Chapter 21
Expression of Cytotoxic T Lymphocyte Antigen-4 in T Cells from Children with Hashimoto's Thyroiditis

Anna M. Kucharska, Elzbieta Gorska, Maria Wasik, and Urszula Demkow

Abstract The cytotoxic T lymphocyte antigen-4 (CTLA-4) (CD152) is a basic negative regulatory molecule of T cell activation and its hypo-function is associated with severe lymphoproliferative syndrome. The aim of the present study was to evaluate the intracellular and surface expression of CTLA-4 on peripheral T cells before and after T cell activation in children with Hashimoto's thyroiditis (HT). Blood samples were obtained from 46 children: 25 with Hashimoto's thyroiditis and 21 controls free of autoimmune disease or thyroid disorders. T cell phenotype was evaluated by flow cytometry with the use of monoclonal antibodies combination: CD4- FITC/ CD28 -PC5/ CD152 -PE and CD8 -FITC/ CD28 -PC5/ CD152 -PE on T cell surface and intracellularly at baseline and after 48 h of T cell culture with the mitogen 48-PHA. We found that the number of T cells with intracellular CD152 expression was comparable in HT patients and controls at baseline and increased after 48-PHA, in CD4 subset only, in both patients and controls. However, the increase was more evident in the HT patients. The number of T cells with the surface expression of CD152 at baseline was significantly lower in the HT patients than in controls ($p < 0.0002$) in non-stimulated CD4+ and CD8+ T cells. After 48-PHA, surface CD152 expression in CD4+T cells increased in both groups; the increase was greater in controls. In conclusion, impaired function of CTLA-4 in HT patients may depend on the imbalance of intracellular/surface expression of CD152 in T cells.

Keywords Children • CTLA-4 • Hashimoto's thyroiditis • Lymphocytes • T cell activation

21.1 Introduction

The cytotoxic T lymphocyte antigen-4 (CTLA-4) (CD152) is a basic negative regulatory molecule of T cell activation (Chambers et al. 1996). It is one of the key molecules which provide co-stimulatory signal during T cell activation. CD28, another co-stimulatory molecule, and CD152 are homological

A.M. Kucharska (✉)
Department of Pediatrics and Endocrinology, Medical University of Warsaw,
Marszalkowska 24 St, 00-756 Warsaw, Poland
e-mail: anna.kucharska@litewska.edu.pl

E. Gorska • M. Wasik • U. Demkow
Department of Laboratory Diagnostics and Clinical Immunology of the Developmental Age,
Medical University of Warsaw, Marszalkowska 24 St, 00-756 Warsaw, Poland

M. Pokorski (ed.), *Respiratory Regulation - The Molecular Approach*,
Advances in Experimental Medicine and Biology 756, DOI 10.1007/978-94-007-4549-0_21,

in about 30% and bind to the same ligands on antigen presenting cells (APC). CD28 is constitutively present on naive CD4+ T cells. In contrast, CD152 is rarely expressed on naive CD4+ T cells, but its expression increases after T cell receptor (TCR) activation. The mRNA of CTLA-4 is detectable as soon as 1 h after TCR activation (Lindsten et al. 1993). The CTLA-4 protein is stored intracellularly and appears in detectable amounts 24 h after activation and is still present on the surface of a T cell 48–72 later (Maszyna et al. 2003). The regulation of the surface expression of CD152 is dependent on the reaction between the clathrin-coated pit adaptor protein (AP-2) and the intracellular thyrosine motif of CTLA-4 (Chuang et al. 1997; Shiratori et al. 1997). The majority of CTLA-4 is stored in clathrin-associated complexes and after activation it is relocalized to the surface of a T cell, where it becomes an integral part of an immunological synapse (Linsley et al. 1996; Darlington et al. 2002; Egen and Allison 2002). Surface expression of CTLA-4 is dynamically regulated by the process of its passing trough the cell membrane into cytoplasm. Alegre et al. (1996) revealed that the surface CTLA-4 after the activation constitutes only 10% of the whole number of the molecules in a T cell and the ratio between the surface and intracellular expression is constant. A direct down-regulation of TCR activation by CTLA-4 is dependent on the surface located molecules, but in the light of recent findings, CTLA-4 plays also an important role as a modulator of function and maturation of T regulatory cells (Treg). In knock-out mice, the loss of CTLA-4 leads to a massive lymphoproliferative syndrome (Tivol et al. 1995; Waterhouse et al. 1995). Impaired function of CTLA-4 is associated with disorders of peripheral autotolerance and autoimmunity in experimental animals and also in humans. One of the most frequent organ specific autoimmune diseases in humans is the autoimmune Hashimoto's thyroiditis (HT). Therefore, HT patients constitute an attractive group for the investigation of T cell function in humans. The aim of the present study was to assess the surface and intracellular expressions of CTLA-4 in T cells isolated from peripheral blood of children with HT at baseline and after *in vitro* T cells activation and to compare it with those in healthy controls.

21.2 Methods

The protocol of the study was approved by the Bioethics Committee of Medical University of Warsaw, Poland. Parents of the patients signed informed consent for the participation in the study.

Blood samples were obtained from 46 children: 25 with HT of the mean age of 14.9 ± 2.3 years and 21 age-matched controls. HT was diagnosed on the basis of the presence of high levels of anti-thyroid antibodies: anti-thyroid peroxidase and/or anti-thyroglobulin antibodies together with disseminated hypoechogenicity of thyroid gland in an ultrasound examination. Children in the control group were free from any infection, autoimmune disease, or thyroid disorders.

The cell preparation before cytometric analysis has been described previously (Kucharska et al. 2009). Briefly, heparinized blood samples obtained from patients and controls were diluted three times with saline, and centrifuged for 30 min at $400 \times g$ on Histopaque 1077-1 density gradient from SIGMA Diagnostics (St. Louis, MO). Isolated PBMCs were incubated with monoclonal antibodies for 30 min at 25°C in darkness. Analysis was performed with the use of monoclonal antibodies combination: CD4- FITC/ CD28 -PC5/ CD152 -PE and CD8 -FITC/ CD28 -PC5/ CD152 -PE from Immunotech Beckman Coulter Company (Beckman Coulter Company, Paris Nord, France). After incubation, samples were fixed and lysed by the reagent set Uti-Lyse (Dako Cytomation, Gdynia, Poland). The T cell phenotype was evaluated using the flow cytometer Beckman Coulter EPICS XL 4C (EPICS XL/XL-MCL, version 2.0, Beckman Coulter Company, Paris Nord, France). T cell phenotype was evaluated at baseline and after 48 h of T cell culture with phytohemagglutinine (48-PHA) as the mitogen activating the T cell.

Results were statistically analyzed by a *t*-test or Mann- Whitney U-test as required. $P < 0.05$ was considered to indicate statistical significance.

21.3 Results

21.3.1 Intracellular Expression of CD152 on T Cells

The number of T cells with intracellular CD152 expression was comparable at baseline in both HT patients and control children: 3.4% *vs.* 2.7%, respectively ($p > 0.05$). The CD4+ subset of T cells positive for CD152 reached 2.2% and 2.1% in the HT and control children, respectively; the difference being insignificant. Likewise, CD8+ cells expressing CD152+ intracellularly did not differ significantly between the patients and controls (Table 21.1).

After stimulation with 48-PHA, the intracellular CD152 increased to comparable values in both HT and control children; from 3.4% to 8.8% and from 2.7 to 6.3, respectively. The CD4+ subset of T cells positive for CD152 reached 6.5% and 4.8% in the HT and control children, respectively. In the CD8+ subset, intracellular CD152 was present in 3.0% and 3.6% of T cells in the HT and control children, respectively. These differences between the patients and controls were not significant (Table 21.1). The alterations of the intracellular phenotype of T cells are illustrated in Fig. 21.1a and b.

21.3.2 The Surface Expression of CD152 on T Cells

At baseline, the number of T cells with surface expression of CD152 was significantly lower in the HT than that in the control children: 2.6% ± 1.8% *vs.* 4.5% ± 1.5%, respectively ($p < 0.001$); the difference remained significant when T cells were divided into CD4+ ($p < 0.001$) and CD8+ subsets ($p < 0.001$) (Table 21.2).

After 48-PHA stimulation, the number of CD152+ T cells increased in both HT and control children, but the increase was higher in CD4+ than in CD8+ subset. In the CD8+ T cells, the difference

Table 21.1 Intracellular CD profile of T cells in Hashimoto's thyroditis (HT) and healthy control children at baseline and after 48 h activation by 48-PHA

		CD4+152+ (%)	CD8+152+ (%)	CD152+ (%)	CD4+ (%)	CD8+ (%)
Baseline	**HT**	2.2 ± 1.5	2.6 ± 3.7	3.4 ± 1.4	37.0 ± 11.1	18.3 ± 4.9
	Control	2.1 ± 1.1	1.8 ± 1.4	2.7 ± 1.4	29.4 ± 10.9	22.9 ± 9.3
48-PHA	**HT**	6.5 ± 3.1	3.0 ± 1.9	8.8 ± 3.8	33.1 ± 10.9	13.8 ± 4.9
	Control	4.8 ± 3.5	3.6 ± 1.6	6.3 ± 3.1	21.9 ± 9.1	15.3 ± 6.1

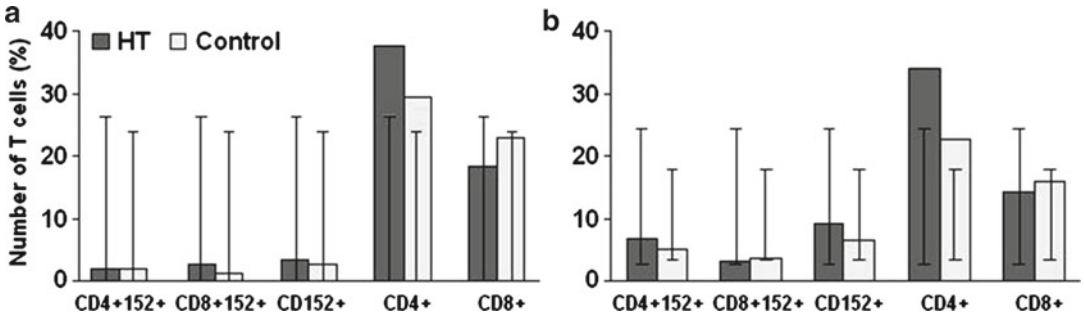

Fig. 21.1 Alterations of the intracellular CD phenotype of T cells before (**a**) and after (**b**) PHA stimulation in Hashimoto's thyroditis children and controls

Table 21.2 Surface CD profile of T cells in Hashimoto's thyroditis (HT) and healthy control children at baseline and after 48 h activation by 48-PHA

		CD4+152+ (%)	CD8+152+ (%)	CD152+ (%)	CD4+ (%)	CD8+ (%)
Baseline	**HT**	1.0 ± 0.8	1.2 ± 1.6	2.6 ± 1.8	23.7 ± 10.1	16.6 ± 8.0
	Control	2.5 ± 1.6	2.5 ± 1.6	4.5 ± 1.5	23.9 ± 9.6	17.0 ± 5.9
48-PHA	**HT**	2.9 ± 1.8	2.0 ± 1.5	4.1 ± 2.2	21.8 ± 11.4	12.2 ± 5.7
	Control	3.4 ± 1.4	4.0 ± 1.5	6.1 ± 0.9	15.6 ± 5.1	14.2 ± 4.4

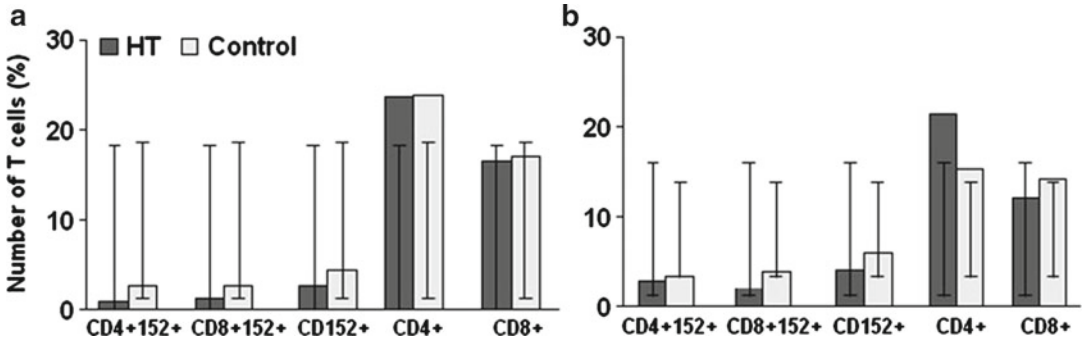

Fig. 21.2 Alterations of the surface CD phenotype of T cells before (**a**) and after (**b**) PHA stimulation

between the number of CD152+ T cells at baseline and after 48-PHA was not statistically significant in either group of children (Table 21.2). Figure 21.2a and b illustrate the alterations of the surface CD phenotype of T cells.

21.4 Discussion

In the present study, the number of CD4+ T cells with the intracellular expression of CD152 was similar in HT and healthy children, but only a part of T cells showed the surface expression of CD152 antigen. This suggests that in HT patients T cells are able to produce the CD152 molecule, but its transport from cytoplasm to cell membrane is hampered. It is unclear why CD152 molecule can not reach the surface of a cell in the course of HT. Anjos et al. (2002) formed an interesting hypothesis that one of the polymorphisms of the CTLA-4 gene associated with autoimmune diseases changes the signal peptide of CTLA-4, alters the early endoplasmic reticulum trafficking or processing of CTLA-4 (CD152), and leads to its differential expression on the cell surface. Thus, a change in a signal peptide can lead to incomplete glycosylation of the protein and results in impaired transit between clathrin-coated vesicles and the cell membrane, resulting in a lower expression of CD152 on the cell surface. Our present data confirm that in HT patients the CD152 molecule lacks the ability of being expressed on T cell surface, even though its intracellular resources are similar to healthy controls. It is unresolved why the basic subsets, CD4+ and CD8+ T cells, have a different pattern of the CD152 antigen response to cell stimulation: an increase in the CD152 on CD4+ T cell subset and no response of CD8+ T cells. This observation was noted only in HT patients. The CD152 antigen in T cells behaved differently in healthy individuals in whom the number of CD152+ T cells increased both intracellularly and on cell surface after stimulation. A dissimilar response of CD8+ CD152+ T cells in HT patients and controls might reflect impaired function of CD152 on regulatory cells (Tregs) and suggests that the alterations are specific for this disease. In HT, the autoimmune process is mainly modulated

by CD8+ effector cells and their cytotoxicity (Weetman 2003; Weetman and McGregor 1994). Nevertheless, it has been recently found that in autoimmune thyroid diseases the naturally occurring regulatory T cells CD4+CD25+ and also CD8+CD122+ play an important role (Marazuela et al. 2006; McLachlan et al. 2007). According to the study of McLachlan et al. (2007), Tregs are a major factor in the intermolecular spreading of the immune response to thyroid antigens and in a shift from Graves' disease and Hashimoto's thyroiditis. Saitoh et al. (2007) supported this idea in excellent animal study. Our present observation on the CD8+ subset can reflect their importance in the pathogenesis of HT in humans. The difference between the CD152 regulation in CD4+ and CD8+ is in concert with the observation of several authors, confirming that induction of CD152 restricts clonal expansion of helper T cells (Doyle et al. 2001) and plays a role in the differentiation and action of Th1 and Th2 cells (Alegre et al. 1998; Bour-Jordan et al. 2003). Under normal conditions, CD152 is constitutively expressed on Tregs (Eggena et al. 2004) and is necessary for their proper function (Takahashi et al. 2000) and generation (Tang et al. 2004; Zheng et al. 2006). On the other hand, hypo-function of CD152 can impair the regulation of the T cell response to a mitogen. Stephens et al. (2001) have found that the CD4+ CD25+ Treg proliferate poorly in response to mitogenic stimulation and suppress the proliferation of $CD4^{+}CD25^{-}$ cells in co-culture. Therefore, impaired function of these cells can explain an increase in the number of CD4+ after mitogen stimulation in our HT patients.

21.5 Conclusions

Hashimoto's thyroiditis is characterized by impaired inhibitory function of CD152. The impairment may depend on imbalance of intracellular/surface CD152 expression on T cells.

Conflicts of interest: The authors declare no conflicts of interest in relation to this article.

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