Survivin Impairs the Apoptotic Machinery in CD4⁺ T Cells of Patients with Ulcerative Colitis

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Keywords

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Abstract

Background: The increase in CD4⁺ T cell infiltration and overproduction of CD4⁺ T cell-associated cytokines have been observed in the inflamed colon mucosa of patients with ulcerative colitis (UC); the underlying mechanisms are not fully understood. Survivin plays a critical role in the interference with apoptotic machinery. This study aims to elucidate the role of survivin in the interference with the apoptotic machinery in CD4⁺ T cells of UC patients. **Methods:** Peripheral blood samples were collected from UC patients (UC group) and healthy subjects (healthy group). The apoptotic status in CD4⁺ T cells was analyzed by flow cytometry. **Results:** We observed that the expression of survivin was significantly higher in CD4⁺ T cells of UC patients than in healthy subjects. UC CD4⁺ T cells were resistant to apoptosis induction. A complex of survivin and c-Myc, the transcription factor of FasL, was detected in CD4⁺ T cells in UC patients, which prevented the binding of c-Myc to the FasL promoter and interfered with the expression of FasL. Increased expression

of survivin prevented the activation-induced CD4⁺ T cells from apoptosis. **Conclusions:** The data indicate that UC CD4⁺ T cells express high levels of survivin, which impairs the apoptotic machinery in CD4⁺ T cells and prevents the activation-induced CD4⁺ T cell apoptosis. Therefore, target therapy against survivin has translational potential in the treatment of UC patients.

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Introduction

The prevalence of inflammatory bowel disease (IBD) is about 0.1–0.5% worldwide and has continued to rise, especially over the last few decades [1]. The pathogenesis of IBD has not been fully elucidated yet. It is recognized that IBD is a chronic inflammatory disease [2]. Published data indicate that profound infiltration of immune cells, including Th1 cells, Th2 cells, eosinophils, mast cells, and macrophages, as well as the proinflam-

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matory cytokines, can be found in the intestinal mucosa of IBD patients [2]. Although research on IBD has advanced rapidly in recent years, the causative factors of IBD still remain elusive [3].

IBD can be categorized into ulcerative colitis (UC) and Crohn disease (CD). In general, CD is dominated by the Th1-type inflammation, while UC is mainly a Th2-type inflammation [4]. It is not fully understood why the number of Th1 or Th2 cells in the intestinal mucosa increases in IBD patients. There are two events that may be associated with this phenomenon: the development of Th2 or Th1 cells may be aberrantly upregulated, or the apoptotic machinery in CD4⁺ T cells may be impaired. The differentiation of Th1 and Th2 cells has been extensively studied [5], while the underlying mechanism in the impairment of the apoptotic machinery in CD4⁺ T cells is less understood.

Published data indicate that survivin plays a critical role in the regulation of apoptosis in cancer cells [6]. By interfering with the apoptotic machinery in cancer cells, survivin suppresses the apoptotic machinery in these cells to render them to escape from the immune surveillance and grow out of control [7]. It promotes T cell differentiation, proliferation, and expansion [8], and is associated with the pathogenesis of immune disorders [9]. Based on the information above, we hypothesize that survivin may interfere with the apoptotic machinery in CD4⁺ T cells in IBD patients, which contributes to the Th1 or Th2 polarization in IBD. To test this hypothesis, blood samples were collected from IBD patients and CD4⁺ T cells were isolated from the samples and analyzed. The results showed that the CD4⁺ T cells expressed higher levels of survivin. Survivin was associated with the impairment of apoptotic machinery in the CD4⁺ T cells.

Materials and Methods

Reagents

The antibodies of survivin, FasL, c-Myc, and Pol II were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). The fluorescence-labeled antibodies of IL-4, IL-5, IL-13, IFN- γ , CD3, and CD4 were purchased from BD Biosciences (Franklin Lakes, NJ, USA). The recombinant IL-4, ELISA kits of IL-4, IL-5, IL-13, and IFN- γ were purchased from R&D Systems (Minneapolis, MN, USA). The annexin V kit, propidium iodide, cisplatin, reagent for immunoprecipitation (IP), and chromatin IP (ChIP) kit were purchased from Sigma Aldrich (St. Louis, MO, USA). The reagents and materials for RT-qPCR and Western blotting were purchased from Invitrogen (Carlsbad, CA, USA). The immune cell isolation kits were purchased from Miltenyi Biotech (San Diego, CA, USA).

Table 1. Demographic data

	UC (n = 26)	CD (n = 6)	Healthy subjects (n = 20)
Age, years	34.4 $\pm$ 8.2	31.5 $\pm$ 6.8	35.8 $\pm$ 8.5
Male	13 (50)	3 (50)	10 (50)
Body weight, kg	59.3 $\pm$ 10.5	58.8 $\pm$ 8.5	61.2 $\pm$ 9.3
Current smoker	2 (10)	0	2 (10)
Duration of disease, years	4.7 $\pm$ 3.1	5.2 $\pm$ 2.8	
MCS	7.8 $\pm$ 2.5	7.6 $\pm$ 1.8	
Partial MCS	6.25 $\pm$ 1.3	6.1 $\pm$ 1.5	
IBDQ score	125 $\pm$ 31	122 $\pm$ 35	
Fecal calprotectin, mg/g	1.12 $\pm$ 1.1	1.16 $\pm$ 0.5	
Rectum and sigmoid colon	4 (15.4)	0	
Left side of colon	7 (28)	3 (50)	
Proximal colon	6 (23.1)	1 (16.6)	
All of the colon	9 (34.6)	2 (33.3)	

Data are presented as the mean $\pm$ SD or n (%). MCS, Mayo Clinic Score; IBDQ, inflammatory bowel disease questionnaire.

Human Subjects

UC patients were recruited into the present study at the gastrointestinal clinic at the Shanghai 10th People's Hospital and the Second Hospital of Zhengzhou University. The diagnosis, obtaining of colon biopsies, and management of the UC and CD patients were carried out by our physicians. The demographic data of the patients are presented in Table 1. Patients with the following conditions were excluded from this study: those suffering from other autoimmune diseases, cancer, and severe organ diseases. In addition, healthy subjects were also recruited into the present study. The use of human tissue in the present study was approved by the Human Ethics Committees at Shanghai Tenth People's Hospital of Tongji University and Zhengzhou University. Informed written consent was obtained from each human subject.

Preparation of Peripheral Blood Mononuclear Cells and Cell Culture

Blood samples were collected from each human subject by an ulnar vein puncture. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples by gradient density centrifugation. The cells were cultured in RPMI1640 medium supplemented with antibiotics (penicillin and streptomycin), 10% fetal bovine serum, and 2 mM glutamine. The medium was changed in 2–3 days. The cell viability was greater than 99% as checked by Trypan blue exclusion assay.

Immune Cell Isolation

The immune cells used in this study were isolated from PBMC or lamina propria mononuclear cells by magnetic cell sorting (MACS) with commercial reagent kits following the manufacturer's instructions. The cell purity was checked by flow cytometry. If the purity did not reach 95% or over, MACS was performed with the cells again.

Fig. 1. High expression of survivin in peripheral CD4⁺ T cells and Th2 polarization in UC patients. Blood samples were collected from healthy subjects ($n = 20$) and UC patients ($n = 20$). PBMCs were prepared from the samples. **a–c** CD4⁺ T cells were isolated from PBMC by MACS and analyzed by RT-qPCR and Western blotting. **a** The scatter dot plots indicate the levels of survivin mRNA in CD4⁺ T cells. **b** The immunoblots indicate the protein levels of survivin in CD4⁺ T cells. **c** Bars indicate the integrated density of immunoblots of panel **b**. **d** The PBMCs (10^6 cells/sample) were analyzed by flow cytometry. The bars (mean $\pm$ SEM;

* $p < 0.01$, compared with the healthy group; t test) indicate the summarized frequency of CD4⁺ T cell phenotypes in PBMCs (the representative histograms are presented in online suppl. Fig. S1). **e** The scatter dot plots show the levels of Th2 and Th1 cytokines in culture supernatant of CD4⁺ T cells after culturing for 48 h in the presence of PMA (50 ng/mL) and ionomycin (100 ng/mL). **f** The scatter dot plots show the correlation (by Pearson correlation test) between survivin expression in CD4⁺ T cells and the frequency of CD4⁺ T cell phenotypes.

Flow Cytometry

Cells were stained with fluorescence-labeled antibodies of interest or isotype IgG for 30 min at 4 °C. In the case of intracellular staining, cells were treated with the fixative/permeable reagents first, then stained with fluorescence-labeled antibodies of interest or isotype IgG for 30 min at 4 °C. After washing with phosphate-buffered saline, the cells were analyzed with a flow cytometer (FACSCanto II, BD Bioscience). The acquired data were analyzed with the software package Flowjo with the isotype IgG-stained data as the gating references.

Enzyme-Linked Immunosorbent Assay

Cytokine levels in the serum and culture supernatant were determined by ELISA with commercial reagent kits following the manufacturer's instructions.

Real-Time Quantitative RT-PCR

The total RNA was extracted from cells collected from relevant experiments and converted to cDNA with a reverse transcription kit following the manufacturer's instructions. The samples were amplified in a real-time PCR device with the presence of SYBR Green Mas-

Fig. 2. The apoptotic machinery in CD4⁺ T cells of UC patients is compromised. Blood samples were collected from healthy subjects ($n = 20$) and UC patients ($n = 20$). PBMCs were prepared from the samples. CD4⁺ T cells were isolated from PBMCs by MACS and cultured in the presence of saline or cisplatin ($5 \mu\text{g/mL}$) for 48 h. **a** The gated dot plots indicate the frequency of apoptotic CD4⁺ T

cells. **b** The bars indicate the summarized data of apoptotic CD4⁺ T cells in panel **a**. **c** The bars indicate the mRNA levels of FasL in CD4⁺ T cells. **d** The immunoblots indicate the protein levels of FasL in CD4⁺ T cells. **e** Bars indicate the integrated density of the immunoblots in panel **d**. * $p < 0.01$, compared with the saline group; # $p < 0.01$, compared with the healthy group.

ter Mix and relevant primers. The primers used in the present study include survivin (cagacttgccagtggttc and acagaaaggaaagcgcaacc), FasL (cccagatctactgggtgga and ctctcttcagggtggcag), and β -actin (cagcagccttcaattacc and gctgtggttcctctcttct). The results are presented as the fold change against the housekeeping gene β -actin.

Western Blotting

The total proteins were extracted from cells collected from relevant experiments and fractioned by SDS-PAGE. The protein fractions were transferred onto a PVDF membrane. After blocking with skimmed milk (5%) for 30 min, the membrane was incubated with antibodies of interest or isotype IgG overnight at 4°C , followed by incubation with the second antibodies (conjugated with peroxidase) for 2 h. The membrane was washed 3 times with Tris-buffered saline Tween-20 after each incubation. The immunoblots on the membrane were developed by the enhanced chemiluminescence and photographed with an imaging device.

Preparation of Protein Extracts

Cells were collected from the relevant experiments and lysed with a lysing buffer. The lysates were centrifuged for 10 min at $10,000 \text{ g}$; the supernatant was collected and used as cytosolic extracts. The pellets were incubated with a nuclear lysing buffer. The lysates were centrifuged for 10 min at $10,000 \text{ g}$; the supernatant was collected and used as nuclear extracts. All the procedures were performed at 4°C .

Immunoprecipitation

The protein extracts were precleared by incubating with protein G agarose beads for 1 h to eliminate the non-specific immune complexes. The samples were incubated with antibodies of interest or isotype IgG overnight. The immune complexes were precipitated by incubating with protein G agarose beads for 2 h. The protein complexes on the beads were eluted with an eluting buffer and analyzed by Western blotting. All the procedures were performed at 4°C .

Chromatin IP

Cells were collected from relevant experiments and fixed with 1% formalin for 15 min. The cells were lysed with a lysing buffer and followed by sonication to shear the DNA into small pieces. The samples were treated with the IP procedures. After eluting, DNA was recovered from the samples with a DNA extracting reagent kit and analyzed by real-time PCR in the presence of the FasL promoter primers (cccactgaccattctctgt and agagcaaacccctggaagt). The results were presented as the fold change against the input.

Statistics

Data are presented as the mean $\pm$ SEM. The difference between two groups was determined by the Student t test or ANOVA followed by the Dunnett t test or SNK test if there were more than two groups. $p < 0.05$ was considered statistically significant.

Fig. 3. Survivin binds c-Myc to form a complex in CD4⁺ T cells. **a** CD4⁺ T cells were isolated from blood samples collected from 20 UC patients. Protein extracts were prepared from the CD4⁺ T cells and analyzed by IP. The immunoblots indicate the complex of survivin and c-Myc in CD4⁺ T cells. **b** HEK293 cells were transfected with the Flag-survivin-expressing plasmids and the His-c-Myc-expressing plasmids. Cell extracts were prepared with the HEK293 cells and processed by IP with anti-Flag and anti-His as the precipitating antibodies. The immunoblots indicate the complex of recombinant survivin and c-Myc in HEK293 cells. The data in **a** represent 20 independent experiments. The data in **b** represent 3 independent experiments.

Results

CD4⁺ T Cells of UC Patients Express High Levels of Survivin

To investigate the role of survivin in the pathogenesis of UC, PBMCs were isolated from blood samples collected from UC patients and healthy subjects. CD4⁺ T cells were isolated from PBMCs and analyzed by RT-qPCR and Western blotting. The results showed that the expression of survivin was detected in the peripheral CD4⁺ T cells, which was higher in the UC group than that in the healthy group (Fig. 1a–c). The PBMCs were also analyzed by flow cytometry. The frequencies of IL-4⁺, IL-5⁺, IL-13⁺, and survivin⁺ CD4⁺ T cells were higher in the UC group than those of the healthy group (online suppl. Fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000500546; Fig. 1d). The frequency of IFN- γ ⁺ CD4⁺ T cells was not significantly different between the UC group and healthy group (online suppl. Fig. S1; Fig. 1d). The isolated CD4⁺ T cells were cultured in the presence of PMA and ionomycin for 48 h. Higher levels of Th2 cytokines, but not IFN- γ , were detected in the culture supernatant of samples collected from the UC group than those collected from the healthy group (Fig. 1e). A positive correlation was detected between the expression of survivin and the frequency of peripheral Th2 cells in UC patients (Fig. 1f). In addition, we obtained colon biopsies from 6 UC patients and 6 CD patients. The biopsies were analyzed by RT-qPCR and Western blotting. The results showed that the expression of survivin was detected in biopsies of both CD samples and UC samples, which was significantly higher in UC samples than

that in CD samples (online suppl. Fig. S2). The results indicate the Th2 polarization status in the peripheral blood system of UC patients. Survivin may be associated with the development of Th2 polarization in UC.

CD4⁺ T Cells from UC Patients Are Resistant to Apoptosis Induction

The Th2 polarization indicates increases in the development of Th2 cells and/or decreases in apoptosis in Th2 cells. The increase in the development of Th2 cells in Th2 polarization has been well investigated [10]. Thus, we tested the response of CD4⁺ T cells to apoptosis induction. The results showed that, after treating with cisplatin (an apoptosis inducer), the apoptotic CD4⁺ T cells detected by flow cytometry were significantly more in those collected from healthy subjects than those from UC patients (Fig. 2a, b). In addition, the expression of FasL in CD4⁺ T cells was lower in the UC group than that in the healthy group (Fig. 2c–e). The results imply that CD4⁺ T cells of UC patients are resistant to the induction of apoptosis.

Survivin Binds the Transcription Factor of FasL in CD4⁺ T Cells of UC Patients

We further analyzed the samples of CD4⁺ T cells collected from UC patients. A complex of survivin and c-Myc, the transcription factor of FasL, was detected in protein extracts of CD4⁺ T cells (Fig. 3a). To corroborate the results, a Flag-survivin-expressing plasmid and a His-c-Myc-expressing plasmid were constructed. The plasmids were transfected into HEK293 cells (obtained from ATCC, Manassas, VA, USA) to make the cells express the recombinant survivin and c-Myc. The cells were harvested 72 h

Fig. 4. Survivin prevents the FasL gene transcription in CD4⁺ T cells. CD4⁺ T cells were collected from healthy subjects ($n = 18$) and UC patients ($n = 18$), treated with the procedures denoted on the x -axis, and exposed to cisplatin in the culture for 48 h. The cells were analyzed by ChIP. The bars indicate the levels of c-Myc (**a–c**) and Pol II (**d–f**) at the FasL promoter locus, which were summarized from 6 independent experiments. Data are presented as the mean $\pm$ SEM. * $p < 0.01$, compared with healthy group (**a, d**) or saline group (**b, c, e, f**).

after transfection. The cell extracts were analyzed by co-IP with anti-Flag and anti-His antibodies as the precipitation antibodies. The results showed that the recombinant survivin and c-Myc formed a complex in HEK293 cells (Fig. 3b). The results demonstrate that survivin can bind the FasL transcription factor to form a complex in CD4⁺ T cells.

Survivin Interferes with the Gene Transcription of FasL in CD4⁺ T Cells

Next, we analyzed the gene transcription of FasL in CD4⁺ T cells. CD4⁺ T cells were isolated from the blood samples collected from UC patients and healthy subjects; the cells were analyzed by ChIP. Lower activities of FasL gene transcription, including lower levels of c-Myc, and Pol II (RNA polymerase II), were detected in the FasL promoter locus in the samples of UC patients as compared to the samples from healthy subjects (Fig. 4). The results imply that the binding of survivin to c-Myc as shown above prevents the c-Myc from binding to the FasL promoter. To test the inference, naive CD4⁺ T cells were collected from healthy subjects. The cells were overexpressed with survivin by transfecting CD4⁺ T cells with survivin-expressing plasmids (online suppl. Fig. S3A) and exposed to

cisplatin for 48 h. The levels of c-Myc and Pol II at the FasL promoter locus were markedly suppressed by the overexpression of survivin (Fig. 4). On the other hand, the CD4⁺ T cells collected from UC patients were treated with survivin RNAi to knock down the expression of survivin (online suppl. Fig. S3B). After exposure to cisplatin for 48 h, the survivin knockdown significantly increased the levels of c-Myc and Pol II at the FasL promoter locus (Fig. 4). The results suggest that survivin prevents the gene transcription activities of FasL in CD4⁺ T cells.

Survivin Prevents CD4⁺ T Cells from Apoptosis

The data reported above suggest that survivin may affect the apoptotic machinery in CD4⁺ T cells. To test this, CD4⁺ T cells from healthy subjects were transfected with survivin-expressing plasmids to overexpress survivin or with control plasmids. The cells were treated with cisplatin. The results showed that overexpression of survivin significantly decreased the frequency of apoptotic cells (Fig. 5a). On the other hand, CD4⁺ T cells collected from UC patients were treated with survivin RNAi to knock down the expression of survivin. The results showed that the suppression of survivin markedly increased the fre-

Fig. 5. Regulating survivin expression modulates the sensitiveness to apoptotic induction in CD4⁺ T cells. **a** CD4⁺ T cells were collected from healthy subjects ($n = 6$) and treated with the procedures denoted above each panel. The gated dot plots indicate the apoptotic cells. The bars indicate the summarized data of 6 experiments. **b** CD4⁺ T cells were collected from UC patients ($n = 6$) and

treated with the procedures denoted above each panel. The gated dot plots indicate the apoptotic cells. The bars indicate the summarized data of 6 experiments. The data are presented as the mean $\pm$ SEM. * $p < 0.01$, compared with the data of panel **a**; # $p < 0.01$, compared with the data of panel **b**.

quency of apoptotic cells (Fig. 5b). The results demonstrate that survivin can prevent CD4⁺ T cells from apoptosis. Inhibition of survivin restores the responsiveness to apoptotic stimuli of CD4⁺ T cells collected from UC patients.

IL-4 and IL-13 Increase the Expression of Survivin in CD4⁺ T Cells

The data in Figures 1–5 suggest that the biased Th2 polarization may modulate the expression of survivin in CD4⁺ T cells. Thus, we collected naïve CD4⁺ T cells from healthy subjects. The cells were cultured in the presence of IL-4 or IL-13 at gradient concentrations for 48 h. As analyzed by RT-qPCR and Western blotting, the exposure to IL-4 or IL-13 increased the expression of survivin in CD4⁺ T cells in a concentration-dependent manner (Fig. 6). The results demonstrate that IL-4 or IL-13 can increase the expression of survivin in CD4⁺ T cells.

Discussion

The present data demonstrate that CD4⁺ T cells from UC patients express high levels of survivin. The levels of survivin in CD4⁺ T cells are positively correlated with the frequency of the peripheral Th2 cells, suggesting that survivin is associated with the pathogenesis of biased Th2 polarization in UC patients.

The present data show the biased Th2 polarization in the peripheral blood system of UC patients. The cytokine profiles of UC are still controversial. Lu et al. [11] reported that the frequency of IL-4⁺ CD4⁺ T cells was increased in UC patients. Itani et al. [12] found that a biased Th2 response was induced in mice with colitis. Our previous studies also found that Th2 polarization in UC patients could be alleviated by specific immunotherapy [13]. High levels of IL-13 and IL-5 in UC patients have also been reported [14, 15]. Thus, it is suggested that a Th2-

Fig. 6. IL-4 increases survivin expression in CD4⁺ T cells. Naïve CD4⁺ T cells were collected from healthy subjects ($n = 6$) and cultured in the presence of IL-4 in the culture for 48 h. The cells were harvested at the end of culture and analyzed by RT-qPCR and Western blotting. **a, b** The bars indicate the mRNA levels of sur-

vivin in CD4⁺ T cells. **c, d** The immunoblots indicate the protein levels of survivin in CD4⁺ T cells. **e, f** Bars indicate the integrated density of immunoblots of panels **c** and **d**. The data represent 6 independent experiments. * $p < 0.01$, compared with the “0” group.

dominant status plays an important role in the pathogenesis of UC [4]. On the other hand, lower levels of IL-4 in UC patients are also reported [16]. Some investigators even aimed to increase serum IL-4 to attenuate UC [17]. IL-17 expression was also found in the intestinal mucosa and serum of UC patients [18]. The present data support the role of a biased Th2 response in the pathogenesis of UC.

We observed that peripheral CD4⁺ T cells express survivin, which is much higher in UC patients than healthy subjects. Survivin is an anti-apoptosis protein. It is mainly found in cancer cells, implying that this molecule is associated with the development of cancer and may be used as a cancer biomarker [6]. It plays a role in T cell development [8]. Lately, it was found that survivin was not only involved in the pathogenesis of cancer, but also in other disorders such as airway allergy [9], rheumatic arthritis [19], and autoimmune diseases [20]. The present data show that survivin is positively correlated with the peripheral Th2 cell population in UC patients, suggesting that survivin may be involved in the pathogenesis of UC, and needs to be further investigated.

The data indicate that CD4⁺ T cells from UC patients express higher levels of survivin as compared to those of healthy subjects. The expression of survivin in CD4⁺ T cells suppresses the expression of FasL. FasL is an important factor in the induction of cell apoptosis [21]. FasL-deficient mice show many more tumor-associated immune cells [22]. The present study provides mechanistic evidence that survivin can bind c-Myc, the FasL transcription factor, to form a complex in CD4⁺ T cells. Such a physical contact between survivin and c-Myc prevents the expression of FasL in CD4⁺ T cells, which compromises the sensitiveness to the apoptosis inducer in CD4⁺ T cells. Thus, inhibiting the expression of survivin can promote the sensitivity to apoptosis induction in CD4⁺ T cells, as shown by the present data.

We also observed that the levels of survivin in colon biopsies were higher in UC patients than in CD patients. The data are in line with the results obtained from the samples collected from peripheral CD4⁺ T cells. A previous report also found the expression of survivin in lymphocytes of IBD patients, in which the expression of survivin was higher in samples collected from CD patients

than in samples collected from UC patients [23]. Another previous report claimed that the expression of survivin was not different between biopsies collected from UC patients and CD patients [24]. The underlying mechanism of the difference between our data and previous reports is not clear at present, and further investigation is necessary.

In conclusion, the present data show that CD4⁺ T cells express high levels of survivin, which is positively correlated with the biased Th2 polarization in UC patients. Survivin interferes with the expression of FasL to compromise the sensitiveness to apoptosis induction. Inhibition of survivin promotes the responsiveness to the apoptosis inducer in CD4⁺ T cells, which has therapeutic potential in the treatment of diseases with skewed Th2 polarization.

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Statement of Ethics

The experimental procedures involving human tissue and animals were approved by the Ethic Committee at Zhengzhou University. An informed written consent was obtained from each human subject.

Disclosure Statement

The authors have no conflicts of interest to declare.

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