SPANDIDOS PUBLICATIONS

CD4+ T cells and the Th1/Th2 imbalance are implicated in the pathogenesis of Graves' ophthalmopathy

NING XIA[1], SUXIAN ZHOU[1], YUZHEN LIANG[1], CHANGQING XIAO[1], HANLEI SHEN[1], HAILIN PAN[1], HONGMING DENG[1], NAIZUN WANG[1] and QINGDI Q. LI[2]

[1]Department of Endocrinology, First Hospital of Guangxi Medical University, Nanning 530021, P.R. China; [2]Mary Babb Randolph Cancer Center, and Department of Microbiology, Immunology and Cell Biology, West Virginia University Health Sciences Center, Morgantown, WV 26506, USA

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Abstract. Graves' ophthalmopathy (GO) is considered to be an organ-specific autoimmune disease. However, the pathogenesis of GO is incompletely understood at the present time. To clarify the immunological differences between newly diagnosed GO and Graves' disease (GD) without ophthalmopathy or healthy controls (HC), we examined T-cell profile and the Th1/Th2 profile cell balance in GO (n=20), GD (n=20) and HC (n=20) using flow cytometry. We also assessed the influence of methimazole on the immunocyte profiles in patients with GO and GD and analyzed the relationship of the immunologic changes with CAS, FT3, FT4, TRAb, TMA and TGA among the three investigated groups. We report in this study that: 1) The percentage of $CD4^+$ T cells and the ratio of $CD4^+/CD8^+$ cells were higher, but the population of $CD8^+$ T cells was lower in both GO and GD than those of HC ($P<0.05$); 2) The percentage of $CD8^-/IFN\gamma^+$ T cells (Th1) and the ratio of $CD8^-/IFN\gamma^+$ to $CD8^-/IL\text{-}4^+$ T cells (Th1/Th2) in GO were considerably higher as compared to those in GD and HC ($P<0.05$). On the contrary, the population of Th1 cells, as well as the ratio of Th1/Th2 cells, was lower in GD than that of GO and HC ($P<0.05$); 3) There were no significant differences in T-cell profile and the Th1/Th2 cell balance in either GO or GD patients before and after methimazole treatment; 4) There was a positive correlation of Th1 cell percentage and the Th1/Th2 cell ratio with the clinical activity score (CAS) in GO ($P<0.05$), whereas CAS in GO had no correlation with the T-cell profile, the percentage of Th2 cells, and TRAb ($P>0.05$); 5) T-cell subset and the ratio of Th1/Th2 cells did not correlate significantly with FT3, FT4, TRAb, TMA, or TGA in GO and GD ($P>0.05$). Finally, 6) there were no statistical differences in TRAb, TMA, and TGA between early GO and GD without ophthalmopathy ($P>0.05$). Collectively, these results indicate that the balance of Th1/Th2 in GO shifts to Th1 dominance and that the cellular immune responses mediated by the Th1-type $CD4^+$ cells might play a dominant role in the pathogenesis of GO, and thus suggest that the Th1 cell percentage and the ratio of Th1/Th2 cell subsets may be potentially utilized as clinical parameters for disease activity, for monitoring the effectiveness of immunosuppressive treatment, or for developing immunospecific forms of therapy for Graves' ophthalmopathy.

Correspondence to: Dr Qingdi Q. Li, 216 Watkins Pond Boulevard, Rockville, MD 20850-5622, USA
E-mail: quentinli2004@yahoo.com

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Introduction

Graves' ophthalmopathy (GO) is considered to be a common organ-specific autoimmune disease with a multitude of factors supposedly involved in the onset of the disease. It is believed that factors such as genes, autoimmunity, alteration of orbital fibroblast activity, environmental factors and smoking are intimately correlated to the pathogenesis of GO. The $CD3^+CD4^+CD8^-$ T lymphocytes are predominant in the T cells derived from orbital tissues and peripheral blood of patients with GO (1,2). Previous investigations reported that T cell-mediated immune reactions by Th1 cells might predominate in the orbit in early GO, whereas humoral immunity and Th2 cells may play a greater role in the later stages of the disease (3). Pappa and colleagues demonstrated that T cells are only significantly present in early disease, but increased HLA-DR antigen expression on fibroblasts is observed at all stages in GO (4). This suggests that T cells are more involved in the early stages of the disease process than the latter (4). Accumulating evidence shows that Th1-like cytokines play an important role in the autoantigen expression, proliferation and differentiation of fibroblasts, transformation of preadipocyte into mature adipocytes, glycosaminoglycan (GAG) synthesis, and the expression of immunomodulatory molecules in the orbital tissues, which lead to the increase in orbital volume and intraocular pressure and cause a series of typical clinical symptoms and signs of GO.

However, the immunologic changes and the role of cell-mediated immunity in the pathogenesis of GO remain largely unknown. To clarify these issues, we have estimated the T cell profile ($CD3^+$ T cell, $CD4^+$ T cell and $CD8^+$ T cell), $CD8^-/IFN\text{-}\gamma^+$ T lymphocytes (IFN-γ represents Th1-like cytokines),

and $CD8^-/IL\text{-}4^+$ T lymphocytes (IL-4 represents Th2-like cytokines) in patients with early GO (5), patients with Graves' disease (GD) without ophthalmopathy, and normal controls by flow cytometry. We show here that $CD4^+$ T cell subpopulation and the ratio of Th1/Th2 cells are markedly greater when compared to GD and normal subjects, illustrating that Th1-dependent immune responses may play a role in the development of early GO.

Patients and methods

Patients and controls. The GO group was comprised of 20 patients with GO, 7 males and 13 females, aged 19-42 years, from the Department of Endocrinology and the Department of Ophthalmology of the First Hospital of Guangxi Medical University (Nanning, P.R. China). The time from onset to diagnose ranged from 2 to 15 months. All GO patients without other ophthalmic diseases or orbital tumor were finally diagnosed by clinical manifestation and laboratory examination. The eye signs of all patients were in Class 2-6. The eye signs of GO were classified by the American Thyroid Association as set forth in Table I (3). The GO group had never been treated with antithyroid drugs, glucocorticoids, or other immunosuppressive drugs. We treated all patients with methimazole (30 mg/day) for one month.

The GD group was composed of 20 patients with GD, 8 males and 12 females, aged 18-40 years, from the Department of Endocrinology and the Department of Ophthalmology of the First Hospital of Guangxi Medical University. The time between onset and diagnose ranged from 1 to 15 months. The eye signs of all patients were in Class 0-1. The GD group of patients had never been treated with antithyroid drugs. We treated all patients with methimazole (30 mg/day) for one month.

The normal control group consisted of 20 healthy volunteers, 6 males and 14 females, aged 22-40 years, from the First Hospital of Guangxi Medical University. They had no diseases of thyroid gland, no autoimmune diseases or family history of autoimmune diseases.

Assessment of GO activity. We utilized the clinical activity score (CAS) reported by Mourits *et al* (6) to assess the activity of GO. The CAS was assessed as follows: i) pain: 1) painful or oppressive feeling on or behind the eye and 2) pain during eye movement; ii) injection: 1) eyelid injection and 2) con-junctival injection; iii) edema: 1) chemosis, 2) eye-hillock edema, a) eyelid edema, and b) increase >2 mm in proptosis in 1-3 months; iv) dysfunction: a) decreases 1 low or above in visual acuity in Snellen chart and b) a decrease of eyeball movement in each direction of 5˚ or above. Each above-mentioned point counts for 1 point. The more points, the higher the activity. Proptosis was determined by using Helter exophthalmometer. The steps and method of measurement strictly followed the manufacturer's instructions.

Antibodies. The following monoclonal antibodies (mAbs) directed against human leukocyte surface markers were used: CD3-fluorescein isothiocyanate (FITC), CD8-FITC as well as anti-human cytokine mAbs: anti-interleukin-4 (anti-IL-4)-PE (rat IgG1), anti-gamma interferon (anti-IFN-γ)-PE (mouse IgG1), and appropriate isotype controls were purchased from Immunotech/Coulter (Marseille, France). Anti-CD3-PE/cyanin 5.1 (Cy5) and appropriate isotype controls were purchased from Immunotech/Coulter.

Table I. Classification of eye changes in Graves' disease.

Class	Definition
0	No signs or symptoms
1	Only signs but no symptoms (signs limited to upper lid, retraction stare, and lid lag)
2	Soft tissue involvement (symptoms and signs)
3	Proptosis (measured with Helter exophthalmometer)[a]
4	Extraocular muscle involvement
5	Corneal involvement
6	Sight loss (optic nerve involvement)

[a]The upper limit of normal differs in races: Oriental, 18 mm; White, 20 mm; Black, 22 mm. Increase in proptosis of 3-4 mm is mild involvement; 5-7 mm, moderate involvement; and >8 mm, severe involvement. Other classes can be similarly graded as mild, moderate, or severe.

Reagents. Ionomycin, monensin, and PMA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The reagents for cell fixation and permeabilization (IntraPrep) were purchased from Immunotech/Coulter.

Cell cultures. Peripheral blood mononuclear cells (PBMCs) were isolated from heparin-treated peripheral blood. PBMCs were separated by lymphocytes separation medium (T.B.D. Co., Tianjing, China) density gradient centrifugation. Cells were suspended in RPMI-1640 medium supplemented with 10% fetal calf serum (S.J.Q. Co., Hangzhou, China), 2 mM glutamine, 100 U of penicillin per ml (Shijiazhuang Pharma. Group ZhongNuo Pharmaceutical Co. Ltd., Shijiazhuang, China), and 100 μg of streptomycin per ml (L.K. Co., Shandong, China) at a density of 10^6/ml. To avoid attachment of monocytes to the tubes, PBMCs were cultured in 1.5 ml polypropylene micro-tubes (Axygen Scientific Inc., Union, USA). Cells were cultured in the absence or presence of the activators PMA (5 ng/ml), ionomycin (1 mM) and monensin (2 mM) for 6 h at 37˚C in a 5% CO_2 incubator.

Immunostaining of cell surface antigens and intracellular cytokine analysis. At the indicated time of culture, cells were harvested and distributed (100 μl per tube) to 1.5 ml polypropylene microtubes (Axygen Scientific Inc.) for immuno-labeling. As some antibodies which recognize cell surface markers may not bind to fixed or denaturated antigens, immunostaining for the surface determinants was performed with unfixed cells prior to staining for intracellular cytokine analysis. For better discrimination of monocytes and lymphocytes in PBMCs during flow cytometry analysis, fluorochrome-conjugated mAbs against

Comparison of the percentages of T cell subsets ...e GO, GD without ophthalmopathy, and control groups.

	GO (n=20)	GD (n=20)	Control (n=20)
$CD3^+$ T cells (%)	63.48±9.54[a]	64.19±6.48[a]	63.57±9.14[a]
$CD4^+$ T cells (%)	40.68±7.70[b]	39.07±5.29[b]	33.49±5.49[b]
$CD8^+$ T cells (%)	19.67±4.55[c]	22.33±3.74[c]	28.31±6.09[c]
$CD4^+/CD8^+$ T cells	2.15±0.54[d]	1.79±0.37[d]	1.22±0.28[d]

[a]Statistical analysis for the comparison of the percentage of $CD3^+$ T cells among the GO, GD without ophthalmopathy and control groups shows no significant differences (P>0.05). [b]Statistical analysis shows that the percentage of $CD4^+$ T cells is higher in the GO and GD groups than in the control group (P<0.05), but there is no significant difference between the GO group and the GD without ophthalmopathy group (P>0.05). [c]Statistical analysis shows that the percentage of $CD8^+$ T cells is lower in the GO and GD groups than in the control group (P<0.05), but there is no significant difference between the GO group and the GD without ophthalmopathy group (P>0.05). [d]Statistical analysis shows that the $CD4^+/CD8^+$ T cell ratio is higher in the GO and GD groups than in the control group (P<0.05), and that the $CD4^+/CD8^+$ T cell ratio is higher in the GO group than in the GD group (P<0.05).

Table III. Comparison of the percentages of $CD8^-/IFN\text{-}\gamma^+$ and $CD8^-/IL\text{-}4^+$ T cells among the GO, GD without ophthalmopathy, and control groups.

	GO (n=20)	GD (n=20)	Control (n=20)
$CD8^-/IFN\text{-}\gamma^+$ T cells(%)	10.09±3.28[a]	4.66±3.77[a]	7.22±1.31[a]
$CCD8^-/IL\text{-}4^+$ cells (%)	1.84±1.77[b]	3.97±2.05[b]	1.68±0.75[b]
$CD8^-/IFN\text{-}\gamma^+/$ $CD8^-/IL\text{-}4^+$ T cells	8.46±5.64[c]	1.71±1.75[c]	5.01±2.19[c]

[a]Statistical analysis shows that the percentage of $CD8^-/IFN\text{-}\gamma^+$ T (Th1) cells is higher in the GO group than in the control group (P<0.05), but there is no significant difference between the GD group and the control group (P>0.05). [b]Statistical analysis shows that the percentage of $CD8^-/IL\text{-}4^+$ T (Th2) cells is higher in the GD group than in the control group (P<0.05), but there is no significant difference between the GO group and the control group (P>0.05). [c]Statistical analysis shows that the Th1/Th2 cell ratio is higher in the GO group than in the GD and control groups (P<0.05).

the surface determinants were added to each tube in the following combinations: CD3-PE/Cy-5 and $CD8^-$FITC. The cells were incubated with mAbs or appropriate FITC- or PE/Cy-5-conjugated isotype controls for 15 min at room temperature (18-25˚C), fixed, and permeabilized with IntraPrep Reagent. Then, the cells were stained for intracellular cytokines by using PE-conjugated mAbs against human IL-4 and IFN-γ (15 min at 18-25˚C). PE-conjugated isotype controls were used in parallel. After the cells were washed in PBS, the cells were suspended in PBS for flow cytometry analysis.

Flow cytometry acquisition and analysis. Samples were analyzed in an EPICS XL flow cytometer (Beckman Coulter, USA) by using System II software. The analysis was conducted by gating $CD3^+$ cells among the 10,000 total events acquired. Isotype controls were used to verify the staining specificity of experimental conditions and as a guide for setting markers to delineate positive and negative populations. Since it has previously been demonstrated that the surface CD4 molecule is rapidly and completely down-regulated in response to phorbol esters, CD4 mAbs could not be used to delineate the $CD4^+$ T-cell subset after >4 h of PMA-ionomycin stimulation. However, we observed that CD8 expression did not diminish after this treatment and that the vast majority of the $CD8^-$ cells were indeed $CD4^+$. Therefore, in all the experiments examining lymphocytes stimulated with PMA, the $CD8^-$ subset was considered equivalent to the $CD4^+$ subset. The types of intracellular cytokines were determined in the following cell subpopulations: $CD3^+$ and $CD3^-$ lymphocytes (dual-color flow cytometry), $CD8^+$ and $CD8^-$ within $CD3^+$ lymphocytes (three-color flow cytometry), or $IFN\text{-}\gamma^+$ and $IL\text{-}4^+$ within $CD8^-CD3^+$ lymphocytes (three-color flow cytometry). It is meant that $IFN\text{-}\gamma^+$ cell within $CD8^-CD3^+$ lymphocytes is type I helper T cell (Th1) and $IL\text{-}4^+$ cell within $CD8^-CD3^+$ lymphocytes is type II helper T cell (Th2).

Detection of T cell subgroups, and $CD8^-/IFN\text{-}\gamma^+$ and $CD8^-/IL\text{-}4^+$ T cells. Disebrin (50 units) was quickly added to patient's blood specimen. All samples were analyzed by flow cytometry (Beckman Coulter) after treatment with phorbol ester (25 ng/ml PMA), ionomycin (1 ng/ml), monensin (1.7 μg/ml), RPMI-1640, CD3-PC5, CD8-FITC, IL-4-PE, IFN-γ-PE, and permeabilization reagent (Immunotech, France).

Other methods. FT3, FT4, and S-TSH were measured by chemiluminescence assay (Beckman Coulter). Thyrotropin receptor antibodies (TRAbs) were assessed by radioimmunoassay (RSR Company, UK). Anti-thyroglobulin antibodies (TG-Abs) and anti-microsomal antibodies (TM-Abs) were detected by radioimmunoassay (Institute of North Biotechnology, Beijing, China).

Statistical analysis. The frequency of cytokine-producing cells was expressed as a mean percentage (± SD) of the labeled cells. The SPSS10.0 was used to evaluate differences between groups: P<0.05 was considered statistically significant.

Results

The percentage of T cell subsets in GO, GD without ophthalmopathy and the controls. As shown in Table II, there were no statistically significant differences in the percentage of $CD3^+$ T cells in the groups studied (P>0.05). The percentage of $CD4^+$ T cells was significantly higher both in GO and GD subjects as compared with the controls (P<0.05), but there was no significant difference in $CD4^+$ T cell population between the early GO group and the GD without ophthalmopathy group (P>0.05) (Table II). By contrast, the percentage of $CD8^+$ T cells was considerably lower in the GO and GD groups in comparison to the controls (P<0.05). Likewise, there was no

Table IV. Comparison of the levels and positive rates of TRAb, TMA, and TGA between the GO group and the GD without ophthalmopathy group.

	TRAb		TMA		TGA	
	Level (U/L)	Positive rate (%)	Titer (%)	Positive rate (%)	Titer (%)	Positive rate (%)
GD[a]	95.4±33.72	70	21.6±11.13	40	31.0±14.41	45
GO[a]	96.0±38.08	75	21.44±4.58	45	30.28±7.24	40

[a]Statistical analysis for the comparison of the levels and positive rates of TRAb, TMA, and TGA shows no significant differences between the GO group and the GD without ophthalmopathy group (P>0.05).

difference in the population of $CD8^+$ T cells between the GO group and the GD without ophthalmopathy group (P>0.05) (Table II). The $CD4^+/CD8^+$ T cell ratio was significantly higher both in GO and GD patients than in the control group (both P<0.05). Also, the $CD4^+/CD8^+$ T cell ratio in the GO group was significantly increased in comparison to the GD without ophthalmopathy group (P<0.05) (Table II).

The populations of $CD8^-/IFN\text{-}\gamma^+$ and $CD8^-/IL\text{-}4^+$ T cells in the GO group, the GD without ophthalmopathy group, and the control group. The percentage of $CD8^-/IFN\text{-}\gamma^+$ T lymphocytes (Th1) was markedly higher in the GO group than in the GD without ophthalmopathy group and the control group (both P<0.05), but there was no difference between the GD without ophthalmopathy group and the control group (P>0.05) (Table III). On the contrary, the population of $CD8^-/IL\text{-}4^+$ T lymphocytes in the GD without ophthalmopathy group was obviously higher than in the GO group and the control group (both P<0.05), and no difference was observed between the GO group and the control group (P>0.05) (Table III). The ratio of $CD8^-/IFN\text{-}\gamma^+$ T (Th1) cells to $CD8^-/IL\text{-}4^+$ T (Th2) cells was markedly higher in GO patients than in GD patients and the control subjects (both P<0.05). Similarly, the $CD8^-/IFN\text{-}\gamma^+$ T (Th1) to $CD8^-/IL\text{-}4^+$ T (Th2) cell ratio in the GD without ophthalmopathy group was substantially higher than in the control group (P<0.05) (Table III).

The analysis of the relationship between CAS and other factors in the GO group. There was a positive correlation between CAS and the percentage of $CD8^-/IFN\text{-}\gamma^+$ T cells (r=0.82, P<0.01) and the ratio of $CD8^-/IFN\text{-}\gamma^+$ T cells to $CD8^-/IL\text{-}4^+$ T cells (r=0.48, P<0.05) in the GO group. However, CAS has no correlation with the percentage of $CD3^+$ T cells, the percentage of $CD4^+$ T cells, the percentage of $CD8^+$ T cells, the $CD4^+$ T/$CD8^+$ T cell ratio, the percentage of $CD8^-/IL\text{-}4^+$ T lymphocytes, and TRAb in GO patients (P>0.05 for all).

The analysis of the relationship between FT3 and other factors in GO patients. FT3 had no correlation with the percentage of $CD3^+$ T cells, the percentage of $CD4^+$ T cells, the percentage of $CD8^+$ T cells, the $CD4^+/CD8^+$ T cell ratio, the percentage of $CD8^-/IFN\text{-}\gamma^+$ T (Th1) cells, the percentage of $CD8^-/IL\text{-}4^+$ T (Th2) cells, and the Th1/Th2 cell ratio in GO subjects (P>0.05 for all).

The analysis of the relationship between FT4 and other factors in GO subjects. FT4 had no correlation with the percentage of $CD3^+$ T cells, the percentage of $CD4^+$ T cells, the percentage of $CD8^+$ T cells, the $CD4^+/CD8^+$ T cell ratio, the percentage of $CD8^-/IFN\text{-}\gamma^+$ T (Th1) cells, the percentage of $CD8^-/IL\text{-}4^+$ T (Th2) cells, and the Th1/Th2 cell ratio in the GO group (P>0.05 for all).

The analysis of the relationship between TRAb and other factors in GO individuals. The levels of TRAb had no correlation with the percentage of $CD3^+$ T cells, the percentage of $CD4^+$ T cells, the percentage of $CD8^+$ T cells, the $CD4^+/CD8^+$ T cell ratio, the percentage of $CD8^-/IFN\text{-}\gamma^+$ T (Th1) cells, the percentage of $CD8^-/IL\text{-}4^+$ T (Th2) cells, and the Th1/Th2 cell ratio in the GO group (P>0.05 for all).

The analysis of other parameters in GO and GD patients. The levels and positive rates of TRAb, TMA, and TGA were measured in patients with early GO and GD without ophthalmopathy (Table IV). There were no statistically significant differences in TRAb, TMA, and TGA between the GO group and the GD without ophthalmopathy group (P>0.05 for all) (Table IV).

The analysis of some parameters in GO and GD patients before and after methimazole treatment. Lastly, we treated patients with early GO and GD without ophthalmopathy with methimazole and assessed the T cell subsets, the populations of $CD8^-/IFN\text{-}\gamma^+$ T lymphocytes and $CD8^-/IL\text{-}4^+$ T lymphocytes, and the ratio of $CD8^-/IFN\text{-}\gamma^+$ T (Th1) cells to $CD8^-/IL\text{-}4^+$ T (Th2) cells in these patients before and after treatment with methimazole. There were no significant differences in these parameters examined in early GO patients and patients with GD without ophthalmopathy before and after methimazole treatment.

Discussion

Although the pathogenesis of GD is not fully understood, cell-mediated immunity is thought to be implicated in the pathogenesis of GO. In the present study, we found by the analysis of human peripheral blood cells, that $CD4^+$ T cells and the $CD4^+$ to $CD8^+$ cell ratio are increased but $CD8^+$ T cells are decreased in patients with early GO and GD without ophthalmopathy (7-10).

T cells are mainly helper T (Th) cells, while $CD8^+$ are mainly cytotoxic T (Tc) cells and suppressor T (Ts) cells. Th cells possess augment functions mediating humoral or cell-mediated immunity, whereas Ts cells possess suppressive functions modulating the activity and/or the transformation of Th cells. The balance between $CD4^+$ and $CD8^+$ T cells maintains the normal immune reaction. The defect in the number and activity of Ts cells in GD leads to the dysfunction of their immune monitoring and regulating functions and fails effectively to sustain the immune surveillance; thus, causing the disturbance of the immune regulatory function in GD and impairing the body normal immune activity. When the body is exposed to external stimulants or internal autoantigen formation (e.g. bacterial, virus or fungus infection results in molecular mimicry and expression of superantigen molecules or autoantigen), the decreased Ts cells fail to properly restrain Th cells; whereas increased Th cell population improperly enhances the humoral immunity and the cell-mediated immunity which activate autoimmune responses against autologous tissues and organs such as the thyroid, retroocular tissues, and pretibial tissues leading to the development of autoimmune inflammation and the formation of a series of clinical symptoms and signs of GD.

In this investigation, we also measured the intracellular IFN-γ and IL-4 in the unit of single cell by flow cytometry to study the constituent of different Th cells and to evaluate the balance between Th1 and Th2 in early GO and GD without ophthalmopathy. We have demonstrated that the percentage of $CD8^-/IFN\text{-}\gamma^+$ T cells and the ratio of $CD8^-/IFN\text{-}\gamma^+$ T (Th1) cells to $CD8^-/IL\text{-}4^+$ T (Th2) cells were markedly higher in early GO patients than in GD patients and the healthy individuals, indicating that the balance of Th1/Th2 shifts to Th1 dominance in early GO (1,2).

$CD4^+/IFN\text{-}\gamma^+$ T cells secrete IFN-γ which represents Th1-type cytokines, while $CD4^+/IL\text{-}4^+$ T cells secrete IL-4 representing Th2-type cytokines (5). Therefore, we can analyze the balance between IFN-γ and IL-4 to reflect the balance between Th1 and Th2 (11). The balance between IFN-γ and IL-4 plays an important role in the process of Th0 cell differentiation into Th1 cells or Th2 cells and further determines whether the immune response is predominantly of a humoral or a cell-mediated pattern.

Th1 cells mediate and facilitate the cell-mediated immune response. Th1 cells and macrophages infiltrate retrobulbar tissues and release a series of cytokines. Th1-type cytokines directly stimulate fibroblast proliferation, the transformation from preadipocyte to adipocyte, glycosaminoglycan synthesis, and the expression of immunomodulatory molecules. The outcome of these changes leads to retrobulbar tissue edema and volume multiplication that cause an increase of intraocular pressure and a series of typical clinical symptoms and signs of GO.

In contrast to Th1 cells, evidence shows that Th2 cells mediate humoral immunity and may play a greater role in the later stages of the disease (3). The percentage of $CD8^-/IL\text{-}4^+$ T (Th2) lymphocytes was higher in GD without ophthalmopathy than in early GO, but the ratio of $CD8^-/IFN\text{-}\gamma^+$ T (Th1) lymphocytes to $CD8^-/IL\text{-}4^+$ T (Th2) lymphocytes was lower in GD than in early GO (12,13), suggesting that the balance between Th1 and Th2 shifts to Th2 and that the humoral immunity plays a dominant role in GD.

Assisted by Th2 cells, B lymphocytes differentiate into plasmocytes and produce a large quantity of autoantibodies, such as thyrotropin receptor antibody (TRAb). The binding of TRAbs to thyrotropin receptors induces thyroid cell proliferation and hypertrophy, as well as thyroid hormone synthesis and release. We found that the symptoms and signs of hyperthyreosis were less in patients with GO than in patients with GD, and thyroid function was even normal in some GO patients, probably due to the fact that $CD4^+$/Th1 cells are predominant in GO which release IFN-γ and other Th1 cytokines. The high levels of Th1 cytokines promote Th0 cells to differentiate into Th1-cytokine-secreting cells while inhibiting the differentiation into Th2 subset and reduce thyroid autoantibody production, leading to suppression of humoral immune reactions.

Methimazole is an antithyroid drug and inhibits thyroid hormone synthesis. Recent research showed that methimazole moderately inhibited the synthesis of immunoglobulins including TSAb (14-20). In this study, we treated GO and GD patients with methimazole and examined the effect of the drug on the T cell subgroups, the populations of Th1 and Th2 cells, and the Th1/Th2 cell ratio in these patients. Our experiments revealed that there were no significant differences in these parameters in early GO patients and patients with GD without ophthalmopathy before and after methimazole treatment. As we treated the patients with methimazole for only one month, to verify the findings relative to methimazole effects in this investigation, additional large-scale studies with a longer time period of methimazole therapy are warranted.

In conclusion, we found that $CD4^+$ T (Th) cells and the $CD4^+/CD8^+$ cell ratio in the GO and GD groups were significantly increased in comparison to the healthy controls. We also demonstrated in this study that Th1 cells and the Th1/Th2 cell ratio were statistically significantly higher in early GO patients than in GD patients without ophthalmopathy, indicating that the balance between Th1 and Th2 shifts to Th1 dominance and that the cell-mediated immunity mediated by Th1 cells is predominant in GO. These data suggest that the immune reaction in early GO is probably unique and different from that in GD without ophthalmopathy, and that Th1 cell-mediated immune response may play an important role in the early stage of GO. The present results also suggest that Th1 cell percentage and the ratio of Th1/Th2 cell subsets could be potentially used as clinical predictors for disease activity and for monitoring the effectiveness of retrobulbar radiotherapy in GO. Finally, our findings may help elucidate the pathophysiologic processes of autoimmune thyroid diseases and develop immunospecific forms of therapy using immunosuppressive agents, specific cytokines, cytokine catastaltics, and cytokine inducers to modulate Th1/Th2 profile balance and to inhibit the autoimmune response and lessen immune-mediated inflammatory injury for Graves' ophthalmopathy.

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References

1. Yang D, Hiromatsu Y, Hoshino T, Inoue Y, Itoh K and Nonaka K: Dominant infiltration of T(H)1-type $CD4^+$ T cell at the retrobulbar space of patients with thyroid-associated ophthalmopathy. Thyroid 9: 305-310, 1999.
2. Foster G, Otto E, Hansen L, Ochs K and Kahaly G: Analysis of orbital T cells in thyroid-associated ophthalmopathy. Clin Exp Immunol 112: 427-434, 1998.
3. Jaroslaw P, Aniszewski R, Valyasevi W and Bahn RS: Relationship between disease duration and predominant orbital T cell subset in Graves' ophthalmopathy. J Clin Endocrinol Metab 85: 776-780, 2000.
4. Pappa A, Lawson JM, Calder V, Fells P and Lightman S: T cells and fibroblasts in affected extraocular muscles in early and late thyroid associated ophthalmopathy. Br J Ophthalmol 84: 517-522, 2000.
5. Rostaing L, Tkaczuk J and Durand M: Kinetics of intracytoplasmic Th1 and Th2 cytokine production assessed by flow cytometry following *in vitro* activation of peripheral blood mononuclear cells. Cytometry 35: 318-328, 1999.
6. Mourits MP, Prummel MF, Wiersinga WM and Koornneef L: Clinical activity score as a guide in the management of patients with Graves' ophthalmopathy. Clin Endocrinol 47: 9-14, 1997.
7. Yang GF, Ding HL, Yan L, Li F and Liu SL: The change of Graves' disease patients periphery blood lymphocyte subgroup and the relation between the change and TRAb. J Pract Med 16: 909-910, 2000.
8. Chen D, Guang M, Huang C and Li Q: Significance of assessment of T-cell subgroups in patients with Graves' disease. J Clin Int Med 17: 279-280, 2000.
9. Dai W, Ye W, Shen G, Zhu H and Su N: Investigation of peripheral blood active T cell subgroups in Graves' disease. Chin J Microbiol Immunol 13: 384-386, 1993.
10. Li S, Feng J, Xiong Z, An Z, Xie Q and Wei S: The role of OKT4/OKT8 in Graves' ophthalmopathy. J West China Med University 16: 206-209, 2001.
11. Abbas AK, Marphy KM and Sher A: Functional diversity of helper T lymphocytes. Nature 383: 787-793, 1996.
12. Kocjan T, Wraber B, Repnik U and Hojker S: Changes in Th1/Th2 cytokine balance in Graves' disease. Pflugers Arch 440 (Suppl): R94-95, 2000.
13. Kallmann BA, Huther M, Tubes M, Feldkamp J, Bertrams J, Gries FA, Lampeter EF and Kolb H: Systemic bias of cytokine production toward cell-mediated immune regulation in IDDM and toward humoral immunity in Graves' disease. Diabetes 46: 237-243, 1997.
14. Nakazato N, Yoshida K and Mori K: Antithyroid drugs inhibit radioiodine induced increases in thyroid autoantibodies in hyperthyroid Graves' disease. Thyroid 9: 775-779, 1999.
15. Feng X, Xu X and Chen F: Methimazole alters the immunogenicity of thyroglobulin. Chin J Immunol 17: 166-168, 2001.
16. Mozes E, Zinger H and Kohn LD: Spontaneous autoimmune disease in (NZB x NZW) F1 mice is ameliorated by treatment with methimazole. J Clin Immunol 18: 106-113, 1998.
17. Takiyama Y, Miyokawa N and Tokusashi Y: Thyroid-stimulating hormone induces interleukin-18 gene expression in FRTL-5 cells: immunohistochemical detection of interleukin-18 in autoimmune thyroid disease. Thyroid 12: 935-943, 2002.
18. Miyauchi S, Matsuura B and Onji M: Increased levels of serum interleukin-18 in Graves' disease. Thyroid 10: 815-819, 2000.
19. Bossowski A and Urban M: Serum levels of cytokines in children and adolescents with Graves' disease and non-toxic nodular goiter. J Pediatr Endocrinol Metab 14: 741-747, 2001.
20. Kim H, Lee TH and Hwang YS: Methimazole as an antioxidant and immunomodulator in thyroid cells: mechanisms involving interferon-γ signaling and H_2O_2 scavenging. Mol Pharmacol 60: 972-980, 2001.