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OVA-specific CD8$^+$T cells do not express granzyme B during anterior chamber associated immune deviation

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Abstract *Purpose:* To examine antigen (Ag)-specific CTL response during anterior chamber associated immune deviation (ACAID). *Methods:* OVA or OVA257-264 peptide was injected into the anterior chamber (AC) of C57BL/6 mice. There were 16 mice in each ACAID group induced with OVA or OVA257-264 peptide. The mice were primed by SC injection with OVA or OVA 257-264 peptide in complete Freund's adjuvant (CFA) on day 7. Ag-specific CD8$^+$T cells in spleens were analyzed on day 14 using Pentamer H-2K^b-SIINFEKL(OVA257-264 peptide). IFN-γ ELISPOT and intracellular granzyme B staining were used to characterize the CTL response. Twelve mice in each group immunized with OVA or OVA257-264 peptide in CFA served as positive controls. Twelve normal mice served as negative controls and 12 receiving injection of CFA as CFA controls for studying the influence of CFA on the Ag-specific CTL response. *Result:* The results showed that anterior chamber inoculation of OVA or OVA257-264 peptide could induce ACAID as evidenced by an impaired DTH response. The frequency of Ag-specific CD8$^+$T cells in ACAID mice was not different from that in mice challenged with Ags in CFA only (positive controls). IFN-γ production by these cells in ACAID mice was not different compared to positive controls. However, Ag-specific CD8$^+$T cells in ACAID mice failed to secrete granzyme B. Mice challenged only with OVA peptide and CFA also showed a granzyme B negative CD8$^+$T cell response. Ag-specific CTL response induced by CFA alone was similar with the negative control. *Conclusion:* These results show that the frequency of Ag-specific CD8$^+$T cells is not altered during ACAID. The Ag-specific CTL response during ACAID is characterized by the absence of granzyme B expression.

Keywords ACAID · Immune tolerance · CD8$^+$T cells · CTL response

Introduction

It is well known that CD8$^+$T lymphocytes are important to the adaptive immune response against viruses, the rejection of allogeneic grafts, etc. [1, 4, 7, 27, 35, 36]. Their role in ACAID, a deviant immune response elicited by intracameral injection of antigen, has however not been well addressed. ACAID is characterized by a deficiency in delayed-type hypersensitivity (DTH) responses and decreased expression of IgG isotypes [24, 28]. Furthermore, allogeneic tumor cells injected into the AC grow progressively, whereas these cells do not expand when injected elsewhere in the body [26]. This progressive growing of tumor cells in the AC has been attributed to ACAID. As CD8$^+$T cells are actively involved in the inhibition of tumor cells, a number of studies have been performed to

investigate their role in ACAID. It has been found that CTL activity is impaired in ACAID [13, 15, 34], thereby resulting in the progressive growth of tumor cells in the AC. Further studies have shown that a CTL response is present in the spleen during ACAID [16], but is not active within the eye.

Most studies addressing the CTL response during ACAID were performed with the conventional technique, i.e. the ^{51}Chromium (^{51}Cr) release assay. However, this assay has been shown to have a number of disadvantages, such as high spontaneous release, inefficient labeling of target cells, a low sensitivity, and health risks associated with gamma irradiation [5, 12, 17, 20, 21, 33]. Novel techniques to quantitatively and qualitatively assay Ag-specific CD8$^+$T cells, for instance Tetramer and cytokine ELISPOT assays, have been developed during recent years. More recently, Pentamer, a more sensitive technique with the same principle as Tetramer, has been used for the study of Ag-specific CD8$^+$T cells. Commercially available SIINFEKL(OVA257-264 peptide) Pentamer contains 5 fluorescent MHC-peptide complexes facing in the same planar orientation, which allows higher avidity and an increase in fluorescent signal. The application of the Pentamer may provide a new insight into the role of Ag-specific CD8$^+$T cells during ACAID and was therefore the subject of the study described here. In this study, the Pentamer was used to enumerate CD8$^+$T cells, an IFN-γ ELISPOT assay and intracellular granzyme B staining were used to functionally evaluate the Ag-specific CD8$^+$T cells. Our results show that the expansion of Ag-specific CD8$^+$T cells expressing IFN-γ is not affected in ACAID, but these cells have lost their capacity to express granzyme B. This may explain the generally observed phenomenon that the CTL response is impaired during ACAID.

Materials and methods

Experimental animals

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

Antigens and adjuvant

OVA (grade VI) was purchased from Sigma-Aldrich (Steinheim, Germany) and dissolved in phosphate-buffered saline (PBS) at a concentration of 20 mg/ml. OVA257-264 peptide (Purity>95%) (Anaspec, Inc., San Jose, Calif., USA) was dissolved in sterile distilled H_2O at three different concentrations (20 mg/ml, 30 mg/ml and 40 mg/ml). CFA containing heat-killed Mycobacterium tuberculosis strain H37Ra was purchased from Sigma-Aldrich.

Induction of ACAID

ACAID was induced as described previously using microinjection of antigen into AC of the eye [25]. Briefly, mice were anesthetized and 5 μl antigens were injected into the AC on day 0 by a glass micropipette with a sterile infant feeding tube mounted onto a 0.1 ml Hamilton (Hamilton, Reno, Nev., USA) syringe. OVA or OVA257-264 peptide (250 μg) emulsified 1:1 in CFA were used for SC immunization on day 7. Normal B6 mice served as negative controls, and those receiving an SC immunization as positive controls. Four mice were used in each group and experiments were repeated at least 3 times.

DTH assay

To determine the DTH response, mice receiving an SC immunization on day 7. On day 14, the mice were challenged with 400 μg OVA or peptide into the left ear pinnae to evoke a DTH response. The right ear pinnae received 20 μl sterile PBS alone and served as a control. The ear thickness was measured using a Mitutoyo micrometer (MTI Corp., Paramus, N.J., USA). The DTH response was determined by the following formula: specific ear swelling= [(24-h-0-h) measurement for the experimental ear−(24-h-0-h) measurement for the negative control ear] μm.

MHC Pentamer reagent, staining and flow cytometry analysis

The mice were killed on day 14. The spleens were removed and dispersed into a single-cell suspension. Erythrocytes were lysed by Tris-NH_4Cl (0.83% in 0.01 M Tris-HCl). The PE-labeled Pentamer (Proimmune, Oxford, UK) was used to assay the OVA-specific CD8$^+$T cells. Fc receptors were blocked with anti-mouse CD16/CD32 Fc-RIIIyII (clone 93; eBioscience, San Diego, Calif., USA) and then stained with Pentamer for 40 min at 4°C. After washing, the cells were costained with anti-CD8a-FITC (Proimmune) and anti-CD3-PEcy5 (eBioscience). Then the samples were resuspended and analyzed by FACScan (Becton Dickinson Immunocytometry Systems, San Jose, Calif., USA) using CELLQUEST software. From a live lymphocyte gate, CD8$^+$T cell gate was drawn and enumerated at least 100,000 CD8$^+$T cells for analysis.

IFN-γ ELISPOT assays and image analysis

IFN-γ-producing CD8$^+$T cells in the spleens were enumerated using mouse IFN-γ-specific ELISPOT assays. The ELISPOT assay was performed according to the manufacture's instruction. Briefly, 96-well PVDF plates

(BD Biosciences Pharmingen, San Jose, Calif., USA) were coated overnight at 4°C with 0.5 μg/well of purified anti-IFN-γ. The plates were then washed once blocked for 2 h with complete RPMI. $CD8^+$T cells (2×10^5) purified by MiniMACS device (Miltenyi Biotec, Bergisch Gladbach, Germany) and APCs (3×10^5) from the syngeneic mice in each well were stimulated with OVA or OVA257-264 peptide (70 μg/ml at final concentration) for 24 h at 37°C in 5% CO_2. The cells were removed by two washes with sterile distilled H_2O and three additional washes with PBS/0.05% Tween20 (PBST). Biotinylated anti-IFN-γ antibody (0.2 μg) was added to each well and incubated at room temperature for 2 h. Horseradish peroxidase-conjugated streptavidin was added and the spots were visualized with the Final Substrate Solution. The spots were automatically analyzed by the SeriesIImmunoSpot Analyzer (Cellular Technology Ltd, Cleveland, Ohio, USA) specifically designed for the ELISPOT assay as described previously [8].

Intracellular granzyme B staining

After preparation of single-cell suspensions and lysis of erythrocytes in these suspensions, the cells were resuspended in complete RPMI 1640 at a density of 2×10^6 cells per ml. For intracellular cytokine staining of granzyme B, the cells (4×10^6) were stimulated with OVA or OVA257-264 peptide (100 μg/ml) and IL-2 (100 IU/ml) at 37°C for 5 h in the presence of Brefeldin A (10 ng/ml) (BioLegend, San Jose, Calif., USA) in the last 4 h before staining. After incubation, the cells were fixed and permeabilized. The cells were then costained with anti-CD3-FITC (eBioscience), anti-CD8-PEcy5 (eBioscience) and anti-granzyme B-PE (eBioscience). The samples were subsequently analyzed on FACScan and $CD8^+$T cells were gated. Matched isotypes served as control.

Statistical analysis

Data were subjected to analysis by ANOVA using SPSS 11.0. A value of $P<0.05$ was considered significantly different.

Results

Induction of ACAID by OVA and OVA257-264 peptide

To investigate the frequency of Ag-specific $CD8^+$T cells in the spleen during ACAID, both OVA and OVA257-264 peptide were used for the induction of ACAID. The DTH response was evaluated. As this peptide had not been used previously for the induction of ACAID, we tested the effect of three different dosages (100 μg, 150 μg and 200 μg). The DTH responses presented in Fig. 1 show that ACAID could be successfully induced following intracameral injection of either OVA or peptide. All OVA peptide dosages tested effectively induced ACAID as shown by the inhibited DTH response.

Frequency of Ag-specific $CD8^+$T cells in the spleens of mice with ACAID

To address the CTL response in ACAID, we first examined the frequency of splenic Ag-specific $CD8^+$T cells using Pentamer H-$2K^b$-SIINFEKL staining. Ag-specific $CD8^+$T cells were observed, respectively, in 0.10% and 0.11% of the total $CD8^+$T cell population in the spleens of ACAID mice induced by OVA or peptide. Ag-specific $CD8^+$T cells were noted in 0.12% and 0.13%, respectively, of the $CD8^+$T cells in positive controls immunized by OVA or peptide (Fig. 2a). There was no significant difference between the ACAID groups and the positive controls as regards the frequency of these cells. The frequency of Ag-specific $CD8^+$T cells was significantly increased in both the ACAID groups and positive controls as compared to those in the negative controls (Fig. 2b).

Secretion of IFN-γ by Ag-specific $CD8^+$T cells in ACAID

As IFN-γproduction may represent, to a great extent, the effector response of Ag-specific $CD8^+$T cells [3, 19, 22], we studied these cells in the spleens of positive controls

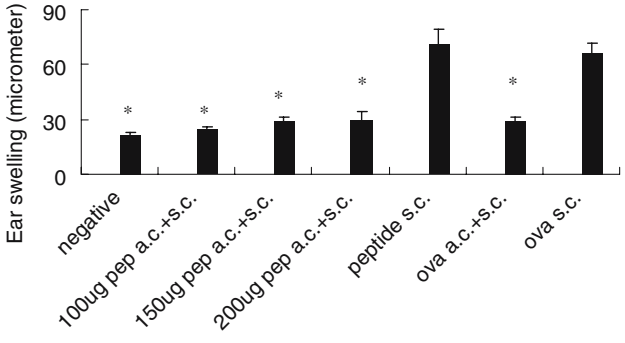

Fig. 1 DTH measurement after the injection of antigens into the AC. Mice received an injection of native OVA (100 μg) or OVA 257–264 peptide (100 μg or 150 μg or 200 μg) into the anterior chamber (*AC*) on day 0. On day 7, mice were primed with either OVA or OVA 257–264 peptide in CFA. Ears of mice were challenged, on day 14, with either OVA or its peptide. DTH was expressed as ear swelling (in micrometers) 24 h after ear challenge. Naive mice were used as negative controls, and mice that received an SC immunization but not an AC injection served as positive controls. *Bars* represent the mean±SEM (n=12) of ear measurements. * Indicates mean values significantly decreased as compared to the positive controls, $P<0.001$

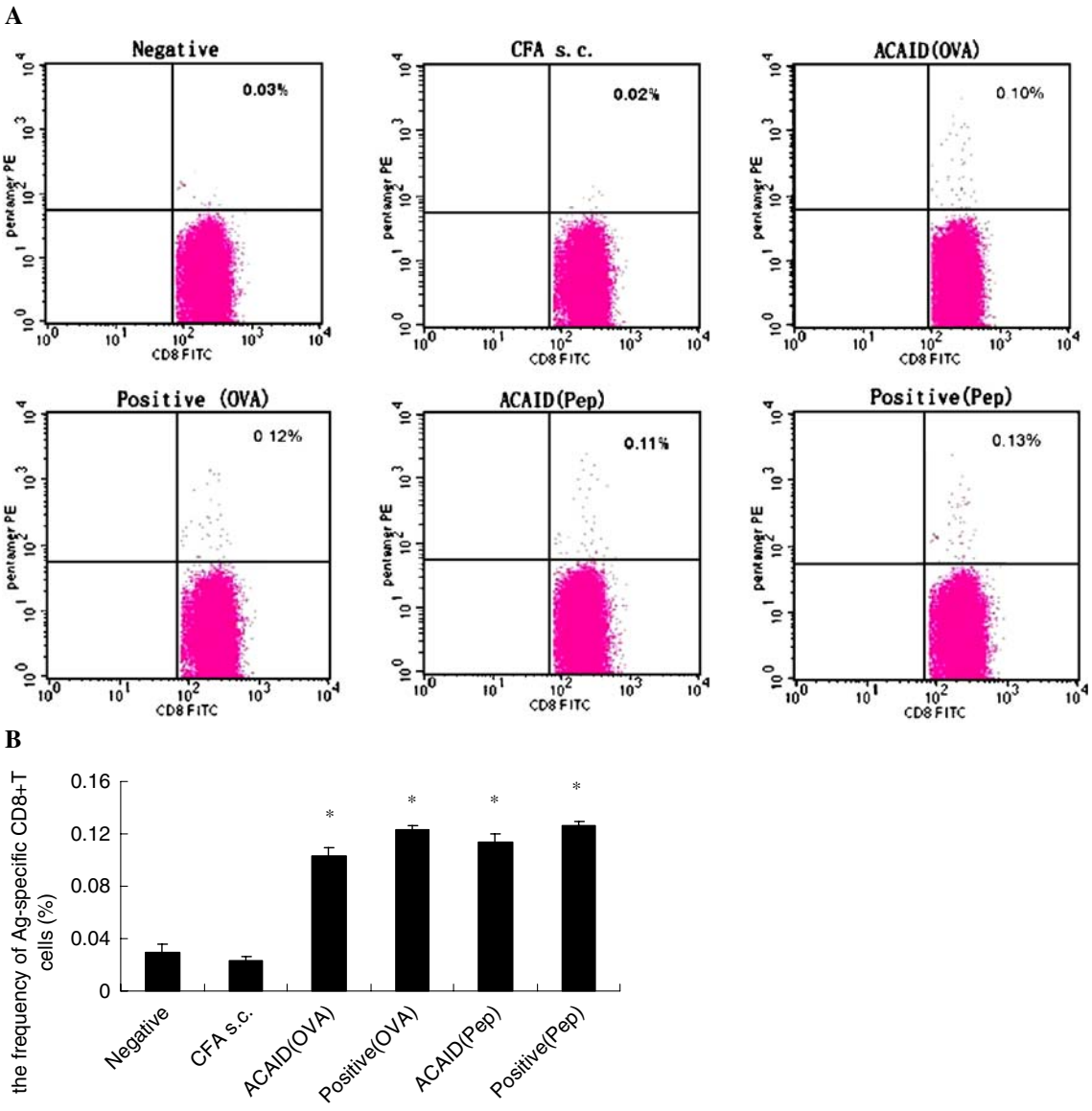

Fig. 2 Flow cytometry analysis of Ag-specific $CD8^+$T cells per 100,000 $CD8^+$T cells in the spleens of different groups. Negative: normal B6 mice. ACAID (OVA/Pep): mice received an AC injection of either OVA or OVA257-264 peptide. Seven days later mice were immunized with OVA or its peptide in CFA. Positive (OVA/Pep): mice received an SC. injection of OVA/OVA257-264 peptide in CFA. CFA was subcutaneously injected and used to examine the influence of adjuvant on the frequency of Ag-specific $CD8^+$T cells. We gated on $CD8^+$T cells and enumerated 100,000 $CD8^+$T cells. FITC-labeled anti-CD8 is shown on the horizontal axis, and PE-labeled Pentamer is shown on the vertical axis. Stained cells in the upper right quadrangle represent antigen specific $CD8^+$T cells. Each analysis was performed at least 3 times to verify the results. Results represent the mean values from 3 separate experiments (4 mice/group). *P<0.001 compared with negative control

immunized by OVA and peptide and found that the average numbers of spots were 167 and 162 per 2×10^5 $CD8^+$T cells, respectively. In the spleens of ACAID mice induced with OVA or peptide, the average numbers were 158 and 160, respectively. There was no significant difference between ACAID groups and the positive controls concerning the number of IFN-γspots (Fig. 3).

Secretion of granzyme B by Ag-specific $CD8^+$T cells in ACAID

As granzyme B is an important index of the lytic function of effector $CD8^+$T cells [10, 11], this enzyme was assayed by intracellular staining to functionally evaluate the Ag-specific $CD8^+$T cells. The results, as displayed in Fig. 4, showed that there was no detectable granzyme B produced by Ag-specific $CD8^+$T cells in the spleens of ACAID mice.

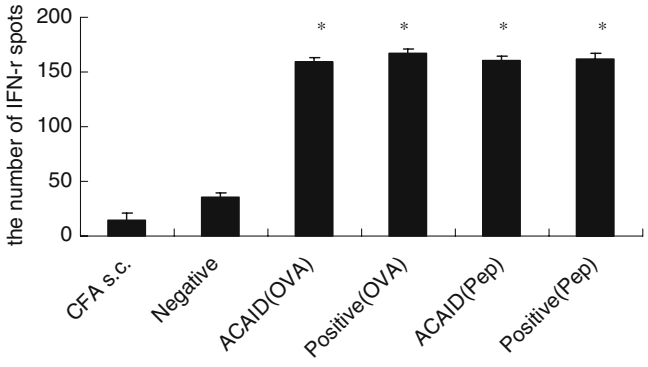

Fig. 3 Frequency of IFN-γ-producing Ag-specific $CD8^+$T cells in the spleens of different groups using ELISPOT assay. There were 2×10^5 $CD8^+$T cells per well and the cells were stimulated by 70 μg/ml OVA or OVA257-264 peptide for 24 h. As shown in this representative graph, ACAID (OVA/OVA257-264 peptide) and positive controls (OVA/OVA257-264 peptide) mice exhibited significantly higher frequencies of IFN-γ-producing Ag-specific $CD8^+$ T cells than the negative controls. The results are representative for three separate experiments. * $P<0.001$ compared with negative control

A similar result was also found in the mice receiving SC immunization with peptide in CFA. In the spleens of mice challenged with OVA in CFA, 0.52% of Ag-specific $CD8^+$T cells were found to produce granzyme B. There was a significant difference when comparing the production of granzyme B by Ag-specific $CD8^+$T cells between the ACAID groups and the positive control receiving OVA immunization.

Discussion

In this study, we show that the CTL response, mediated by Ag-specific $CD8^+$T cells in ACAID induced by OVA or

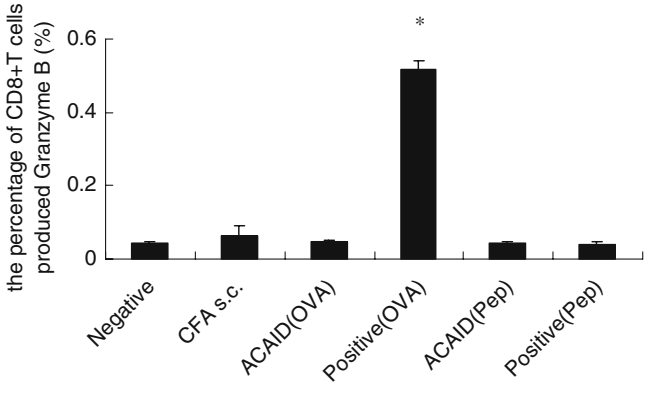

Fig. 4 Frequency of $CD8^+$T cells producing granzyme B in the spleens of the different groups of mice as identified by flow cytometry analysis. Only the positive controls receiving OVA in CFA showed a detectable granzyme B response. The results are representative for three independent experiments. * $P<0.001$ compared with negative control

OVA257-264 peptide, is characterized by the absence of granzyme B. Granzyme B is released by cytotoxic T cells and leads to apoptosis in target cells through cleavage of caspase-3. Using the Pentamer H2-K^b-SIINFEKL technique, we found that the frequency of splenic Ag-specific $CD8^+$T cells could be induced by an SC challenge with antigen in CFA, but that a previous intracameral antigen injection did not alter this frequency. Similar results were obtained using either OVA or its peptide. The capacity to express IFN-γ by splenic Ag-specific $CD8^+$T cells was also not altered in ACAID mice. These findings suggest that the expansion of Ag-specific $CD8^+$T cells is not influenced by the intracameral injection of antigens and that the previously presumed clonal deletion, i.e. absence or decreased frequency of Ag-specific $CD8^+$T cells, may not be the reason for the impaired CTL response in ACAID. Our findings support earlier studies by the group of Kapp [14], who followed antigen specific $CD8^+$T cells during ACAID and showed that this population of cells expanded in vivo but lost their cytotoxic function. As the production of granzyme B generally represents the killing function of $CD8^+$T cells, it is important to co-stain granzyme B and Pentamer for FACS analysis. However, we did not do this experiment due to the very limited cell numbers of Ag-specific $CD8^+$T cells identified with Pentamer was not sufficient for this analysis. Intracellular staining for granzyme B was, therefore, only performed to compare its production in ACAID mice and positive controls. Our study failed to demonstrate the production of granzyme B in ACAID mice. These data suggest that the absence of granzyme B production may be responsible for the impairment of the killing function of Ag-specific $CD8^+$T cells during ACAID. It is interesting to note that the frequency of granzyme B^+CD8^+T cells identified by FACS analysis was much higher than that of Ag-specific $CD8^+$T cells measured by Pentamer. The explanation could be that in vitro measurement of the frequency of granzyme B^+CD8^+T cells is performed after exposure of $CD8^+$T cells to OVA257-264 peptide and IL-2, which could result in extra expansion of effector $CD8^+$T cells secreting granzyme B. Further studies are needed to correlate the absence of granzyme B and cytotoxic activity of $CD8^+$T cells during ACAID. As granulysin [23], perforin [23] and Fas-FasL pathway [30] are also involved in the mechanisms by which $CD8^+$T cells kill the target cells, a further point of interest would be to address their roles in the CTLs in mice undergoing ACAID.

Loss of granzyme B staining in $CD8^+$T cells was also observed in our experimental group challenged SC. with OVA peptide in the presence of CFA (Fig. 4). Peptide immunization did result in the generation of antigen specific $CD8^+$T cells and the expression of IFN-γ. An interesting result has been reported by Porgador et al [18] in which a weak CTL response in the spleen was observed after immunization with OVA257-264 peptide emulsified in IFA. The reason for the difference in the CTL response

between the IFA group in the study of Porgador et al [18] and CFA groups in our study is not known. A previous study has suggested that microenviroments induced by different adjuvants might be responsible for this difference in CTL response [10]. Further studies are needed to investigate whether similar mechanisms are involved in the impaired granzyme B expression seen in ACAID and that following SC peptide immunization.

Effector $CD8^+$T cells are normally defined by their capability of producing high amounts of IFN-γ and lysing virus-infected cells [11]. It has been established that $CD8^+$T cells lyse target cells by utilizing perforin and granzyme B. Both proteins are constitutively expressed within the preformed granules of effector $CD8^+$T cells [2, 6, 13, 29, 32]. Evidence has emerged that granzyme B may exert cytolytic activity in the absence of the perforin channel and can even penetrate the cells to activate the apoptotic cascade [18].

In this study, we found that the Ag-specific $CD8^+$T cells secreted large amount of IFN-γ almost equal to that produced in the positive controls. Therefore, these Ag-specific $CD8^+$T cells seem not to be the regulatory cells characterized as little IFN-γ production [9]. It is not clear yet which mechanisms are involved in the process whereby these Ag-specific $CD8^+$T cells lose their cytotoxic capacity or their ability to express granzyme B.

In summary, our study showed, at a single cell level, that the expansion of Ag-specific $CD8^+$T cells is not influenced during ACAID, but that the ability to express granzyme B is impaired. The deficiency in granzyme B production by these Ag-specific $CD8^+$T cells may be one of reasons for the impaired killing function generally observed in animals undergoing ACAID. This study may open an avenue for the further investigation of mechanisms involved in ACAID and perhaps the manipulation of intraocular tumors.

References

1. Doherty PC, Christensen JP (2000) Accessing complexity: the dynamics of virus-specific T cell responses. Annu Rev Immunol 18:561–592
2. Grayson JM, Murali-Krishna K, Altman JD, Ahmed R (2001) Gene expression in antigen-specific $CD8^+$T cells during viral infection. J Immunol 166:795–799
3. Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV (1996) Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity 4:25–36
4. Harty JT, Tvinnereim AR, White DW (2000) $CD8^+$T cell effector mechanisms in resistance to infection. Annu Rev Immunol 18:275–308
5. Heo DS, Park JG, Hata K, Day R, Herberman RB, Whiteside TL (1990) Evaluation of tetrazolium-based semiautomatic colorimetric assay for measurement of human antitumor cytotoxicity. Cancer Res 50:3681–3690
6. Kaech SM, Wherry EJ, Ahmed R (2002) Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol 2:251–262
7. Kagi D, Ledermann B, Burki K, Zinkernagel RM, Hengartner H (1996) Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. Annu Rev Immunol 14:207–232
8. Karulin AY, Hesse MD, Tary-Lehmann M, Lehmann PV (2000) Single-cytokine-producing CD4 memory cells predominate in type 1 and type 2 immunity. J Immunol 164:1862–1872
9. Kezuka T, Streilein JW (2000) In vitro generation of regulation $CD8^+$T cells similar to those found in mice with anterior chamber-associated immune deviation. Invest Ophthalmol Vis Sci 41:1803–1811
10. Kleen TO, Asaad R, Landry SJ, Boehm BO, Tary-Lehmann M (2004) Tc1 effector diversity shows dissociated expression of granzyme B and interferon-γ in HIV infection. AIDS 18:383–392
11. Klein JR, Raulet DH, Pasternack MS, Bevan MJ (1982) Cytotoxic T lymphocytes produce immune interferon in response to antigen or mitogen. J Exp Med 155:1198–1203
12. Kruger-Krasagakes S, Garbe C, Kossman P, Orfanos CE (1992) A rapid and sensitive fluorometric microassay for determining cell mediated cytotoxicity to adherent growing cell lines. J Immunol Methods 156:1–8
13. Ksander BR, Streilein JW (1989) Analysis of cytotoxic T cell response to intracameral allogeneic tumors. Invest Ophthalmol Vis Sci 30:323–329
14. McKenna KC, Xu YJ, Kapp JA (2002) Injection of soluble antigen into the anterior chamber of the eye induces expansion and functional unresponsiveness of antigen-specific $CD8^+$T cells. J Immunol 169:5630–5637
15. Niederkorn JY, Streilein JW, Shadduck JA (1981) Deviant immune responses to allogeneic tumors injected intracamerally and subcutaneously in mice. Invest Ophthalmol Vis Sci 20:355–363
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. Papadopoulos NG, Dedoussis GV, Spanakos G, Gritzapis AD, Baxevanis CN, Papamichail M (1994) An improved fluorescence assay for the determination of lymphocyte-mediated cytotoxicity using flow cytometry. J Immunol Methods 177:101–111
18. Porgador A, Gilboa E (1995) Bone marrow-generated dendritic cells pulsed with a class I-restricted peptide are potent inducers of cytotoxic T lymphocytes. J Exp Med 182:255–260
19. Power CA, Grand CL, Ismail N, Peters NC, Yurkowski DP, Bretscher PA (1999) A valid ELISPOT assay for enumeration of ex vivo, antigen-specific, IFN-γ -producing T cells. J Immunol Methods 227:99–107
20. Sheehy ME, McDermott AB, Furlan SN, Klenerman P, Nixon DF (2001) A novel technique for the fluorometric assessment of T lymphocyte antigen specific lysis. J Immunol Methods 249:99–110

21. Slezak SE, Horan PK (1989) Cell-mediated cytotoxicity. A highly sensitive and informative flow cytometric assay. J Immunol Methods 117:205–214
22. Slifka MK, Rodriguez F, Whitton JL (1999) Rapid on/off cycling of cytokine production by virus-specific $CD8^+$T cells. Nature 401:76–79
23. Stegelmann F, Bastian M, Swoboda K, Bhat R, Kiessler V, Krensky AM, Roellinghoff M, Modlin RL, Stenger S (2005) Coordinate expression of CC chemokine ligand 5, granulysin, and perforin in $CD8^+$T cells provides a host defense mechanism against *Mycobacterium tuberculosis*. J Immunol 175:7474–7483
24. Stein-Streilein J, Streilein JW (2002) Anterior chamber associated immune deviation (ACAID): regulation, biological relevance, and implications for therapy. Int Rev Immunol 21:123–152
25. Streilein JW, Neiderkorn JY, Shadduck JA (1980) Systemic immune unresponsiveness induced in adult mice by anterior chamber presentation of minor histocompatibility antigens. J Exp Med 152:1121–1125
26. Streilein JW, Niederkorn JY (1981) Induction of anterior chamber-associated immune deviation requires an intact, functional spleen. J Exp Med 153:1058–1067
27. Streilein JW, Ksander BR, Taylor AW (1997) Commentary: immune privilege, deviation and regulation in the eye. J Immunol 158:3557–3560
28. Streilein JW (2003) Ocular immune privilege: the eye takes a dim but practical view of immunity and inflammation. J Leukoc Biol 74:179–185
29. Teague TK, Hildeman D, Kedl RM, Mitchell T, Rees W, Schaefer BC, Bender J, Kappler J, Marrack P (1999) Activation changes the spectrum but not the diversity of genes expressed by T cells. Proc Natl Acad Sci USA 96:12691–12696
30. Uchiyama H, Kishihara K, Minagawa R, Hashimoto K, Sugimachi K, Nomoto K (2002) Crucial Fas-Fas ligand interaction in spontaneous acceptance of hepatic allografts in mice. Immunology 105:450–457
32. Veiga-Fernandes H, Walter U, Bourgeois C, Mclean A, Rocha B (2000) Response of naive and memory $CD8^+$T cells to antigen stimulation in vivo. Nat Immunol 1:47–53
33. Volgmann T, Klein-Struckmeier A, Mohr H (1989) A fluorescence-based assay for quantitation of lymphokine-activated killer cell activity. J Immunol Methods 119:45–51
34. Whittum JA, Niederkorn JY, Streilein JW (1982) Alloantigen presentation to the anterior chamber of the eye subverts specific in vitro cell-mediated immune responses. Transplantation 34:190–195
35. Wong P, Pamer EG (2003) $CD8^+$T cell responses to infections pathogens. Annu Rev Immunol 21:29–70
36. Zinkernagel RM (1996) Immunology taught by viruses. Science 271:173–178