DECREASED PERCENTAGE OF $CD4^+CD25^+$ REGULATORY T CELLS AND GITR GENE EXPRESSION IN THE SPLEEN OF FLUOROSED MALE MICE

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ABSTRACT: In view of the important role of the glucorticoid-induced tumor necrosis factor-related receptor (GITR) in the inhibiting function of $CD4^+CD25^+$ regulatory T cells, a study was undertaken to evaluate the effects of the fluoride ion (F) on $CD4^+CD25+$ regulatory T cells and the expression of the GITR gene in the spleen in mice. Forty-eight healthy 7-week-old male Kunming mice were divided randomly into four groups and exposed to F at 0 (control), 50, 100, and 150 mg/L NaF in their drinking water for 90 days. At the end of 90 days, the $CD4^+CD25^+$ regulatory T cells were measured by flow cytometry (FCM). With the help of quantitative real-time polymerase chain reaction (QRT-PCR), the spleen GITR gene expression levels of the four groups were quantified. The results indicated that the percentage contents of $CD4^+CD25^+$ regulatory T cells in $CD4^+$ T cells in peripheral blood were reduced in the F-treated groups. Compared with the control group, the GITR gene expression in mice spleen in the groups treated with 50, 100, and 150 mg/L NaF decreased by 15.6%, 42.5%, and 69.9%, respectively.

Keywords: $CD4^+CD25^+$ regulatory T cells; Fluoride; GITR gene; Mice; Spleen.

INTRODUCTION

Fluorosis causes widespread injuries in various tissues and organs of animals, plants, and human beings.[1-4] This could be due to an association with a leak or breakdown in the immune system. Recently we found that fluorosis significantly increased the expression level of Foxp3 in the immune system of mice.[5] With the same mice and methods, we have now quantified another important gene, the GITR (glucocorticoid-induced tumor necrosis factor receptor) gene, which is expressed on $CD4^+CD25^+$T cells.

GITR is a member of the tumor necrosis factor receptor family. Two independent research teams reported that GITR has a vital role to play in the immunosuppression effect mediated by $CD4^+CD25^+$ T cells. Shimizu et al.[6] found that GITR expression had a relatively high level in peripheral blood $CD4^+CD25^+$ T cells and thymus $CD4^+CD25^+$ T cells. Neutralizing GITR by anti-GITR monoclonal antibody could antagonize the immunosuppression mediated by $CD4^+CD25^+$ T cells. Specific autoimmune disease was caused by removing the T cells with GITR expression or giving anti-GITR monoclonal antibody.[6] McHugh

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et al.[7] analyzed GITR gene expression in $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells by DNA chip technology and found that GITR gene expression was higher in $CD4^+CD25^+$ T cells.[7] The distribution of GITR in $CD4^+CD25^+$ regulatory T cells, effector T cells, and antigen presenting cells has a great impact on the functioning of $CD4^+CD25^+$ regulatory T cells.[8]

In this study, with the help of quantitative real-time polymerase chain reaction (QRT-PCR), we quantified the expression levels of the splenic GITR gene of the four groups of male mice with 0, 50, 100, and 150 mg/L NaF in their drinking water.

MATERIALS AND METHODS

Experimental animals: Forty-eight healthy 7-week-old male Kunming mice, supplied by the Chinese Academy of Medical Sciences of Beijing, were used for the experiments, The mice were randomly divided into four groups with free access to standard pellet feed (provided by Experimental Animal Center of Shanxi Medical University) and drinking water as in Table 1.

Table 1. Fluoride ion (F) levels in the drinking water and pellet feed

Group (n=12)	NaF in drinking water (mg/L)	F in drinking water (mg/L)	F ion in feed (mg/kg)
Control	0	0	18±1.12
50 mg/L NaF	50	22.62	18±1.12
100 mg/L NaF	100	45.25	18±1.12
150 mg/L NaF	150	67.87	18±1.12

Conventional analysis: The changes in the daily water intake were observed during the experiment. On the 90th day of feeding, the mice were anesthetized by 20% urethane and sacrificed. The spleens were collected and the spleen index calculated.

$$\text{Spleen index} = \frac{\text{Spleen weight (SW)}}{\text{Body weight (BW)}}$$

After embedding in paraffin, the spleens were serially sectioned to widths of 5 μm and stained with HE. The appropriate dyeing time for the HE staining was determined to obtain the best conditions for visualizing the distinctive spleen structure.

Flow cytometry (FCM): We took blood by enucleating the eyes and adding ethylenediamine tetraacetic acid (EDTA). After this anticoagulation process, we diluted 1mL of blood with an equal volume of Hanks solution. The diluted blood was then trickled slowly down the side of a centrifuge tube containing 3 mL of lymphocyte separation medium and centrifuged at 1000 rpm for 20 min. We then extracted mononuclear cells from the boundary between the two liquids, added 3 mL of Hanks solution, centrifuged at 1500 rpm for 5 min, and then discarded the supernatants. We re-suspended the cells with 0.5 mL RPMI-1640 medium,

passaged the cells at 1.0×10^6/mL dilution, washed the cells in PBS, and then discarded the supernatants. Anti-mouse FITC-CD4/PE-CD25 antibody (eBioscience, San Diego, CA, USA) was introduced and incubated for 20 min at 25ºC. Next, we added 2 mL RBC lysate, stirred mildly, and then incubated for 10 min at 25ºC. After washing in PBS, we added 300 μL of Binding Buffer and monitored the mixture by FCM in the dark at room temperature.

Total RNA extraction and quantitative real-time polymerase chain reaction (QRT-PCR): The spleens were crushed up in liquid nitrogen and the total RNA was extracted according to the Trizol (Invitrogen, USA) manufacturer's instructions for QRT-PCR. According to the alignments of the published mRNA sequences of the β-actin and GITR genes in mice from Genbank, two pairs of specific primers were designed by the Primer 3 software (Table 2). The primers of the GITR gene were designed to amplify an 85 base pairs (bp) transcript. The endogenous house-keeping gene β-actin was used as a control to normalize the quantity of GITR transcripts with its primers designed to amplify an 83 bp transcript.

Table 2. Primer sequences with their corresponding PCR product size and position

Gene	Primers (5'→3')	Primer locations	Product (base pairs)	Genebank accession NO.
β-actin	GATCATTGCTCCTCCTGAGC ACATCTGCTGGAAGGTGGAC	1063–1145	83	NM_007393.
GITR	TCCCCAGTTCTCATTCCATC CTCTGCCCTTTGAGGACTTG	19–103	85	NM_183391.2.

The expression level of GITR gene was quantified by the method of Yan et al.[9] QRT-PCR was conducted by using the Mx3000P™ QRT-PCR system (Stratagene, USA) and two-Step SYBR® QRT-PCR kit (Takara, China). The QRT-PCR protocol included reverse transcription at 42ºC for 5 min and an initial denaturation at 95ºC for 10 sec. This was followed by 40 PCR cycles consisting of a denaturation step at 95ºC for 5 sec, an annealing step at 62ºC for 15 sec, and an extension step at 72ºC for 6 sec. Finally, the melting curve analysis was performed at 95ºC for 15 sec, at 60°C for 1 min, and at 95ºC for 15 sec as in the protocol for the three reaction steps. The amplified products were analyzed by agarose gel electrophoresis.

Statistical analysis: Experimental data were expressed as mean values±SD or SEM. Independent sample T-tests (Statistical Package for the Social Sciences, SPSS 11.5) were performed to analyze differences, for which $p<0.01$ was considered statistically significant.

RESULTS

Water intake and spleen index: The water intake and spleen index decreased with increased dietary fluoride ion (F), but not to a statistically significant extent by the T-test ($p>0.05$, Table 3).

Table 3. Water intake and spleen index of the mice in the four groups (Mean±SD)

Group (n=12)	Water intake (mL/day)	Spleen index (g/kg)
Control	5.7415±1.1063	3.26±0.58
50 mg/L NaF	6.5061±1.0186	3.19±1.25
100 mg/L NaF	5.6565±0.8806	2.94±0.44
150 mf/L NaF	5.7334±0.9684	2.22±0.91

HE staining: The results of HE staining (Figures 1A-1D.) were in agreement with our previous studies. HE staining showed that compared with the control group, the number of lymphocytes in the lymph nodes and lymphatic sheath around the white pulp central artery in spleen were both reduced, and the red pulp was filled with a lot of lymphocytes in the F-exposed groups. With increasing doses of F, the spleen showed more severe pathological damage.

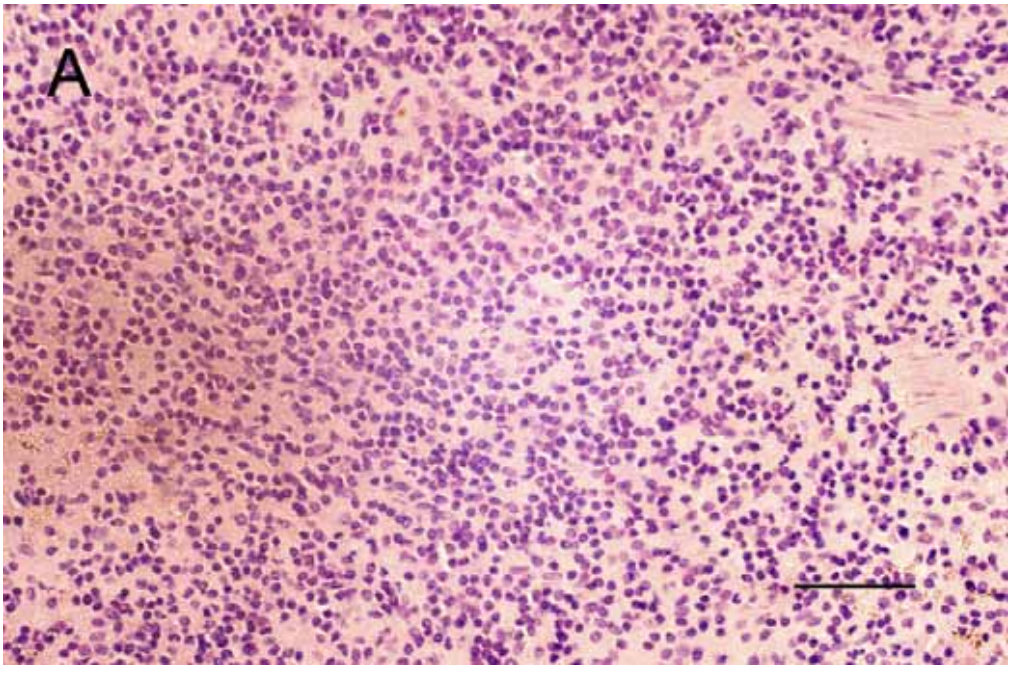

Figure 1A. Photomicrograph showing HE staining of the spleen structure of the male mice in the control group with 0 mg/L NaF. Scale bar=50 μm.

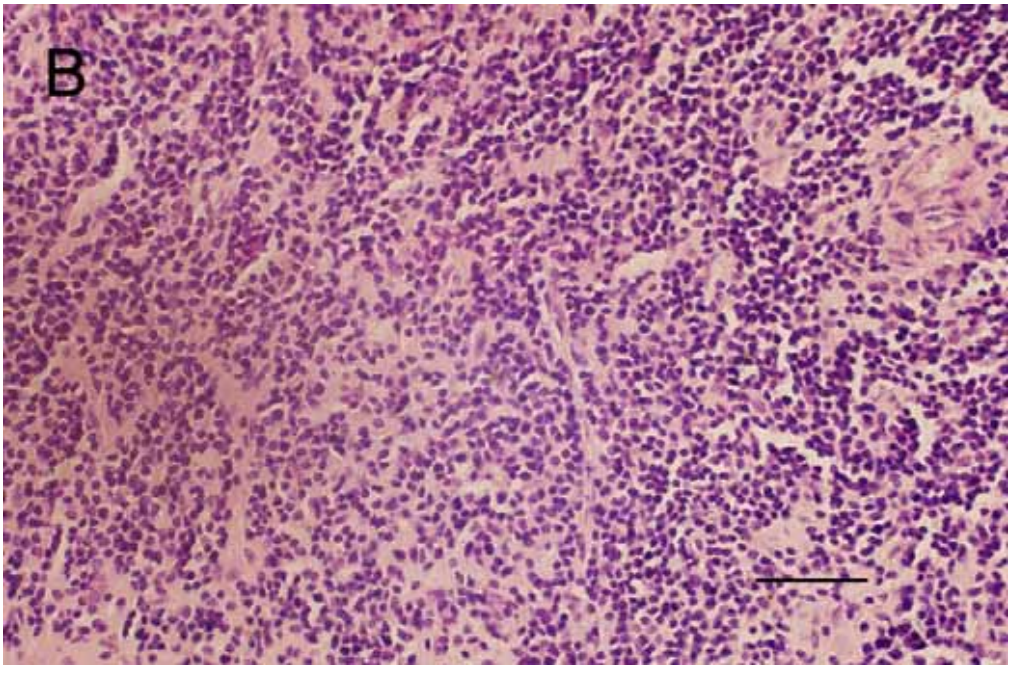

Figure 1B. Photomicrograph showing HE staining of the spleen structure of the male mice in the 50 mg/L NaF group. Scale bar=50 μm.

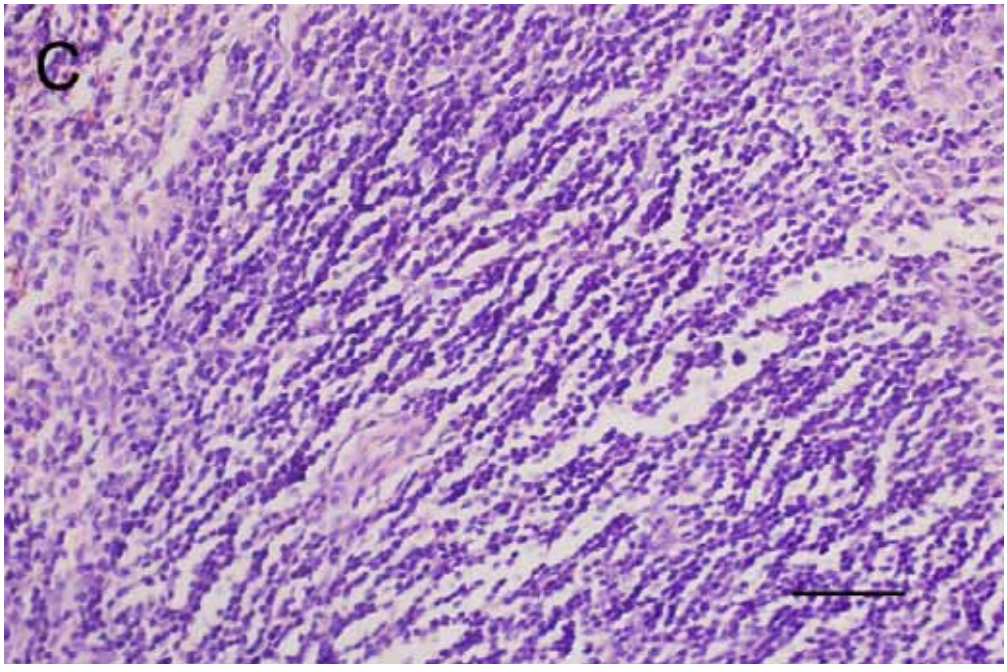

Figure 1C. Photomicrograph showing HE staining of the spleen structure of the male mice in the 100 mg/L NaF group. Scale bar=50 μm.

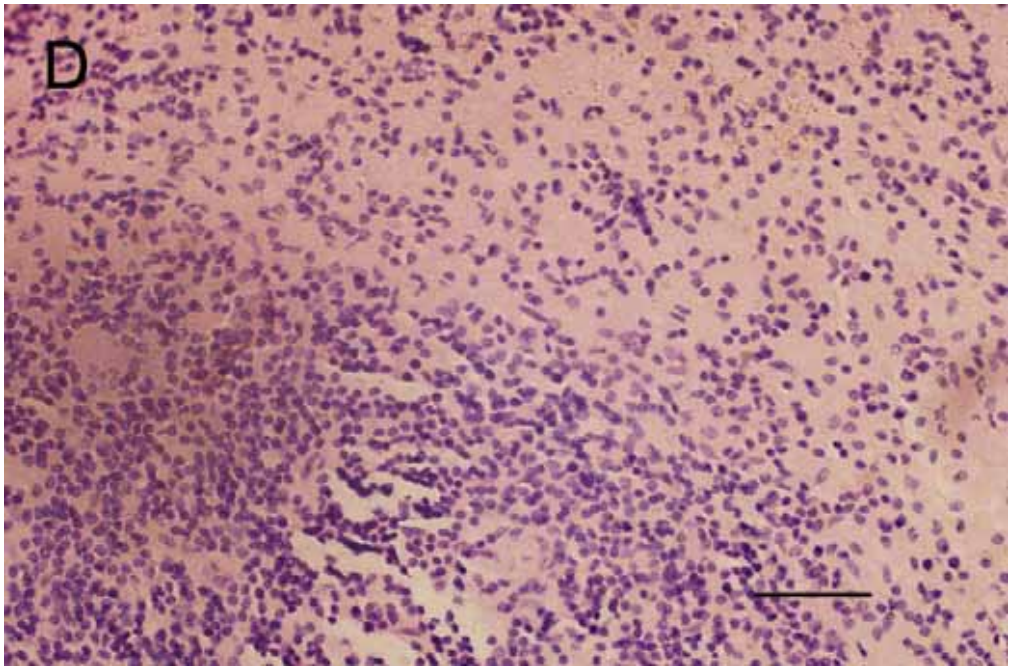

Figure 1D. Photomicrograph showing HE staining of the spleen structure of the male mice in the 150 mg/L NaF group. Scale bar=50 μm.

Flow cytometry (FCM): We picked up the fluorescent signal logarithmically using Cellqust and made scatter diagrams with two sets of parameters by using FSC/SSC. (Figures 2A-2D).

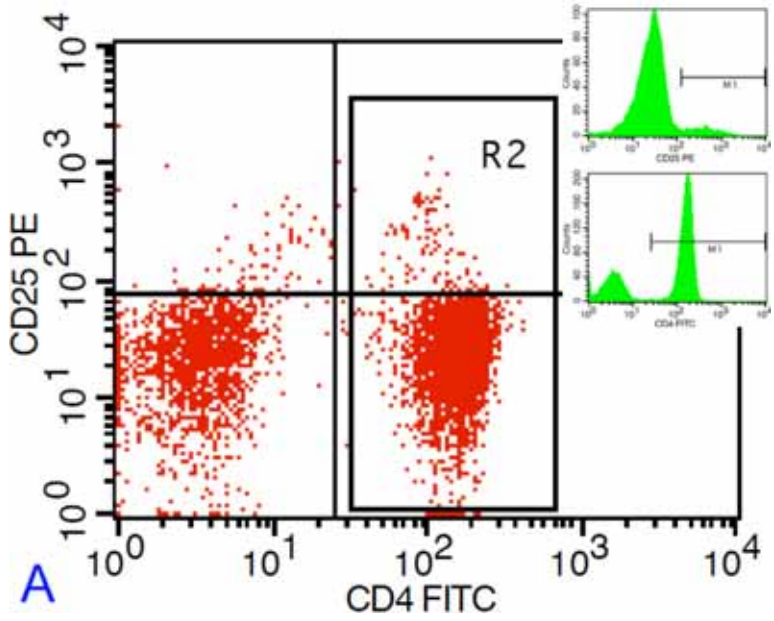

Figure 2A. Scatter diagram of $CD4^+CD25^+$ regulatory T cells of the male mice in the control group with 0 mg/L NaF.

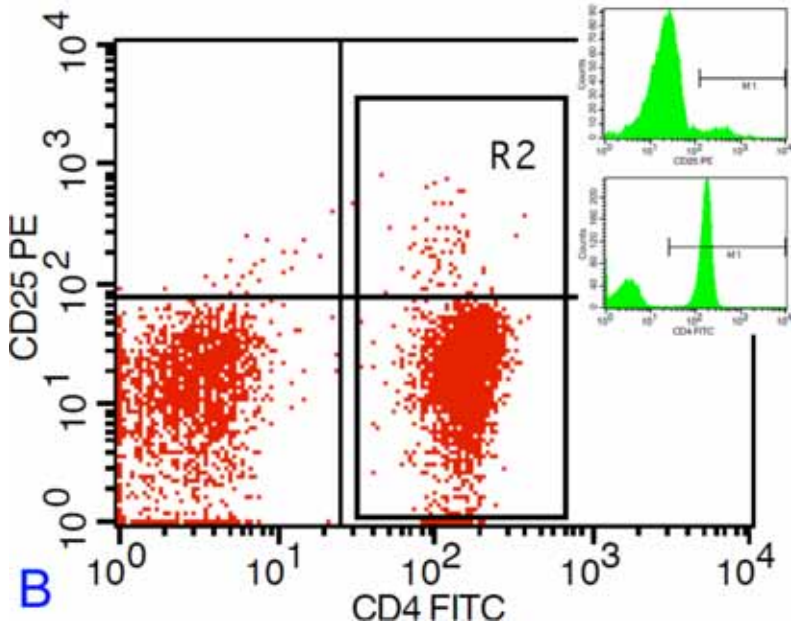

Figure 2B. Scatter diagram of $CD4^+CD25^+$ regulatory T cells of the male mice in the 50 mg/L NaF group.

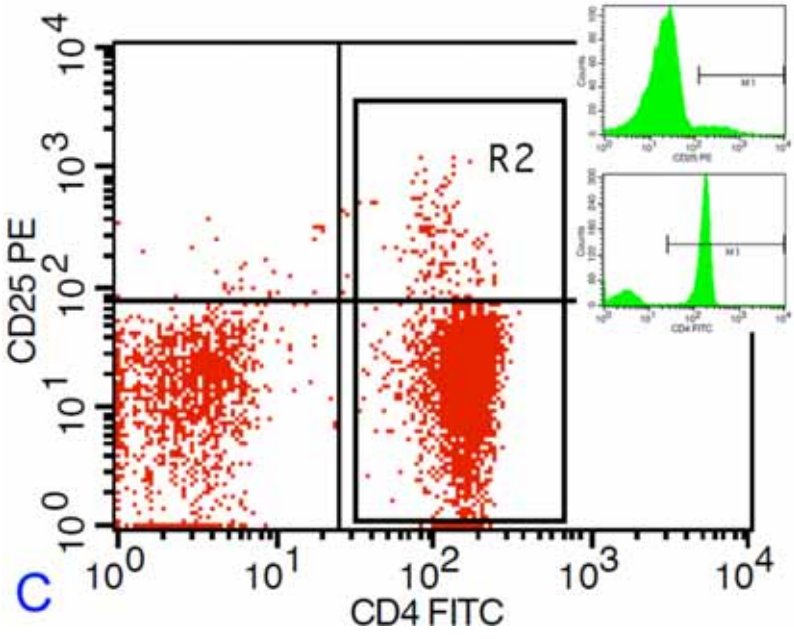

Figure 2C. Scatter diagram of $CD4^+CD25^+$ regulatory T cells of the male mice in the 100 mg/L NaF group.

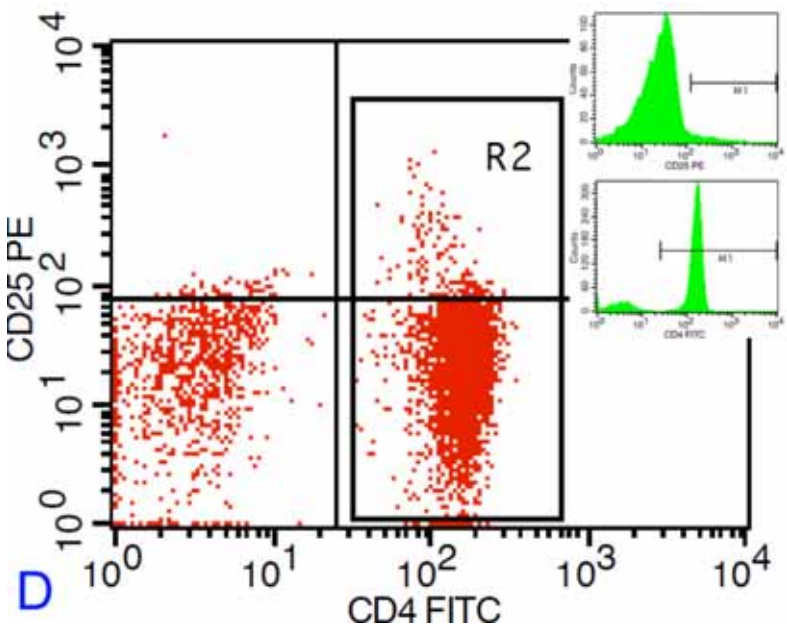

Figure 2D. Scatter diagram of $CD4^+CD25^+$ regulatory T cells of the male mice in the 150 mg/L NaF group.

We detected green fluorescence in 10^4 cells in photon counting histograms with FL1. The median fluorescence intensity (MFI) was calculated by using Cellqust (Table 4.). The percentage content of $CD4^+CD25^+$ regulatory T cells was significantly reduced in the F-exposed groups but was not significantly related to the F dose.

Table 4. The percentage contents of $CD4^+CD25^+$ regulatory T cells in the $CD4^+$ T cells of the male mice in the four groups (Mean±SD)

Group (n=12)	Percentage of $CD4^+CD25^+$ regulatory T cells in the $CD4^+$ T cells (%)	Relative values
Control	7.72±1.95	1
50 mg/L NaF	5.59±0.46†	0.724
100 mg/L NaF	5.98±0.59*	0.775
150 mg/L NaF	5.80±2.01†	0.751

Compared with the control group: *$p<0.05$, $^{\dagger}p<0.01$.

Quantification of GITR gene expression: The amplification plots were obtained after serial dilutions of total RNA, starting from 1 (undiluted, leftmost curve) to 0.015625 (1:64 diluted, rightmost curve). In the melting curves, the single peak at 83ºC for the β-actin gene and 79.5ºC for the GITR gene indicated that no other transcripts were amplified in the real-time RT-PCR. The standard curves for the β-actin gene (Y = –3.678 × LOG(X) + 9.55, RSq = 0.990, Eff = 87.0%) and the GITR gene (Y= –2.997 × LOG(X) +19.05, RSq = 0.999, Eff = 115.6%) were obtained by the correlation of the Ct values (threshold cycles) with the dilution series of the total RNA. The amplified products were analyzed by agarose gel electrophoresis (Figure 3).

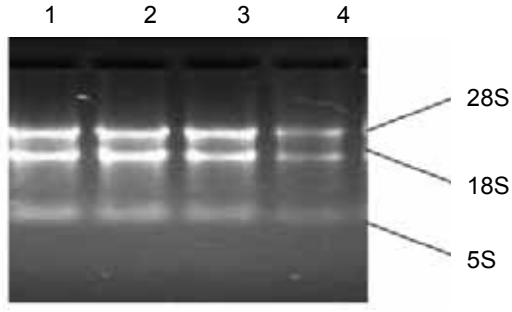

Figure 3. Electrophoresis of total RNA from male mice spleen. Lanes 1, 2, 3, and 4 show the total RNA of the 0 (control), 50, 100, and 150 mg/L NaF groups, respectively.

The relative expression levels of GITR mRNA in the spleen of the mice are shown in Figure 4. In comparison with the control group, the expression level of the GITR gene in the spleen in the 50, 100, and 150 mg/L NaF groups were reduced by 15.6%, 42.5%, and 69.9%, respectively.

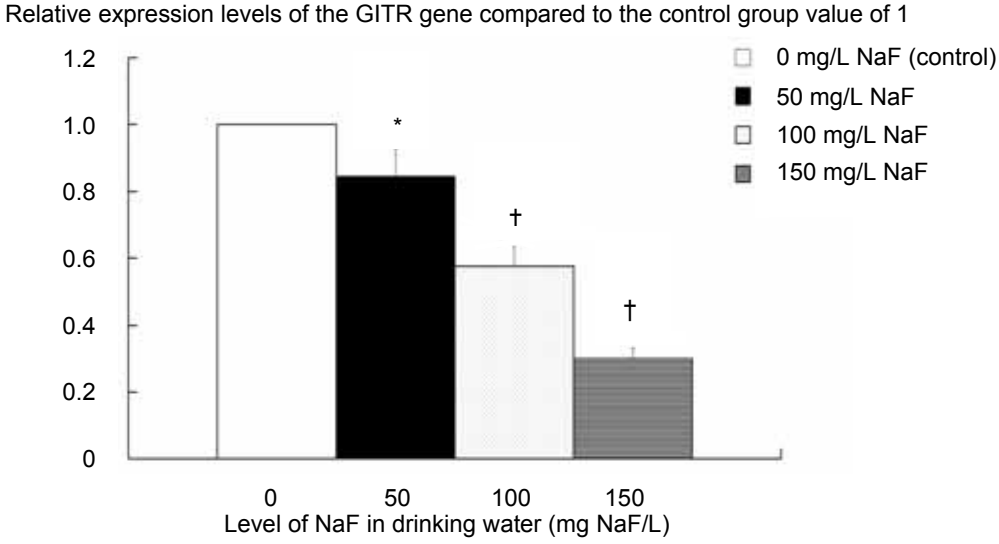

Figure 4. The relative expression levels of the GITR gene in the spleen of the male mice exposed to 50, 100, and 150 mg/L NaF in their drinking water compared to the control group, with 0 mg/L NaF in their drinking water, which has a value of 1. Compared with the control group: *$p<0.05$, †$p<0.01$.

DISCUSSION

The effects of high fluoride ion exposure on the immune function is a new hot spot in fluoride toxicity research. Various degrees of F toxicity to the immune organs and cells have been demonstrated.[10] In recent years, many studies of the toxicity of F on immune function in rats, human beings, rabbits, mice, etc. have been carried out using different methods, and most of the results show that the toxicological effect of F on immune function is broad and complex.[11-13] These findings indicate that F has a toxic effect on immune cells and related factors, and can lead to visible morphological changes in the thymus and spleen.[14]

The regulatory T cell is also a focus in current immunological research. $CD83^+$ T cells share important features with regulatory T cells, identifying CD83 as a novel lineage marker to discriminate between different T cell populations.[15] SAP-1 also inhibited IL-17 and the $CD4^+CD25^+$ cell population showing that it had a suppressive effect on the Th-17 pathway as well as on the T-regulatory cells.[16] Chen et al.[17] suggested that activation of the $CD8^+$ T population exerted feedback regulation in the modification of dentritic cells and then attenuated the $CD4^+$ T mediated immune response. The study demonstrated that $CD4^+$ T cells enriched in n-3 PUFA also exhibited a depleted plasma membrane non-raft PI (4,5) P2 pool as detected by decreased co-clustering of Src (N15), a non-raft marker, and PH (PLC-d), a PI (4,5) P2 reporter.[18]

$CD4^+CD25^+$ regulatory T cells come from the thymus and express CD25, CTLA-4, GITR, and Foxp3, etc. on their surfaces. Research has shown that GITR and Foxp3 affect the suppression function of $CD4^+CD25^+$ regulatory T cells. Active GITR is expressed on regulatory T cells and activated T cells. GITR may play an important role in maintaining the immune tolerance of regulatory T cells.[19-20] The $CD4^+CD25^+$ regulatory T cell is one of the most important regulatory T cells. It can inhibit the cell proliferation of autoreactive T cells by negative regulatory cytokines. Foxp3 and GITR are relevant to the inhibiting function of $CD4^+CD25^+$ regulatory T cells. Liu et al. reported that the expression level of GITR was significantly correlated with its ligand GITRL in peripheral blood.[21] Yang et al. reported that the expression level of Foxp3 was significantly correlated with GITR and GITRL.[22]

The results of our study indicate that excess fluoride ion can obviously reduce the expression of GITR mRNA. These results offer a basis for further exploring the mechanisms by which the fluoride ion affects immunoregulatory function. However, the changes in the expression level did not follow a similar trend. Further studies of this observation are needed to determine the possible mechanism involved.

CONCLUSION

In conclusion, from the present and our previous study,[5] excess F exposure has been found to produce adverse effects on the immune function of male and female mice by reducing the expression of GITR and Foxp3. The changes of the percentage contents of $CD4^+CD25^+$ regulatory T cells do not appear to be directly

connected with the reduced expression level of GITR and Foxp3, while the percentage contents of $CD4^+CD25^+$ regulatory T cells were significantly reduced in the fluoride-exposed groups, and the down trend was consistent with GITR and Foxp3.

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