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BASIC SCIENCE

Splenic CD8$^+$ T cells secrete TGF-β1 to exert suppression in mice with anterior chamber-associated immune deviation

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Abstract
Background CD8$^+$ regulatory T cells (Treg) have been considered to be involved in a model of ocular-induced tolerance, known as anterior chamber-associated immune deviation (ACAID). The mechanisms of suppression by CD8$^+$ T cells in ACAID remain only poorly understood. TGF-β1 is considered as an inhibitory cytokine for immunosuppression in some models. The production of TGF-β1 by CD8$^+$ T cells in ACAID, and whether CD8$^+$ T cells exert suppression through TGF-β1, is unknown.
Methods The suppressive effect of CD8$^+$ T cells in ACAID mice was determined by a local adoptive transfer (LAT) assay. The production of TGF-β1 by CD8$^+$ T cells was measured by enzyme-linked immunosorbent assay (ELISA). Anti-TGF-β1 antibodies were used in the LAT assay to test if they could block the inhibitory effect of CD8$^+$ T cells.
Results CD8$^+$ T cells from ACAID mice were shown to block the delayed-type hypersensitivity (DTH) response in an antigen-specific manner in a LAT assay. These CD8$^+$ T cells secreted TGF-β1, and their suppression could partially be blocked by anti-TGF-β1 antibodies.
Conclusions Our study confirms that CD8$^+$ T cells from ACAID mice possess inhibitory properties. This population exerts part of its suppressive function via the production of TGF-β1.

Keywords Anterior chamber-associated deviation · CD8$^+$ regulatory T cells · Transforming growth factor β1

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Introduction

An injection of soluble protein antigen (Ag) into the anterior chamber (AC) of the eye induces a kind of immune tolerance termed anterior chamber-associated immune deviation (ACAID), which is characterized by impairment of the delayed-type hypersensitivity (DTH) response. There are two distinct populations of regulatory T cells (Tregs) involved in the suppression of DTH responses in ACAID. The efferent regulatory cells involved in the impaired expression of DTH are considered to be antigen-specific CD8$^+$ T cells [1].

Transforming growth factor-β (TGF-β) is a regulatory cytokine with a pivotal role in regulating immune responses [2]. There are three homologous TGF-β isoforms in mammals, TGF-β1, 2, and 3, encoded by different genes. TGF-β1 is the predominant isoform expressed by the immune system, although all three isoforms have similar properties in vitro [3]. Recent studies have suggested that TGF-β1 produced by CD4$^+$ Tregs may serve as an effective mechanism of suppression of these cells, possibly via

binding to Treg cell TGF-β receptors [4]. Moreover, it plays an essential role in the maintenance of Foxp3 expression in $CD4^+$ $CD25^+$ Tregs in the periphery [5]. The production of TGF-β1 by $CD8^+$ T cells and the exact role of this cytokine derived from splenic $CD8^+$ T cells during ACAID is unknown and was, therefore, the subject of our study. Here we show that splenic $CD8^+$ T cells from ACAID mice produce a large amount of TGF-β1, and that this cytokine is involved in the suppression exerted by $CD8^+$ T cells during ACAID.

Materials and methods

Mice

Female 6–8 week old C57BL/6 (B6; H-2^b) mice were purchased from the animal facility at Sun Yat-sen University, Peoples Republic of China. All mice were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Treatment of mice

ACAID was induced as described previously using micro-injection of ovalbumin (OVA; Sigma, St. Louis, MO, USA) into the AC of the eye [6]. Primed mice received a subcutaneous (s.c.) injection of 250 μg of OVA or BSA (Sigma-Aldrich, Steinheim, Germany) emulsified 1:1 in Complete Freund's adjuvant (CFA, Sigma-Aldrich, Steinheim, Germany) in a total volume of 200 μl. The numbers of mice used in the various experiments are mentioned in the figure legends.

DTH assay

The ear-swelling response was measured to indicate the DTH response to OVA or BSA as described previously [7]. Briefly, 7 days after subcutaneous immunization, mice were challenged respectively by intradermal injection of Ag (200 μg of OVA/10 μl of PBS) and an equal volume of sterile PBS into the right and left ear pinnae. Ear pinnae of experimental and control animals were measured with a Mitutoyo engineer's micrometer (Mitutoyo, Japan) immediately before challenge and 24 h later.

Preparation of peritoneal exudate cells (PECs)

PECs were collected from C57BL/6 mice following intraperitoneal injection with thioglycolate (Sigma-Aldrich) as described previously [8]. More than 90% of the adherent cells were identified as being $F4/80^+$ cells, using flow cytometry (FCM).

Cell isolation and culture

Purification of $CD8^+$ T cells from the spleen of the mice was described previously [9]. The purity of $CD8^+$ T cells was identified to be >95% according to FCM analysis. For cytokine assay, purified $CD8^+$ T cells (2×10^5 cells/well) were cultured in 96-well culture plates containing isolated PECs (2×10^5 cells/well) in the presence of 200 μg/ml OVA or BSA at 37°C in complete RPMI 1640 medium for 24 hours. Cultures containing PECs (2×10^5 cells/well) and OVA (200 μg/ml OVA) were used as control.

TGF-β1 and IL-10 assay

TGF-β1 and IL-10 in the collected supernatants of cell cultures were analyzed using the R&D Duoset ELISA development system, according to the manufacturer's instructions. For TGF-β1, samples were activated by incubation with 1 N HCL for 10 min, and subsequently neutralized with 1.2N NaOH/0.5 M HEPES. The background level of TGF-β1 from PECs was determined separately, and subtracted from these samples. The limits of TGF-β1 and IL-10 detection were 31.2 pg/ml and 15.6 pg/ml respectively.

LAT assay

A LAT assay, as described previously [10], was developed to test suppressor cells during ACAID. Briefly, putative suppressor cells consisted of purified $CD8^+$ T cells separated from splenocytes of ACAID mice. Responder cells were collected from splenocytes of OVA or BSA primed mice. Both suppressor cells and responder cells were harvested on day 7 after AC-inoculation and conventional immunization, and were suspended at 5×10^7 cells/ml in 10 mg/ml OVA or BSA. The immune and suppressor cells populations were then mixed 1:1 in the presence of OVA or BSA (10 mg/ml). Then, the cell mixture was injected (20 μl) into the ear pinnae of naïve C57BL/6 mice. Ear swelling was measured 24 hours later to evaluate DTH. In order to clarify whether TGF-β1 has an effect on suppression mediated by $CD8^+$ T cells from ACAID mice, additional LAT assays were performed after blocking with mouse anti-TGF-β1 mAb (100 μg/ml) or matched isotype (100 μg/ml) (R&D Systems, Inc., USA).

Statistical analyses

Statistical analyses were performed by one-way ANOVA using SPSS 11.0. Values of $p<0.05$ were considered significant.

Results

$CD8^+$ T cells from ACAID mice specifically inhibit DTH response in vivo

The LAT assay was used to study the inhibitory function of antigen-specific efferent suppressor cells from ACAID mice. Our results showed that the ear-swelling responses of mice that received responder cells mixed with $CD8^+$ cells from normal mice (positive control) displayed significant ear swelling indicative of DTH. This ear-swelling response induced by OVA-primed responder cells was significantly reduced when $CD8^+$ T cells from OVA-induced ACAID mice were co-injected with these cells. However, OVA-specific $CD8^+$ T cells did not inhibit the DTH response induced by BSA primed responder cells. The results of a typical experiment are shown in Fig. 1.

$CD8^+$ T cells from ACAID mice secreted TGF-β1

Previous studies have indicated that TGF-β and IL-10 play an important role as immunosuppressive cytokines in ACAID [11]. In this study, we examined the production of these cytokines by $CD8^+$ T cells during ACAID. As shown in Fig. 2, $CD8^+$ T cells from ACAID mice pulsed with OVA produced a higher level of TGF-β1 than those from normal mice. However, when an irrelevant antigen, BSA, was used, there was no difference between these two groups concerning the production of TGF-β1. The levels of IL-10 secreted by $CD8^+$ T cells from both ACAID mice and normal mice were below the detection limit of the assay.

Anti-TGF-β1 antibody partially blocked the suppression by ACAID $CD8^+$ T cells

As $CD8^+$ T cells could secrete TGF-β1, a further experiment was performed to examine whether the inhibitory effect of these cells was mediated by TGF-β1. Neutralizing anti-TGF-β1 antibodies or matched isotype was used in the blocking study. The result of a representative experiment is presented in Fig. 3. The mice receiving an injection of neutralizing anti-TGF-β1 antibodies showed an ear-swelling response that was approximately half of that observed in the positive control. Isotype control did not affect the impaired ear-swelling response.

Discussion

In this study, we showed that $CD8^+$ T cells from ACAID mice could specifically inhibit the expression of the DTH response. In vitro experiments showed that these cells were able to produce TGF-β1 in an antigen-specific manner. The suppressive effect of $CD8^+$ T cells from ACAID mice could be partially blocked by anti-TGF-β1 antibodies in a LAT assay. All these results suggest that secretion of TGF-β1 may be an important suppressive property of $CD8^+$ T cells in ACAID.

It has been shown that $CD8^+$ T cells are necessary in the development of ACAID [12]. In a previous study [9], we showed increased frequencies of $CD8^+$ T cells as well as $CD8^+$ $Foxp3^+$ T cells in the spleens of ACAID mice. The inhibitory property of these $CD8^+$ T cells was shown by a

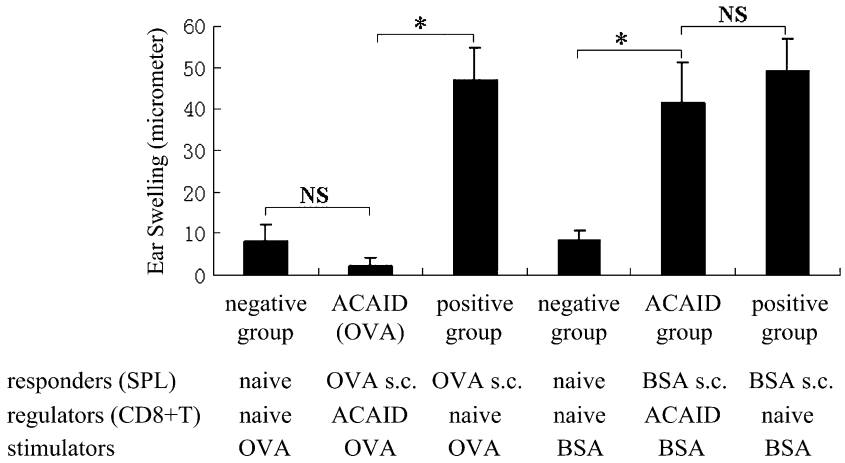

Fig. 1 Effect of $CD8^+$ T cells from ACAID mice on expression of DTH in vivo. Regulator cells consisted of purified $CD8^+$ T cells separated from splenocytes of OVA-induced ACAID mice. Responder cells were collected from splenocytes(SPL) of OVA- or BSA-primed mice. As a negative control, naïve spleen cells were used as responder cells and purified $CD8^+$ T cells from untreated mice were used as regulatory cells. Primed spleen cells were used as responder cells and purified $CD8^+$ T cells from naive mice were used as regulatory cells for a positive control. Ear swelling was measured at 24 hours. Mean ± SD ear-swelling responses are presented (n=5). The experiments were repeated twice with similar results. *p<0.05; NS, p>0.05

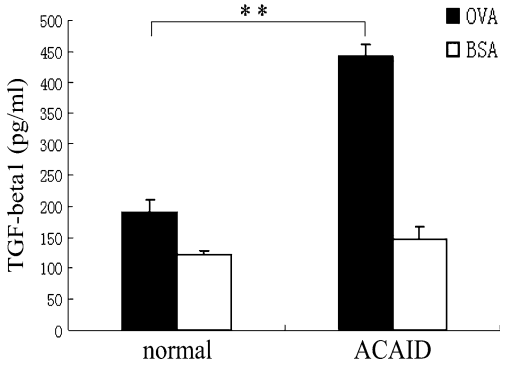

Fig. 2 TGF-β1 production by $CD8^+$ T cells from ACAID mice. Purified $CD8^+$ T cells (2×10^5 cells/well) from normal mice and ACAID mice were incubated in the presence of PECs (APC, 2×10^5 cells/well) and pulsed with OVA or BSA for 24 hours. TGF-β1 secreted in the supernatant was measured by standard ELISA. Cultures stimulated with OVA are represented by *black bars*; cultures stimulated with BSA are represented by *white bars*. Five mice were used in each group in one experiment. Results are represented as mean ± SD. The experiments were repeated twice. $^{**}p<0.01$

decreased DTH response in a LAT assay. The present study confirmed our previous observation in another group of mice using the LAT assay. Furthermore, it extends these previous observations focusing on TGF-β1. Our results suggest that $CD8^+$ T cells may function as inhibitory cells, generally known as Tregs.

TGF-β and IL-10 have been shown to be the suppressive cytokines in immune tolerance models including ACAID [13–22]. TGF-β1, a major component of the TGF-β family, along with TGF-β2 has been proven to be involved in the immunoregulation. In general, TGF-β1 is implicated in the regulating autoimmune and inflammatory diseases and converting $CD4^+$ $CD25^-$ T cells into Treg in vitro [3, 23–25]. Whereas TGF-β2 mainly contributes to the intraocular immunosuppressive microenvironment and endows APC with ACAID-inducing capability [8, 12, 26], Kezuka et al. [8] demonstrated that TGF-β2 was not required for the suppression mediated by in vito-activated ACAID-like $CD8^+$ Treg. In this study we tested whether $CD8^+$ T cells could produce TGF-β1, a cytokine important to the Treg. Our results showed that splenic $CD8^+$ T cells from ACAID mice could secrete a large amount of TGF-β1 upon stimulation with OVA, an antigen used for induction of ACAID. This result suggests that primed $CD8^+$ T cells are able to secrete TGF-β1 in an antigen-specific manner. Blocking experiments with anti-TGF-β1 antibodies could partially inhibit the function of $CD8^+$ Tregs. This result is consistent with that observed by Weiner et al. [27] in an oral tolerance model. They found that both CD4 and CD8 regulatory cells mediated their down-regulatory effect through secreting TGF-β. It has also been reported that induced $CD8^+$ Tregs from another immune tolerance model in lupus-prone mice, in which artificial peptide is injected intravenously, secrete abundant TGF-β1. Antibodies to TGF-β can abrogate the suppression of these $CD8^+$ Tregs [28]. All these results suggest that TGF-β1 is a predominant cytokine involved in the function of $CD8^+$ T cells from ACAID model as well as other immune tolerance models.

It has been shown that the generation of the efferent $CD8^+$ Tregs in ACAID is dependent on the production of IL-10 by NKT cells and δγ T cells [29, 30], and that IL-10 is necessary for APC to acquire the ability of inducing ACAID [11]. However, it is unknown whether IL-10 functions as a suppressive factor in the effector phase by $CD8^+$ Treg. We further detected the IL-10 secretion by splenic $CD8^+$ T cells from ACAID mice using ELISA. However, the level of IL-10 secreted by these cells was below the detection limit of the assay (15.6 pg/ml). One of the reasons may be that IL-10 is not required for $CD8^+$ Treg-mediated suppression. Another possibility could be that this assay is not sensitive enough to define the different level of IL-10 secretion by $CD8^+$ T cells between ACAID mice and normal mice. A more sensitive technique is expected to clarify this issue.

Our result differs from that presented by Kosiewicz et al. [31], who showed that TGF-β1 was produced primarily by the splenic $CD4^+$ T and non-T cells, but not by $CD8^+$ T cells, during ACAID. This difference may be due to the different culture conditions used in the experiments. $CD4^+$ and $CD8^+$ T cells were not separated from each other in their study. The response of $CD8^+$ T cells to OVA

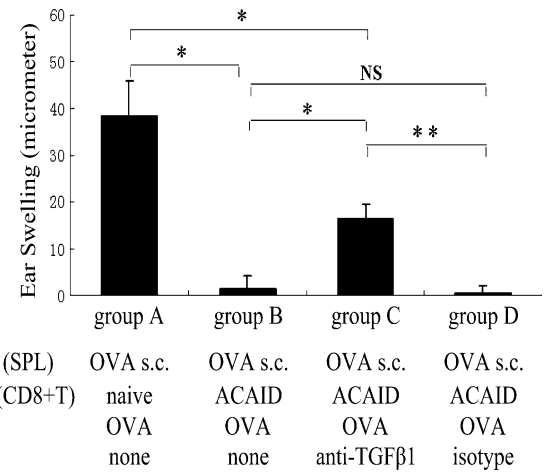

Fig. 3 Capacity of anti-TGF-β1 antibody to restore the suppressed DTH response in vivo by $CD8^+$ T cells from ACAID mice. Regulator cells consisted of purified $CD8^+$ T cells separated from splenocytes of ACAID mice. Responder cells were collected from splenocytes of OVA-primed mice. *Group A*: positive control; *Group B*: ACAID group; *Group C*: anti-TGF-β1 antibody can partially block the suppressive effect of $CD8^+$ T cells from ACAID mice; *Group D*: isotype control for anti-TGF-β1 antibody. All results are reported as mean ear swelling ±SD. Each group represents five animals. $^{**}p<0.01$;$^{*}p<0.05$; NS, $p>0.05$

stimulation cultured alone may be different from those cultured with $CD4^+$ T cells. Another study showed that the neutralizing anti-TGF-β2 antibodies didn't reverse the suppressive effect of in vitro-generated ACAID-like $CD8^+$ Tregs [8], while we found that anti-TGF-β1 antibodies could reverse the suppressive effect of $CD8^+$ Tregs in ACAID. The discrepancy may be due to the different blocking antibodies used in the experiments. TGF-β2 has been considered as a crucial immunomudulatory factor within the eye. It may contribute to the induction of ACAID, whereas the TGF-β1 produced by $CD8^+$ T cells possibly exerts its effect in the expression phase. Our results are also different from the findings by Kapp et al. [32]. They found that in vitro TGF-β2-treated APC activated OT-1 Treg, ACAID-like $CD8^+$ Treg, exert suppression in a TGF-β-independent manner. The difference may be due to the origin of $CD8^+$ Tregs. The in vitro and in vivo models may result in different consequences. As TGF-β2 is only one of the important immunomodulatory cytokines existing in AC, the environment in the AC in vivo is much more complex than the experimental condition used in vitro. Additionally, we found that the anti-TGF-β1 antibodies could only partially reverse the inhibition of DTH response by $CD8^+$ Tregs. In our preliminary experiment, three concentrations (200 μg/ml, 100 μg/ml and 50 μg/ml) were used. This study showed a similar result concerning the first two concentrations, although a lower effect was found in the concentration of 50 μg/ml (data not shown). All these results suggest that TGF-β1, is not a sole factor involved in the inhibition of $CD8^+$ Tregs, and that other factors, for instance other isoforms of TGF-β or other cytokines, may also be implicated in this inhibition. A recent study showed that interferon-γ was required for the inhibitory activity of these cells [33]. More studies are needed to clarify the in vivo mechanisms involved in the suppressive effects mediated by $CD8^+$ Tregs during ACAID.

In conclusion, our study revealed an inhibitory effect of $CD8^+$ T cells in a LAT assay, and increased production of TGF-β1 by these cells. Experiments using anti-TGF-β1 antibodies were able to partially reverse the suppressive effect of $CD8^+$ Tregs. These results suggest that TGF-β1 may be one of the important cytokines involved in the suppressive effect mediated by these cells. However, the form of TGF-β1, soluble, membrane-bound, or both of them, concerned with this function should be addressed, and the exact mechanisms involved in the function of efferent $CD8^+$ Tregs during ACAID needs to be determined.

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Declaration All the listed authors have participated actively in the study, and have read and approved the submitted manuscript. None of the authors has any potential financial conflict of interest related to this manuscript.

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