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Original article

Deficiency in the frequency and function of Tr1 cells in IgAV and the possible role of IL-27

Lu Pan[1,*], Jinghua Wang[1,*], Jinxiang Liu[1], Lishuang Guo[1] and Sirui Yang [1]

Abstract

Objective. Type 1 regulatory T (Tr1) cells are involved in the pathogenesis of numerous immune-mediated diseases. However, little is known about whether and how Tr1 cells affect the development of IgA vasculitis (IgAV). We aimed to investigate this question in IgAV patients.

Methods. . Tr1 cells in peripheral blood and kidney tissue of IgAV patients were analysed by multi-parametric flow cytometry and immunofluorescence techniques. An *in vitro* assay of suppression of T cell proliferation and cytokine release was performed to evaluate the function of Tr1 cells. Real-time PCR and cell stimulation *in vitro* were used to explore the roles of IL-27 and early growth response gene 2 (EGR2).

Results. The frequency of Tr1 cells was decreased in peripheral blood but increased in kidney tissue from IgAV patients. A defective suppressive function of Tr1 cells in IgAV was observed. The frequency of Tr1 cells and the cytokines secreted by them were up-regulated in the presence of recombinant IL-27 *in vitro*. Moreover, IL-27 also increased the expression of EGR2. Furthermore, lower frequency of Tr1 cells during remission had a higher recurrence rate.

Conclusion. Tr1 cells are involved in the pathogenesis of IgAV. The low IL-27 in IgAV is responsible for impaired frequency and function of Tr1 cells, and EGR2 may be the specific transcription factor involved in the progression. Tr1 may be a risk factor for IgAV recurrence.

Key words: immunoglobulin A vasculitis, Type 1 regulatory T (Tr1) cell, interleukin-27, interleukin-10, transforming growth factor-β, early growth response gene 2

BASIC SCIENCE

Rheumatology key messages

- Type 1 regulatory T cells play a potential role in the pathogenesis of IgA vasculitis.
- Low IL-27 in IgA vasculitis was a potential cause of impaired Type 1 regulatory T cells.
- Type 1 regulatory T cells in the remission phase were associated with the recurrence of IgA vasculitis.

Introduction

IgA vasculitis (IgAV), formerly known as Henoch–Schönlein purpura, is the most common form of vasculitis affecting children, characterized by IgA deposition in small vessels with dysregulated immune response. Clinical manifestations of IgAV vary according to the involved organs, including skin, gastrointestinal tract, joints and kidney [1]. IgAV is a self-limiting disease and the prognosis is generally good; however, the recurrence rate is high among children (2.7–66.2%) [2]. There are no specific biomarkers for predicting the prognosis and recurrence of IgAV.

The pathogenesis of IgAV remains unclear. In addition to the critical role of galactose-deficient IgA1, both innate and adaptive immune cells are known to contribute to the development of IgAV [3–5]. Tregs play a critical role in the pathogenesis of autoimmune and chronic inflammatory diseases through their immunosuppressive functions, including IgAV [6–8].

The Type 1 regulatory T (Tr1) cell is a special type of Treg, which maintains immune tolerance predominantly by secreting immunosuppressive cytokines, such as IL-10 and TGF-β [9, 10]. Specific biomarkers for Tr1 cells were not identified until 2013, limiting their study and clinical application. Currently, human and murine Tr1 cells are characterized by co-expression of CD49b and

[1]Department of Pediatric Rheumatology and Allergy, The First Hospital of Jilin University, Changchun, China

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Correspondence to: Sirui Yang, 71 Xinmin Street, Changchun, Jilin, China. E-mail: sryang@jlu.edu.cn

*Lu Pan and Jinghua Wang contributed equally to this work.

lymphocyte activation gene 3 (LAG-3) [11]. Since their discovery, Tr1 cells have been found to be involved in the pathogenesis of numerous of immune-mediated and inflammatory diseases [12]. Moreover, multiple molecular mechanisms were investigated. IL-27 is an anti-inflammatory cytokine found in infectious and autoimmune animal models [13]. Several studies have demonstrated that the differentiation of Tr1 cells is facilitated by IL-27 via multiple molecular pathways. However, the role of IL-27 in IgAV is paradoxical and little is known about whether and how Tr1 cells affect the development and prognosis of IgAV. This study aimed to address these questions in patients with IgAV.

Methods

Study subjects

Fifty patients fulfilling the EULAR/PRINTO/Pediatric Rheumatology European Society criteria for IgAV [14] were randomly recruited between May 2019 and May 2020 from the First Hospital of Jilin University. According to presenting symptoms, the recruited patients were grouped into five subtypes: skin type ($n = 13$), abdominal type ($n = 11$), joint type ($n = 10$), kidney type ($n = 4$) and mixed type ($n = 12$). Twenty gender-, age- and ethnicity-matched healthy volunteers were recruited as healthy controls (HCs). Peripheral blood samples were collected from HCs and patients with IgAV during the acute phase. Among IgAV patients, 28 remission-phase blood samples were also collected. Our study complied with the declaration of Helsinki and was approved by the ethics committee of the First Hospital of Jilin University. Written informed consent was obtained from all participants.

Laboratory evaluation

Basic laboratory tests were performed on all subjects. The levels of white blood cells, lymphocytes and platelets were measured with an XS-800i blood analyser from Sysmex (Kobe, Japan). Serum immunoglobulin and complement levels were measured by specific protein analyser (BN-II; Siemens, München, Germany). Serum CRP level was measured by the QuikRead go CRP kit (Orion Diagnostica, Espoo, Finland).

Cell preparations

Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll-Hypaque density gradients (Amersham Biosciences, Little Chalfont, UK) from all subjects. Tr1 cells were depleted from PBMCs by cell sorting using a cell sorter (FACSAria II, Beckton Dickinson, San Diego, CA, USA).

Flow cytometry analysis

Tr1 cells were evaluated by multicolour flow cytometry (FACSAria II). After washing, PBMCs were prepared for surface staining. Samples were incubated for 30 min at 4°C in the dark with the following mAbs: anti-human CD3 (HIT3a), CD4 (A161A1), LAG-3 (11C3C65) and CD49b (P1E6-C5) from Biolegend (San Diego, CA, USA). Intracellular IL-10 (JES3-9D7) and TGF-β1 (TW4-2F8) were measured after stimulation with elltimulation ocktail (plus protein transport inhibitors; BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. After fixing and permeabilization, the cells were incubated with corresponding human antibodies (Biolegend, San Diego, CA, USA). Data were analysed with FlowJo software (v5.7.2, Tree Star Inc., Ashland, OR, USA).

Functional studies of Tr1 cells

Suppressive function of Tr1 cells was analysed by an assay of inhibition of $CD4^+$ T cell proliferation. PBMCs isolated from IgAV patients and HCs were labelled with anti-human CD3, CD4, LAG-3 and CD49b mAbs. Then, $CD3^+CD4^+LAG3^+CD49b^+$ Tr1 cells were depleted from PBMCs by cell sorting using a cell sorter (FACSAria II), to obtain a cellular purity ≥95% (supplementary Fig. S1, available at *Rheumatology* online). Then, depleted and non-depleted PBMCs were labelled with carboxyfluorescein succinimidyl ester (CFSE). CFSE was used at a concentration of 2.5 μM (BD Biosciences, San Jose, CA, USA). Cells labelled with CFSE were stimulated with plate-bound anti-CD3 (2 μg/ml, clone OKT3) and anti-CD28 (1 μg/ml, clone CD28.2) (both Biolegend, San Diego, CA, USA). At day 5, T cells were harvested and proliferation was analysed using flow cytometry. The suppressive capacity of Tr1 cells was determined as the relative inhibition of cell proliferation and was calculated as follows: (1 – % divided cells in the presence of Tr1/% divided cells in the absence of Tr1) × 100 [15].

Function of IL-27 in inducing Tr1 cells

To determine the role of IL-27 on Tr1 cells in IgAV, PBMCs from IgAV patients were stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) in 96-well plates at a density of $2–5 \times 10^5$ cells per well (200 μl). Thereafter, cells were cultured in RPMI 1640 medium (Gibco-Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Hyclone, South Logan, UT, USA) supplemented with 2 mM L-glutamine, 0.05 mM β-mercaptoethanol, 100 U/ml of penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 for 5 days in the presence or absence of human IL-27 (100 ng/ml, PeproTech, Rocky Hill, NJ, USA).Then, the cells were collected for flow cytometry and real-time quantitative PCR. Cell culture supernatant was collected for and stored at –20°C until bulk analysis.

Real-time quantitative RT-PCR

Total RNA was extracted from PBMCs alone and PBMCs stimulated by IL-27, using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The first-strand cDNA was reverse-transcribed using Trans Script All-in-one First-Strand cDNA Synthesis SuperMix for qPCR (TransGen

Biotech, Beijing, China) according to the manufacturer's instructions. Real-time PCR was performed using the QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and FastStart Universal SYBR-Green Master kit (Roche, Mannheim, Germany). The mRNA expression levels were quantified using primers for *IL-10*, *IL-27*, *early growth response gene 2* (*EGR2*), *TGF-β1* and *TGF-β3*, and *actin* was used as an internal control for normalization by standard $2^{-\triangle\triangle CT}$ calculation, as previously described [16]. Primer sequences are listed in supplementary Table S1, available at *Rheumatology* online.

Measurement of cytokines

Cytokine levels were measured in serum of IgAV patients, HCs, and culture supernatants of PBMCs depleted or not of Tr1 cells. Levels of IL-10 and IL-27 were measured by human Magnetic Luminex screening assay (R&D Systems Inc., Minneapolis, MN, USA). Levels of TGF-β1 and TGF-β3 were measured using the Meso Scale Discovery human TGF-β1 and TGF-β3 kits (Meso Scale Diagnostics, Rockville, MD, USA).

Immunofluorescence analysis

Renal tissues from IgAV nephritis patients and volunteers were prepared as frozen tissue sections and stained with rabbit anti-LAG3 (Abcam, Cambridge, UK) and mouse anti-CD49b (Abcam, UK) mAbs, or isotype control antibodies (that is, rabbit IgG and mouse IgG2 for LAG3 and CD49b), followed by a Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 647-conjugated anti-rabbit IgG, respectively. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, BD Bioscience). Sections were analysed using a Leica TCS-SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Data were expressed as mean (S.D.). Intergroup comparisons were performed using two-tailed *t*-test, whereas multiple-group comparisons were performed using one-way analysis of variance followed by the Newman–Keuls test. The Spearman rank test was used for analysis of correlation. Receiver operating characteristic (ROC) curve was performed to find the optimal cut-off value. Kaplan–Meier survival estimates model was assessed by log-rank test. A *P*-value <0.05 was considered significant. Statistical analyses were performed on GraphPad Prism 6.0 software.

Results

Circulating Tr1 cells are decreased in IgAV patients

As a first step in investigating the role of Tr1 cells in IgAV, we examined the frequency of circulating Tr1 cells in IgAV patients and HCs. PBMCs were collected from 50 IgAV patients in the acute phase [26 females and 24 males, aged 7.745 (3.124) years] and 20 HCs [11 females and 9 males, aged 7.211 (3.521) years]. The complete clinical and demographic data of the participants are shown in supplementary Table S2, available at *Rheumatology* online. We identified Tr1 cells as $CD3^+CD4^+LAG3^+CD49b^+$ cells (Fig. 1A). Compared with HCs, IgAV patients showed a significant decrease in the percentage of Tr1 cells [0.368 (0.208)% *vs* 1.398 (0.691)%, $P<0.001$, Fig. 1B]. As mentioned above, IgAV was further divided into five subtypes; all the subtypes showed a relative decrease in the percentage of Tr1 cells compared with HCs ($P<0.001$, $P<0.001$, $P<0.001$, $P=0.003$, $P<0.001$, respectively, Fig. 1C). Moreover, a negative correlation was observed between Tr1 cells and serum IgE ($r=0.5703$, $P=0.002$, Fig. 1D). In contrast, Tr1 cells were positively correlated with serum IgG ($r=0.4936$, $P=0.004$, Fig. 1E). Interestingly, Tr1 cells showed no relation with serum IgA ($r=0.1042$, $P=0.0514$, data not shown).

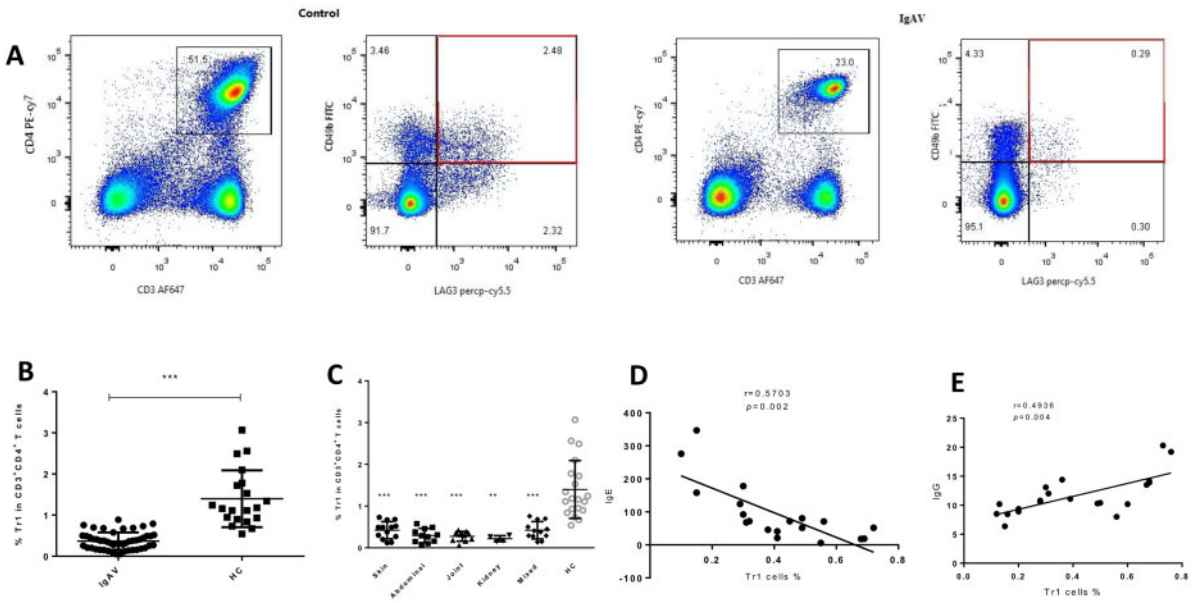
The frequency of Tr1 cells is increased during remission phase

To support that Tr1 cells play a role in IgAV, we collected the peripheral blood from 28 IgAV patients during remission from among the recruited active IgAV patients. During disease remission, the frequency of Tr1 cells was increased from the corresponding value in the acute stage [0.37 (0.203)% *vs* 1.365 (1.039)%, $P<0.001$, Fig. 2A]. The IgAV patients were also divided into five subtypes to detect the alterations. Except for the kidney type ($P=0.1521$, Fig. 2E), the frequency of Tr1 cells in the skin type ($P=0.0111$, Fig. 2B), abdominal type ($P=0.0454$, Fig. 2C), joint type ($P=0.0333$, Fig. 2D) and mixed type ($P=0.0484$, Fig. 2F) was increased compared with the acute stage. The opposite result in the kidney stype may be due to the small number of subjects.

The levels of IL-27, IL-10 and TGF-β are decreased in IgAV patients

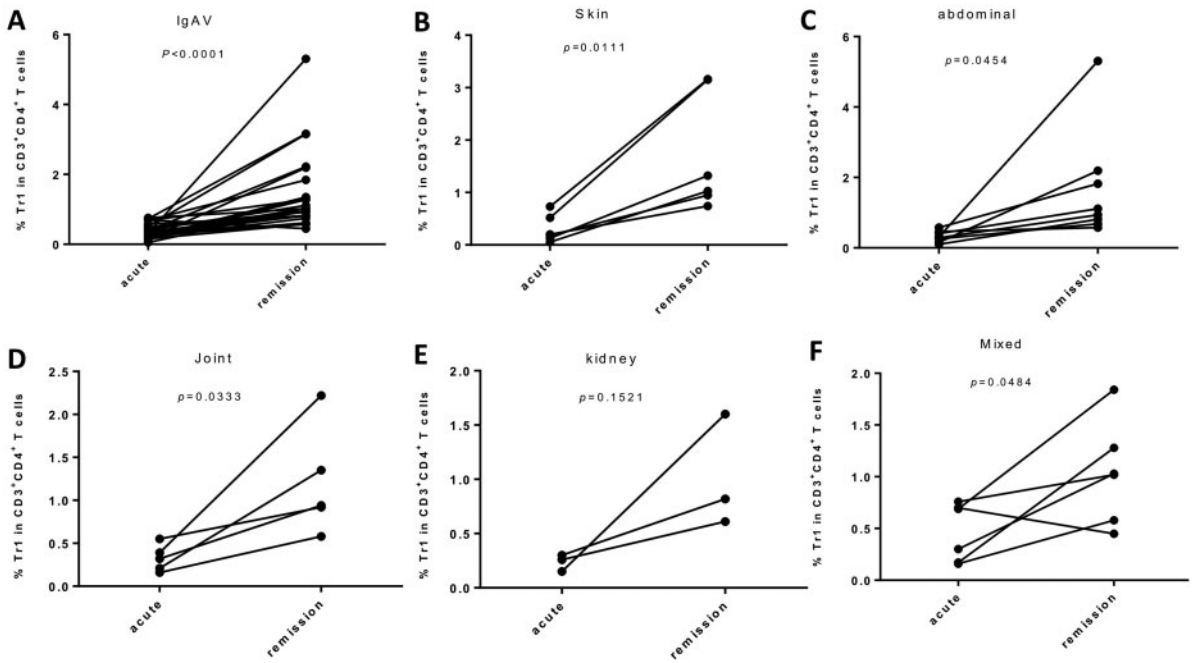
Tr1 cells were reported to produce high levels of IL-10 and TGF-β, through which they execute suppressor functions [12, 17, 18]. Furthermore, IL-27 plays a critical role in the differentiation of Tr1 cells [19, 20]. To evaluate IL-10, IL-27 and TGF-β, PBMCs were subjected to real-time quantitative PCR analysis and serum samples were subjected to Magnetic Luminex screening assay or Meso Scale Discovery. The results showed that serum level and mRNA expression level of *IL-27* were decreased in IgAV patients compared with HCs ($P=0.0021$, $P<0.0001$, respectively, Fig. 3A). TGF-β1 and TGF-β3 showed the same trend ($P=0.0287$, $P<0.0001$, $P=0.0002$, $P<0.0001$, respectively, Fig. 3B and C), especially TGF-β3. Consistently, the IgAV patients had statistically lower serum level of IL-10 in comparison with the HCs ($P=0.0158$, Fig. 3D), but the mRNA expression level of *IL-10* showed no significant difference ($P=0.0158$, $P=0.3021$, respectively, Fig. 3D).

Fig. 1 The frequency of peripheral blood Tr1 cells and correlation analysis



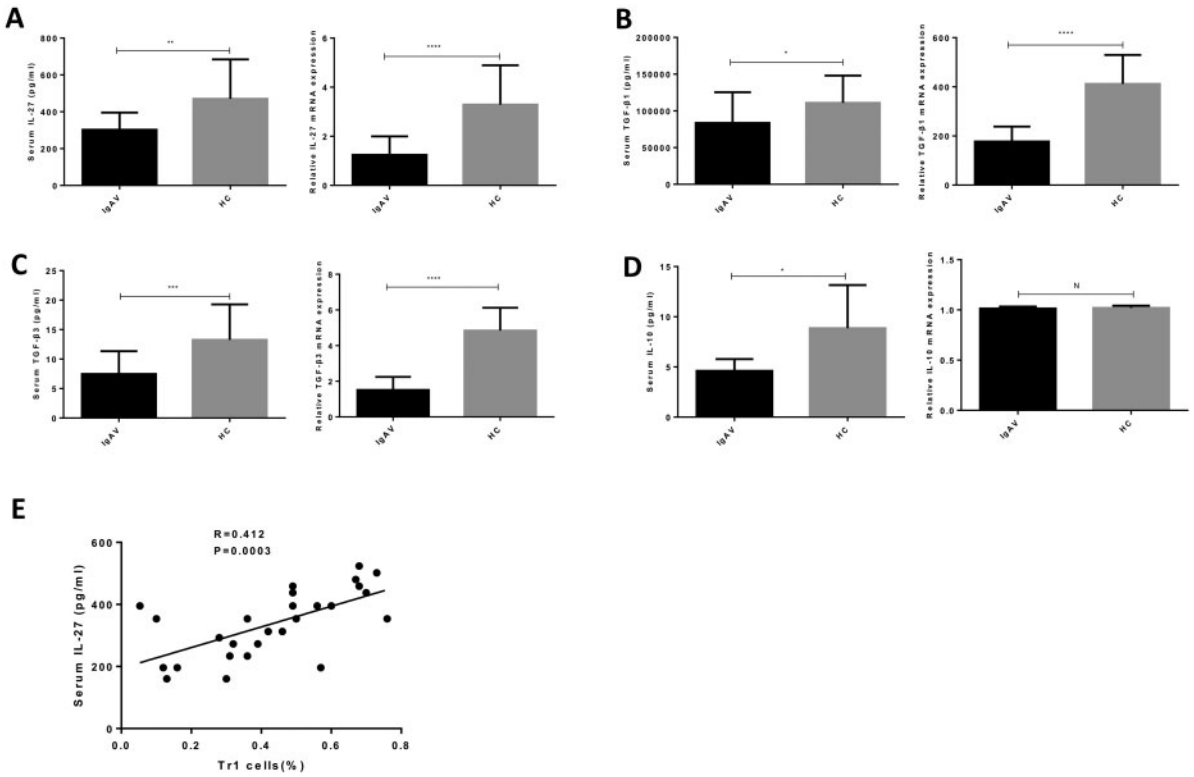
Tr1 cells were gated as $CD49b^{+}LAG3^{+}$ cells in pregated $CD3^{+}CD4^{+}$ T cells. (**A**) Representative dot plots of a healthy control and an IgAV patient. (**B**) Comparison of Tr1 cells between IgAV patients and HCs. (**C**) Comparison of Tr1 cells between the patients with different subtypes of IgAV and HCs. (**D**) Relationship between the percentage of Tr1 cells and serum IgE. (**E**) Relationship between the percentage of Tr1 cells and serum IgG. $^{**}P < 0.005$, $^{***}P < 0.001$. Tr1: Type 1 regulatory T cells; HCs: healthy controls; IgAV: IgA vasculitis.

Fig. 2 Treatment-induced alterations of Tr1 cells in IgAV



(**A**) The percentage of Tr1 cells in IgAV patients during acute and remission phases ($n = 28$). (**B–F**) The percentage of Tr1 cells in skin type ($n = 6$), abdominal type ($n = 8$), joint type ($n = 5$), kidney type ($n = 3$) and mixed type ($n = 6$) IgAV patients during acute and remission phases. Tr1: Type 1 regulatory T cells; IgAV: IgA vasculitis.

FIG. 3 Levels of IL-27, TGF-β and IL-10 in IgAV and the relationship with Tr1 cells



Serum levels (left panel, IgAV $n = 23$ and HCs $n = 20$) and mRNA expression levels in PBMCs (right panel, IgAV $n = 4$ and HCs $n = 17$) of *IL-27* (**A**), *TGF-β1* (**B**), *TGF-β3* (**C**) and *IL-10* (**D**) in IgAV patients and HCs. (**E**) The correlation analysis between serum IL-27 and Tr1 cells. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$, $^{****}P < 0.0001$, N: not significant. Tr1: Type 1 regulatory T cells; HCs: healthy controls; IgAV: IgA vasculitis; PBMCs: peripheral blood mononuclear cells.

Similar results were observed in all subtypes (supplementary Fig. S2, available at *Rheumatology* online). In addition, serum IL-27 was positively correlated with Tr1 cells ($r = 0.412$, $P = 0.0003$, Fig. 3E).

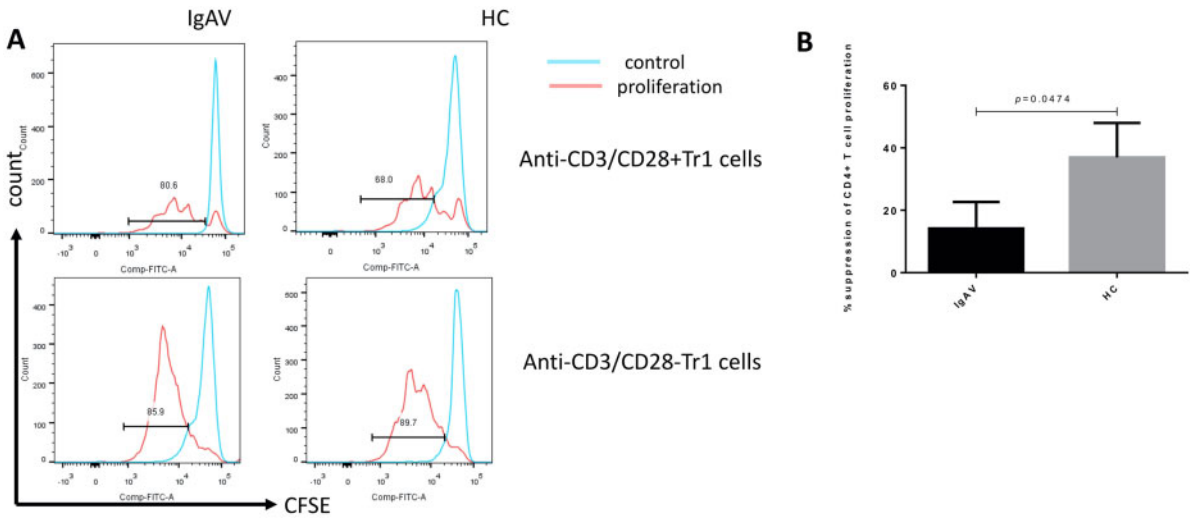
Tr1 cells show impaired suppressive function in IgAV patients

Tr1 cells modulate immune responses mainly through their suppressive function [21]. Therefore, we investigated the inhibitory capacity of Tr1 cells on $CD4^+$ T cell proliferation. CFSE-labelled PBMCs from IgAV patients and HCs with or without Tr1 cells were stimulated by anti-CD3/CD28 for 5 days. Stimulation of $CD4^+$ T cells in the presence of Tr1 cells resulted in suppression of T cell proliferation in IgAV and HC groups. Meanwhile, the whole PBMCs from IgAV patients showed a higher proliferation of $CD4^+$ T cells than HCs (Fig. 4A). Thus, T cell proliferation was inhibited by Tr1 cells both in IgAV and HCs. However, the percentage of suppression by Tr1 cells was lower in IgAV patients compared with HCs [14.17 (8.52)% *vs* 36.93 (11.03)%, $P = 0.0474$, Fig. 4B]. Collectively, these data showed that Tr1 cells had impaired percentage and function in IgAV.

Recombination IL-27 promotes the expansion of Tr1 cells and the production of IL-10 and TGF-β in Tr1 cells *in vitro*

Next, we explored the possible underling mechanism for Tr1 cell alteration in IgAV. Previous studies have revealed that IL-27 promoted Tr1 cell differentiation [19, 22, 23]. Forced expression of EGR2 could convert naïve $CD4^+$ T cells to Tr1 cells [24]. The aforementioned results showed a decreased expression of IL-27 in IgAV, and a positive correlation of IL-27 and Tr1 cells. We therefore hypothesized that low IL-27 affected the generation of Tr1 cells, and then down-regulated the secretion of IL-10 and TGF-β. We found that the mRNA expression levels of *IL-27*, *EGR2*, *TGF-β1* and *TGF-β3* were decreased in the acute phase of IgAV and increased in the remission phase (supplementary Fig. S3, available at *Rheumatology* online). In addition, IL-27 increased the frequency of Tr1 cells and reached a better result at a concentration of 100 ng/ml (supplementary Fig. S4, available at *Rheumatology* online). These results suggested that our hypothesis was reasonable. Then, in an *in vitro* experiment, IL-27 (100 ng/ml) was added or not to PBMCs from IgAV patients stimulated with anti-CD3/CD28 mAbs for 5 days. As shown in Fig. 5A [0.74 (0.2406)% *vs* 3.72 (0.743)%, $P = 0.0023$], IL-27

Fig. 4 Suppressive functional assessment of Tr1 cells



Proliferated fraction was determined by CFSE dilution. Gating strategy for T cells identified as $CD3^+CD4^+$ T cells. (**A**) T cell proliferation of PBMC with or without Tr1 cells in IgAV patients (left panel) and HCs (right panel). (**B**) Percentage suppression of T cell proliferation in IgAV patients ($n = 7$) and HCs ($n = 7$). Tr1: Type 1 regulatory T cells; CFSE: carboxyfluorescein succinimidyl ester; HCs: healthy controls; IgAV: IgA vasculitis.

enhanced the percentage of Tr1 cells in IgAV. The supernatant levels of IL-10, TGF-β1 and TGF-β3 were up-regulated by IL-27 ($P = 0.0455$, $P = 0.0182$, $P = 0.0352$, respectively, Fig. 5B). Flowcytometry analysis revealed that IL-10 ($P = 0.0079$) and TGF-β1 ($P = 0.0174$) expressed in Tr1 cells from PBMCs were increased by IL-27 stimulation (Fig. 5C and D). It is noteworthy that *EGR2* mRNA expression in PBMC was also up-regulated ($P = 0.0153$, Fig. 5E). In addition, the serum level of IL-27 was positively correlated to the mRNA level of *EGR2* ($r = 0.53$, $P = 0.034$, Fig. 5F).

Lower frequency of Tr1 cells during remission phase had a higher recurrence rate

The recurrence rate of IgAV is high in children, but the risk factors for recurrence are inconsistent [25, 26]. We next explore whether Tr1 cell was a predictor of IgAV recurrence. ROC analysis showed that Tr1 cells in the remission (area under the curve = 0.769) but not in the acute phase (area under the curve = 0.528), is a potential predictor of IgAV recurrence (supplementary Fig. S5, available at *Rheumatology* online). The optimal cut-off value proposed by ROC analysis for Tr1 cells in remission was 1.02%. Patients were further divided into two groups (high and low) based on this value. The univariate Kaplan–Meier analysis to identify the potential association between Tr1 cells and IgAV recurrence showed that the cumulative incidence of IgAV recurrence was higher in the Tr1-low group than in the Tr1-high group ($\chi^2 = 3.875$, $P = 0.049$, Fig. 6A), indicating that low Tr1 cells in remission phase were associated with the recurrence of IgAV. Other clinical factors that may affect the prognosis were also examined using Kaplan–Meier method. However, no statistical significance was found (supplementary Table S3, available at *Rheumatology* online).

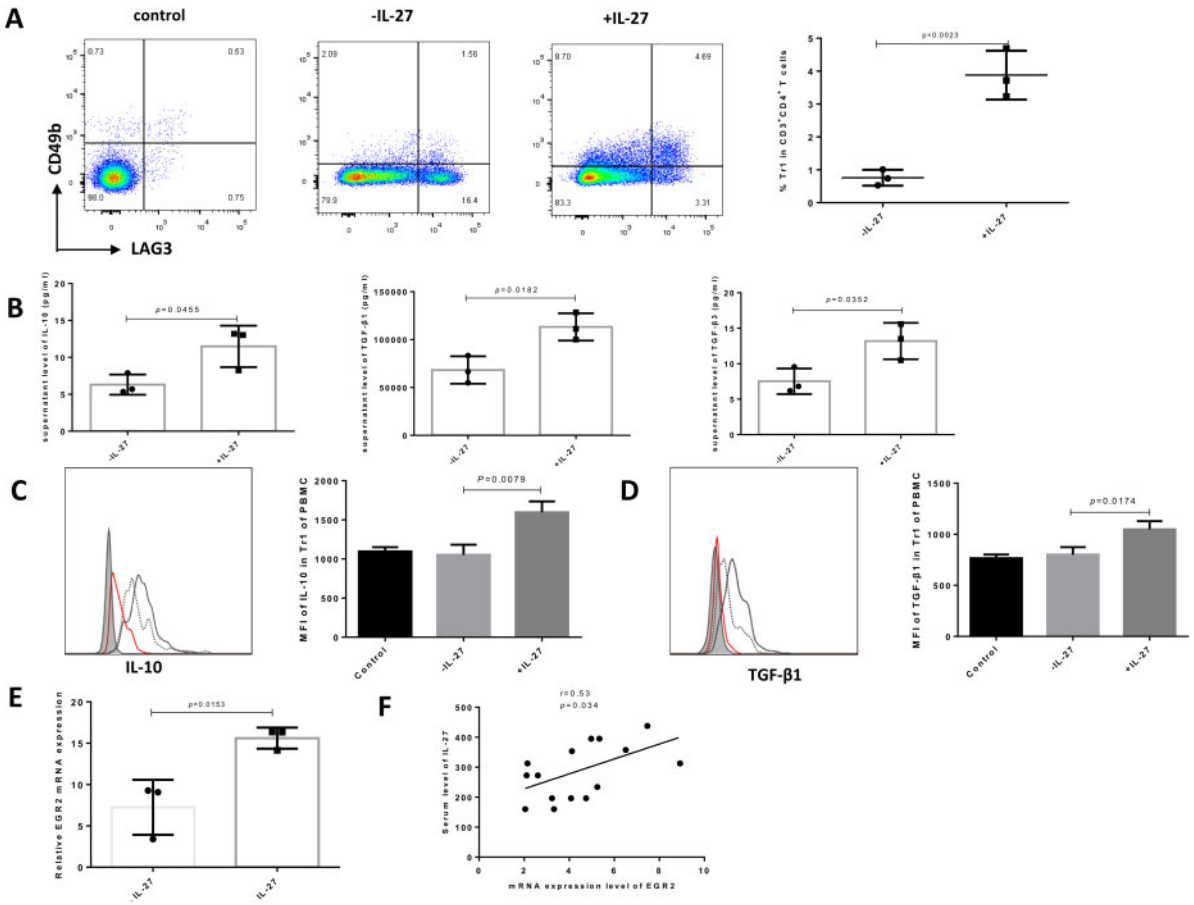
Tr1 cells in kidney tissues from IgAV nephritis patients

The possible presence of Tr1 cells in kidney tissue was analysed by immunofluorescence microscopy. Immunohistology staining revealed the presence of CD49b and LAG3 in the kidney biopsy samples from all IgAV patients examined. Both CD49b and LAG3 were detected in the glomeruli and tubuli (Fig. 6B). In contrast, the co-expression of CD49b and LAG3 was rare in HCs, especially in glomeruli (Fig. 6B and supplementary Fig. S6, available at *Rheumatology* online).

Discussion

Dysregulation of the immune system results in immune-mediated and inflammatory diseases. Tregs are engaged in the maintenance of immune tolerance and homeostasis [27]. Given that IgAV is an IgA-mediated dysregulated immune response to an antigen [28, 29], researchers have explored the potential role of $Foxp3^+$ Tregs in IgAV [7, 8, 30]. However, other subsets of Tregs have not been explored in IgAV. Tr1 cells, a special subset of Tregs, are known to be involved in several immune-mediated diseases [31–34]. Similar to autoimmune diseases [15, 35], in this study we found a diminished percentage of Tr1 cells in the peripheral blood during acute IgAV. Following disease remission, the Tr1 cells were elevated. Interestingly, the expression of Tr1 cells in kidney tissue of IgAV nephritis patients was increased compared with healthy individuals. This

FIG. 5 IL-27-induced expansion of Tr1 cells and production of IL-10 and TGF-β in Tr1 cells

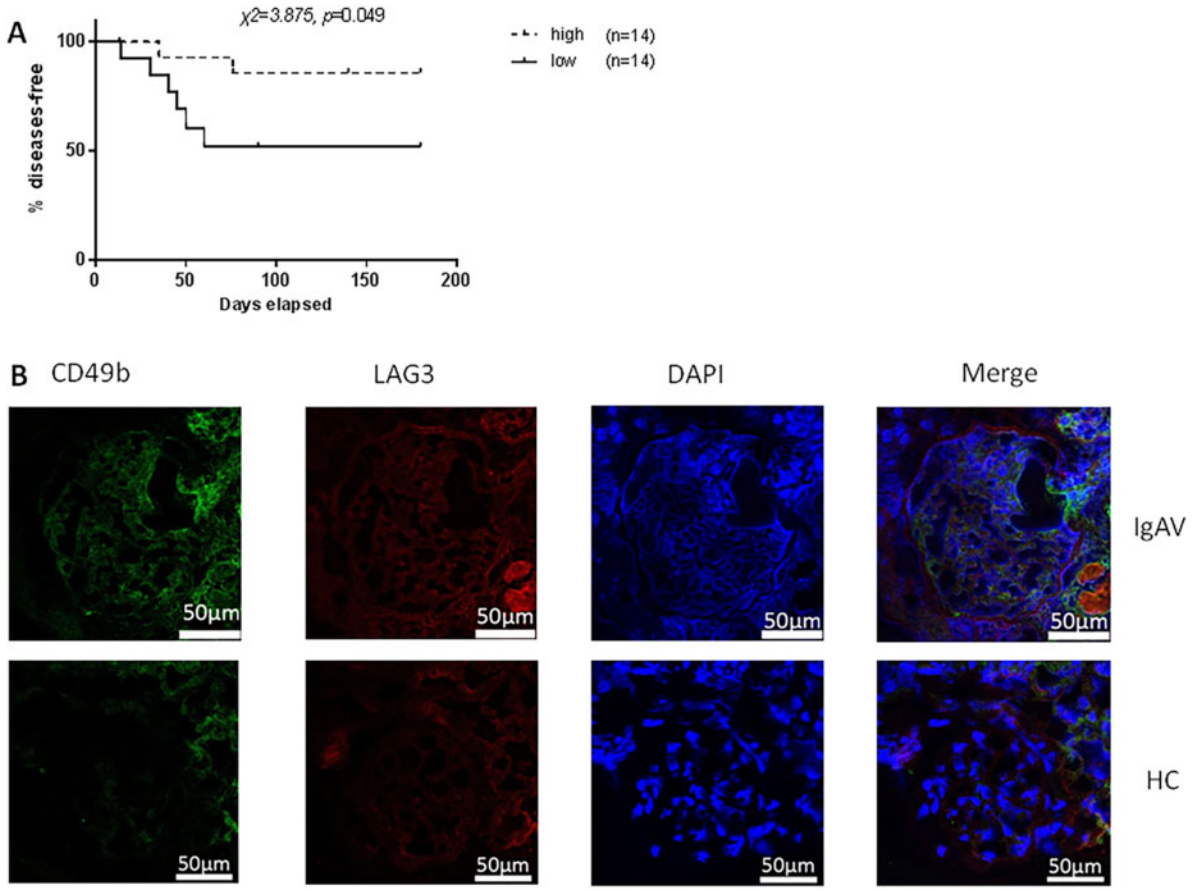


PBMCs from IgAV patients were cultured with anti-CD3/CD28 mAbs with or without IL-27 for 5 days. (**A**) Flow cytometry of Tr1 cells with or without IL-27 stimulation. (**B**) Supernatant levels ($n = 3$ per group) of IL-10 (left panel), TGF-β1 (middle panel) and TGF-β3 (right panel). Flow cytometry of IL-10 (**C**) and TGF-β1 (**D**) in Tr1 cells. Grey: isotype; red solid: control (without CD3/CD28 and IL-27); dotted: CD3/CD28 only; grey solid: IL-27. Data are representative of three independent experiments with similar results. Quantification of signal is shown in bar graphs, and error bars represent mean ± S.D. (**E**) The mRNA expression of *EGR2* in PBMCs ($n = 3$ per group). (**F**) The relationship between the mRNA expression of *EGR2* and serum IL-27. Tr1: Type 1 regulatory T cells; PBMCs: peripheral blood mononuclear cells; IgAV: IgA vasculitis.

was not consistent with the change of Tr1 cells in peripheral blood. It may be due to the migratory behaviour of cells. As IgAV progresses, Tr1 cells are gradually deposited in the tissues, resulting in the decrease of these regulatory lymphocytes in the peripheral blood. This hypothesis needs to be verified in further studies using more samples and different tissues. Collectively, these data suggested that Tr1 cells participate in the pathogenesis of IgAV. Furthermore, the correlation analysis showed a negative correlation of IgE with Tr1 cells and a positive correlation of IgG with Tr1 cells. Interestingly, no correlation was observed between IgA and Tr1 cells. The galactose-deficient IgA1 results in an increased amount of IgA immune complex in circulation, which plays a major role in IgAV [36]. Even so, IgA was found to be elevated in half of the children, and there was no correlation with disease severity [37], which may partially explain our results. Moreover, Meiler *et al.* [38] demonstrated that Tr1 cells suppressed IgE and induced IgG, but the production of IgA was not influenced by Tr1 cells, which strongly supported our results.

Tr1 cells secrete high levels of IL-10 and TGF-β, low levels of IFN-γ and IL-2, and no IL-4 [39, 40]. In addition to TGF-β1, Tr1 cells secrete large amounts of TGF-β3, which mediates immune suppression through programmed cell death 1 (PD-1). Some previous studies showed decreased levels of IL-10 and TGF-β1 in IgAV patients [7, 8], while other studies showed an increased level of IL-10 [41]. The present study showed that serum IL-10, TGF-β1 and TGF-β3 were decreased in IgAV. The suppressive functional analysis of Tr1 cells showed an impaired ability to inhibit the proliferation of T cells in IgAV. Together, these results suggested that Tr1 cells

FIG. 6 Kaplan–Meier analysis and renal immunofluorescence of Tr1 cells



(**A**) Tr1 cells is a risk factor for IgAV recurrence. Tr1 cell frequency was determined in the blood samples of patients during remission. The patients were divided into the high Tr1 group and the low Tr1 group according to ROC analysis (1.02%). Events of IgA recurrence were monitored for six months. Log-rank test. (**B**) Immunofluorescence staining of kidney biopsy samples from IgAV nephritis patients ($n = 4$, upper panel). Renal biopsy samples from healthy subjects were used as controls ($n = 3$, lower panel). Blue fluorescence corresponds to DAPI, green fluorescence to CD49b and red fluorescence to LAG3. Tr1 cells were identified as $DAPI^+CD49b^+LAG3^+$ cells in sections. Original magnification, ×20. Tr1: Type 1 regulatory T cells; HCs: healthy controls; IgAV: IgA vasculitis; ROC: receiver operating characteristic.

had an impaired function in IgAV. These findings are in agreement with previous studies that have reported quantitative and qualitative deficiencies of Tr1 cells in organ transplantation [42], cancer [43] and autoimmune diseases [44]. Notably, IL-10 and TGF-β are produced not only by Tr1 cells but also by other cell types, including Th17, Th1, B cells, etc. [45, 46]. Further studies are necessary to confirm the impaired secretion function of Tr1 cells in IgAV.

IL-10 is a key factor for the induction of murine and human Tr1 cells [47]. IL-27, a member of the IL-12/IL-23 cytokine family, plays a pivotal role in the resolution of inflammation [48]. Several recent studies have shown that IL-27 is a potent inducer of Tr1 cell differentiation in mice via a plethora of potential mechanisms [49–51]. However, the role of IL-27 in inducing human Tr1 cells remains unclear. In this study, we found a positive correlation between IL-27 and Tr1 cells. Recombinant IL-27 enhanced the expansion of Tr1 cells from IgAV *in vitro*, accompanied by an increased expression of IL-10 and TGF-β1 in Tr1 cells. This was consistent with previous studies that during IL-27-mediated induction of Tr1 cells, IL-10 promoter was activated by c-Maf, arl hydrocarbon receptor (AhR) [22], eomesodermin [52], Interferon regulatory factor 1 (IRF1) and Basic leucine zipper transcription factor ATF-like (BATF) [53], resulting in increased production of IL-10. These data suggested that IL-27 directly or indirectly contributed to the generation of Tr1 cells and the cytokines secretion by Tr1 cells in IgAV, indicating that low IL-27 in IgAV was responsible for the impaired frequency and function of Tr1 cells.

Until now, the molecular mechanisms underlying the development of Tr1 cells were unclear. No master

transcription factor defining Tr1 cells has been described [54]. A previous study [55] revealed high level of EGR2 expression in Tr1 cells, which is barely expressed in other T cell subsets [56]. A recent study demonstrated that EGR2 is induced by IL-27 in a STAT3-dependent manner and directly bound to the B lymphocyte induced maturation protein 1 (Blimp-1) promoter to induce IL-10 expression [57]. In line with previous observations, this study showed that IL-27 upregulated the mRNA expression of *EGR2*, and the two parameters had a positive correlation. Given that EGR2 is also essential for effective secretion of TGF-β from $LAG3^{+}$ Tregs [27], we inferred that in IgAV, low IL-27 may impair Tr1 cells via EGR2. However, *EGR2* mRNA expression was measured in PBMCs from IgAV patients, and the expression level did not represent EGR2 in Tr1 cells, which is a limitation of this study.

Since Tr1 cells are associated with long-term transplantation tolerance, we investigated the association of Tr1 cells with the prognosis of IgAV. Interestingly, compared with Tr1 in acute phase, Tr1 in remission phase was more suitable for prognosis prediction. The results showed that the patients with lower Tr1 cells in remission were more likely to recur. Tr1 cells in IgAV exert a defective regulatory function, so the lower Tr1 cells were not conducive to re-establish immune homeostasis. The persistent dysregulation of the immune system might contribute to the high recurrence rate. Further exploration found no other clinical factors affecting the recurrence of IgAV. This suggested that Tr1 cells may be a risk factor for IgAV recurrence, and targeting Tr1 cells may offer potential therapeutic strategies for the treatment of HSP to reduce the recurrence rate. However, it should be noted that the number of patients in this study was small, the follow-up time was short and the sampling was not random. Hence, there is a need to explore more patients in a more rigorous design in the future.

In summary, this study revealed impaired frequency and function of circulating Tr1 cells in IgAV, but an increased expression in kidney tissue, suggesting a potential role of Tr1 cells in IgAV pathogenesis. The low IL-27 in IgAV was responsible for impaired frequency and function of Tr1 cells, and EGR2 may be the specific transcription factor involved in the progression of IgAV. Furthermore, Tr1 cells may be a risk factor for IgAV recurrence.

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Data availability statement

Data are available upon reasonable request by any qualified researchers who engage in rigorous, independent scientific research, and will be provided following review and approval of a research proposal and Statistical Analysis Plan (SAP) and execution of a Data Sharing Agreement (DSA). All data relevant to the study are included in the article.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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