The Amsterdam autoimmune thyroid disease cohort
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Chapter 6

A reduced Il2R (CD25) expression level in first and second-degree female relatives of AITD patients. A sign of a poor capability to preserve tolerance?

Abstract

There is room for immune markers other than TPO-Abs to identify an increased risk to develop autoimmune thyroid disease (AITD). Our aim was to test the hypothesis that activation of CD4+ T cells is such a marker in relatives of AITD patients, who have an increased risk to develop AITD. We established a controlled study on 20 TPO-Ab positive and 20 TPO-Ab negative euthyroid female relatives. All these cases had at least one 1st or 2nd degree relative with a documented autoimmune hyper- or hypothyroidism in whom we studied the percentages of circulating subsets of activated (MHC class-II, CD25 (Il-2R), CD71, CD69+) CD4+ T cells and the level of the soluble (s)-IL2R in serum.

We found that euthyroid female relatives did not show an activation of their T cell system, but a reduced expression of CD25 on CD4+ T cells. The level of the shed IL-2R in serum was also lower in comparison with levels found in healthy control females. A reduced T cell activity was found in both TPO-Ab positive and negative relatives.

In conclusion, female relatives with at least one 1st or 2nd degree relative with an AITD show signs of a reduced expansion capability of their T cell pool. It is hypothesized that this reduced expansion capability may affect T cell tolerance mechanisms more than T effector mechanisms.
Introduction

The etiology of autoimmune thyroid disease (AITD), encompassing Graves’ hyperthyroidism and Hashimoto’s thyroiditis, is multifactorial [1]. Genetics play an important role in the development of AITD, illustrated by the concordance rate for Graves’ disease and Hashimoto’s hypothyroidism in monozygotic twins, which is much higher than in dizygotic twins [2,3]. In addition, many patients with AITD have family members affected by this disorder. However, it has been estimated that at best 79% of the liability to develop AITD can be attributed to genetics [3] and, therefore, environmental and hormonal risk factors must also be involved [4]. A follow-up cohort study (the Amsterdam AITD Cohort) was initiated to determine risk factors involved in new cases of AITD. To increase the likelihood of diagnosing new patients during a 5-year follow-up, subjects were included who had at least one relative with documented AITD and who were in self-proclaimed good health. In a previously reported baseline study, evidence for autoimmune thyroiditis was found in a fairly large proportion of euthyroid relatives (i.e., relatives with a serum TSH within the normal range): 183/759 (24%) of such relatives had autoantibodies to thyroid peroxidase (TPO). This value lies between the prevalence value in the general population (10–15%) and prevalence rates of 48% [5] and 43% [6] previously reported in first-degree relatives of AITD patients. It was also reported that the TPO antibody titre in the euthyroid subjects of our study cohort correlated positively with TSH levels ($r = 0.386; P < 0.001$) [7]. In addition 86% of 29 hypothyroid relatives detected in the cohort (i.e., relatives with a TSH above the normal range) had detectable levels of TPO antibodies. Taken together, the findings of the Amsterdam AITD Cohort are in line with the notion that subjects with a family history of AITD are at increased risk for AITD and that TPO-Abs are reasonably good markers to predict such development [8–10]. Interestingly and unexplainably, smoking and estrogen use were negatively correlated with the presence of TPO antibodies in the Amsterdam AITD Cohort [7]. Although TPO-Abs are reasonably good markers for the development of AITD, there is room for immune markers other than the abs to establish the increased chance to develop AITD. Such immune markers are likely to be found in the T cell system. Thyroid antigen specific CD4+ T cells and CD8+ T cells are required for the thyroid autoimmune reaction to develop [11]. Autoreactive CD8+ T cells are capable of directly killing thyrocytes, while autoreactive CD4+ T cells have various helper functions. Autoreactive CD4+ Thelper2 cells (producing IL-4 and IL-5) help autoreactive B cells to switch to IgG-autoantibody producing plasma cells, whilst CD4+ Thelper1 cells (producing IFN-γ) activate
macrophages to destroy thyrocytes. Apart from these immune stimulating and effector functions, T cells are able to down-regulate immune responses and at present various subsets of T cells are recognized as being capable of exerting such immune regulatory function [12,13]. For this report a controlled study was initiated in which the apportioning of the various circulating T cell subsets was studied in 20 TPO-Ab positive and 20 TPO-Ab negative euthyroid female relatives, randomly selected out of the cohort of the 803 characterized subjects from the Amsterdam AITD Cohort [7] at baseline. Subjects were stratified by age, current smoking habits and current estrogen medication. Apart from measuring the circulating subsets of T helper cells (CD3+ CD4+), the T cytotoxic cells (CD3+CD8+) and the T helper memory cells (CD4+CD45RO+), the studies concentrated on the determination of T cell activation markers on the CD4+ T cells in the circulation, i.e. of MHC class-II, CD25 (the IL2 receptor), CD71 (the transferring receptor) and CD69, since a previous study found that a raised number of circulating activated T cells, i.e. cells expressing MHC class-II, is indicative for a progression of TPO-Ab positive pregnant women to post-partum thyroiditis [14]. The level of the soluble (s)-IL2R in the serum of these relatives was also tested since this molecule represents the shed CD25 from T cells and can easily be determined.

Subjects and Methods

Subjects

Subjects in the present study are

a. Forty euthyroid female subjects selected from the 803 subjects of the Amsterdam AITD Cohort (7). The subjects in the Amsterdam AITD cohort have at least one 1st or 2nd degree relative with a documented autoimmune hyper- or hypothyroidism and are in self-proclaimed good health, without a history of thyroid disease, and between 18 and 65 years of age. Current pregnancy is an exclusion criterion. Information on smoking habits (current and past), use of oral contraceptives or other estrogens (current and past) is available, because all subjects have been asked to fill in questionnaires at the time blood samples (to obtain serum and circulating mononuclear leukocytes to be stored) were collected. Current smoking is defined as smoking now, or having stopped smoking within one year prior to visiting our clinic. Current estrogen usage is defined as presently on exogenous estrogen medication.
The actual selection of the 40 subjects from the cohort was carried out as follows: initially 10 euthyroid TPO-Ab positive relatives were randomly selected from the cohort. For each selected subject all possible euthyroid TPO Ab negative counterparts matched for age +/- 5 years, smoking status and current estrogen use were searched. If more than one possible match existed a single match was randomly selected. This procedure was repeated, but starting by randomly selecting 10 euthyroid TPO-Ab negative subjects and matching TPO-Ab positives to these subjects. Of two of the selected subjects, stored blood cells later appeared not to be viable and, therefore, only the results of 38 subjects could be analyzed and will be provided here. The mean age of the selected subjects was 36.8 years (range 20-58 years). Twenty of the 38 studied relatives were smokers, 20 of the 38 used oral contraceptives.

b. Female controls in self-proclaimed good health, excluding afflictions such as infections, asthma and endocrine disorders, consisted of Dutch interns, hospital and laboratory staff and healthy female twins selected as controls for a non-related twin study. The family history needed to be negative for thyroid disorders and subjects positive for TPO-Abs were excluded from the study. Serum and mononuclear leukocytes needed to be collected in the same time period (1997-2003) as those of the relatives of the AITD patients using the same density isolation and storage protocol. Stratification for smoking habits and estrogen usage had not taken place in the healthy controls. From 65 of such healthy female subjects (ages 22–56, mean age 36.8 years) serum was available, of these only 5 were smokers and 22 used estrogens. Stored mononuclear leukocytes were available from 9 other healthy females (ages 22–62 years, mean age 38.6 years), of these 2 were smokers and 3 used estrogens.

The local Medical Ethics committees of our Institutions had approved the study. All subjects gave written informed consent.

**Blood samples**

Serum samples were stored at -20°C until determination of the study parameters. Mononuclear cells were obtained from heparinized blood by using Ficoll (Pharmacia, Uppsala, Sweden; density 1.077 g/ml) density gradient centrifugation. The isolated cells were frozen with 10% dimethylsulfoxide (Sigma-Aldrich Chemie, Steinheim, Germany) in RPMI 1640 medium with 25mM HEPES and L-glutamine (BioWhittaker Europe, Verviers, Belgium) containing ultraglutamine (UG) (2mM, BioWhittaker), 100U/ml penicillin
Low IL-2R expression in relatives of thyroid autoimmune patients

(BioWhittaker), 100µg/ml streptomycin (BioWhittaker) and 10% heat inactivated fetal calf serum (BioWhittaker) and stored at –70°C until analysis.

*TSH, fT4, TPO Ab*

In all subjects free Thyroxine (fT4; time-resolved fluoroimmunoassay, Delfia, Turku, Finland) and thyrotropin (TSH; Delfia) levels were measured, as well as antibody titers against TPO using a chemiluminescence immunoassay (LUMI-test, Brahms, Berlin, Germany). For this study, euthyroidism was defined as a TSH value of ≥0.40 mU/L and ≤5.70 mU/L, in combination with a fT4 value of ≥9.3 pmol/L and ≤20.1 pmol/L. TPO antibody levels of ≥100 kU/L were considered to be positive.

*Soluble IL-2R*

T cell activation was determined by measuring serum soluble IL-2 receptor (sIL-2R).

The soluble IL-2R levels were measured by automatic Immulite chemiluminescent enzyme immunimetric assay (DPC, Los Angeles, CA, USA) according to the manufacturer’s instruction. Enzyme immunoasay (ELISA) was used to determine the serum levels of soluble (s) ICAM-1 (Bio-source ELISA KH5412 high sensitive, Camarillo, CA, USA).

*Flowcytometry*

Marker staining and analysis were done at the same time in the same institution, i.e. the Erasmus MC, on all frozen mononuclear cell samples using the same protocol and FACS analysis settings.

The following monoclonal antibodies (mAbs) were used as control mAbs for flowcytometry: anti-IgG1 Fluorescein isothiocyanate (FITC), anti-IgG1 Phycoerythrin (PE), anti-IgG1 Peridin chlorophyl protein (Percp) (all three 1:10, Becton Dickinson (BD), San Jose, CA, USA), anti-CD3 FITC (1:20, BD), anti-CD8 PE (1:20, BD), anti-CD4 Percp (1:10, BD), anti-CD25 FITC (1:10, BD), anti-HLA-DR PE (1:200, BD), anti-CD28 FITC (1:10, Immunotech, Marseille, France), anti-CD45RO PE (1:5, Dako Cytomation, Glostrup, Denmark), anti-CD71 FITC (1:10, BD), and anti-CD69 PE (1:100, Serotec, Oxford, England).

Thawed mononuclear cells were incubated for double staining with 25µl mAb / 10⁵ cells (of a FITC-labeled antibody (Ab), of a PE-labeled Ab, and/or 25 _L of a PerCP-labeled Ab) for 15 minutes and washed twice with phosphate buffered saline (PBS) pH 7.4 (Biowittaker) with 0,1% bovine serum albumin (BSA) (Bayer, Kankakee, IL, USA).
Routinely 10,000 cells in the lymphocyte or monotypic gate were measured immediately following cell staining using a FACScan flowcytometer and analyzed using CellQuestPro (BD). The background staining was determined by the staining of the cells with the isotype antibody controls alone and the threshold for background positivity staining was arbitrarily set between 1 and 2% of cells positive for the isotype control. Using this fixed threshold the percentage of lymphocytes positive for the specific anti-CD antibody was determined and the arbitrarily fixed background staining (obtained with the isotype control of 1-2%) was subtracted from the values found with the specific CD staining. The data obtained was mainly expressed as mean ± SD percentages of positive lymphocytes found in the individual patients of the various patient and control group.

Statistics
Because of the abnormal distribution of the variables, statistical analyses were performed using the nonparametric two independent-sample-tests; the Mann Whitney U test. With use of a standard statistical software package (SPSS 11.0) a two-tailed significance value was determined between cases and controls. A p-value of <0.05 was considered as statistically significant.

Results

Soluble IL2-R levels in serum
As expected and as a result of the randomization and stratification procedure, healthy first- and second-degree female relatives of AITD patients with TPOAb were no different from those without TPO-Ab regarding age, current smoking behavior and current estrogen medication. As described previously, the TPO Ab positive subjects had slightly, but significantly higher TSH levels (Table I and [7]).

Figure 1 shows, that the healthy relatives had statistically significantly reduced serum levels of sIL-2R as compared to that of the healthy female controls. This reduced sIL-2R level was present in both TPOAb positive and TPO-Ab negative relatives. Interestingly there was a statistically significant difference between smoking and non-smoking relatives. While the non-smoking relatives had significantly reduced serum sIL-2R levels as compared to our healthy controls, smoking relatives had near normal serum levels of sIL-2R (Figure 1). It is of note that a large proportion of our healthy control females were non-smokers (55 out of 62, i.e. 89%). Probably due to the low number of smokers in the
Table I. Age, thyroid status and the outcomes of various lymphocyte phenotypic studies in 9 healthy female controls and 38 female euthyroid relatives of AITD patients grouped for TPO antibody status (n = 19 in each group)

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls</th>
<th>All relatives</th>
<th>TPO Ab neg</th>
<th>TPO Ab pos</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38 ± 14</td>
<td>38 ± 12</td>
<td>37 ± 11</td>
<td>36 ±12</td>
</tr>
<tr>
<td>TSH (mU/L)</td>
<td>&gt;0.4, &lt;5.7</td>
<td>1.7 (0.7–4.7)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.6 (0.7–2.9)</td>
<td>2.6 (1.3–4.7)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Free T4 (pmol/L)</td>
<td>&gt;9.3, &lt;20.1</td>
<td>12.5 (9.8–19.7)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.4 (10.4–17.5)</td>
<td>12.5 (9.8–19.7)</td>
</tr>
<tr>
<td>CD3 + CD4&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>57 (7)</td>
<td>47 (13)</td>
<td>48 (10)</td>
<td>47 (15)</td>
</tr>
<tr>
<td>CD3 + CD4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>23 (6)</td>
<td>20 (5)</td>
<td>20 (5)</td>
<td>20 (5)</td>
</tr>
<tr>
<td>CD4 + CD45RO&lt;sup&gt;+&lt;/sup&gt;</td>
<td>34 (9)</td>
<td>29 (9)</td>
<td>28 (8)</td>
<td>30 (10)</td>
</tr>
<tr>
<td>CD4 + MHCclassII&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.4 (0.9)</td>
<td>2.0 (0.9)</td>
<td>2.1 (0.8)</td>
<td>2.0 (1.0)</td>
</tr>
<tr>
<td>CD4 + CD71&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.9 (0.4)</td>
<td>2.3 (1.0)</td>
<td>2.3 (1.1)</td>
<td>2.2 (0.9)</td>
</tr>
<tr>
<td>CD4 + CD69&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.0 (0.8)</td>
<td>1.0 (1.0)</td>
<td>1.1 (1.3)</td>
<td>0.8 (0.7)</td>
</tr>
</tbody>
</table>

a In years, medians ± standard deviations are given.
b Means and ranges are given.
c There exists a statistical significant difference between this value and the 1.6 value of the TPO-Ab negative relatives (see text).
d Percentages of lymphocytes positive for the given markers, means and standard deviations are given.

healthy control group it was not possible to detect a statistical significant difference between the smoking and nonsmoking healthy controls: smoking and non-smoking healthy controls

Figure 1. The serum soluble IL2-receptor (R) levels (pg/ml) in healthy female controls (n = 65), and 1st and 2nd degree female relatives of AITD patients (relatives, in total n = 38). Female relatives are subdivided in TPO-Ab positive (n = 19) and TPO-Ab negative (n = 19) female relatives, smoking (n = 18) and nonsmoking (n = 20) female relatives and female relatives using (n = 20) and not using (n = 18) oral estrogens. See for further explanations text. “a” represents a p-value of <0.05 versus healthy controls. “b” represents a p-value of <0.05 versus smoking relatives.
showed sIL-2R levels of 434 (mean) ± 68 (standard deviation) and 461 ± 190 pg/ml respectively (data not shown in Figure 1). Other studies generally show that smoking raises the level of serum sIL-2R in healthy individuals [15–17].

Estrogen usage had no effects on the serum level of sIL-2R levels and both estrogen using female relatives and non-users of estrogen showed reduced s-IL-2R levels and there was no statistically significant difference in sIL-2R serum levels between these two groups of relatives (Figure 1). There was also no such difference between estrogen using female healthy controls and non-estrogen using healthy controls: levels of 461 ± 190 pg/ml (n = 22) and 434 ± 68 pg/ml (n = 43) were found in these healthy control groups respectively (data not shown in Figure 1).

Figure 2. The percentage of circulating lymphocytes expressing CD4CD25 in female healthy controls (n = 9) and in 1st and 2nd degree female relatives of AITD patients (relatives, in total n = 38). Female relatives are subdivided in TPO-Ab positive (n = 19) and TPO-Ab negative (n = 19) female relatives, smoking (n = 18) and non-smoking (n = 20) female relatives and female relatives using (n = 20) and not using (n = 18) oral estrogens. See for further explanations text. “a” represents a p value of ,0.05 versus healthy controls, “b” represents a p value of ,0.05 versus relatives not using oral estrogens.
Sub-sets of circulating T cells, in particular that of CD4 + CD25 + T cells

The percentage of CD3+CD4+ (helper) T cells was reduced in the circulation of the female relatives of AITD patients (Table I, mean 47.4 ± 12.6%) as compared to the percentage found in the healthy females (i.e. mean 56.7 ± 6.7%), but this difference was not considered to be statistically significant (p = 0.23). Within the subset of CD4+ T helper cells the percentage of T helper cells expressing the IL2-R (CD4+CD25+ cells) was, however, significantly reduced in the female relatives of AITD patients (Figure 2). Percentages CD4+CD25+ cells were equally low in the TPO-Ab positive and TPOAb negative female relatives. In addition smoking and non-smoking relatives both showed reduced percentages of CD4+CD25+ cells (Figure 2) and there was no statistically significant difference in percentages of CD4+CD25+ cells in the smoking and non-smoking female relatives. There was such a difference, however, in the smoking and non-smoking healthy control subjects, who showed values of 17.4 ± 1.2% (n = 2) and 11.1 ± 3.5% (n = 7) respectively (p < 0.01, data not shown in Figure 2) supporting the view that smoking activates the CD25 expression on T cells (see also previous remarks on sIL-2R levels in smoking healthy subjects and [15–17]). The CD4+CD25+ T cell subset was significantly lower in female relatives using estrogens versus that of female relatives not taking estrogens, while both groups were lower as compared to the values found in the healthy controls (Figure 2). In regard to comparison with the healthy controls it must be noted that our female control group took oral estrogens in one third of all cases, but in this group we were unable to detect a statistically significant difference between the estrogen non-using female healthy controls (11.4 ± 3.7%, n = 6) and the estrogen-using healthy controls (14.7 ± 4.8%, n = 3). Subsets of CD3+CD8+ (cytotoxic) T cells, CD4+CD4+5RO (memory) T cells, CD4+ MHCclass- II + , CD4+CD69+ and CD4+CD71+ T cells were not different between female relatives and healthy controls (Table I).

Discussion

This study shows that euthyroid females with an heightened risk of developing thyroid autoimmunity, i.e. females with at least one 1st or 2nd degree relative with a documented autoimmune hyper- or hypothyroidism, exhibited a reduced serum level of sIL-2R and reduced percentages of circulating CD4+ T cells expressing the sIL-2R (CD25) as compared to healthy control females. It is important to note that these signs of reduced T cell activity were evident in all female relatives, irrespective of their TPO-Ab status.
The data must thus be viewed as contradictory to a concept that the T cell system is activated in high-risk female relatives of AITD patients. The data support a notion of a low expansion activity of the T cell system in these relatives, since IL-2 is particularly involved in the proliferation of T cells and the expansion of the T cell pool. Hence, the data reported here can be taken to support the following point of view: the lower the T cell system is triggered to expand via IL-2/IL-2R interactions, the higher the risk of developing thyroid autoimmunity. Can such a view be supported with literature data? The textbook view of the IL-2/IL-2R interaction still remains that it is the most critical cytokine for promoting the clonal expansion of T cells and hence a stimulator of effector T cell functions. This perceived importance of IL-2/IL-2R interactions has made this cytokine and its receptor a prime target for interventions to suppress unwanted immune responses such as those occurring in autoimmunity. Therefore, an attempt has been made to knock-out genes for IL-2 or for its receptor in autoimmune-prone mice in order to cure the mice from disease. (18, 19). An unexpected and counterintuitive result occurred: the knock-out mice were not cured from autoimmunity, but in all cases had an aggravation of their autoimmunity and died of massive lympho-proliferative syndrome.

These experiments introduced the new and current concept of experts that IL-2/IL-2R interactions are essential earlier for autoimmune responses to end than to be initiated (19). To explain this concept, it was at first assumed that the IL-2/IL-2R interaction was a key element for activation-induced T cell death (AITCD) of autoreactive T cells. In such a view, a failure of an optimal IL-2/IL-2R interaction (as is likely the case in the relatives due to the low IL-2R expression) hampers an appropriate check of (in this case thyroid-) autoreactive T cells escaped from thymic negative selection in the periphery (19). However, the IL2/IL-2R system might also be linked to another mechanism of tolerance induction, i.e. that induced via CD4+CD25+ T regulator (reg) cells (13). The actual T cells with a regulatory function form a sub-group within the CD4+CD25+ T cell population, develop in the thymus, are “anergic” to TCR ligation, but capable of suppressing the activation of (bystander) CD4+ and CD8+ cells. Ligation of surface molecules such as CD152 (CLTA-4) and GITR, as well as secretion of anti-inflammatory cytokines, such as TGF-β, IL-4 and IL-10, have all been implicated in the regulation or mediation of the suppressive activity of CD4+CD25+ Treg cells (13). Is it possible that the low percentages of CD4+CD25+ T cells found in the female relatives are a reflection of low numbers of this specific subset of thymus-derived Treg cells? Clearly more experiments are needed in the relatives focussing on the actual functional immune suppressive
capability of the cells and on additional marker analyses (CTLA-4, GITR and Fox-P3) to reliably identify the suppressor character of the T cells.

A weak point of this study is that subgroups of smoking and estrogen-taking relatives and healthy controls were rather small and, (in the case of the healthy individuals), not evenly distributed. Nevertheless some conclusions may be drawn. Interestingly, relatives who smoked had near normal sIL-2R serum levels and slightly raised percentages of circulating CD4+CD25+ T cells (such raised percentages were also present in the smoking healthy controls). It is well known that smoking activates the IL-2/IL-2R system [15–17]. Hence smoking may have corrected the reduced T cell functions in the relatives of AITD patients, including the defective T cell tolerance mechanisms. The smoking subgroup of relatives is indeed special, because it was previously reported that smoking is associated with a reduced risk in these relatives to be positive for TPO-Abs [7]. With regard to estrogen usage, female relatives taking estrogens showed particularly reduced percentages of circulating CD4+CD25+ T cells, which was not reflected by an extra reduced serum sIL-2R level in this estrogen taking women. This observation is in accordance with the view that estrogens are immune suppressive in autoimmune diseases [20] and that they suppress IL-2 and the IL-2R [21]. However, effects of estrogen usage were not evident in our healthy controls. The observation also does not support the above expressed view of “the lower the T cell system is triggered to expand via IL-2/IL2R interactions, the higher the risk to develop thyroid autoimmunity”, since estrogen taking women have a lower chance of developing thyroid autoimmunity [7]. Clearly estrogen-sensitive mechanisms other than T cell tolerance mechanisms must be operative in preventing thyroid autoimmunity in these female relatives.

In conclusion, the study shows that females with an heightened risk of developing thyroid autoimmunity, i.e. females with at least one 1st or 2nd degree relative with a documented autoimmune hyper- or hypothyroidism, exhibited various signs of a reduced T cell activation, in particular signs of a reduced expansion of the T cell pool.

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References