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The role of X-linked *FOXP3* in the autoimmune susceptibility of Turner Syndrome patients

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Abstract

Turner Syndrome patients have an absent second sex chromosome and a predisposition to autoimmune disease. We hypothesized that the autoimmune susceptibility in Turner Syndrome may be due to an alteration in the expression of the X-linked *FOXP3* gene. *FOXP3* is important in the development of regulatory T cells, and complete loss of *FOXP3* expression has been shown to result in severe autoimmunity. To test this hypothesis, we characterized the regulatory T cells and performed immunophenotyping on the peripheral blood leukocytes of a cohort of Turner Syndrome patients. These patients retained regulatory T cell frequency and function despite an increased prevalence of autoimmunity. Immunophenotyping revealed a decrease in the ratio of CD4 to CD8 lymphocytes. These findings suggest that the autoimmune predisposition in Turner Syndrome is not due to alterations in regulatory T cells but may be associated with a change in the proportion of T cell subsets.

Keywords

Turner Syndrome; *FOXP3*; Regulatory T cells; Autoimmunity

Introduction

Turner Syndrome (TS) patients have an absent second sex chromosome and a number of physical findings including short stature and absent pubertal development [1]. Interestingly, TS patients also have an increased incidence of autoimmune disease [1]. Hashimoto thyroiditis is the most common autoimmune disease with a prevalence of approximately 20% in TS patients [2]. Other reported autoimmune diseases include Graves' hyperthyroidism [3], alopecia [1], inflammatory bowel disease [4], and psoriasis [1]. Why TS patients are predisposed to autoimmune disease, however, is not known.

Some of the clinical findings associated with TS are believed to be due to an altered dosage of certain X chromosome genes [1]. Short stature in TS, for example, has been attributed to decreased expression of the X-linked *SHOX* gene in TS [5]. We therefore asked whether the predisposition to autoimmunity seen in TS could be due to altered dosage of an X-linked gene, *FOXP3*.

FOXP3 is a transcription factor that is important for the development and maintenance of regulatory T cells (Tregs) (reviewed in [6]). Tregs are a CD4⁺ T cell lymphocyte subset that plays a key role in preventing autoimmunity. Humans and mice with mutations in *FOXP3* lack Tregs and develop severe lymphoproliferation and autoimmunity [7]. Quantitative decreases in *FOXP3* expression in mice have also been shown to alter Treg function and result in autoimmune disease [8]. Notably, the thyroid autoimmunity in TS has been mapped to Xp11.2–p22.1 [9], a chromosomal region containing the *FOXP3* gene.

To investigate whether the increased incidence of autoimmune diseases seen in TS may be due to quantitative changes in the Tcell subset expressing *FOXP3* expression, we examined Tregs in the peripheral blood of a cohort of TS patients. We found that TS patients do not have an alteration in the frequency of Tregs and retain the ability to suppress effector T cell function. TS patients, however, did have a decrease in the CD4 to CD8 lymphocyte ratio in the peripheral blood, suggesting an immune alteration that may be associated with autoimmunity.

Materials and methods

Participants

A total of 30 participants (20 TS patients and 10 controls) were recruited through the University of California, San Francisco (UCSF) Endocrinology Clinics. The diagnosis of TS was verified by medical chart review. Blood samples were obtained with informed consent and/or assent under an Institutional Review Board approved protocol at UCSF.

Peripheral blood mononuclear cell (PBMC) isolation

PBMCs were isolated at the UCSF Pediatric Clinical Resarch Center using Ficoll–Hypaque. Whole blood was diluted in PBS at a 1:1 ratio and overlaid onto an equivalent volume of Ficoll–Hypaque. The gradient was centrifuged at 400×g for 30 min and PBMCs removed. The PBMCs were then washed twice with PBS, counted and cryopreserved.

Antibodies

Antibodies used for staining (PERCP-conjugated anti CD4, APC-conjugated anti CD25, PE-conjugated anti CD127, PECy-conjugated anti CD8, PERCP-conjugated anti CD19, FITC-conjugated anti CD14, and PE-conjugated anti CD45) and sorting (FITC-conjugated anti CD4, APC-conjugated anti CD25, and PE-conjugated anti CD127) were provided by Becton Dickinson (BD Biosciences). Alexa 488-conjugated anti FOXP3 (Clone 206D) was purchased from Biolegend.

Flow cytometry

Frozen PBMCs were removed from liquid nitrogen and warmed to 37 °C. Cells were washed twice in 10% fetal calf serum in Dulbecco's Modified Eagle's Medium and counted. FOXP3 intracellular staining was performed according to manufacturer's instructions (Biolegend). For quantitation of immune cell subsets, 10 µl of pre-mixed panels of antibodies were used per 10⁶ cells. Flow cytometry was performed using the LSR II Flow Cytometer (BD Biosciences).

Sorting of Tregs

Sorting of Tregs was performed as previously described [10]. In brief, frozen PBMCs were removed from liquid nitrogen and warmed to 37 °C. Cells were washed twice in PBS, counted and resuspended at a concentration of 100×10⁶ per ml for cell staining. Tregs (CD4⁺, CD127lo/–, CD25⁺) cells were sorted using a Moflo cell sorter (Beckman Coulter) into 50% human serum.

Suppression assays

Suppression assays were performed using 30,000 sorted Tregs and 100,000 allogeneic PBMCs as responders in round-bottom 96 well plates. Antigen presenting cells were allogeneic PBMCs irradiated with 40 Gy. Antigen presenting cells were plated at a concentration of 100,000 cells/well. Total volume per well was 200 μ l. Anti-CD3 and anti-CD28 were added to the wells at final concentration of 1 μ g/ml. On day 4, 1 μ Ci of 3 H-thymidine was added for the final 16 h. Plates were harvested using a Tomtec cell harvester and 3 H-thymidine incorporation was determined using a 1450 microbeta Wallac Trilux liquid scintillation counter. Percent suppression was calculated as $[1 - (\text{cpm co-cultures} / \text{cpm co-cultures of responders alone})] \times 100$.

Statistics

Results were analyzed by Mann–Whitney test using Prism 4 software (GraphPad). *p* values <0.05 were considered significant.

Results

Turner Syndrome patients have a high prevalence of autoimmune disease

We collected a cohort of 20 TS and 10 control patients and obtained medical histories from the patients and from available medical charts (Table 1). Ages ranged from 8 to 62 years in TS patients and 7 to 62 years in control patients. Mean ages were not significantly different between TS (22.8 ± 13.1 years) and control (30.4 ± 19.7 years) patients. Similar to previous reports [1], a high prevalence (40%) of autoimmune disease was seen in TS patients. 3 of the 20 (15%) TS patients had clinically significant thyroid disease, and 1 additional TS patient had detectable thyroid autoantibodies (Table 1). A variety of other autoimmune diseases, including psoriasis, idiopathic thrombocytopenic purpura (ITP), and Crohn's Disease, was also seen in our cohort (Table 1).

The frequency and suppressive function of regulatory T cells is not altered in Turner Syndrome

We utilized flow cytometry to determine whether the percentage of Treg subsets (defined as the percentage of FOXP3+ cells within CD4+ lymphocytes) was decreased in TS patients as a possible cause of increased autoimmunity. FOXP3+ cells were easily detected in the peripheral blood of both TS and control patients (Fig. 1A). The average percentage of FOXP3+ in the CD4+ gate was not significantly different between the cohort of TS ($4.03\% \pm 1.43$) and control ($4.62\% \pm 1.53$) patients (Fig. 1B). Furthermore, the subgroup of TS patients with known autoimmune disease showed no difference in the percentage of Tregs ($4.34\% \pm 1.22$) when compared with controls ($4.62\% \pm 1.53$) (Fig. 1B, right).

We next determined whether the suppressive function of Tregs in TS patients might be altered. To do this, we performed allogeneic responder cell suppression assays with a polyclonal stimulus using sorted Tregs and responders at decreasing ratios. Tregs were sorted based on the cell surface markers CD4+, CD127lo/–, CD25+ as has previously been described [10]. We have previously shown that this sorting scheme results in a Treg population that is typically greater than 94% FOXP3+ [10]. As shown in Fig. 1C, Tregs from TS patients were able to suppress effector T cell proliferation at comparable levels to those in control patients. At a 1:2 ratio of Tregs to responders, the average percent suppression was $36.9\% \pm 6.5\%$ in Turners patients and $42.3\% \pm 8.8\%$ in control subjects ($p=0.34$).

Turner Syndrome patients have a decreased CD4 to CD8 ratio in peripheral blood

Previous studies have yielded conflicting reports on how the cellular immunophenotype of TS patients may differ from controls [11–14]. Interestingly, two studies have reported a decrease

in the CD4 to CD8 lymphocyte ratio in the peripheral blood of TS patients [12,14]. Consistent with these two previous reports, we also found that TS patients have a decreased CD4 to CD8 ratio compared with controls ($p=0.02$, Figs. 2A and B). While the average percent of CD4+ T cells in the lymphocyte population was not significantly different between TS patients and controls (Fig. 2C), the average percent of CD8+ T cells was higher in TS patients than in controls ($p=0.04$, Fig. 2D). We did not find any significant differences in the frequency of B cells (Fig. 2E), macrophages (Fig. 2F), or NK cells (Fig. 2G) in TS patients compared with controls.

Discussion

In this study, we show that the frequency and suppressive function of Tregs in TS patients do not appear to be altered despite an increased predisposition to autoimmunity. The role of Tregs in the pathogenesis of autoimmune diseases has been the subject of intensive investigation in recent years (reviewed in [15]). While loss of Tregs due to *FOXP3* mutations has been well-linked to the Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome [16], the connection between more subtle changes in Tregs and other autoimmune diseases are less clear. For example, whether alterations in Treg number and function contribute to the pathogenesis of Type I Diabetes Mellitus (T1DM) has been controversial [17,18].

A limitation of our study is the heterogeneity of our study group. Our cohort consisted of TS patients with a number of different karyotypes (Table 1) and with a large spread in ages, which may obscure any differences that may exist. Indeed, there is evidence that the frequency of Tregs may be largely dependent on age [17].

TS patients may be predisposed to autoimmunity for other reasons besides Treg alterations. First, aneuploidy itself may predispose to autoimmunity. A number of aneuploid conditions, including Down Syndrome (Trisomy 21) and Klinefelter's Syndrome (XXY), have also been associated with increased risk of autoimmunity [1]. Second, other genes on the X chromosome may be playing a role in the predisposition to autoimmunity in TS. A number of immune-related genes, including those involved in Wiskott–Aldrich Syndrome and Bruton's Agammaglobulinemia, are linked to the X chromosome [19]. Third, TS patients may have a defect in thymic negative selection of self-reactive T cells. Recent studies have highlighted the importance of this mechanism in the maintenance of self-tolerance in a number of diseases, including Autoimmune Polyendocrinopathy Syndrome Type 1 [20,21], Type 1 Diabetes Mellitus [22], and Myasthenia Gravis [23].

Consistent with two previous reports [12,14], we have also noted a decrease in the CD4 to CD8 ratio in our Turner Syndrome cohort. This derangement in the immune phenotype of Turner patients may be related to the increased autoimmunity. Further studies will have to be performed to understand the significance of this finding.

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Figure 1.

TS Patients Retain Treg Frequency and Suppressive Function. (A) Representative flow cytometric plots of PBMCs from a TS patient (upper panels) and a control patient (bottom panels). The percentage of FOXP3+ cells within CD4+ gated lymphocytes (left) and isotype control (right) is shown. As previously shown, many FOXP3+ cells are also CD25+ [24]. (B) Cumulative data showing the percentage of FOXP3+ cells within CD4+ gated lymphocytes for TS patients (circles), control patients (triangles), and TS patients with a history of autoimmune disease (squares). Each shape represents an individual subject. Lines represent the average percent for each group. AID=autoimmune disease. (C) Suppression assays performed using sorted Tregs, allogeneic PBMC responders, and allogeneic PBMC antigen presenting cells. 1:1 indicates 30,000 Tregs:100,000 responder cells. Responder cell numbers were kept constant in each well with decreasing amounts of Tregs in each dilution. Cells were plated at 1:2 (15,000 Tregs), 1:4 (7500 Tregs), 1:8 (3750 Tregs), 1:16 (1875 Tregs), 1:32 (937 Tregs), and 1:64 (468 Tregs) dilutions. The average percent suppression ($\pm$ S.D.) of TS (squares) and control (diamonds) patients of 3 separate experiments are shown.

Figure 2.

TS Patients Have a Decreased CD4 to CD8 Ratio in the Peripheral Blood. (A) Representative flow cytometric plots of PBMCs showing percentage of CD4+ and CD8+ cells within lymphocytes in a TS (left) and control (right) subject. (B) Ratio of the percent of CD4+ cells to the percent of CD8+ cells within the lymphocyte population. (C) through E) Percentages of CD4+ T cells (C), CD8+ T cells (D), and CD19+ B cells (E) within the lymphocyte population. (F and G) Percentages of CD14+ macrophages (F) and CD56+ NK cells (G) within total PBMCs. For B through G, TS (squares) and control (triangles) patients are shown. Each shape represents an individual subject. Lines represent the average percent for each group. (*) indicates statistically significant differences.

Table 1

Patient demographics

Age	Karyotype	Autoimmune history
<i>Turner Syndrome (n=20)</i>		
8	45,X/46,Xr(X)	
10	45,X/46,X,idic(Y)	
12	45,X/46,XY	
12	45,X/46,Xr(X)	Hashimoto thyroiditis
12	45,X/46,XX	
13	45,X/46,X,i(X)q	Idiopathic thrombocytopenic purpura
15	45,X	
15	46,X,i(X)q	Psoriasis
16	45,X/46,XX	
16	45,X	Psoriasis
21	45,X/46,X,i(X)q	Hashimoto thyroiditis
21	45,X	
26	45,X	
28	Not available	
30	45,X	
33	Not available	Blepharitis
35	45,X	Crohn's disease
35	45,X	Positive thyroid antibodies
36	45,X/46,XX	
62	45,X	Graves' hyperthyroidism
<i>Control (n=10)</i>		
7	–	
8	–	
11	–	
13	–	
29	–	
37	–	
42	–	
45	–	
50	–	
62	–	