig. 3b). As in the ELISA data, the EGFP-Ad group showed low TBI values throughout. In contrast, the TBI values in A-sub-Ad-injected monkeys began to increase after the first injection, stabilized between the second and fourth injections, and peaked at $63.3 \pm 14.2\%$ ($\pm$ S.E.M.), after the fifth injection (Fig. 3a). A-sub-Ad-injected monkeys had higher TBI value after five injections than after three ($P=0.04$, independent samples *t*-test, $n=6$; Fig. 3b).

Determination of anti-adenovirus neutralizing antibody titer

One of the concerns regarding adenovirus vaccines, in general, is the effect of preexisting anti-adenovirus neutralization antibodies on the performance of vaccines. Therefore, we determined the level of anti-adenovirus antibody elicited by the initial vaccination and its effect on the subsequent booster vaccine (Fig. 4b). To do so,

anti-adenovirus antibodies were measured at multiple time points. Before receiving their first vaccination, all animals were negative, but after receiving their second inoculation, all animals developed antibodies, with end-point titers ranging from 200 to 800. These titers steadily increased when the animals received the remaining dose of adenovirus, peaking at 6400 (Fig. 4a). When comparing TRAb and anti-adenovirus antibodies, we observed that TRAb showed a slight increase during the first four injections and sharply peaked after the fifth injection, while the anti-adenovirus antibodies rose in a straight line from the first to the fifth injection. This result showed that the neutralizing antibody's effect on TRAb is very small in the case of repeated administration. This finding was similar to that in Chen's report (Chen *et al.* 2000).

Physiology and biochemistry parameters

The body temperature of rhesus showed no significant difference between the two groups either in the morning or evening ($P=0.58$, $P=0.78$, $n=6$; Supplementary Figure 2, see section on supplementary data given at the end of this article). In addition, blood pressure was normal throughout the experiment. Biochemistry parameters, including ALT, AST, ALP, LDH, and CK, were monitored at several time points, and no significant differences were observed between the two groups (Supplementary Table 2). In addition, neither inflammation nor other pathological changes, including cellular swelling or necrosis, could be observed from the histology of liver and heart (Supplementary Figure 4). These findings indicated that during the experiment, hyperthyroidism does not affect the physiology or biochemistry of rhesus monkeys.

Furthermore, we can confirm the safety of adenovirus as an immune vector throughout the five repeated injections.

Th1/Th2 balance

Th1 and Th2 cells produce IFN- γ and IL4 respectively. The levels of IFN- γ and IL4 were higher in A-sub-Ad-injected monkeys than in EGFP-Ad-injected monkeys, no matter which cytokines were detected in serum ($P=0.07$, $P=0.17$, independent samples t -test) or splenocyte-culture supernatants ($P=0.05$, $P=0.04$, independent samples t -test). The ratio of cytokine IFN- γ to IL4 showed no statistical difference in A-sub-Ad-injected monkeys vs EGFP-Ad-injected monkeys (Fig. 5a). Similar to the cytokine concentrations, mRNA levels of *TBX21* in both PBMCs ($P=0.04$, independent samples t -test) and splenocytes ($P=0.04$, independent samples t -test) increased in A-sub-Ad-injected monkeys compared with the EGFP-Ad-injected monkeys. There was no statistical difference in the proportion of *TBX21* to *GATA3* in both PBMCs and splenocytes ($P=0.11$, $P=0.11$, independent samples t -test) (Fig. 5b).

Th17/Treg balance

Treg cells (Tregs), a subset of CD4+ lymphocytes expressing the surface marker CD25 as well as the transcription factor FOXP3, downregulate Th1, Th2, and Th17 responses and decrease acute inflammation in autoimmune diseases. The percentages of CD4+CD25+ from CD4+T cells were significantly lower in A-sub-Ad-injected monkeys in PBMC at $6.70 \pm 1.09\%$ ($\pm$ S.E.M.) and splenocyte at $5.93 \pm 1.13\%$ ($\pm$ S.E.M.) compared with EGFP-Ad-injected monkeys at $9.18 \pm 0.95\%$ ($\pm$ S.E.M.) and $9.83 \pm 0.44\%$ ($\pm$ S.E.M.). The ratio of CD4+CD25+ Foxp3+ on CD4+ cells in PBMC and splenocyte of monkeys treated with A-sub-Ad at $0.70 \pm 0.11\%$ ($\pm$ S.E.M.) and $0.81 \pm 0.10\%$ ($\pm$ S.E.M.) also declined significantly compared with EGFP-Ad-injected monkeys at $1.34 \pm 0.12\%$ ($\pm$ S.E.M.) and $2.35 \pm 0.53\%$ ($\pm$ S.E.M.) (Fig. 6a). No significant difference was observed in the proportion of Tregs between PBMCs and splenocytes. As in the flow cytometry data, the level of *FOXP3* mRNA in PBMCs and the spleen was lower in A-sub-Ad-injected monkeys (0.76 ± 0.24 , 0.90 ± 0.10) compared with the EGFP-Ad-injected monkeys (1.00 ± 0.34 , 1.11 ± 0.16 ; Fig. 6b).

Th17, a newly identified effector T-cell subset, has recently been observed to play a role in numerous autoimmune diseases. In the flow cytometry data, no significant difference was observed between the A-sub-Ad-injected monkeys and EGFP-Ad-injected monkeys in the

Figure 5

Th1/Th2 balance between A-sub-Ad and EGFP-Ad group (mean $\pm$ S.E.M.). (a) The levels of cytokines IFN- γ and IL4 from A-sub-Ad vs EGFP-Ad immunized macaques ($P=0.07$, $P=0.04$ in PBMCs and splenocytes of IFN- γ , respectively; $P=0.17$, $P=0.04$ in PBMCs and splenocytes of IL4, respectively). (b) mRNA expression of *TBX21* and *GATA3* from the peripheral blood and the spleen investigated both in A-sub-Ad macaques and EGFP-Ad macaques ($P=0.04$, $P=0.03$ in PBMCs and splenocytes of *TBX21*, respectively, independent-sample t -test, $n=6$). * $P < 0.05$.

proportion of IL17+ cells to CD4+ cells (Fig. 6a). As with the flow cytometry data, the expression of *ROR-γt* mRNA in PBMCs and the spleen did not increase significantly after immunization with A-sub-Ad ($P=0.05$, $P=0.22$, independent samples *t*-test; Fig. 6b). However, the ratio of Th17 cells to Tregs cells in A-sub-Ad-injected monkeys group was significantly higher than in the EGFP-Ad-injected monkeys (Fig. 6c).

Dose-dependent inhibition of CD4+CD25+ Treg cells following the proliferation of CD4+CD25- T cells and cytokine production

Tregs typically do not respond to polyclonal and allogeneic stimuli, and they inhibit the response of

CD4+CD25- cells to these stimuli. At ratios of 1:1, 1:4, and 1:16, Treg cells from A-sub-Ad-injected monkeys suppressed CD4+CD25- T-cell proliferation by an average of 36.46, 24.88, and 7.72%, respectively, generating levels significantly lower than the figures observed in the EGFP-Ad-injected monkeys ($P=0.02$, $P=0.03$, $P=0.02$, independent samples *t*-test; Fig. 7a). In addition, we quantified the dose-dependent inhibition of CD4+CD25+ Treg cells. In the three groups, by decreasing the ratio of CD4+CD25+ Treg cells to CD4+CD25- T cells, the inhibitory ability of CD4+CD25+ Treg cells was gradually reduced (Fig. 7b).

As Th1, Th2, and Th17 cells play an important role in the development of autoimmune disease, we investigated whether the CD4+CD25+ Treg cells regulated CD4+

Figure 6

Th17/Treg balance between the A-sub-Ad and EGFP-Ad groups (mean $\pm$ S.E.M.). (a) The percentages of CD4+CD25+, CD4+CD25+ Foxp3+ from CD4+T cells and the proportions of Th17 cells in PBMC and splenocyte from A-sub-Ad vs EGFP-Ad immunized macaques. (b) mRNA expression of *FOXP3* and *ROR-γt* mRNA from the peripheral blood and the spleens of A-sub-Ad

macaques and control macaques ($P=0.03$, $P=0.003$ in PBMCs and splenocytes of *FOXP3* independent-sample *t*-test, $n=6$). (c) The ratio of Th17 to Treg in A-sub-Ad vs con-Ad immunized macaques ($P=0.002$, $P=0.003$ in PBMCs and splenocytes of Th17/Treg, respectively, independent-sample *t*-test, $n=6$). * $P<0.05$; ** $P<0.01$.

Figure 7

Dose-dependent inhibition of the proliferation of CD4+CD25- T Cells by CD4+CD25+ Treg cells. (a) The proliferation of various cell-to-cell ratios and the suppressive effect of CD4+CD25+ Treg cells on the proliferation of CD4+CD25- T cells (mean $\pm$ S.E.M.). (b) Mixed lymphocyte reaction. CD4+

CD25- cells (2.5×10^4 /well) were cultured with CD4+CD25+ cells with plate-bound anti-CD3 in 96-well round-bottom plates. Antigen-presenting cells were added to all cultures for 72 h. (c) Cytokine production and inhibition by CD4+CD25+ cells (mean $\pm$ S.E.M.). * $P < 0.05$; ** $P < 0.01$.

CD25- T cells through the modulation of IFN- γ , IL4, and IL17A. These cytokines are considered to be the representative of Th1, Th2, and Th17 cells respectively. Levels of the cytokines IFN- γ and IL17A were significantly higher in the A-sub-Ad group than in EGFP-Ad group, independent of dose-dependent inhibition of CD4+CD25+ Treg cells. When CD4+CD25- T cells were cultured with CD4+CD25+ Treg cells, IFN- γ production was significantly decreased in A-sub-Ad monkeys at ratios of 1:0, 1:1, and 1:4. Compared with the IFN- γ and IL17A at ratio of 1:16, their production declined significantly at ratios of 1:0 and 1:1 (Fig. 7c).

Discussion

Various protocols have been reported for the rodent model of GD and repeated injection of A-sub-Ad is the most widely used method. Essential clinical and immunological features of a high-quality GD model should consider criteria such as thyrotoxicosis, goiter, and TRAb

(McLachlan *et al.* 2005). In this study, when compared with EGFP-Ad-injected monkeys, half of the A-sub-Ad-injected monkeys had increased TT₄ and FT₄, 66.7% developed goiter, and all had significant higher TRAb levels. In contrast to the rodent model of GD, in which TRAb was detected at levels far higher than those observed in the typical Graves' patient (Chen 2003a, Wu *et al.* 2006), this macaque model had lower TRAb levels. Additionally, there was a significant decrease in the weight of hyperthyroid monkeys, which more closely approximates human GD. It has been uncertain for a long time whether GD could occur in nonhuman primates (McLachlan *et al.* 2011), and we have demonstrated in this study that GD could be induced in rhesus monkeys.

Tregs are involved in the maintenance of homeostatic mechanisms that suppress autoreactive T cell proliferation and promote autoreactive T cell anergy (Wang *et al.* 2009). In this study, both the proportion and the capacity of CD4+CD25+ Treg cells declined in A-sub-Ad-injected monkeys. Th17, a newly identified effector T-cell subset,

has recently been found to play a role in numerous autoimmune diseases, including type-1 diabetes, arthritis and experimental autoimmune encephalitis (Weaver *et al.* 2006, Steinman 2007). The ratio of IL17+ cells to CD4+ cells did not differ between the A-sub-Ad and Ad-control groups. This result was supported by Nicté Figueroa-Vega's report (Figueroa-Vega *et al.* 2010) and Takashi Nanba's clinical trial (Nanba *et al.* 2009). Th17 immune response plays distinct roles in Graves' hyperthyroidism in mice with different genetic backgrounds (Horie *et al.* 2011). The commonly observed GD-susceptible Balb/c strain is not Th17 dependent. In contrast, the non-susceptible non-obese diabetic (NOD)-H2^{h4} mouse model is IL17 dependent. Even though we do not have a commendable explanation for our result that Th17 is not associated with this GD model, it is feasible that it could be due, at least in part, to the different genetic background of the individuals studied, the presence or absence of ophthalmopathy, and the stage of the disease. The balance between Treg and Th17 cells may control inflammation and be important in the pathogenesis of GD (Zhou *et al.* 2012). To assess whether this balance was broken in an animal model of Graves' hyperthyroidism, we detected Th17/Treg functions on different levels including cell frequencies and key transcription factors. Although there was no difference in Th17 levels between the A-sub-Ad and Ad-control groups, we observed a significantly higher ratio of Th17 to Treg cells in the A-sub-Ad group compared with the Ad-control group. This result was also confirmed by real-time PCR analysis of *ROR-γt* and *FOXP3*. The Th17/Treg ratio may be a useful marker with which to assess the severity of diseases in animal models and human diseases (Weaver & Hatton 2009). This result may indicate that lower Treg proportions and an increased Th17:Treg ratio are associated with GD.

In summary, our data indicated that Graves' hyperthyroid has been successfully induced in macaques. In contrast to the rodent model used previously, this macaque model was the first nonhuman primate model of GD and thus more closely approximates human GD.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-13-0279>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Y W, L W, B S, P H, and M J designed the project. Y W, L W, and B S wrote the manuscript. X G performed blood collection. L X, C G, and P C carried out TSHR antibody TBI assay. J F and H L performed sample collection. All authors discussed the results and commented on the manuscript.

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