GITR/GITRL interaction promotes the expansion of T helper 9 and T helper 17 in psoriatic arthritis

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Abstract

Objective

Psoriatic arthritis (PsA) is a chronic inflammatory disease characterised by the involvement of multiple targets. Accumulating evidence suggests the key role played by T helper (Th)9 and Th17 cells in PsA. Recently, the ability to activate GITR in promoting differentiation and proliferation of Th17 and Th9 cells has been investigated in several inflammatory conditions. We aimed to evaluate the effects of GITR/GITRL interaction in the immune responses underlying the disease, including the main PsA target sites.

Methods

Twenty-one PsA patients with active disease, naive to disease-modifying anti-rheumatic drugs, were enrolled. Peripheral blood mononuclear cells and synovial fluid (SF) mononuclear cells were collected to assess GITR and GITRL expression by flow cytometry. An in vitro functional assay with recombinant GITR agonist was performed to detect the effect on T cell subsets. Synovial and ileal biopsies were obtained to evaluate GITR and GITRL expression by immunofluorescence. Healthy subjects and osteoarthritis patients were enrolled as controls.

Results

We reported an increased in vitro expression of GITR among $CD4^+$ T cells and its cognate ligand GITRL on antigen-presenting cells in PsA peripheral blood. In vitro, the addition of the GITR agonist resulted in increased expansion of Th9 and Th17 cells. Increased expression of GITR and GITRL was found even in PsA SF, synovium and ileum.

Conclusion

Our results suggest a novel role of GITR/GITRL in promoting the expansion of Th9 and Th17 in PsA-inflamed tissues.

Key words

psoriatic arthritis, spondyloarthritis, T cells, inflammation, lymphocytes

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Received on November 2, 2024; accepted in revised form on July 28, 2025.

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Competing interests: none declared.

Introduction

Psoriatic arthritis (PsA) is a chronic inflammatory disease that belongs to Spondyloarthritis (SpA) (1) affecting multiple target tissues (2). IL-17 and IL-9 cytokine axis play a pivotal role in PsA pathogenesis, with Th17 and Th9 being expanded in peripheral blood and infiltrating skin, enthesis, gut and synovium of patients.

Recently, several studies have demonstrated a strong correlation between IL-9 and glucocorticoid-induced tumour necrosis factor-related receptor (GITR), a member of tumour necrosis factor (TNF) receptor superfamily (TNFRSF) (3-5).

GITR is expressed on tissue-infiltrating macrophages, dendritic cells (DC), natural killer cells (NK), effector T (Teff) cells $CD4^+$, $CD8^+$ and T regulatory (Treg) cells, while GITR ligand (GITRL) is mainly expressed on antigen presenting cells (APC), endothelial and parenchymal tissue cells (6), (7). Specifically, GITR signalling acts as a costimulatory axis for both $CD4^+$ e $CD8^+$ T cells, enhancing their proliferation and activation and promoting the differentiation of Th0 in IL-9-producing $CD4^+$ T (Th9) cells (8).

Taking into account the key role of these two cell subsets, Th17 and Th9, in the immunopathogenesis of PsA, coupled with the proinflammatory function of GITR activation in autoimmune diseases, we aimed to study the effects of GITR/GITRL interaction in the immune responses underlying PsA (6, 7).

Materials and methods

Patients

Twenty-one active, naive PsA patients, 16 healthy controls (HC) and 16 osteoarthritis (OA) patients, consecutively referred to the Policlinico Paolo Giaccone University Hospital of Palermo, were enrolled (Table I). Blood samples and synovial fluid samples were collected. Synovial fluid was analysed to exclude potential infectious causes of effusion.

Ethical considerations

The study was approved by local Ethical Committee of the University of Palermo and complied with the dictates of the Declaration of Helsinki (registration no. 04/2019). Informed consent was obtained from all participants.

Isolation of peripheral blood mononuclear cells (PBMC) and SF cells

After collection of blood and SF samples, PBMC and SFMC were isolated by density gradient centrifugation on Ficoll-Hypaque. Cell viability was always >95% (9).

In vitro functional assay

PBMC of patients and controls, and SFMC of PsA patients were cultured in: i. RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine and antibiotics, ii. complete RPMI medium plus T Cell TransAct (T cell activation via CD3 and CD28), for 48 hours at 37°C and 5% of CO2, in the presence of 10 mg/ml of monensin. Cells were stimulated with the human recombinant protein TNFSF18 for 48 hours at 37°C and 5% of CO2 plus 10 mg/ml of monensin.

SFMC from PsA and OA were *ex vivo* labelled. 2 x 10^6 cells were used for each experimental condition.

Cell staining and flow cytometric analysis

After *in vitro* incubation, cells were stained with the following conjugated monoclonal antibodies (mAbs): APC-Vio 770 anti-human CD45; FITC anti-human CD3, PE anti-human GITR, PerCp-Vio 700 anti-human CD4, Pe-Vio 615 anti-human CD8, PerCP/Cyanine5.5 anti-human IL-9, Pe-Vio 770 anti-human CD4, Pe-Vio 615 anti-human IL-17, VioGreen anti-human CD3, FITC anti-human CD14, PE anti-human CD11c, PerCp anti-human GITRL, Pe-Vio 770 anti-human HLA-DR, APC anti-human CD19, Alexa Fluor®405 anti-human $\alpha4\beta7$.

The cells were permeabilised for intracellular staining using the 'inside stain kit' for the determination of Th9 ($CD4^+CD3^+IL9^+$) and Th17 ($CD4^+CD3^+IL17^+$), and acquired on FACSAria II flow cytometer. At least 100.000 cells (events) were acquired for each sample. The data were analysed with FlowJo software (v. 10.5.3 Treestar Inc., Ashland, OR, USA).

Table I. Demographic and clinical characteristics of patients and controls.

	PsA (n=21)	OA (n=16)	HC (n=16)
Age mean, years (range)	50.4 (32–70)	61 (45–79)	47.2 (31–60)
Female sex, n (%)	9 (42.8)	8 (50)	6 (37.5)
Disease duration, months (range)	84 (6–240)	–	–
CRP mg/l, mean (range)	10.2 (4–25.2)	3.4 (0–4.6)	3.5 (1–5.2)
DAPSA score, mean (range)	21.6 (14.3–35.1)	–	–

CRP: C-reactive protein; DAPSA: Disease Activity in PSoriatic Arthritis; HC: healthy control; OA: osteoarthritis; PsA: psoriatic arthritis.

Immunofluorescence staining

Immunofluorescence staining was performed on 5-μm-thick paraffin-embedded sections of PsA and HC ileum and PsA and OA synovium, obtained from the Hospital biobank. Synovial samples were obtained from prosthetic knee surgery. The sections were treated to remove paraffin. Antigens were unmasked after rehydration using Dako Target Retrieval Solution. All sections were incubated with rabbit polyclonal TNFSF18 antibody and processed by secondary staining with goat anti-rabbit FITC. Then, the sections were stained with monoclonal mouse anti-human CD19 and mouse anti-human CD11c. Sections stained with mouse anti-human CD68 were previously permeabilised with 0.1% Triton X-100 for 10 minutes. Primary antibodies were incubated overnight at 4 °C. Sections were incubated with rabbit anti-mouse Alexa Fluor®555 according to the manufacturer's guideline. Nuclei were counterstained with Hoechst 33342 for 15 min at room temperature. Sections rehydrated, fixed and stained with only secondary antibodies were used as negative control. Lif files of images were collected by confocal laser-scanning microscope DMI6000 with Leica Application Suite X.

Statistical analysis

All data were analysed using GraphPad

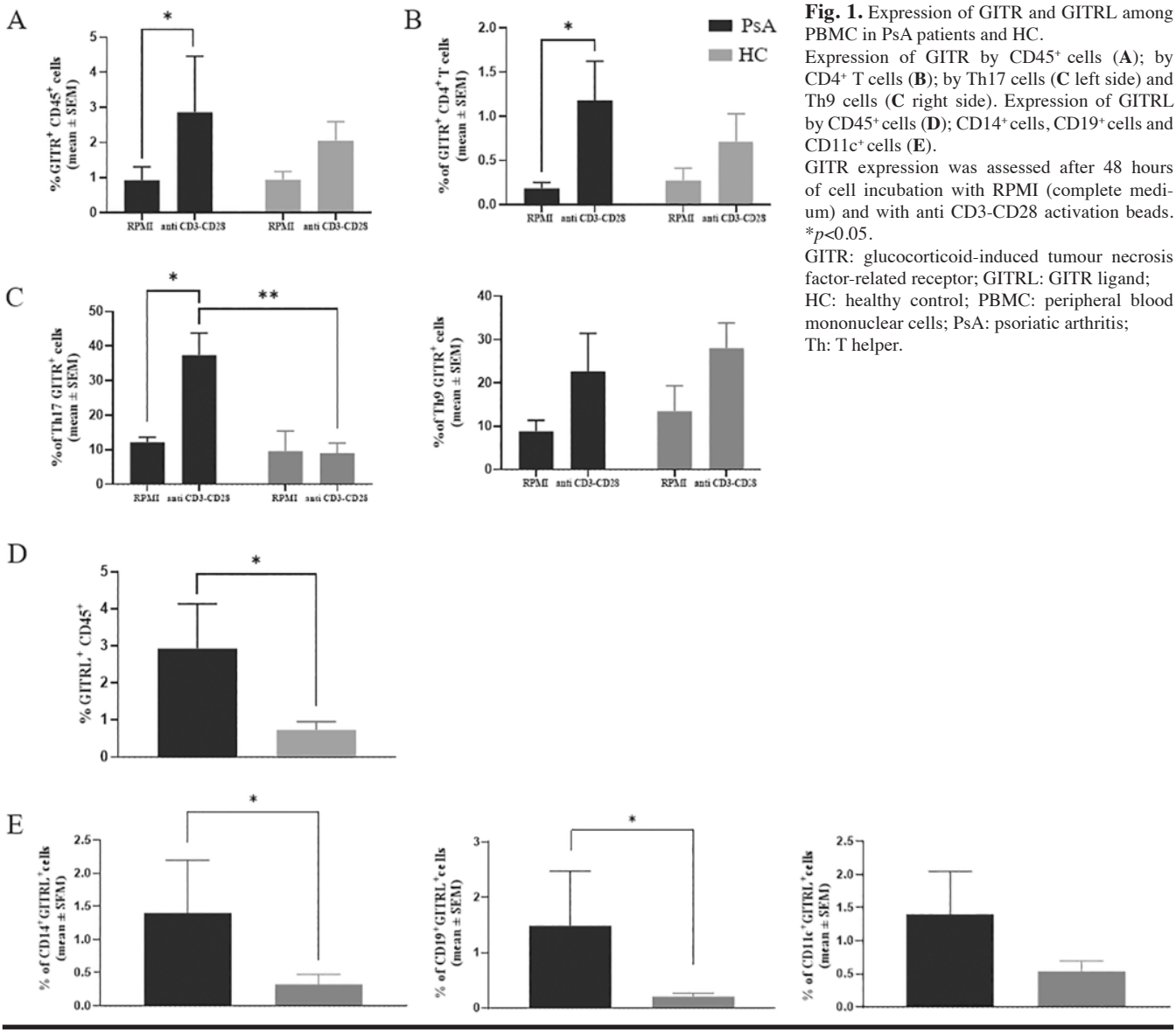

Fig. 1. Expression of GITR and GITRL among PBMC in PsA patients and HC.
Expression of GITR by $CD45^+$ cells (**A**); by $CD4^+$ T cells (**B**); by Th17 cells (**C** left side) and Th9 cells (**C** right side). Expression of GITRL by $CD45^+$ cells (**D**); $CD14^+$ cells, $CD19^+$ cells and $CD11c^+$ cells (**E**).
GITR expression was assessed after 48 hours of cell incubation with RPMI (complete medium) and with anti CD3-CD28 activation beads. *$p<0.05$.
GITR: glucocorticoid-induced tumour necrosis factor-related receptor; GITRL: GITR ligand; HC: healthy control; PBMC: peripheral blood mononuclear cells; PsA: psoriatic arthritis; Th: T helper.

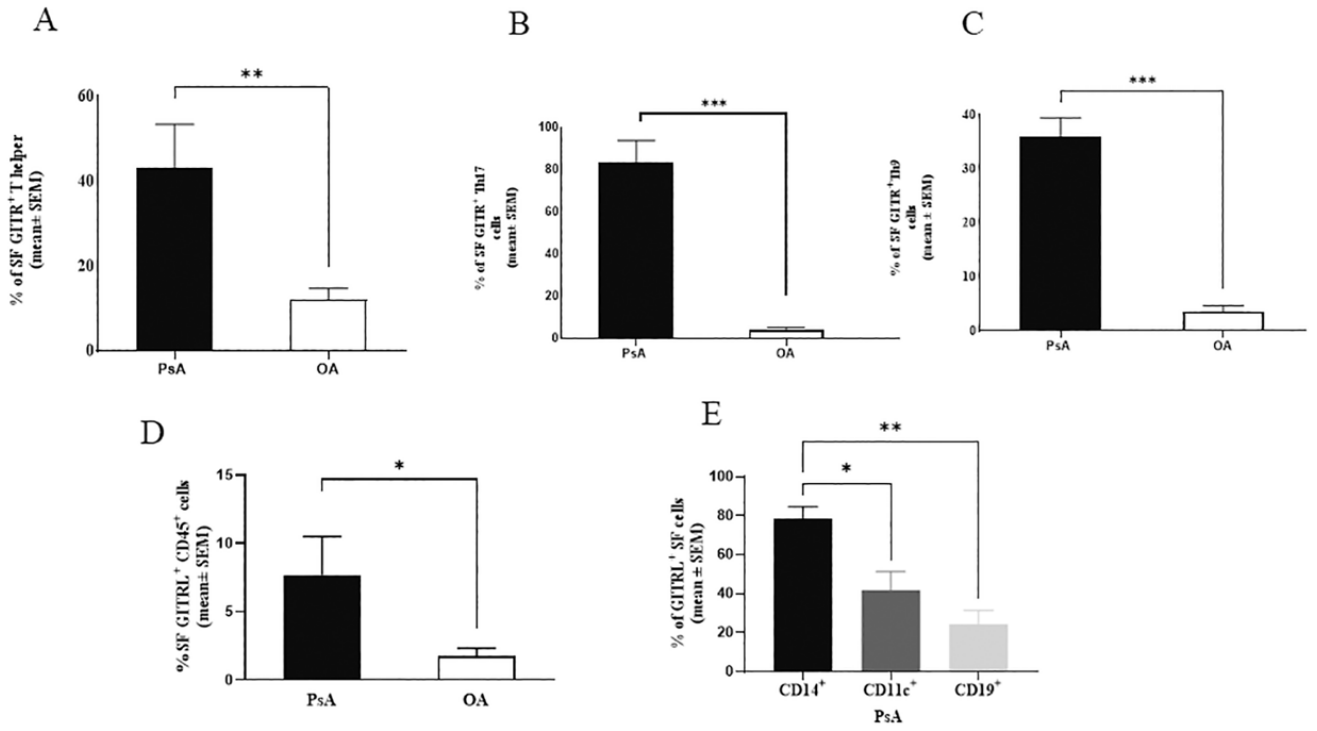

Fig. 2. GITR and GITRL *ex vivo* expression by SF cells of PsA and OA patients.
Expression of GITR by Th cells (**A**), Th17 cells (**B**) and Th9 cells (**C**);
Expression of GITRL by CD45+ cells of PsA and OA patients (**D**); by CD14+ cells, CD11c+ cells and CD19+ cells of PsA patients (**E**). *p<0.05 **p<0.005.
GITR: glucocorticoid-induced tumour necrosis factor-related receptor; GITRL: GITR ligand;
OA: osteoarthritis; PsA: psoriatic arthritis; SF: synovial fluid; Th: T helper.

Prism version 8.0.1. Statistical analysis was performed using t-test and ANOVA; p-values <0.05 were considered significant.

Results

GITR and GITRL expression is enhanced on CD4+ T cells and APC in PsA patients

Among PBMC, flow cytometry analysis showed an enhanced expression of GITR on CD45+ cells and CD4+ T cells of PsA patients compared with HC after *in vitro* stimulation for 48 hours with anti-CD3-CD28 mAb (Fig. 1A-B). Th17 showed an enhanced GITR expression statistically significant after stimulation, while GITR expression in Th9 did not change between PsA and HC (Fig. 1C).

Assessing GITRL expression, CD45+ cells of PsA patients showed an increased expression of GITRL compared with HC (Fig. 1D). Specifically, GITRL was up-regulated on CD14+, CD19+ and CD11c+ cells in PsA *versus* HC, statistically significant in the first two cell subsets (Fig. 1E).

The frequency of cell subsets and the expression levels of human leukocyte antigen (HLA)-DR, constitutionally expressed on CD14+, CD11c+ and CD19+ cells were not different between the two groups.

GITR and GITRL expression is increased in PsA SF, synovium and ileum and peripheral GITR+ Th cells recirculate from the gut

An increased GITR expression was detected on Th cells, especially Th17 and Th9, in SF of PsA patients *versus* OA patients (Fig. 2A-B-C), together with an increase in GITRL expression on CD45+ cells in PsA SF (Fig. 2D). Among APC, GITRL was more expressed by CD14+ cells in PsA SF (Fig. 2E).

Immunofluorescence on PsA synovial and ileal samples revealed an overexpression of both GITRL and GITR in PsA samples *versus* controls (Fig. 3A-B). The expression of α4β7, as marker of intestinal homing, was assessed on peripheral GITR+ and GITR- Th cells evidencing a significant higher expression of such integrin on peripheral GITR+ Th PsA derived cells (Fig. 3C).

GITR stimulation induces Th9 and Th17 expansion in vitro in PsA patients

Given the up-regulation of GITRL on APC and GITR on CD4+ cells in PsA samples, we stimulated PBMC from PsA with the recombinant GITR agonist. *In vitro* stimulation with recombinant GITR agonist for 48 hours resulted in an increased expansion of PsA-derived Th9 and Th17 cells, assessed by flow cytometry analysis, in presence of anti-CD3-CD28 stimulation beads, statistically significant compared with HC (Fig. 4A-B).

The effect of GITR agonist stimulation was also evaluated on Th9 and Th17 cells from SF, resulting in an increased frequency of SF Th9 and Th17 in PsA *versus* OA (Fig. 4C-D).

Discussion

The proinflammatory function of GITR activation in autoimmune diseases has recently been highlighted (10); GITR stimulation can promote the differentiation and proliferation of Th17 and Th9 (7), and the administration of recombinant GITRL exacerbated the pro-

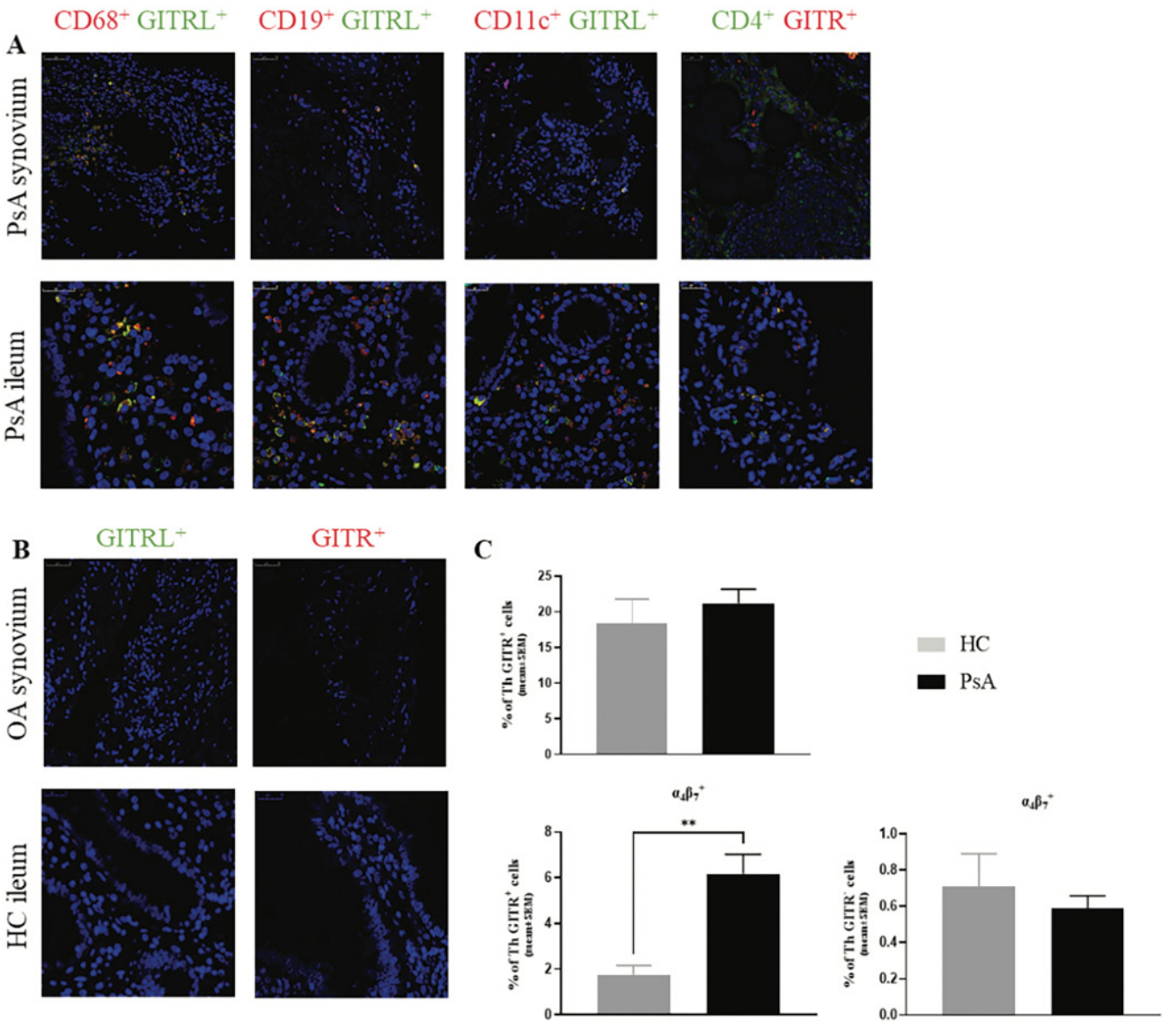

Fig. 3. GITRL and GITR evaluation on synovium and ileum of PsA patients and controls and α4β7 expression among Th GITR$^+$ and GITR$^-$ cells in PB.
Representative merge panel of PsA synovial and ileal tissue (**A**): GITRL$^+$ (green) - CD68$^+$ (red) - nuclei (blue); GITRL$^+$ (green) - CD19$^+$ (red) - nuclei (blue); GITRL$^+$ (green) - CD11c$^+$ (red) - nuclei (blue); CD4$^+$ (green) - GITR$^+$ (red) - nuclei (blue).
Representative merge panel of OA synovial tissue and HC ileal tissue (**B**): GITRL$^+$ (green) - nuclei (blue); GITR$^+$ (red) - nuclei (blue).
Expression of GITR among PB Th cells from PsA and HC (**C**, upper part).
Expression of α4β7 among PB Th GITR$^+$cells from PsA and HC (**C**, bottom left)
Expression of α4β7 among PB Th GITR$^-$ cells from PsA and HC (**C**, bottom right)
GITR: glucocorticoid-induced tumour necrosis factor-related receptor; GITRL: GITR ligand;
HC: healthy control; OA: osteoarthritis; PB: peripheral blood; PsA: psoriatic arthritis; Th: T helper.

gression of arthritis in CIA mice, confirming the role of the GITR/GITRL axis in determining joint inflammation (11).

In this study, we provided the first evidence for the role of GITR/GITRL interaction in the immunopathogenesis of PsA. Our results evidenced an increased GITR expression among peripheral CD4$^+$ T cells, specifically after stimulation, in line with the inducible nature of the receptor, and a concomitant increased GITRL expression on APC in PsA patients *versus* HC.

Considering the systemic nature of the inflammatory response in PsA (12), we assessed GITR/GITRL expression in multiple target tissues. The finding of an enhanced GITR/GITRL expression in PsA SF, synovium and ileum corroborates the fascinating hypothesis of the gut-joint axis as a pivotal mechanism in the development of PsA. Specifically, GITR/GITRL may cooperate in the activation of immune cells in the gut, favouring the interaction between APC and T cells that can then recirculate and reach target sites of inflammation (9). This hypothesis is further supported by the expression of α4β7, marker of intestinal homing, on the peripheral GITR$^+$ Th cells in PsA. Interestingly, we assessed whether GITR/GITRL interaction could contribute in driving the expansion of Th9 and Th17 cells in PsA.

After the addition of the recombinant

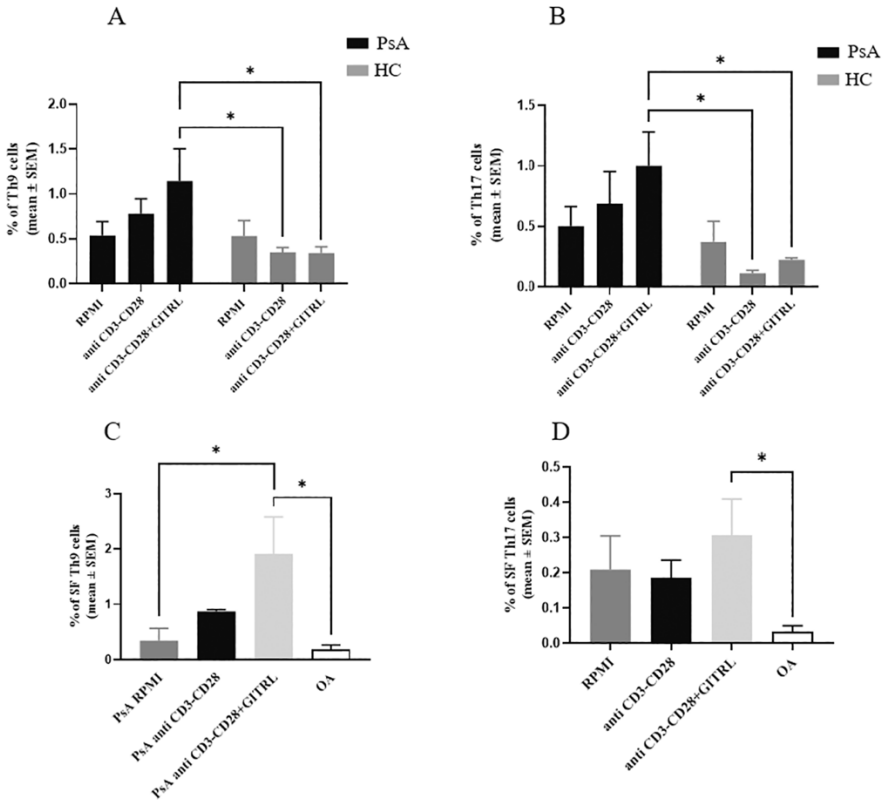

Fig. 4. Effects of recombinant GITRL on Th9 and Th17 cells frequency from PB and SF.
Frequency of Th9 cells (**A**) and Th17 cells (**B**) in PsA patients and HC from PB.
Frequency of Th9 cells (**C**) and Th17 cells (**D**) of PsA and OA patients from SF.
Cells of PsA patients and HC were incubated with RPMI (complete medium) alone, anti CD3-CD28 activation beads alone and anti CD3-CD28 + GITRL. Percentages of Th9 and Th17 were evaluated for OA samples in absence of any stimulation. *p<0.05.
GITR: glucocorticoid-induced tumour necrosis factor-related receptor; GITRL: GITR ligand; HC: healthy control; OA: osteoarthritis; PB: peripheral blood; PsA: psoriatic arthritis; SF: synovial fluid; Th: T helper.

GITR agonist, an increased expansion of peripheral Th9 and Th17 cells was detected. Our findings are in line with our previous data on the strong Th9 polarisation as the predominant immunological feature in PsA (9) and the robust data on Th17 expansion in PsA inflammatory sites (13).

Recently, expression of GITR together with OX40 was found on the surface of pathogenic Th17 cells in SF from active ankylosing spondylitis and the authors demonstrated that the simultaneous blockade of GITR and OX40 suppressed clinical arthritis in the murine model of SpA (14), pointing out the potential role of this double signal in the aberrant immune responses evidenced in SpA.

Taken together, our results let us suppose that GITR/GITRL may contribute to shape a strong proinflammatory milieu in PsA through the activation of pathogenic cell subsets and pave the way for exploring whether manipulation of this pathway may be useful in the treatment of inflammatory joint disease.

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