Graefe's Arch Clin Exp Ophthalmol (2007) 245:1549–1557
DOI 10.1007/s00417-007-0591-8

LABORATORY INVESTIGATION

$CD4^+CD25^+$Tregs express an increased LAG-3 and CTLA-4 in anterior chamber-associated immune deviation

Xuefei Zhu · Peizeng Yang · Hongyan Zhou · Bing Li · Xiangkun Huang · Qianli Meng · Li Wang · Aize Kijlstra

Received: 19 November 2006 / Revised: 30 March 2007 / Accepted: 5 April 2007 / Published online: 31 May 2007

Abstract
Background Regulatory $CD4^+CD25^+$ T cells have been proven to be essential for maintenance of peripheral tolerance and autoimmune diseases. ACAID is a model of immune privilege in the eye. Relatively little is known about the role and phenotype of these regulatory $CD4^+CD25^+$ T cells in ACAID.
Methods Injection of OVA into the anterior chamber of BALB/C mice was performed to induce ACAID. The frequencies of splenic $CD4^+CD25^+$ Tregs and the expression of CTLA-4 and LAG-3 on these cells were determined by flow cytometry. Magnetic cell sorting was used to isolate $CD4^+CD25^+$ and $CD4^+CD25^-$T cells. The function of $CD4^+CD25^+$ T cells was detected by in vitro immunosuppression assays and in vivo adoptive transfer.
Results ACAID was successfully induced following an i.c. injection of OVA. Frequencies of $CD4^+CD25^+$ and Tregs were significantly increased in ACAID mice as compared to those in controls. The $CD4^+CD25^+$ T cells stimulated with OVA in ACAID mice showed a stronger suppressive ability in vitro than those seen in non-ACAID mice. $CD4^+CD25^+$ T cells from ACAID mice, but not from non-ACAID mice, were able to suppress DTH responses in an antigen-specific manner following adoptive transfer. The frequencies of CTLA-4 or LAG-3 on Tregs in ACAID mice were higher as compared with those in naive mice.
Conclusion Splenic $CD4^+CD25^+Foxp3^+$T cells expressing CTLA4 and LAG3 play an important role in the induction of ACAID.

Keywords Regulatory T cells · Immune regulation · Foxp3 · ACAID

This study is supported by the Fund for the National Natural Science Foundation (30400487), Research Group Fund of Guangdong Province Natural Science (2005-04) and Innovation Research Groups of China (30321004).

X. Zhu · P. Yang (✉) · H. Zhou · B. Li · X. Huang · Q. Meng · L. Wang
State Key Laboratory of Ophthalmology,
Zhongshan Ophthalmic Center, Uveitis Study Center,
Sun Yat-sen University,
54 South Xianlie Road,
Guangzhou 510060, People's Republic of China
e-mail: peizengy@126.com

A. Kijlstra
Animal Sciences Group van Wageningen UR Divisie Veehouderij,
Lelystad, The Netherlands

A. Kijlstra
Department Ophthalmology, Eye Research Institute Maastricht,
University of Maastricht,
Maastricht, The Netherlands

Introduction

Anterior chamber-associated immune deviation (ACAID), a model of systemic immune tolerance, is characterized by an antigen-specific suppression of delayed type hypersensitivity (DTH) and a selective deficiency of complement-fixing antibodies (Abs) [16, 17]. DTH is inhibited by two types of suppressor cells: one is a $CD4^+$ T cell population that inhibits the induction and the other is a $CD8^+$T cell population that suppresses DTH expression [20, 24].

$CD4^+$T suppressor cells from ACAID mice are able to transfer immune tolerance to naïve mice [21]. Further study proved that $CD4^+$T cells expressing CD25 and producing IL-10 are required for the development of ACAID [19]. $CD4^+CD25^+$ regulatory T cells include two populations: one naturally exists in thymus [10], the other is induced in

the periphery [13]. A recent report suggests that development of ACAID is independent of the naturally occurring $CD4^+CD25^+$ regulatory T cell population [11]. This suggests a possible role for antigen-specific $CD4^+CD25^+$ regulatory T cells during ACAID. In the study presented here, we focused on a possible role of these regulatory T cells from the spleen in the development of ACAID. This was performed by quantifying $CD4^+CD25^+$ T cells during the development of ACAID using a set of novel T cell markers such as Foxp3, LAG-3 and CTLA-4.

Foxp3 is considered as an important marker for regulatory T cells, and $CD4^+CD25^+Foxp3^+$ T cells are preferentially regarded as regulatory T cells (Tregs) [3, 4, 8]. LAG-3 selectively marks the induced regulatory $CD4^+CD25^+$T cells [9], and CTLA-4 modulates the suppressive activity of $CD4^+CD25^+$ T cells by providing co-stimulatory signals [22].

Our results showed that Tregs were increased in ACAID mice. Over 90% $CD4^+CD25^+$ T cells from ACAID mice, positive controls, negative controls or naïve mice were Foxp3 positive. Splenic Tregs expressed high levels of CTLA-4 and LAG-3 in ACAID mice. Furthermore, we showed that $CD4^+CD25^+$ T cells from the spleens of ACAID mice could transfer tolerance when adoptively transferred into naïve mice.

Materials and methods

Mice

Female 6- to 8-week old BALB/c mice were used in the experiments. Mice were obtained from the Animal Center of the Sun Yat-sen University (Guangzhou, China). Mice were treated humanely and in accordance with the ARVO statement for the use of animals in vision and ophthalmology research.

Induction of ACAID and DTH assay

ACAID was induced as previously described [16]. In brief, ACAID mice received an injection of 2-μl ovalbumin (OVA, 20 mg/ml, Sigma, St. Louis, MO) dissolved in PBS into the anterior chamber (AC) and were immunized on day 7 by subcutaneous (s.c.) injection 250 μg/200 μl of OVA or bovine serum albumin (BSA, Amresco, USA) emulsified in Complete Freudn's adjuvant (CFA, Sigma, St. Louis, MO). Mice only receiving a s.c. injection of OVA in CFA alone served as positive controls. Mice receiving an intracameral injection of 2 μl sterile PBS alone acted as negative controls. Untreated mice were used as naïve controls.

DTH to specific alloantigen was measured using a conventional ear swelling assay [16]. Ag (200 μg of OVA/10 μl of PBS) and an equal volume of sterile PBS were respectively injected 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.

Phenotypes of Tregs determined by flow cytometry

Splenocytes were suspended in PBS and 0.1% BSA at a final concentration of 1×10^6 cells/100 μl. These cells were incubated with FITC-anti-CD3 mAb, FITC or APC-anti-CD4 mAb, PE-cy7-anti-CD25 mAb and PE-anti-LAG-3 mAb for 30 min in the dark at 4°C. Cells were washed, fixed and permeabilized and stained with APC or PE-anti-Foxp3 mAb, PE-anti-CTLA-4 mAb or matched isotype according to the manufacturer's instructions and analyzed by flow cytometry (FCM). All conjugated antibodies were purchased from eBioscience and BD Pharmingen (San Diego, CA).

$CD4^+CD25^+$ and CD4+$CD25^-$T-cell selection

$CD4^+CD25^+$ T cells were isolated from mouse splenocytes with a $CD4^+CD25^+$ Regulatory T Cells Isolation kit (Miltenyi Biotec, Auburn, CA). Briefly, spleen cell suspensions were incubated with a cocktail of biotin-conjugated antibodies and anti-biotin microbeads for negatively sorting $CD4^+$T cells. The enriched $CD4^+$T cells were incubated with PE-labeled $CD25^+$ and anti-PE microbeads for positively sorting $CD4^+CD25^+$T cells. The purities of isolated $CD4^+CD25^+$ or $CD4^+CD25^-$T cells identified by flow cytometric analysis both were shown to be higher than 90%.

In vitro suppression assay of $CD4^+CD25^+$T cells

Splenocytes isolated from naïve BALB/c mice were used as antigen presenting cells (APCs) and were treated with 50 μg/ml mitomycin-C (MMC, Sigma, St. Louis, MO) for 45 min at 37°C.These cells were washed three times with RPMI-1640 (Gibco, USA) before incubation in proliferation assays. Purified $CD4^+CD25^-$T cells from positive controls served as effector cells, and purified $CD4^+CD25^+$T cells acted as regulator cells. Effector cells (2×10^4 cells/well) cultured with or without OVA (200 μg/ml), MMC-treated splenocytes (8×10^4 cells/well) and regulator cells (2×10^4 cells/well) from each group were incubated in RPMI-1640 medium, supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate and 1% streptomycin/penicillin at 37°C for 72 h and pulsed with 1 μCi/well [^{3}H]thymidine (Shanghai Institute of Applied Physics, Chinese Academy of Sciences, China) during the

last 16 h of incubation. T cell proliferation was determined by the incorporation of [^{3}H]thymidine. Proliferation data were the mean value of triplicate wells and represented three independent experiments.

In vivo suppression assay of $CD4^+CD25^+$T cells

The isolated $CD4^+CD25^+$T cells or $CD4^+CD25^-$T cells were washed three times with PBS, resuspended in PBS and subsequently injected intravenously into the tail vein of naive BALB/c mice at a concentration of 5×10^5 or 5×10^6/200 μl. Twenty-four hours later, recipients were s.c. immunized with OVA in CFA or BSA in CFA. On day 7, DTH responses were assayed according to the aforementioned method.

Statistics

The results were expressed as the mean±SEM. Data were analyzed by ANOVA using SPSS11.0. A value of $P<0.05$ was considered significantly different.

Results

Frequency of splenic $CD4^+$Tregs is increased in ACAID mice

$CD4^+CD25^+$T cells have been shown to play a critical role in immune tolerance and immune responses in various diseases and experimental models of disease in rodents and humans [5, 7, 12]. Our study aimed to examine whether $CD4^+CD25^+$T cells were increased in ACAID, an eye-derived immune tolerance model. The results showed that ACAID was successfully induced by injection of OVA into the anterior chamber of the mice as evidenced by a markedly decreased delayed type hypersensitivity response (Fig. 1). The frequency of splenic $CD4^+CD25^+$ T cells in $CD4^+$T cells from ACAID mice was significantly higher than that from non-ACAID groups ($P<0.01$) (Fig. 2a).

As CD25 is not a definitive marker for regulatory T cells, Foxp3 was used to further identify these cells [3, 4, 8]. The results showed that the frequency of splenic $CD4^+CD25^+Foxp3^+$ in $CD4^+$T cells in ACAID mice were slightly, but significantly increased as compared with that observed in the control groups ($P<0.05$) (Fig. 2b), although the frequencies of Foxp3 in $CD4^+CD25^+$T cells were similar (all being more than 90%) (Fig. 2c). Foxp3 was also found to be expressed by approximately 7% of $CD4^+CD25^-$T cells. There was no significant difference among these four groups concerning the frequency of $CD4^+CD25^-Foxp3^+$T cells in the spleen.

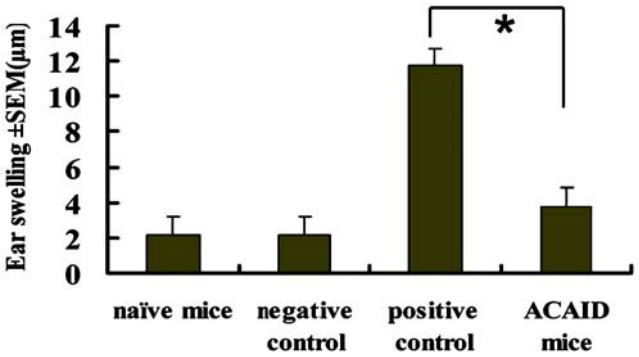


Fig. 1 ACAID was successfully induced. ACAID mice received an intracameral injection of OVA followed by s.c. immunization with OVA in CFA after 7 days. Positive controls were immunized s.c. with OVA in CFA. Negative controls received an intracameral injection of sterile PBS. Naïve mice did not receive any stimulation. Mice were challenged 14 days later by injection of antigen into the right ear pinnae and an equal volume of sterile PBS into the left ear pinnae. Delayed-type hypersensitivity responses were measured 24 h later. The results shown are the means±SEM of three experiments performed. In the ACAID group (n=4), DTH responses were significantly suppressed compared with those in the positive control group (n=4). *$P<0.001$

Splenic $CD4^+CD25^+$T cells from ACAID mice have a stronger suppressive ability in vitro

To characterize the function of $CD4^+CD25^+$T cells and $CD4^+CD25^-$ T cells in vitro, these two populations were purified from naïve mice, the positive control and the ACAID group, and proved to be over 90% pure (Fig. 3a). Purified $CD4^+CD25^+$T cells from three groups were unresponsive to stimulation with OVA and APCs in vitro. A slight proliferation of $CD4^+CD25^-$T cells was observed upon exposure to APC alone, whereas these $CD4^+CD25^-$T cells showed vigorous proliferation after stimulation with OVA and APCs. We subsequently examined whether the splenic $CD4^+CD25^+$T cells from ACAID mice had a stronger regulatory activity than those from other groups. The results revealed that $CD4^+CD25^+$T cells from each group could inhibit the proliferation of effector cells ($CD4^+CD25^-$T cells) in the presence or absence of OVA. There was no significant difference concerning the suppressive ability among these three groups without OVA. However, $CD4^+CD25^+$T cells from ACAID mice had a much stronger suppressive ability than those obtained from naïve mice ($P<0.01$) or positive controls ($P<0.01$) in the presence of OVA. There was no difference concerning the suppressive ability between negative controls and positive controls (Fig. 3b).

Adoptively transferred $CD4^+CD25^+$T cells from ACAID mice significantly suppress DTH responses

The isolated $CD4^+CD25^+$T cells (5×10^5 cells per mouse) or $CD4^+CD25^-$T (5×10^5 or 5×10^6 cells per mouse) from

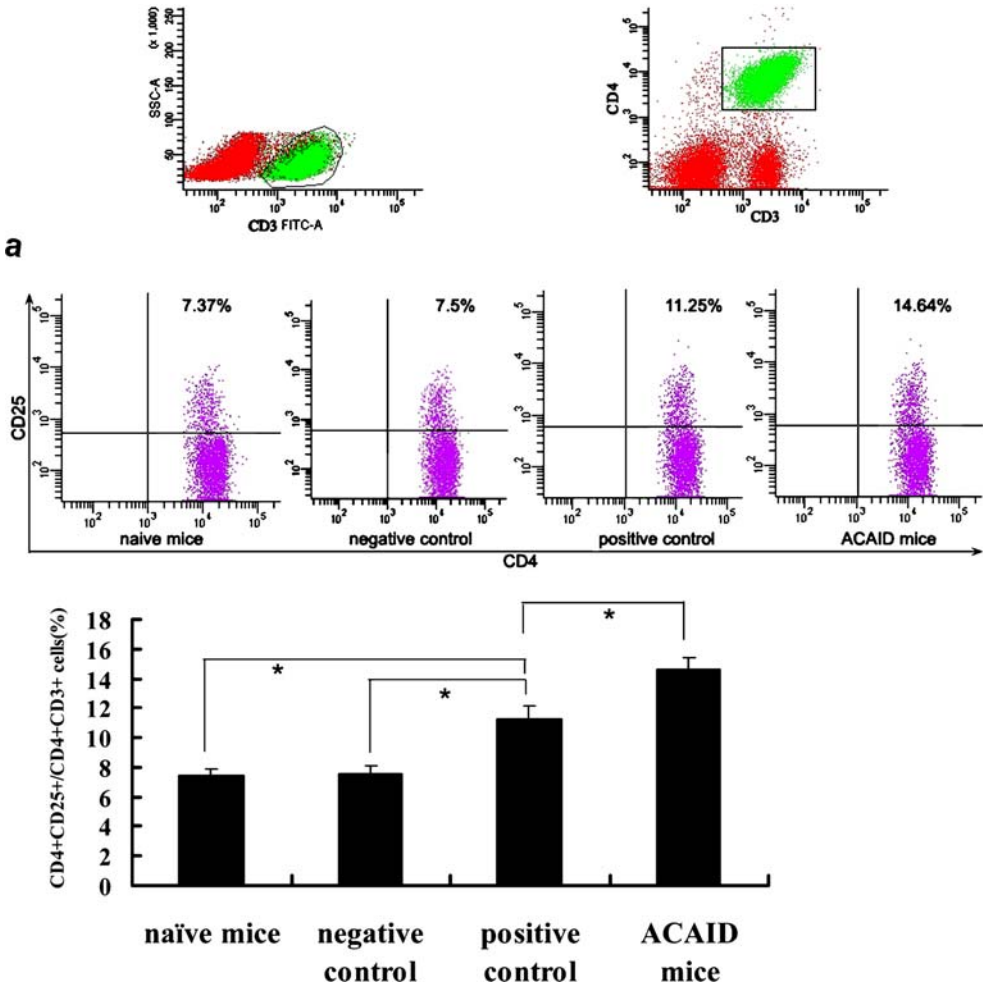


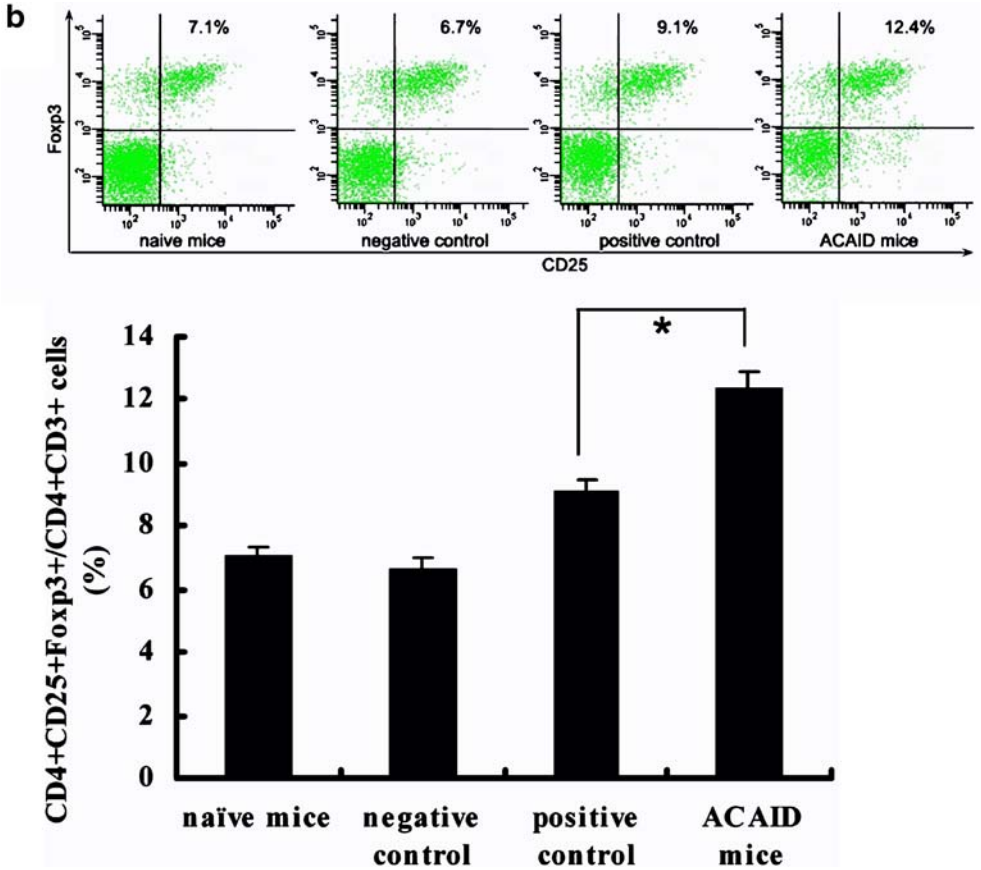
Fig. 2 The frequencies of $CD4^+CD25^+$ and $CD4^+CD25^+Foxp3^+$T cells among $CD4^+$T cells. All the spleens from each group were harvested on day 7 after the last treatment. Splenocytes were prepared as described in the methods section and stained with anti-CD3, anti-CD4, anti-CD25 mAb and anti-Foxp3 mAb. Data in the quadrant indicate the percentage of positive cells, and derive from gating on $CD3^+CD4^+$ cells. **a**: The frequency of $CD4^+CD25^+$ among $CD4^+$T cells; **b**: the frequency of $CD4^+CD25^+Foxp3^+$ among $CD4^+$T cells; **c**: the percentages of $CD4^+CD25^+Foxp3^+$ on $CD4^+CD25^+$ T cells or $CD4^+CD25^-Foxp3^+$ on $CD4^+CD25^-$T cells. The frequencies of $CD4^+CD25^+$ and $CD4^+CD25^+Foxp3^+$T cells among $CD4^+$T cells in the ACAID group (n=4) are significantly increased compared with those in the positive control (n=4). There is no significant difference in the percentages of $CD4^+CD25^+Foxp3^+$ on $CD4^+CD25^+$ T cells or $CD4^+CD25^-Foxp3^+$ on $CD4^+CD25^-$T cells between the ACAID group and the positive control group. One representative dot blot of four independent experiments is shown. The results shown in the histogram are the means±SEM of four independent experiments. *$P<0.05$

naïve mice, positive control or ACAID mice were adoptively transferred into the tail vein of syngeneic mice. These recipients were s.c. immunized with OVA in CFA or BSA in CFA 24 h later. On day 7 DTH responses were assayed. The results, as shown in Fig. 4, revealed that the $CD4^+CD25^+$T cells from ACAID mice, but not from non-ACAID mice, could adoptively transfer DTH inhibition. When a large number of $CD4^+CD25^-$ T cells (5×10^6 cells per mouse) from ACAID mice were transferred, DTH responses could also be suppressed, but not as strongly as when $CD4^+CD25^+$T cells had been transferred. Moreover, transferred $CD4^+CD25^+$T cells from the ACAID mice induced by OVA did not show any effect on the ear swelling of those mice receiving a s.c. injection of BSA in CFA and challenged with BSA, showing the antigen specificity of the transferred cells ($P<0.01$).

Expression of CTLA-4 and LAG-3 on $CD4^+$Tregs in ACAID mice is increased

As CTLA-4 and LAG-3 have been shown to be involved in the function of regulatory $CD4^+CD25^+$T cell, we went on to study the expression of CTLA-4 and LAG-3 on Tregs in ACAID [9, 22]. The results showed that the frequency of

Fig. 2 (continued) **b**



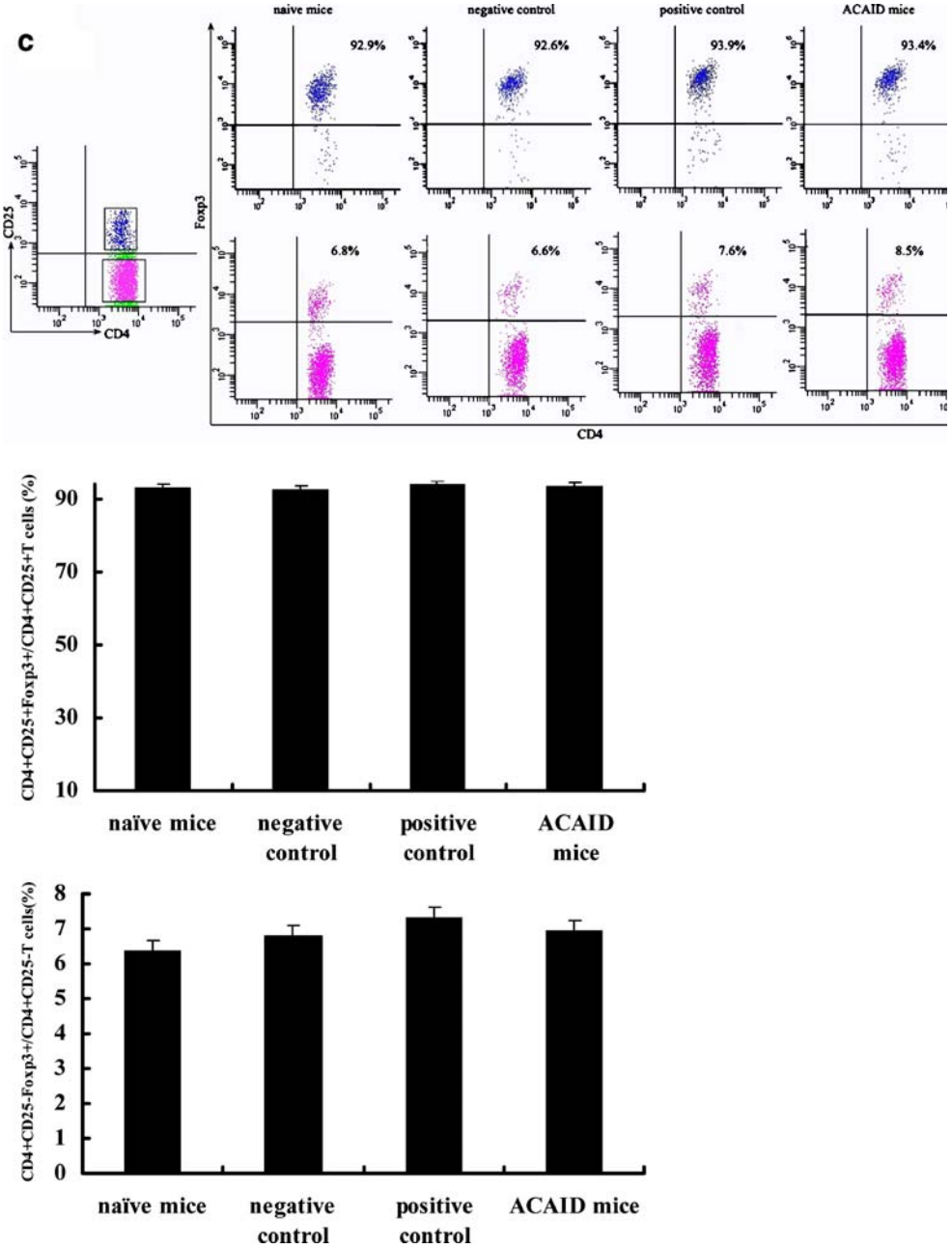
Foxp3$^+$CTLA-4$^+$ in CD4$^+$CD25$^+$T cells (Fig. 5a) was significantly increased in ACAID mice and positive controls as compared with that in naïve and negative controls ($P<0.01$). There was no significant difference concerning the frequency of Foxp3$^+$CTLA-4$^+$CD4$^+$CD25$^+$T cells between ACAID mice and positive controls, although a tendency for a light increase was observed in the former. The frequency of Foxp3$^+$LAG-3$^+$ in CD4$^+$CD25$^+$T cells (Fig. 5b) in ACAID mice was significantly higher than that in naïve, negative controls and positive controls ($P<0.01$).

Discussion

The results presented in this paper provide strong evidence for an important role for CD4$^+$CD25$^+$Foxp3$^+$T cells (Tregs) expressing a high level of CTLA-4 and LAG-3 in the development of ACAID. Our earlier work has shown that CD4$^+$PD-1$^+$T cells played a potent regulatory role in ACAID. PD-1, a negative regulatory molecule in the CD28/B7 family, may be a marker for CD4$^+$ regulatory cells [14]. In the present paper, our experiments showed that the frequency of Tregs was significantly increased in ACAID mice compared to the non-ACAID groups. A vast majority of CD4$^+$CD25$^+$T cells (>90%) in all groups were Foxp3 positive. CD4$^+$CD25$^+$T cells from ACAID mice displayed a stronger inhibitory ability to CD4$^+$CD25$^-$T cells in vitro as compared with that observed in naïve mice and positive controls. CD4$^+$CD25$^+$T cells from ACAID mice, but not from non-ACAID mice, were able to adoptively transfer tolerance to naïve mice. Our further phenotypic analysis of Tregs in ACAID mice showed that these cells expressed higher levels of both CTLA-4 and LAG-3. Taken together, these results suggest that both CD4$^+$PD-1$^+$T cells and CD4$^+$CD25$^+$Foxp3$^+$T cells may be the subpopulations of the CD4$^+$ suppressor cell in ACAID.

It has been shown that ACAID is a phenomenon mediated or maintained by generating an active regulatory T cell population to suppress DTH [15]. Regulatory CD4$^+$T cells are one of the suppressive populations responsible for the development of ACAID [2]. These CD4$^+$ T cells are highly enriched with a subpopulation expressing the IL-2Rα chain (CD25) [13]. In our study, the frequencies of CD4$^+$CD25$^+$ and CD4$^+$CD25$^+$Foxp3$^+$T cells were significantly higher in ACAID mice as compared with those in non-ACAID groups. In an attempt to exclude the influence of intracameral injection on the frequency of CD4$^+$CD25$^+$T cells as well as CD4$^+$CD25$^+$Foxp3$^+$T cells, an experiment

Fig. 2 (continued)



with PBS injection into the anterior chamber was performed. This experiment showed that this manipulation did not influence the frequency of these cells. All these results suggest that an upregulation of Tregs occurs during ACAID.

Tregs are clearly shown to be increased following an intracameral injection of OVA in our study. It is important to test if these cells have a suppressive property. In vitro experiments revealed that this population without stimulation had a suppressive ability on $CD4^+CD25^-$ effector T cells, no matter if they were obtained from ACAID mice, positive mice or naïve mice. Upon stimulation with OVA, these $CD4^+CD25^+$T cells from ACAID mice showed a stronger suppressive activity than those from the other two groups. These results suggest that OVA-specific $CD4^+CD25^+$Tregs are induced during ACAID. Our in vivo experiment showed that DTH responses were impaired by adoptively transferring splenic $CD4^+CD25^+$T cells from ACAID mice, but not from non-ACAID mice. These results indicate that splenic $CD4^+CD25^+$T cells in ACAID mice are not identical to natural Tregs in naïve mice, and seem to have a stronger immunosuppressive capacity. As ACAID is

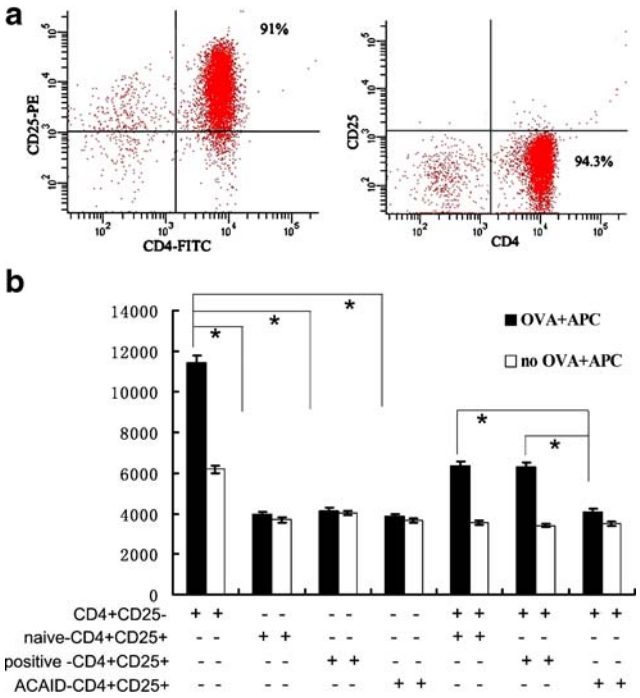


Fig. 3 Suppressive effect of $CD4^+CD25^+$T cells on $CD4^+CD25^-$ effector T cells in vitro. **a**: The purity of isolated of $CD4^+CD25^+$ (left) and $CD4^+CD25^-$ T (right) cells by MACS; **b**: the suppressive effect of $CD4^+CD25^+$T cells on the proliferation of $CD4^+CD25^-$ T cells. Splenocytes from naïve mice were treated with mitomycin-C as APC. $CD4^+CD25^-$ effector T cells (2×10^4 cells/per well) from positive control were cultured with or without OVA (200 μg/ml), APC (8×10^4 cells/per well) and equal numbers of $CD4^+CD25^+$T cells (regulator cells, 2×10^4 cells/per well), respectively, from naïve mice, positive controls and ACAID mice. The cells were cultured for 72 h and pulsed with [^{3}H]thymidine for the last 16 h of culture. Splenic $CD4^+CD25^+$T cells stimulated with OVA have a stronger suppressive ability when obtained from ACAID mice than from the other two groups.Data are the means±SEM of triplicate samples and are representative of three independent experiments. *P<0.01

an immune tolerance induced by foreign antigens, it is presumed that these $CD4^+CD25^+$T cells with an antigen-specific inhibitory function (Tregs) play a crucial role in the development of this immune deviation. Future studies using $Foxp3^{-/-}$ mice may further clarify their role. Additionally, time response studies should be performed in the future to determine the half-life of the suppressive activity of $CD4^+CD25^+$T cells generated during ACAID. It is interesting to note that a ten-fold higher number of $CD4^+CD25^-$T cells from ACAID mice, when adoptively transferred to naïve mice, could also suppress DTH responses, although the suppression was weaker than when transferring $CD4^+CD25^+$T cells. This result suggests that a subset of regulatory T cells with a weak suppressive property or in a very small frequency also exists in the $CD4^+CD25^-$T cells subpopulation. It has been found that a small proportion of $CD4^+CD25^-$T cells express Foxp3 [4]. In our experiment we also found that about 7% of these T cells express Foxp3. It is possible that the observed suppressive activity could be ascribed to these Foxp3-expressing $CD4^+CD25^-$T cells This is consistent with a recent report that both $CD4^+CD25^+$ and $CD4^+CD25^-$ regulatory cells mediate dominant transplantation tolerance [6]. We cannot rule out the possibility that contamination with $CD4^+CD25^+$T cells may also be involved in the inhibition of the DTH response observed when using high numbers of $CD4^+CD25^-$T cells. Activation of $CD25^-$ cells into $CD25^+$ cells following injection can also explain the observed findings. Further studies are needed to clarify this issue.

As $CD4^+CD25^+Foxp3^+$T cells in ACAID mice showed an increased frequency and suppressive ability, we further explored the phenotype of this regulatory population.

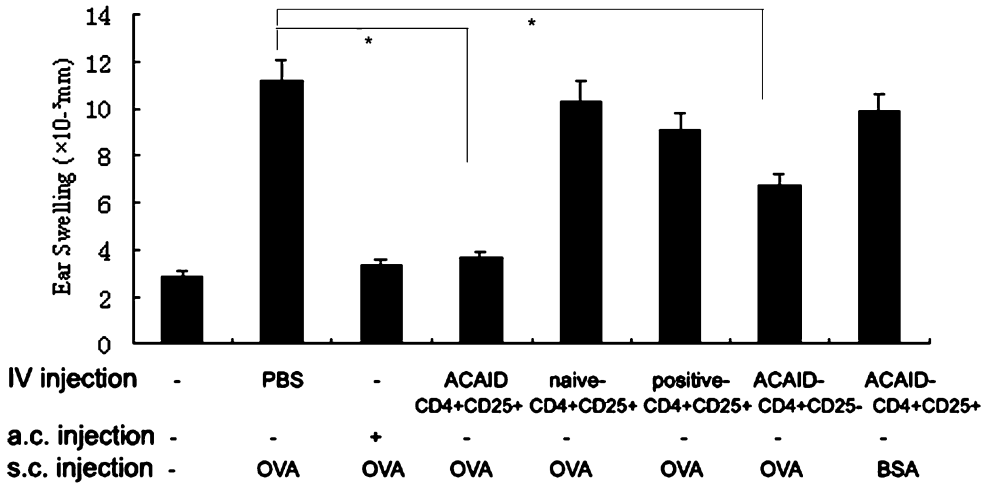


Fig. 4 DTH responses to adoptive transferred cells. Splenic $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from naïve mice, positive controls and ACAID mice were sorted by MACS. Isolated $CD4^+CD25^+$T cells (5×10^5 cells/per mouse), $CD4^+CD25^-$T cells (5×10^6 cells/per mouse) or PBS were injected intravenously into the tail vein of syngeneic mice. At 24 h, the recipients received a s.c. injection of OVA or BSA in CFA. On day 7, the mice were challenged by injection of OVA (10 μl) into the right ear pinnae and an equal volume of sterile PBS into the left. DTH responses were measured 24 h later. Splenic $CD4^+CD25^+$ T cells or large numbers of $CD4^+CD25^-$ T cells from ACAID mice transferred suppression of DTH responses. Each group contained four animals. The experiments were repeated two times. All results are expressed as mean±SEM. * P<0.01

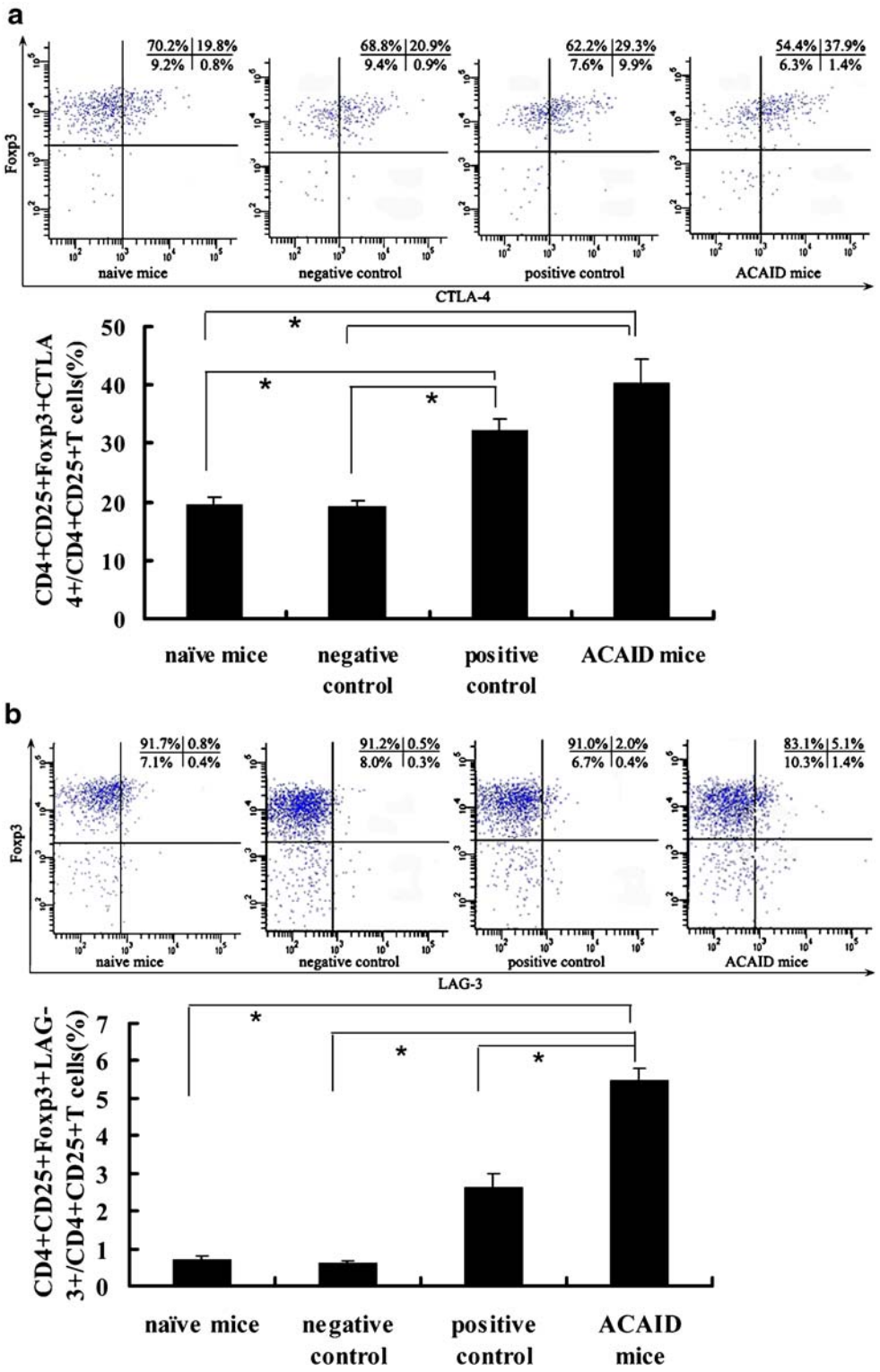


Fig. 5 The frequencies of CTLA-4$^+$Foxp3$^+$ or LAG-3$^+$Foxp3$^+$cells among CD4$^+$CD25$^+$T cells. All the spleens from each group were harvested on day 7 after the last treatment. Splenocytes were prepared as described in the methods section and CD4$^+$T cells were required by negative sorting. CD4$^+$T cells were stained with anti-CD4, anti-CD25, anti-Foxp3 and anti-CTLA-4 or anti-LAG-3 mAb. Data in the quadrants indicated the percentage of positive cells that derived from gating on CD4$^+$CD25$^+$T cells. **a**: The frequencies of CTLA-4$^+$Foxp3$^+$ among CD4$^+$CD25$^+$T cells; **b**: the frequencies of LAG-3$^+$Foxp3$^+$cells among CD4$^+$CD25$^+$T cells. The frequency of CTLA-4$^+$Foxp3$^+$ or LAG-3$^+$Foxp3$^+$cells among CD4$^+$CD25$^+$T cells in ACAID group (n=4) was markedly increased compared with the negative control group (n=4). One representative dot blot of four independent experiments is shown. The results shown in the histogram are the means±SEM of four independent experiments. *P<0.01

CTLA-4 and LAG-3, two important molecules for Tregs, were studied for their expression in Tregs from ACAID mice as well as other groups. Our study revealed an increased expression of LAG-3 in Tregs in ACAID mice as compared with that seen in the various control groups used, suggesting that LAG-3 may be involved in the specific regulation in this immune tolerance. The results also showed an increased expression of CTLA-4 in Tregs in ACAID mice as well as in the positive group, although a little higher expression was observed in the former. As CTLA-4 is a marker of activated Tregs, it is likely that these CTLA-4-expressing Tregs in the positive control are

those primed by complete Freund's adjuvant used in this study. As the number of LAG-3$^+$ T cells are very low and CD4$^+$CD25$^+$Foxp3$^+$CTLA-4$^+$ T cells are not isolated by MACS, further studies with CTLA-4 or LAG-3 knockout mice or using anti-CTLA-4 or anti-LAG-3 antibodies to block the corresponding pathway will greatly help us to clarify the exact role of CTLA-4 or LAG-3 in the induction of ACAID. A number of regulatory molecules, such as GITR, PD-1 and BTLA, have been recently documented [1, 2, 18, 23]. It is also necessary to investigate their role in Tregs in the development of ACAID.

In conclusion, our results suggest that CD4$^+$CD25$^+$Foxp3$^+$T cells expressing a high level of CTLA-4 and LAG-3 represent an important subset of CD4$^+$Tregs in ACAID. However, further studies on the phenotype and function of regulatory cells will provide more profound insight into the development of ACAID.

Acknowledgments The authors thank Prof. Dr. Changyou Wu for his constructive suggestions and Dr. Haining Zhang for her technical assistance.

References

1. Ansari MJ, Salama AD, Chitnis T, Smith RN, Yagita H, Akiba H, Yamazaki T, Azuma M, Iwai H, Khoury SJ, Auchincloss H Jr, Sayegh MH (2003) The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. J Exp Med 198:63–69
2. Carreno BM, Collins M (2003) BTLA: a new inhibitory receptor with a B7-like ligand. Trends Immunol 24:524–527
3. Fontenot JD, Gavin MA, Rudensky AY (2003) Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. Nat Immunol 4:330–336
4. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY (2005) Regulatory T cell lineage specification by the forkhead transcription factor Foxp3. Immunity 3:329–341
5. Gangi E, Vasu C, Cheatem D, Prabhakar BS (2005) IL-10-producing CD4$^+$CD25$^+$ regulatory T cells play a critical role in granulocyte-macrophage colony-stimulating factor-induced suppression of experimental autoimmune thyroiditis. J Immunol 174:7006–7013
6. Graca L, Thompson S, Lin CY, Adams E, Cobbold SP, Waldmann H (2002) Both CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ regulatory cells mediate dominant transplantation tolerance. J Immunol 168:5558–5565
7. Heuer JG, Zhang T, Zhao J, Ding C, Cramer M, Justen KL, Vonderfecht SL, Na S (2005) Adoptive transfer of in vitro-stimulated CD4$^+$CD25$^+$ regulatory T cells increases bacterial clearance and improves survival in polymicrobial sepsis. J Immunol 174:7141–7146
8. Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. Science 299:1057–1061
9. Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, Hipkiss EL, Ravi S, Kowalski J, Levitsky HI, Powell JD, Pardoll DM, Drake CG, Vignali DA (2004) Role of LAG-3 in regulatory T cells. Immunity 10:503–513
10. Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, Sakaguchi S (1999) Thymus and autoimmunity: production of CD25$^+$CD4$^+$ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. J Immunol 162:5317–5326
11. Keino H, Takeuchi M, Kezuka T, Hattori T, Usui M, Taguchi O, Streilein JW, Stein-Streilein J (2006) Induction of eye-derived tolerance does not depend on naturally occurring CD4$^+$CD25$^+$ T regulatory cells. Invest Ophthalmol Vis Sci 47:1047–1055
12. Kohm AP, Carpentier PA, Anger HA, Miller SD (2002) Cutting edge: CD4$^+$CD25$^+$ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. J Immunol 169:4712–4716
13. Maloy KJ, Salaun L, Cahill R, Dougan G, Saunders NJ, Powrie F (2003) CD4+CD25+TR cells suppress innate immune pathology through cytokine-dependent mechanisms. J Exp Med 197:111–119
14. Meng QL, Yang PZ, Li B, Zhou HY, Huang YK, Zhu LX, Ren Y, Kijlstra A (2006) CD4$^+$PD-1$^+$T cells acting as regulatory cells during the induction of anterior chamber-associated immune deviation. Invest Ophthalmol Vis Sci 47:4444–4452
15. Nakamura T, Terajewicz A, Stein-Streilein J (2005) Mechanisms of peripheral tolerance following intracameral inoculation are independent of IL-13 or STAT6. J Immunol 175:2643–2646
16. Niederkorn JY, Streilein JW (1983) Alloantigens placed into the anterior chamber of the eye induce specific suppression of delayed-type hypersensitivity but normal cytotoxic T lymphocyte and helper T lymphocyte responses. J Immunol 131:2670–2674
17. Niederkorn JY (2002) Immune privilege in the anterior chamber of the eye. Crit Rev Immunol 22:13–46
18. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S (2002) Stimulation of CD25$^+$CD4$^+$ regulatory T cells through GITR breaks immunological self-tolerance. Nat Immunol 3:135–142
19. Skelsey ME, Mayhew E, Niederkorn JY (2003) CD25+, interleukin-10-producing CD4+T cells are required for suppressor cell production and immune privilege in the anterior chamber of the eye. Immunology 110:18–29
20. Streilein JW, Niederkorn JY (1985) Characterization of the suppressor cell(s) responsible for anterior chamber-associated immune deviation (ACAID) induced in BALB/c mice by P815 cells. J Immunol 134:1381–1387
21. Takahashi M, Ishimaru N, Yanagi K, Saegusa K, Haneji N, Shiota H, Hayashi Y (1999) Requirement for splenic CD4$^+$T cells in the immune privilege of the anterior chamber of the eye. Clin Exp Immunol 116:231–237
22. Teft WA, Kirchhof MG, Madrenas J (2006) A molecular perspective of CTLA-4 function. Annu Rev Immunol 24:65–97
23. Totsuka T, Kanai T, Makita S, Fujii R, Nemoto Y, Oshima S et al (2005) Regulation of murine chronic colitis by CD4+CD25-programmed death-1+ T cells. Eur J Immunol 35:1773–1785
24. Wilbanks GA, Streilein JW (1990) Characterization of suppressor cells in anterior chamber-associated immune deviation (ACAID) induced by soluble antigen. Evidence of two functionally and phenotypically distinct T-suppressor cell populations. Immunology 71:383–389