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Original Article

Ubiquitin A20 maintains activation-induced CD4$^+$ T cell death

Mingyang Li[1*], Min Zhu[2*], Enqiang Linghu[1], Fan Feng[3], Bing Zhu[4], Cheng Wu[5], Mingzhou Guo[1]

[1]Department of Gastroenterology and Hepatopathy, Chinese PLA General Hospital, Beijing 100853, PR China; [2]Department of Medical Oncology, Division of South Building, Chinese PLA General Hospital, Beijing 100853, PR China; [3]Department of Pharmacy, General Hospital of Shenyang Military Command, Shenyang 110016, PR China; [4]Liver Failure Treatment and Research Center, 302nd Military Hospital, Beijing 100039, PR China; [5]Department of Digestive Endoscopy, Division of South Building, Chinese People's Liberation Army General Hospital, Beijing 100853, PR China. [*]Equal contributors.

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Abstract: Aberrant T helper (Th)2 polarization plays a critical role in allergic diseases; the underlying mechanism is to be further investigated. Ubiquitin E3 ligase A20 (A20) plays an important role in the immune regulation. This study aims to test a hypothesis that A20 plays an important role in the activation-induced cell death (AICD) in CD4$^+$ T cells. In this study, a T cell AICD cell culture model was developed. T cell apoptosis was assessed by flow cytometry used as the indicator of AICD. A food allergy (FA) mouse model was developed. The results showed that the frequency of AICD CD4$^+$ T cells was significantly less in FA mice as compared to control mice. Much less A20 expression was detected in CD4$^+$ T cells in FA mice than in control mice. Interaction of TIM1 (T cell immunoglobulin mucin domain-1) and TIM4 suppressed the expression of A20 in CD4$^+$ T cells. Overexpression of A20 promoted the AICD in CD4$^+$ T cells. In conclusion, A20 plays a critical role in the T cell AICD. To promote A20 expression can be a potential remedy in the regulation of T cell AICD.

Keywords: Food allergy, T helper cell, apoptosis, flow cytometry, cell culture

Introduction

The T helper (Th) 1 and Th2 cells are the two major fractions of CD4$^+$ T cells. Th2 cells play a critical role in the humoral immunity by producing Th2 cytokines, including interleukin (IL)-4, IL-5, IL-6, IL-9, IL-13 and IL-25 [1, 2]. These Th2 cytokines mediate the activation and maintenance of the humoral, or antibody-mediated, immune response against extracellular parasites, bacteria, allergens, and toxins [3, 4]. In allergic environment, profound infiltration of Th2 cells and high levels of Th2 cytokines can be detected in the local tissue, such a pathological condition is designated Th2 polarization [5, 6]. On the other hand, Th1 polarization also contributes to some types of allergic inflammation, such as some atopic dermatitis [7, 8] and delayed hypersensitivity [9]. Yet, although the research in this field was advanced rapidly in the recent decades, the pathogenesis of T helper cell polarization is still less understood.

The activation-induced cell death (AICD) indicates a condition that soon after activation, T cells become death [10]. The underlying mechanism of AICD is caused by the interaction of Fas receptors (Fas, CD95) and Fas ligands (FasL, CD95 ligand) [10, 11]. AICD is a negative regulator of activated T lymphocytes that results from repeated stimulation of their T-cell receptors (TCR) and helps to maintain peripheral immune tolerance. Alteration of the process may lead to immune diseases, such as autoimmune diseases or other immune disorders [12]. Whether the AICD mechanism in subjects with allergic disease is compromised is less clear.

The ubiquitin editing enzyme A20 (hereinafter, A20) is a protein that in humans is encoded by the TNFAIP3 gene [13]. The A20 gene was identified as a gene that is induced by the tumor necrosis factor [14]. It is reported that A20 can suppress nuclear factor-κB to regulate immune

inflammation [15]. Recent reports indicate that farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells [16]. Yet, the detail mechanism by which the deregulation of A20 involving inducing allergic disorders is to be further elucidated. Based on the above information, we hypothesize that deregulation of A20 may affect AICD on T cells, which may result in the $CD4^+$ T cell polarization. In this study, we found that $CD4^+$ T cells from FA mice expressed less A20 and were less sensitive to the AICD stimulation, which may be associated with the Th2 polarization in the body.

Materials and methods

Mice

Male BALB/c mice were purchased from the Beijing Experimental Animal Center. The mice were maintained in a pathogen-free environment with accessing food and water freely. The animal experiments were approved by the Animal Care Committee at Chinese PLA General Hospital. The experiments were performed in accordance with the approved guidelines.

Food allergy (FA) mouse model

Following published procedures [17], mice were treated with ovalbumin (0.2 mg/mouse) mixed with cholera toxin (20 μg/mouse) in 0.3 ml saline via gavage-feeding weekly for 4 consecutive weeks. Control group mice were fed with saline. The mice were sacrificed in week 5. FA parameters were evaluated in each mouse with the published procedures [17].

Enzyme-linked immunosorbent assay (ELISA)

The serum levels of OVA-specific IgE and IL-4 were determined by ELISA with commercial reagent kits (Dakewe Biomart; Beijing, China) following the manufacturer's instructions.

Isolation of $CD4^+$ T cells

Naive $CD3^+$ $CD4^+$ $CD25^-$ T cells were isolated from the mouse spleen by magnetic cell sorting (MACS) with commercial reagent kits (Miltenyi Biotech) following the manufacturer's instructions. The purity of isolated cells was greater than 96% as assessed by flow cytometry.

Cell culture

The isolated immune cells were cultured in RPMI1640 medium supplemented with 10% fetal serum albumin, 100 U/ml penicillin, 0.1 mg streptomycin and 2 mM L-glutamine. The medium and agents were changed in 2 to 3 days. The cell viability was assessed by Trypan blue exclusion assay.

Assessment of antigen-specific $CD4^+$ T cells

Spleen $CD3^+$ $CD4^+$ T cells ($CD4^+$ T cells hereinafter) were isolated from spleen cells with magnetic cell sorting (MACS) reagent kits (Miltenyi Biotech; San Diego, CA) following the manufacturer's instructions. The $CD4^+$ T cells were labeled with CFSE (Carboxyfluorescein diacetate succinimidyl ester; 1 μmol/ml; Invitrogen) for 8 min at 37°C). The $CD4^+$ T cells were cultured with DCs (T cell: DC = 10^5 T cells: 2×10^4 DCs/well) in the presence of specific antigen OVA (5 μg/ml; or BSA, used as an irrelevant antigen) for 3 days. The cells were analyzed with a flow cytomter (FACSCanto II; BD Biosciences, Lincoln Park, NJ).

AICD procedures

Following published procedures [18], mouse spleen $CD4^+$ T cells were incubated with 5 μg/ml anti-CD3 mAb plus 5 μg/ml CD28 mAb (Santa Cruz Biotech), and followed by adding 5 μg/ml of goat anti-mouse IgG-Fc to the culture for cross-linking as well as adding IL-2 (10 ng/ml) to the culture to maintain the proliferation and differentiation for 48 h. To induce AICD, the cells were cultured in an anti-CD3 mAb coated microplate to directly crosslink TCR for 24 h at 37°C. Apoptotic cell fractions were assessed by annexin V-FITC and Propidium Iodide (PI) (Sigma Aldrich; St. Louis., MO) staining according to the manufacturer's instructions. The cells were analyzed on a flow cytometer (FACSCanto II).

Flow cytometry

The expression of A20 in $CD4^+$ T cells was assessed by flow cytometry via the intracellular staining. Cells were fixed with 2% paraformaldehyde and permeabilized in 0.5% saponin for 1 h. The cells were incubated with anti-A20 antibody (or an isotype IgG used as a control) at

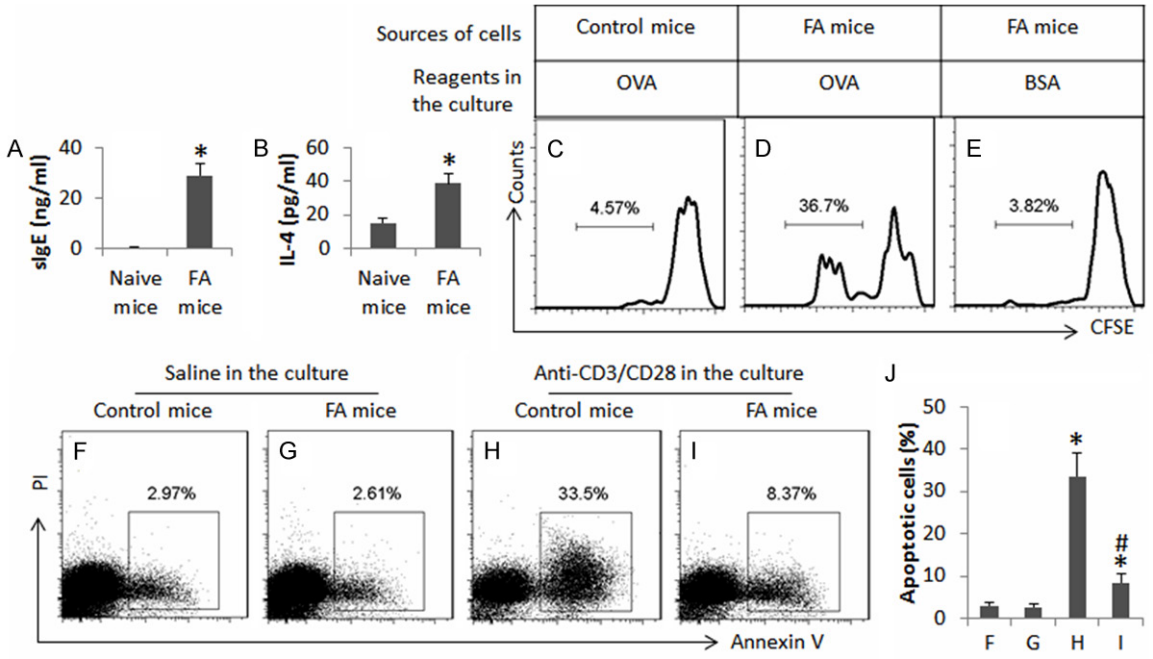

Figure 1. Less AICD in CD4$^+$ T cells from FA mice. The sera and spleen CD4$^+$ T cells and DCs were isolated from control mice and FA mice. (A, B) The bars indicate the serum levels of specific IgE and IL-4 (by ELISA). (C-E) The histograms indicate the frequency of proliferating CD4$^+$ T cells (by CFSE-dilution assay). (F-I) The gated dot plots indicate the apoptotic CD4$^+$ T cells. (J) The bars indicate the summarized data of F-I. Data of bars are presented as mean ± SD. *, $p<0.01$, compared to control mice (A, B) or (F, J). #, $p<0.01$, compared with (H). Each group consists of 6 mice. Samples from individual mice were processed separately.

4°C for 30 min. After washing with PBS, the cells were analyzed with a flow cytometer. The frequency of A20$^+$ cells was recorded. The mean fluorescence intensity of each cell was calculated with software flowjo.

Real time quantitative RT-PCR (RT-qPCR)

RNA was extracted from cells using TRIzol reagent (Invitrogen; Carlsbad, CA) according to manufacturer's instructions. Total RNA was reverse transcribed into first-strand cDNA with a reverse transcription kit (Invitrogen). The cDNA was amplified in a real time PCR device (CFX96 Touch; Bio Rad; Hercules, CA) with the SYBR Green Master Mix (Invitrogen). The primers of A20 used in the present study are tcttccttcgtcacagccat and ccagtcctagcacgcatttc. The results were calculated with the $2^{-\Delta\Delta Ct}$ method and presented as folds of change against controls.

Western blotting

Proteins were extracted from cells, fractioned SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred onto a PVDF membrane. The membrane was blocked by 5% skim milk for 30 min, incubated with the primary antibodies (or isotype IgG) overnight at 4°C, and incubated with the second antibodies (labeled with peroxidase) for 1 h at room temperature. Washing with TBST (Tris-buffered saline Tween 20) for 3 times was performed after each time of incubation. The membrane was developed with ECL (enhanced chemiluminescence). The results were photographed with an image processing system (UVI, Cambridge, UK). The integrated density of the immune blots was determined by ImageJ.

RNA interference (RNAi)

The TIM1 gene in CD4$^+$ T cells was knocked down by RNAi with a TIM1 shRNA kit (Santa Cruz Biotech; Santa Cruz, CA) following the manufacturer's instructions. The effect of RNAi was examined by Western blotting.

A20 overexpression

The A20 plasmids were constructed by the Genescript (Nanjing, China) and transfected to CD4$^+$ T cells with lipofectamine 2000 (Invitro-

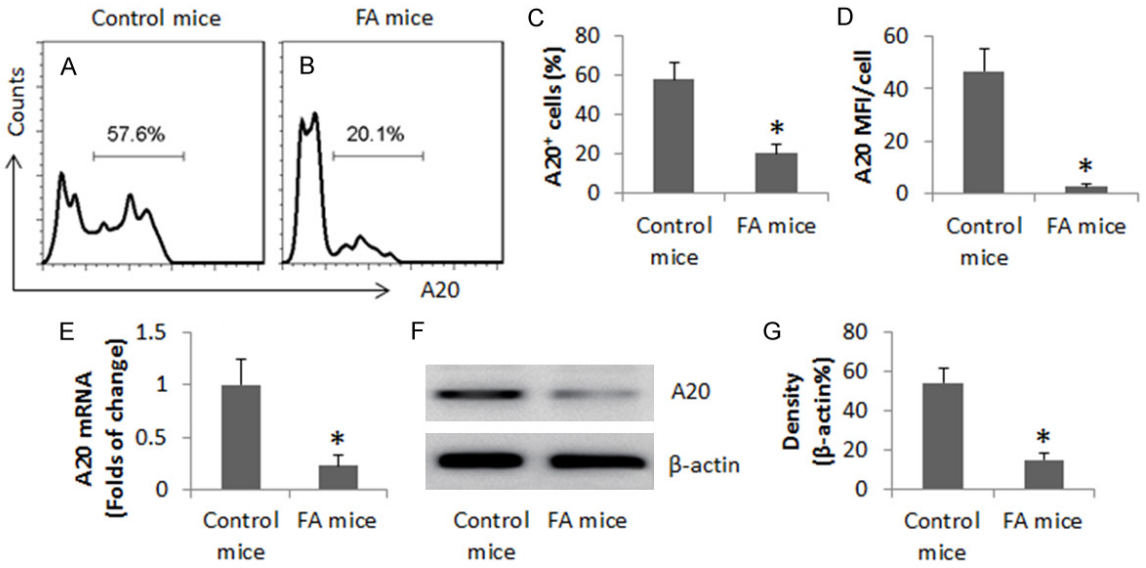

Figure 2. CD4+ T cells of FA mice express less A20. Spleen CD4+ T cells were isolated from control mice and FA mice. (A-D) The CD4+ T cells were analyzed by flow cytometry. The histograms indicate the frequency of A20+ CD4+ T cells (A, B). (C) The bars indicate the summarized data of (A, B). (D) The bars indicate the MFI of the CD4+ T cells. (E) The bars indicate the levels of A20 mRNA in CD4+ T cell extracts. (F) The Western blots indicate the protein levels of A20 in CD4+ T cell extracts. (G) The bars indicate the integrated density of the immune blots of F. Each group consists of 6 mice. Samples from individual mice were processed separately. Data of bars are presented as mean ± SD. *, $p<0.01$, compared with the control mouse group (D) or (E).

gen) following the manufacturer's instructions. The effect of A20 overexpression was examined by Western blotting.

Statistics

Data are presented as mean ± SD. Difference between two groups was determined by Student t test or ANOVA if more than two groups. A p value less than 0.05 was set as a significant criterion.

Results

CD4+ T cells from FA mice show less AICD

To elucidate if allergy affects AICD, we developed an FA mouse model. The FA mice showed higher levels of antigen-specific IgE (**Figure 1A**) and IL-4 (**Figure 1B**) in the sera than control mice. Higher frequency of antigen-specific CD4+ T cell was detected in the spleen cells of FA mice as compared to control mice (**Figure 1C-E**). The results indicate the mice were sensitized. We then treated spleen CD4+ T cells with the AICD procedures. Higher frequency of apoptotic cells was detected in the control group as compared to the FA group (**Figure 1F-J**). The results suggest that CD4+ T cells in the FA mouse are less sensitive to AICD.

CD4+ T cells from FA mice show less A20 expression

To evaluate the levels of A20 in antigen-specific CD4+ T cells, we isolated spleen CD4+ T cells from food allergy (FA) mice and naive BALB/c mice. The cells were analyzed by flow cytometry. The results showed that CD4+ T cells from FA mice had a lower frequency of A20+ CD4+ T cells compared to control mice (**Figure 2A-C**). The A20-specific fluorescence intensity of each A20+ CD4+ T cell was much weaker in FA group than in the control group (**Figure 2D**). To strengthen the data, we prepared cell extracts from the CD4+ T cells. As analyzed by RT-qPCR and Western blotting, the levels of A20 mRNA (**Figure 2E**) and proteins (**Figure 2F**, **2G**) were much higher in the control group than in FA group. The results demonstrate that CD4+ T cells express less A20 in FA mice.

Interaction of TIM1/TIM4 represses A20 level expression in CD4+ T cells

We next investigated the effect of interaction of TIM1/TIM4 on the expression of A20 in CD4+ T cells. We isolated CD4+ T cells from the spleen of naive mice. The cells expressed low levels, but detectable, of TIM1, which was increased

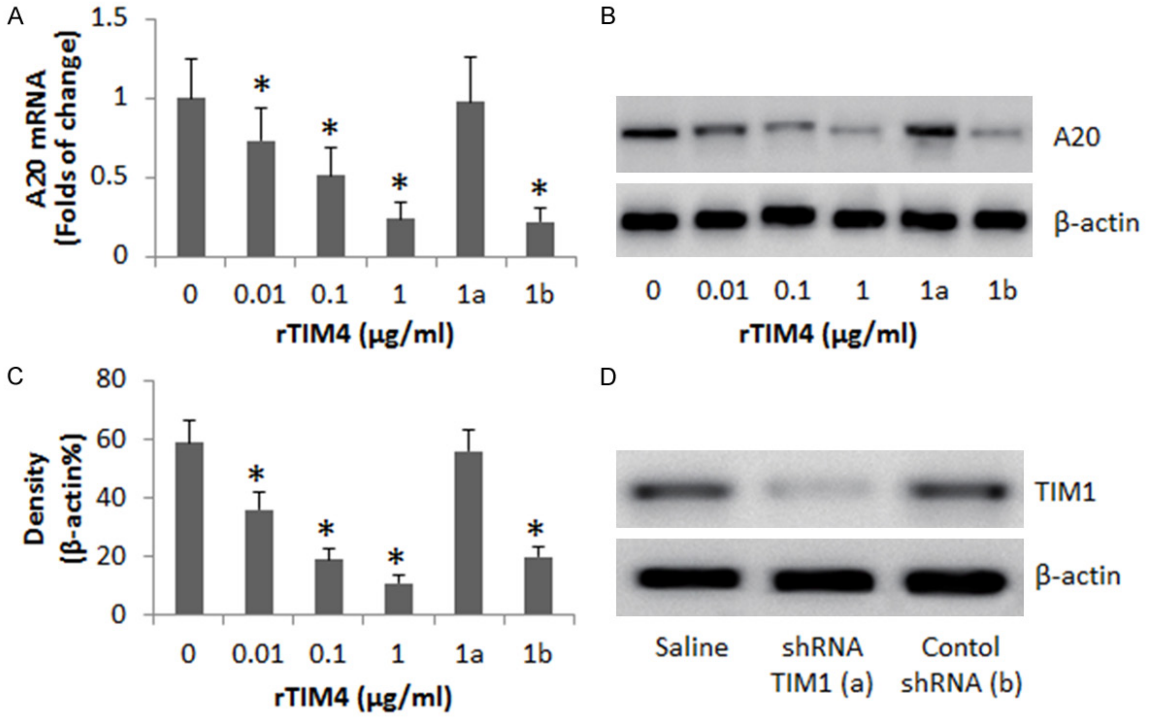

Figure 3. Interaction of TIM1/TIM4 suppresses A20 in CD4⁺ T cells. Naive CD4⁺ T cells were treated by Th2 polarization condition. The conditioned CD4⁺ T cells were exposed to rTIM4 for 48 h in the culture with doses as denoted in the figures. A. The bars indicate the mRNA levels of A20. B. The Western blots indicate the protein levels of A20. C. The bars indicate the integrated density of the immune blots of B. D. The immune blots show the TIM4 RNAi results. Data of bars are presented as mean ± SD. *, p<0.01, compared with the "0" group. The data are representatives of 3 independent experiments.

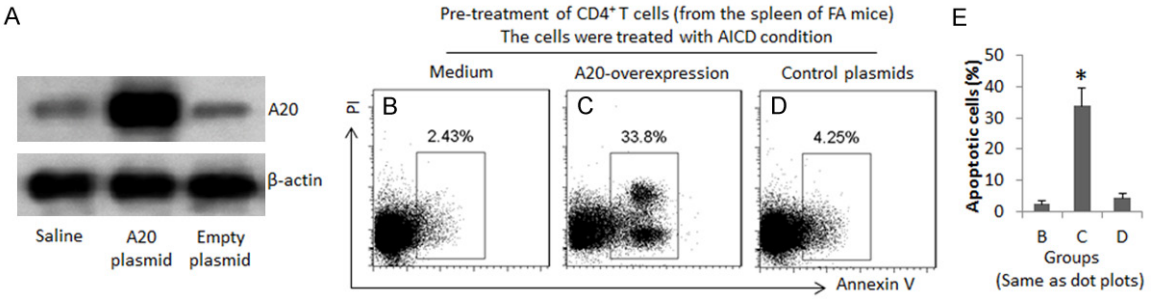

Figure 4. Overexpression of A20 promotes AICD in CD4⁺ T cells. Spleen CD4⁺ T cells were isolated from FA mice. A portion of the cells were overexpressed A20 (A). The CD4⁺ T cells were treated with the conditions denoted above the dot plot panels. The gated dot plots indicate the frequency of apoptotic cells (B-D). (E) The bars indicate the summarized data of B-D. Data of bars are presented as mean ± SD. *, p<0.01, compared with group B. The data are representatives of 3 independent experiments.

after treating with the Th2 polarization condition. We then exposed the CD4⁺ T cells to rTIM4 in the culture for 48 h. The expression of A20 in the CD4⁺ T cells was assessed by RT-qPCR and Western blotting. The results showed that rTIM4 suppressed the levels of A20 in CD4⁺ T cells in an rTIM4 dose-dependent manner, which was abolished by knocking down the TIM1 gene in the CD4⁺ T cells (**Figure 3**). The results suggest that the interaction between TIM1 and TIM4 may be an important causative factor in the suppression of A20 in CD4⁺ T cells.

Overexpression of A20 promotes AICD in CD4⁺ T cells from FA mice

We next sought to elucidate if overexpression of A20 could promote AICD in CD4⁺ T cells of allergic subjects. We isolated spleen CD4⁺ T cells from FA mice. A portion of the cells were overexpressed A20. The CD4⁺ T cells were treated with the AICD procedures. As analyzed by flow cytometry, the A20-overexpressing CD4⁺ T cells had much more apoptotic cells than in the CD4⁺ T cells treated with control plasmids (**Figure 4**). The results suggest that A20 can increase CD4⁺ T cell's sensitivity to AICD.

Discussion

CD4⁺ T cell polarization plays an important role in a large number of immune diseases [19]. To find the mechanism by which CD4⁺ T cells abnormally increase in the body can enrich our knowledge in understanding the pathogenesis of immune diseases. The present study showed impaired mechanism of AICD in the CD4⁺ T cells from FA mice. These CD4⁺ T cells had low levels of A20. Inhibition of A20 impaired the AICD in CD4⁺ T cells. Overexpression of A20 promoted the AICD in CD4⁺ T cells. The data pinpoint the importance of A20 in the maintenance of the biological homeostasis of CD4⁺ T cells.

Published data indicate that profound infiltration of inflammatory cells in the local tissue of the organs suffering with allergic disorders [20, 21]. Such a phenomenon contradicts to the concept of AICD, by which the CD4⁺ T cells should die after activation [10]. However, in allergic disorders, the absolute numbers of CD4⁺ T cells increase [21]. The present data showed much less frequency of apoptotic CD4⁺ T cells in FA group as compared to control mice, suggesting dysfunction of AICD that may contribute to the CD4⁺ T cell polarization in allergic conditions. Kwon et al indicated that the nuclear factor of activated T cells (NFAT) was involved in the process of AICD [22]. They provided evidence to demonstrate that the expression of NFAT was increased in mice with allergic dermatitis [22]. Yang et al reported that the increase in IL-13 in allergic subjects played a critical role in the compromise of AICD [23]. Our data reveal another factor in interfering with AICD that suppression of A20 is associated with AICD dysfunction.

FasL is the major death receptor in triggering apoptosis in activated T cells. The interaction of Fas/FasL is the major mechanism of apoptosis [24]. The present data reveal another aspect of the mechanism of apoptosis, in which A20 plays a role in the initiation of apoptosis. A20 mainly suppresses nuclear factor-κB to regulate immune response [15]. In this study, we observed that the levels of A20 were lower in CD4⁺ T cells from FA mice together with the reduced rate of apoptotic cells in response to AICD procedures. Such a phenomenon should not be a coincidence because we also found that knockdown of A20 in CD4⁺ T cells from control mice also reduced the AICD procedure-induced rate of apoptotic cells; overexpression of A20 promoted the apoptotic cell rate in response to AICD procedures. Therefore, we conclude that A20 plays a critical role in the maintenance of CD4⁺ T cell AICD.

In summary, the present data indicate that the interaction of TIM1 and TIM4 suppresses A20 expression in CD4⁺ T cells, indicating to interrupt the TIM1/TIM4 interaction may have therapeutic potential in the treatment of allergic disorders.

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Disclosure of conflict of interest

None.

Authors' contribution

MYL, MZ, FF, BZ, CW and MZG performed experiments, analyzed data and reviewed the manuscript. EQL designed the project, supervised experiments and wrote the manuscript.

Address correspondence to: Dr. Enqiang Linghu, Department of Gastroenterology and Hepatopathy, Chinese PLA General Hospital, Beijing 100853, PR China. E-mail: enqiangrlinghu@sina.com

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