Diminished Frequency and Function of CD4+CD25high Regulatory T Cells Associated with Active Uveitis in Vogt-Koyanagi-Harada Syndrome

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PURPOSE. CD4+CD25high regulatory T (Treg) cells have been shown to be involved in the pathogenesis of autoimmune diseases. Vogt-Koyanagi-Harada (VKH) syndrome is an organ-specific autoimmune disease. This study was designed to phenotypically and functionally characterize peripheral blood CD4+CD25high Treg cells in VKH patients with active uveitis.

METHODS. Blood samples were taken from 30 patients with active VKH, 19 patients with inactive VKH, and 26 healthy controls. Peripheral blood mononuclear cells were subjected to flow cytometry for analysis of phenotypes of the CD4+CD25high Treg cells. For functional analysis, CD4+CD25high Treg cells and CD4+CD25− T cells were separated by means of magnetic-assisted cell sorting and subsequently cocultured for 6 days. The proliferation of CD4+CD25− T cells was measured by [3H] thymidine incorporation assay. The levels of IFN-γ, IL-17, and IL-13 in the supernatants were determined by enzyme-linked immunosorbent assay.

RESULTS. Significantly decreased frequencies of CD4+CD25high Treg cells and percentages of FOXP3+ cells in these Treg cells were shown in patients with active VKH. Treg cells from patients with active VKH showed a significant deficiency in suppressing the proliferation of CD4+CD25− T cells and inhibiting the production of IFN-γ and IL-13 by CD4+CD25− T cells. CD4+CD25high Treg cells from VKH patients or healthy controls did not markedly inhibit or promote IL-17 production.

CONCLUSIONS. A significantly decreased frequency and diminished function of CD4+CD25high Treg cells is associated with active uveitis in patients with VKH syndrome. These results suggest that these dysfunctional CD4+CD25high Treg cells may play a role in the pathogenesis of uveitis in VKH syndrome. (Invest Ophthalmol Vis Sci. 2008;49:3475–3482) DOI:10.1167/iovs.08-1793

Initially, CD4+CD25high regulatory T (Treg) cells were identified in the mouse and were characterized by their immunoregulatory ability to inhibit the development of certain autoimmune diseases in animal models.1,2 It has been reported that approximately 5% to 10% of the human CD4+ T-cell subpopulation from peripheral blood expresses CD25 (IL-2 receptor α chain) and that these cells only 1% to 2% express high levels of CD25. These CD4+CD25high cells have regulatory properties and are designated CD4+CD25high regulatory T (Treg) cells.3,4 Winged-helix/forkhead transcription factor foxp3 (FOXP3) has been a reliable marker for CD4+CD25+ Treg cells and is critical for maintaining immune tolerance and preventing autoimmune diseases.5,6 Convincing evidence has established that certain mutations in the FOXP3 gene in humans leads to the IPEX syndrome (immunodysregulation, polyendocrinopathy, and enteropathy X-linked syndrome).7 Apart from FOXP3, human glucocorticoid-induced tumor necrosis factor receptor (GITR), intracellular cytotoxic T lymphocyte antigen (CTLA)-4, and CD45RO are potential markers for identifying CD4+CD25high Treg cells. CD69 is an early activation marker of T cells, but it is hardly expressed on CD4+CD25high Treg cells.

The involvement of CD4+CD25high Treg cells in human autoimmune disease has been studied recently. Either reduced frequency or impaired function of CD4+CD25high Treg cells has been reported in patients with a number of autoimmune diseases, including multiple sclerosis, psoriasis, systemic lupus erythematosus, and rheumatoid arthritis.8–11 Vogt-Koyanagi-Harada (VKH) syndrome is an organ-specific autoimmune disease characterized by chronic bilateral granulomatous panuveitis and involvement of the central nervous, auditory, and integumentary systems.12,13 Whether an altered frequency and impaired function of CD4+CD25high Treg cells is possibly associated with the pathogenesis of VKH syndrome must be addressed and was, therefore, the subject of the study described here. Our results showed decreased percentages of CD4+CD25high Treg cells and CD4+CD25high FOXP3+ T cells in patients with active VKH. Furthermore, these CD4+CD25high Treg cells in patients with active VKH showed a diminished function in proliferation and cytokine production experiments. This loss of function coincided with a temporal decrease in the percentage of Foxp3+ cells within CD4+CD25high Treg cells. These results suggest that decreased frequency and diminished function of CD4+CD25high Treg cells are associated with the active uveitis seen in VKH patients.

MATERIALS AND METHODS

Patients

Forty-nine VKH patients were referred to the uveitis study center of Sun Yat-sen University were included in this study. The diagnosis of
VKH syndrome was made according to the diagnostic criteria revised for VKH syndrome by an international committee on nomenclature. \(^{14}\) Thirty VKH patients (18 males, 12 females; age range, 21–52 years) had active uveitis, as evidenced by mutton fat keratic precipitates, cells in the anterior chamber and vitreous body in association with sunset glow fundus, and Dalen-Fuchs nodules. The systemic findings included headache (6 patients), scalp supersensitivity of skin (16 patients), tinnitus (8 patients), dysacusis (7 patients), poliosis (11 patients), alopecia (9 patients), and vitiligo (9 patients). These patients did not use immunosuppressive agents at least for 1 week or used only a low dosage of corticosteroids (<20 mg/day) before blood sampling. Nineteen VKH patients (13 males, 6 females; age range, 23–55 years) had no active intraocular inflammation. No suppressive agent was used at least for 1 month before blood sampling. Twenty-six healthy persons (17 males, 9 females; age range, 20–55 years) served as controls. All procedures followed the tenets of the Declaration of Helsinki.

**Flow Cytometry**

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly drawn heparinized blood by Ficoll-Hypaque gradient centrifugation and were stained by direct immunofluorescence using four-color flow cytometry (FCM; FACSaire; BD Biosciences, Franklin Lakes, NJ). To analyze cell surface molecule expression, the PBMCs were incubated with the appropriate monoclonal antibodies (mAbs), followed by fixation in 4% paraformaldehyde. Alternatively, cells were permeabilized and fixed using fixation/permeabilization solution (eBioscience, San Diego, CA) and were stained with the appropriate mAbs for examination of the expression of intracellular molecules. The mAbs used in this study were FITC-anti-CD3, PEcy5-anti-CD4, PE-anti-CD25, PE-anti-CTLA-4, antigen-presenting cell (APC)-anti-FOXP3 staining set, APC-anti-GITR, FITC-anti-CD69 and APC-anti-CD45RO (purchased from eBioscience, San Diego, CA) and were stained with the appropriate mAbs for examination of the expression of intracellular molecules. The mAbs used in this study were FITC-anti-CD3, PEcy5-anti-CD4, PE-anti-CD25, PE-anti-CTLA-4, antigen-presenting cell (APC)-anti-FOXP3 staining set, APC-anti-GITR, FITC-anti-CD69, and APC-anti-CD45RO purchased from eBioscience, San Diego, CA, APC-anti-CD3, FITC-anti-CD4, and PEcy5-anti-CD25 purchased from Beckman Coulter, Fullerton, CA).

**Cell Purification**

CD4^+^ CD25^high^ Treg cells and CD4^+^ CD25^−^ T cells were isolated by MACS using a human CD4^+^ CD25^+^ regulatory cell isolation kit (Miltenyi Biotec, Palo Alto, CA). PBMCs were fractionated into two aliquots with adequate cell numbers, complying with the requirements of the experiments. One portion was used to separate CD4^+^ CD25^−^ T cells according to the manufacturer’s instructions to acquire highly purified CD4^+^ CD25^+^ T cells. The other portion was used to separate CD4^+^ CD25^high^ Treg cells using a reduced volume of anti-CD25 beads. To obtain a highly purified CD4^+^ CD25^high^ Treg cell population, we reduced the recommended volume of anti-CD25 beads with the use of the method described previously. \(^{15}\) As a result, the anti-CD25 beads at a concentration of 0.25 μL/1×10^7^ PBMCs could yield 2% to 3% CD4^+^ CD25^high^ Treg cells with greater than 95% purity from VKH patients and healthy controls, similar to that obtained by fluorescence-activated cell sorting (FACS) (see Figs. 3A, 3B). \(^{14,15}\) Therefore, this concentration of anti-CD25 beads was used for the isolation of CD4^+^ CD25^high^ Treg cells from VKH patients and healthy controls in the following experiments. In addition, we also determined the suppressive function of CD4^+^ CD25^high^ Treg cells isolated from the same healthy controls by MACS and FACS (FACSaire; BD Biosciences). The results showed that immunomagnetically purified and sorted CD4^+^ CD25^high^ Treg cells inhibited the proliferation of autologous CD4^+^ CD25^−^ T cells by 70%. APCs were isolated from PBMCs by negative depletion of CD3^+^ T cells using anti-CD3 magnetic beads (Miltenyi Biotec) according to the manufacturer’s instructions.

**Proliferation Assay**

To test the inhibition of CD4^+^ CD25^high^ Treg cells on the proliferation of alloantigen-specific CD4^+^ CD25^−^ T-cell proliferation, 1×10^4^ CD4^+^ CD25^−^ T cells in the presence of 4×10^4^ autologous APCs were cultured with or without various amounts of CD4^+^ CD25^high^ Treg cells in triplicate in 200 μL RPMI 1640 medium supplemented with 2% penicillin/streptomycin and 10% fetal bovine serum for 6 days in a U-bottom, 96-well plate. For co-culturing experiments, 1×10^4^ CD4^+^ CD25^−^ T cells from healthy controls in the presence of 4×10^4^ autologous APCs were mixed at a ratio of 1:1 with CD4^+^ CD25^high^ Treg cells from patients with active VKH and vice versa. To test the inhibitory capacity of CD4^+^ CD25^high^ Treg cells on the proliferation of CD4^+^ CD25^−^ T cells in response to polyclonal stimulation (anti-CD3 mAb, clone UCHT1; BD Biosciences Pharmingen, San Diego, CA), purified 1×10^4^ CD4^+^ CD25^−^ T cells in the presence of 4×10^4^ autologous APCs were stimulated with soluble anti-CD3 mAb at a concentration of 0.01 μg/mL or plate-bound anti-CD3 mAb at a concentration of 5 μg/mL and cultured with or without 1×10^4^ autologous CD4^+^ CD25^high^ Treg cells for 6 days. Mitomycin C (Sigma-Aldrich, St. Louis, MO)–treated and CD3^+^ T cell–depleted PBMCs were used as APCs. Cells were pulsed with 1 μCi/well [3H] thymidine for 16 hours before harvesting, and the amount of incorporated [3H] thymidine was determined by liquid scintillation spectroscopy (Tri-carb2900; Packard Bioscience, Meriden, CT).

**ELISA**

To analyze cytokine production, 100 μL supernatant was removed from cultures of proliferation and additional co-culturing experiments stimulated with immobilized anti-CD3 mAb. Levels of IL-17, IFN-γ, and IL-13 were measured using human ELISA development kits (Duoset; R&D Systems, Minneapolis, MN) with detection limits of 15 pg/mL, 15 pg/mL, and 90 pg/mL, respectively.

**Statistical Analysis**

Data are expressed as mean ± SD. Statistical analysis was performed using Student’s t-test. P < 0.05 was considered statistically significant.

**Results**

**Decreased Frequency of CD4^+^ CD25^high^ Treg Cells in Active VKH**

Our data showed a significantly decreased percentage of CD4^+^ CD25^high^ Treg cells in patients with active VKH compared with healthy controls (P = 0.002) and patients with inactive VKH (P = 0.010; Figs. 1A, 1B). Furthermore, the intensity of CD25 expression of CD4^+^ T cells indicated by mean fluorescence intensity was also decreased in patients with active VKH (Fig. 1C). The frequency of the total CD4^+^ CD25^+^ T-cell population was not altered in patients with active VKH (Fig. 1B).

**Decreased Expression of FOXP3 in Active VKH Patients**

The results of our further experiments showed that FOXP3 was expressed by most (82%–95%) CD4^+^ CD25^high^ Treg cells in VKH patients and healthy controls. A significantly decreased frequency of Foxp3^+^ cells in CD4^+^ CD25^high^ Treg cells was detected in patients with active VKH compared with healthy controls (P = 0.001) and patients with inactive VKH (P = 0.023; Fig. 2A).

**Similar Expression of CD69, CD45RO, CTLA-4, and GITR in CD4^+^ CD25^high^ Treg Cells from VKH Patients and Healthy Controls**

To characterize the phenotypes of CD4^+^ CD25^high^ Treg cells in VKH patients, flow cytometry was performed to study the
surfaces and intracellular markers in CD4^+CD25^high Treg cells, including CD45RO, CD69, GITR, and CTLA-4. The results of FCM analysis showed that CD4^+CD25^high Treg cells expressed significantly higher levels of CTLA-4 (50%), GITR (30%), and CD45RO (85%) than CD4^+CD25^low and CD4^+CD25^- T-cell subsets in patients and controls. There was no difference regarding levels of CD45RO, CTLA-4, or GITR expression in CD4^+CD25^high Treg cells between VKH patients and healthy controls (Fig. 2B). In addition, a very low percentage (<1%) of CD4^+CD25^high Treg cells expressed CD69 in VKH patients and healthy control subjects.

**Diminished Inhibitory Function of CD4^+CD25^high Treg Cells in Active VKH Patients**

In this study, we investigated the functional properties of CD4^+CD25^high Treg cells from healthy controls and VKH patients in response to alloantigen-specific T-cell stimulation. The activity of CD4^+CD25^high Treg cells in inhibiting autologous CD4^+CD25^- T cells was severely dysfunctional in patients with active VKH. At a ratio of 1:1, CD4^+CD25^high Treg cells from patients with active VKH inhibited autologous CD4^+CD25^- T-cell proliferation by an average of 35.9% that was significantly decreased compared with healthy controls (P < 0.001) and patients with inactive VKH (P = 0.001; Fig. 3C).

Comixing experiments were performed simultaneously to further validate the diminished alloantigen-specific regulatory activity of the CD4^+CD25^high Treg cells in patients with active VKH. The inhibitory effect of CD4^+CD25^high Treg cells from patients with active VKH showed a significantly deficient effect on the proliferation of allogeneic CD4^+CD25^- T cells (P = 0.019, P = 0.003, respectively; Fig. 3D).

Subsequently, we performed polyclonal stimulation assays. Results showed that CD4^+CD25^high Treg cells from VKH patients and healthy controls did not proliferate in response to polyclonal stimulation, confirming the anergic property of CD4^+CD25^high Treg cells. CD4^+CD25^high Treg cells from patients with active VKH inhibited the proliferation of autologous CD4^+CD25^- T cells in response to soluble or immobilized anti-CD3 mAb, but this inhibition was markedly lower when compared with that of CD4^+CD25^high Treg cells from patients with inactive VKH (P = 0.003, P = 0.004, respectively) or healthy controls (P = 0.003, P = 0.007, respectively; Fig. 3E).

These results showed that weak and suboptimal polyclonal TCR stimulation could elicit a significantly diminished suppressive function in CD4^+CD25^high Treg cells from patients with active VKH.

Our experiments further quantified the inhibitory function of CD4^+CD25^high Treg cells. CD4^+CD25^- T cells were cocultured in the presence of autologous APCs with autologous CD4^+CD25^high Treg cells at various ratios. At a ratio of 1:1, CD4^+CD25^high Treg cells from patients with active VKH inhibited the proliferation of CD4^+CD25^- T cells by 36%, which was equal to that observed at ratios of 1:16 to 1:8 in patients with inactive VKH and in healthy controls (Fig. 3F).

**Inefficient Inhibition of CD4^+CD25^high Treg Cells from Active VKH Patients in the Production of IFN-γ and IL-13 by Allogeneic CD4^+CD25^- T Cells**

Supernatants from immobilized anti-CD3 mAb–stimulating cultures of CD4^+CD25^- T cells in the presence of autologous APCs and allogeneic CD4^+CD25^high Treg cells were removed to detect levels of IFN-γ and IL-13. Results showed that the IFN-γ level was significantly higher in patients with active VKH than in healthy controls (P < 0.001) and patients with inactive VKH (P = 0.001). The production of IFN-γ was significantly reduced in the presence of CD4^+CD25^high Treg cells in patients with active VKH (P = 0.002), healthy controls (P < 0.001), and patients with inactive VKH (P < 0.001). However, IFN-γ levels in the coculture supernatants were significantly higher in patients with active VKH than in healthy controls (P < 0.001) and patients with inactive VKH (P < 0.001). These results showed that CD4^+CD25^high Treg cells from patients with active VKH had less efficient suppression in IFN-γ production (Fig. 4A). Although IL-13 levels in CD4^+CD25^- T cells culture supernatants were not different between VKH patients and healthy controls, a significantly increased level was noted in the coculture supernatants from patients with active VKH compared with healthy controls (P = 0.018) and patients with inactive VKH (P = 0.044; Fig. 4B). Together with the results of comixing tests, a reduced ability of CD4^+CD25^high Treg cells in suppressing the production of IFN-γ and IL-13 was observed in patients with active VKH (Figs. 4C, 4D).
**FIGURE 2.** Immunophenotyping of CD4⁺CD25<sup>high</sup>, CD4⁺CD25<sup>low</sup>, and CD4⁺CD25<sup>-</sup> T-cell subsets from freshly drawn peripheral blood by FCM. (A) Representative FCM diagrams illustrate frequencies of FOXP3<sup>+</sup> cells within CD4⁺ T-cell subsets. Asterisks indicate the significantly lowered expression of FOXP3 in CD4⁺CD25<sup>high</sup>-Treg cells in patients with active VKH. (B) FCM analysis of cell surface and intracellular molecules expressed in CD25<sup>high/low/-</sup> T-cell subsets.
Inability of CD4⁺CD25<sup>high</sup> Treg Cells To Suppress the Production of IL-17 by Cocultured Allogeneic CD4⁺CD25<sup>-</sup> T Cells in VKH Patients or Healthy Controls

Th17 cells have been linked to autoimmune disease. To elucidate whether CD4⁺CD25<sup>high</sup> Treg cells were involved in the regulation of IL-17 in VKH syndrome, we also measured the level of IL-17. Our results showed that the production of IL-17 by CD4⁺CD25<sup>-</sup> T cells was significantly higher in patients with active VKH than in patients with inactive VKH (P = 0.003) and healthy controls (P = 0.008). CD4⁺CD25<sup>high</sup> Treg cells did not secrete IL-17 in VKH patients or healthy controls.

DISCUSSION

In this study we observed a significantly reduced frequency of CD4⁺CD25<sup>high</sup> Treg cells and a decreased expression of CD25 molecules on the surfaces of Treg cells in patients with active VKH. A deficiency in CD4⁺CD25<sup>high</sup> Treg cell numbers and a diminished function of Treg cells has been reported to be associated with organ-specific autoimmunity in humans, and
our data in VKH syndrome are the first to show this phenomenon in an autoimmune ocular disease.

Our results revealed a decreased frequency of CD4⁺CD25high Treg cells that correlated with active uveitis in VKH patients. In addition, we found a decreased expression of CD25 (IL-2 receptor α chain) on CD4⁺CD25high Treg cells at the single-cell level. Recent studies have shown that signaling through the high-affinity IL-2 receptor is critical for the differentiation, survival, and function of CD4⁺CD25high Treg cells. It is, therefore, presumed that the decreased expression of CD25 is associated with the dysfunction of these Treg cells, as demonstrated by our later experiments.

Expression of the transcription factor FOXP3 has been implicated as a key element for murine CD4⁺CD25+ and human CD4⁺CD25high Treg cell expansion and function. A decreased frequency of FOXP3⁺ cells in CD4⁺CD25high Treg cells was shown in patients with active VKH. This result suggested a diminished function of these cells in active VKH syndrome. The decreased expression of FOXP3⁺ cells in CD4⁺CD25high Treg cells may be associated with the dysfunction of these Treg cells, as demonstrated by our later experiments.

Our experiments were designed to test whether there was a diminished function of these cells in active VKH syndrome. First, we assayed the inhibitory effect of CD4⁺CD25high Treg cells on the proliferation of CD4⁺CD25+ T cells on stimulation with autologous APCs loaded with allogeneic antigen. The results showed a significantly less alloantigen-specific inhibitory activity of CD4⁺CD25high Treg cells in patients with active VKH. However, this result warrants further validation using allogeneic APCs as stimulators. Second, we conducted a coexisting exper-
iment to test whether the decreased inhibition observed in the experiments with alloantigen stimulation in patients with active VKH was caused by an increased resistance of the effector CD4^{+}CD25^{-} T cells. The result did not show any increased resistance of these effector T cells to CD4^{+}CD25^{high} Treg cells in patients with active VKH compared with that in healthy controls. Third, we tested the inhibitory effect of CD4^{+}CD25^{high} Treg cells on the proliferation of CD4^{+}CD25^{-} T cells after polyclonal stimulation. Similarly, we observed a diminished function of CD4^{+}CD25^{high} Treg cells in patients with active VKH. Given that the experiment with polyclonal stimulation could exclude the possibility that the difference in CD4^{+}CD25^{high} Treg cell function might have been caused by diverse alloantigens responses in CD4^{+}CD25^{-} T cells, this experiment validated the result disclosed by the study with alloantigen stimulation. Fourth, we evaluated the inhibitory efficiency of CD4^{+}CD25^{high} Treg cells on the proliferation of CD4^{+}CD25^{-} T cells using different ratios of these two cell populations. The results showed at least eightfold decreased inhibitory function in the CD4^{+}CD25^{high} Treg cells of patients with active VKH compared with those of patients with inactive VKH and healthy controls. All the results demonstrated that functionally diminished CD4^{+}CD25^{high} Treg cells were indeed present in patients with active VKH and that the inhibitory function of these Treg cells was markedly decreased. Our study did not address whether the recovery of CD4^{+}CD25^{high} Treg cells functionally and quantitatively resulted from immunosuppressive treatment in these patients or whether recovery was a consequence of disease quiescence. More studies are needed to clarify this issue.

Previous studies have suggested that Th1 responses are predominant in the development of VKH syndrome. Some have shown that Th17 cells are predominant in various aspects of human autoimmune disease, including VKH syndrome. To understand whether the CD4^{+}CD25^{high} Treg cells exert their regulatory function on CD4^{+}CD25^{-} T cells through the modulation of cytokines, we investigated the production of cytokines, including IFN-γ, IL-13, an important cytokine of Th2 cells, and IL-17, and the influence of the CD4^{+}CD25^{high} Treg cells on the in vitro production of these cytokines. The results showed significantly increased IFN-γ and IL-17 production in patients with active VKH, suggesting the involvement of both Th1 and Th17 cells in this disease. CD4^{+}CD25^{high} Treg cells were able to significantly inhibit the production of IFN-γ. Importantly, CD4^{+}CD25^{high} Treg cells from patients with active VKH exerted a far less inhibitory effect. We did not find an inhibitory effect of CD4^{+}CD25^{high} Treg cells in patients with healthy controls in the production of IL-17 by allogeneic CD4^{+}CD25^{-} T cells. This result is consistent with that presented recently by another group, who found that the proliferation of a Th17 clone in response to allogeneic stimulation exhibited a significantly lower susceptibility to the suppressive activity of an autologous CD4^{+}CD25^{FOXp3}^{+} Treg cell clone. Taken together, the results demonstrated that CD4^{+}CD25^{high} Treg cells in patients with active VKH were dysfunctional in inhibiting the production of IFN-γ but not IL-17. Therefore, these dysfunctional Treg cells disclosed by proliferation experiments may be involved in the pathogenesis of VKH syndrome because of their inefficient suppressive effects on cytokine production.

A recent study has shown that FOXP3 is transiently expressed in polyclonally stimulated human CD4^{+}CD25^{-} T cells and that these activated cells do not have a suppressive function in vitro. Although our study did not exclude the possibility that a slight contamination of activated CD4^{+}CD25^{-} T cells may exist and a minor influence of low-dose corticosteroids was present, our results concerning the frequency and function of CD4^{+}CD25^{high} Treg cells in VKH syndrome are generally consistent with those observed in other autoimmune diseases. Functionally diminished CD4^{+}CD25^{high} Treg cells are found in these studies, though there are differences between the studies concerning the reported frequency of these Treg cells. It is likely that functional impairment of the CD4^{+}CD25^{high} Treg subpopulation is one of the common final pathways involved in these autoimmune diseases, though the initial factors and insulting autoantigens may be different.

In conclusion, our study revealed significantly decreased frequency and diminished function of CD4^{+}CD25^{high} Treg cells in patients with active VKH. These findings suggest that quantitatively abnormal and functionally diminished CD4^{+}CD25^{high} Treg cells may be important for the development of active intraocular inflammation in VKH syndrome.

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**References**


