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Role of GITR/GITRL interaction in modulating T helper 9, T helper 17 and T regulatory cells response in psoriatic arthritis

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ABSTRACT

Objective: Psoriatic arthritis (PsA) is a systemic chronic inflammatory disease characterized by the involvement of multiple target sites. 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. Aim of the study was to evaluate the effects of GITR/GITRL interaction in the immune responses underlying the disease in different inflamed tissues.

Methods: Twenty-one PsA patients with active disease, naïve to disease modifying antirheumatic 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. Quantitative real-time reverse transcription–polymerase chain reaction was also performed. Synovial and ileal biopsies were obtained to evaluate GITR and GITRL expression by immunofluorescence. Healthy subjects and osteoarthritis patients were enrolled as controls.

Results: An increased *in vitro* expression of GITR among $CD4^+$ T cells and its cognate ligand GITRL on antigen-presenting cells in PsA peripheral blood was evidenced. *In vitro,* the addition of the GITR agonist resulted in increased expansion of Th9 and Th17 cells, and reduced suppressive capacity of T regulatory 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 with a concomitant impairment in Treg functions.

CHAPTER 1

PSORIATIC ARTHRITIS

1.1 Introduction

Psoriatic arthritis (PsA) is a multifaceted, chronic inflammatory arthritis that affects up to 30% of patients with psoriasis, a common skin condition frequently accompanied by systemic complications (1). Initially regarded as a relatively benign condition, PsA is now recognized for its potential to cause substantial functional impairment and a reduction in quality of life. Clinically, PsA is characterized by diverse phenotypes, ranging from symmetrical polyarthritis and asymmetrical oligoarthritis to distal interphalangeal (DIP) joint involvement, axial disease, and the severe deforming subtype known as arthritis mutilans. These varied manifestations, along with hallmark features like enthesitis, highlight the diagnostic complexity of PsA, often resulting in delayed treatment. Since the original descriptions by Moll and Wright in 1973 (2), advances in genetic research, imaging, and a deeper understanding of disease mechanisms have greatly enhanced the knowledge of PsA. Classification tools, such as the Classification Criteria for Psoriatic Arthritis (CASPAR) criteria introduced in 2006, have further refined the clinical identification of PsA, aligning it more closely with other forms of spondyloarthritis (SpA) (3).

In PsA, T cell subsets play a critical role in driving the inflammatory and autoimmune processes underlying the disease. Both $CD4^+$ and $CD8^+$ T cells contribute to pathogenesis through their cytokine production and interactions with other immune cells, fostering a pro-inflammatory environment within joints and entheses. Specifically, T helper (Th)1, Th17, and Th22 subsets have been shown to be key players, with Th17 cells, in particular, producing IL-17 and IL-22, cytokines that are central to the inflammation and tissue damage characteristic of PsA (4). These T cell subsets also contribute to the activation

of other immune cells, like macrophages and dendritic cells (DC), amplifying inflammatory cascades and perpetuating tissue damage (5). An additional layer of complexity arises from the imbalance between pro-inflammatory Th cells and T regulatory (Treg) cells in PsA. Treg typically function to suppress immune responses and maintain immune tolerance, yet their numbers or function appear to be compromised in PsA, resulting in unchecked Th cell activity. In particular, recent studies suggest that diminished Treg function or stability may allow Th17-driven inflammation to persist, worsening disease severity. Moreover, tissue-resident memory T cells in the skin and synovium of PsA patients may act as long-lasting reservoirs of inflammation, linking skin lesions and joint inflammation. Targeting specific T cell subsets, the Th/Treg balance, or their cytokines has become a promising therapeutic approach, as evidenced by the efficacy of IL-17 and IL-23 inhibitors in managing PsA (6). A deeper understanding of T cell subsets dynamics in PsA, particularly the interplay between Treg and Th cells, not only sheds light on disease mechanisms but also opens avenues for developing targeted, more effective therapies.

1.2 Epidemiology

PsA affects between 6 and 30% of individuals with psoriasis, which itself impacts approximately 3% of the U.S. population (7). The prevalence of PsA varies widely across studies due to differences in methodologies, geographic regions, case definitions, and criteria used to diagnose the disease. For example, prevalence estimates range from 30 to 100 cases per 10,000 with an annual incidence between 2 and 3% in patients with psoriasis (8). Population-based studies show varying rates: a recent meta-analysis across 28 studies indicated a prevalence of 133 cases per 100,000 and an incidence of 83 per

100,000 person-years (PY). Geographical disparities also play a significant role; for example, North America reports PsA prevalence rates around 158 per 100,000, while Northern Europe sees rates as high as 670 per 100,000 (9,10). In contrast, Asian and Southern European populations show lower rates, at 38 and 28 per 100,000, respectively. These differences are often attributed to genetic, environmental, and lifestyle factors, including smoking, infections, obesity, alcohol use, and the prevalence of psoriasis in different regions (11).

The timeline of PsA diagnosis has also evolved. Studies indicate that PsA prevalence and incidence rates have risen over time, possibly due to increased awareness, improved screening tools, and advanced imaging techniques that allow for earlier detection (12). For instance, studies from the U.S. and Denmark reveal rising PsA incidence rates from the 1970s through the early 2000s, suggesting that the increase may result from better diagnostic practices rather than an actual rise in disease frequency (13). Conversely, studies from Israel show stable incidence rates, illustrating how epidemiological trends can vary even within similar populations (14).

In psoriasis patients, the cumulative incidence of PsA is higher than in the general population. Studies in the U.S. and Canada reported incidence rates of 2.7 cases per 1,000 PY among psoriasis patients, emphasizing that these individuals are at a significantly higher risk. Moreover, approximately 15% of psoriasis patients are estimated to have undiagnosed PsA, with prevalence ranging from 4.2% in Germany to 33.6% in the U.S. (15).

Increased screening efforts, particularly in dermatology and general practice, are helping identify cases earlier, yet undiagnosed PsA remains a substantial issue (16,17).

Demographically, PsA typically manifests between ages 30 and 50, with no clear gender predominance according to recent meta-analyses, though some

studies report a slight male or female predominance. The disease can also begin during childhood and presents in two clinical subtypes: oligoarticular PsA, often affecting young girls and associated with antinuclear antibodies and uveitis, and a more generalized subtype with a balanced sex ratio and higher rates of enthesitis, nail pitting, and axial involvement (18,19).

Despite increased awareness and improved treatments, PsA remains associated with considerable psychological and functional burdens, comparable to rheumatoid arthritis (RA) and axial SpA. The disease frequently leads to work absenteeism and reduced productivity, with impacts closely tied to disease activity and physical function.

Mortality rates for PsA patients have improved over time, aligning with the general population in many regions, though cardiovascular-related mortality remains slightly elevated (20,21).

1.3 Pathogenesis

The pathogenesis of PsA is complex and multifactorial, involving the interplay of genetic, immune, and environmental factors that contribute to the initiation and progression of the disease. It is characterized by an aberrant immune response, where both the innate and adaptive immune systems become dysregulated, leading to chronic inflammation in the joints, entheses, and skin. Genetic factors, including specific human leukocyte antigen (HLA) alleles, notably HLA-B27, have been implicated in predisposition to PsA. Environmental triggers, such as infections, trauma, and possibly smoking, may initiate or exacerbate disease activity in genetically susceptible individuals (22).

Immune dysregulation in PsA is largely driven by T cells, particularly Th1, Th17, and Th22 subsets, which produce pro-inflammatory cytokines like

tumor necrosis factor (TNF)-α, IL-17, and IL-22. These cytokines not only promote inflammation in the joints and entheses but also contribute to the development of skin lesions in psoriasis. Furthermore, the involvement of other immune cells such as DC, macrophages, and neutrophils perpetuates inflammation, leading to tissue damage and remodeling in affected areas (23). Understanding the intricate mechanisms behind PsA pathogenesis is critical for better characterizing PsA patients and consequently for developing new treatment strategies.

1.3.1 Genetic factors

PsA is a highly heritable, polygenic disease with a strong familial component, where a family history of psoriasis or PsA significantly increases the risk of developing the disease (24). Case-control studies have demonstrated that individuals with a family history of PsA have an odds ratio (OR) of 20.5 for developing the disease, and first-degree relatives of PsA patients have a 30 to 55 times greater risk compared to the general population (25).

The presence of a family history not only affects the likelihood of disease onset but also influences the severity and phenotype of PsA, with a higher risk for joint deformities and a distinct skin disease phenotype when compared to psoriasis alone (26). A variety of genes have been implicated in the pathogenesis of PsA, particularly those involved in inflammatory pathways, although their individual effect sizes are generally modest, limiting their utility in predicting the disease. Polymorphisms within the IL-17/IL-23 pathway (including IL23R and IL12B), NF-κB signaling pathways (such as TNFAIP3), and other inflammatory pathways (such as NOS2, IFIH1, TNF-α-238A/G and -857T/C) have been associated with PsA. Additionally, deletions in LCE3C/B, MICA A9, and polymorphisms in PTPN22 have also been found

to contribute to disease susceptibility (27,28).

Despite these associations, the precise functional implications of these genetic variants remain unclear. The most notable genetic markers for PsA are located in the human HLA region, with specific alleles linked to increased risk for the disease. HLA-B*27* is particularly associated with severe forms of PsA, including enthesitis, peripheral joint damage, and axial involvement, but not with psoriasis itself (29,30). Other alleles, such as HLA-B38 and HLA-B39, are specifically linked to peripheral polyarticular involvement. Although HLA-C06 is the strongest genetic marker for psoriasis, its frequency is significantly lower in PsA patients compared to those with psoriasis alone, and it is associated with a longer interval between the onset of skin and joint disease (31,32). Importantly, while these genetic findings provide insights into the pathogenesis of PsA, they are not sufficiently robust to recommend genetic screening for diagnosis due to the small effect sizes and lack of sufficient evidence to improve diagnostic accuracy (25). These genetic data, however, contribute to a deeper understanding of the complex interactions between genetic susceptibility and environmental triggers in the development and progression of PsA.

1.3.2 Environmental factors

There are several environmental risk factors for PsA (33). These include obesity; severe psoriasis; scalp, genital, and inverse psoriasis; nail disease; and trauma or deep lesions at sites of trauma, defined as Koebner's phenomenon (34).

Biomechanical stress is increasingly recognized as a significant factor in the onset and progression of PsA, particularly in individuals with a genetic predisposition (35). This stress can be both physical, due to repetitive joint

movements or mechanical loading, and structural, related to inflammation at the sites where tendons, ligaments, joint capsules, and pulleys attach to bones, known as entheses (36,37). Enthesitis, a hallmark feature of PsA, is believed to be triggered or exacerbated by biomechanical stress, leading to inflammatory changes that affect the synovium, bone, and surrounding soft tissues (38). Physical trauma, such as injury or repetitive strain, may activate the immune system at these sites, potentially initiating the inflammatory cascade that contributes to the development of PsA (39). The Koebner phenomenon, which is characterized by the appearance of psoriatic lesions at sites of injury or trauma, suggests that mechanical stress at the skin level may also play a role in deep tissue inflammation (40). Furthermore, studies have shown that patients with psoriasis who engage in activities involving heavy lifting or excessive joint loading are at a higher risk of developing PsA (41). This suggests that mechanical overload on joints may not only cause localized damage but also promote the immune system's inappropriate activation, leading to chronic inflammation and joint involvement (42). In addition to direct mechanical forces, altered posture, gait abnormalities, and uneven distribution of weight can further exacerbate stress at enthesial sites, influencing disease progression (43,44). Therefore, biomechanical stress, particularly at sites of enthesis and joint movement, likely plays a crucial role in both the initiation and chronicity of PsA, underscoring the need for careful management of physical stressors in susceptible individuals (45).

In addition to physical factors, metabolic abnormalities, such as obesity, hyperlipidaemia, and hyperuricemia, have been recognized as significant contributors to PsA risk (46). Studies show that a higher body mass index (BMI), particularly from early adulthood, increases the likelihood of developing PsA in individuals with psoriasis (47). Moreover, weight reduction

through interventions like gastric bypass surgery has been associated with a decreased risk of developing both psoriasis and PsA, as well as improved disease prognosis, highlighting the potential role of lifestyle modifications in disease prevention (48,49). The role of smoking in PsA remains controversial. Some studies have suggested a paradoxical protective effect in psoriasis patients, while others have found that smoking may increase the risk of PsA in the general population, with some reports indicating smoking as a risk factor specifically for axial PsA (axPsA) (50). Alcohol consumption has also been implicated, with heavy drinking being associated with an elevated risk of PsA, particularly in women, while moderate alcohol intake seems to have a protective effect, although this relationship is still under investigation (51,52). The role of drugs in the development and progression of PsA has been explored in several studies, with some medications showing potential associations with an increased risk of disease onset (53). A large cohort study of U.S. women found that long-term use of acetaminophen (hazard ratio [HR] 3.60) and nonsteroidal anti-inflammatory drugs (NSAIDs) (HR 2.10) may be linked to a higher risk of developing PsA. Interestingly, no association was found between aspirin use and PsA risk in this cohort. Another study identified the use of corticosteroids within two years prior to the onset of psoriasis as a potential risk factor for PsA development, with an OR of 4.33 (54). This suggests that corticosteroids might influence the pathogenesis of PsA in genetically predisposed individuals. Retinoid medications were also associated with an increased risk of PsA, with a relative risk (RR) of 3.42, according to a prospective cohort study (55). However, the relationship between drugs and PsA is complex, as patients with PsA may experience a preclinical phase of joint pain before formal diagnosis, which could lead them to use medications like acetaminophen or NSAIDs. In such cases, the use of

these drugs might be more of an indicator of ongoing disease activity, rather than a direct causative factor. Therefore, while these studies suggest potential links between certain drugs and PsA, the causality remains difficult to establish, as confounding factors such as preclinical symptoms and medication use could bias the findings.

Recently, the microbiome, particularly the gut microbiome, has emerged as a crucial factor in the pathogenesis of PsA, highlighting the intricate connection between microbial communities and immune regulation (56). Studies have shown that patients with PsA exhibit altered microbiota composition, with reduced microbial diversity and an increased presence of pro-inflammatory bacteria compared to healthy controls (HC) (57). This dysbiosis is believed to contribute to systemic inflammation, potentially triggering the immune system in genetically predisposed individuals (58). Specific microbial species, including *Firmicutes* and *Bacteroidetes*, have been implicated in PsA, influencing immune responses that exacerbate both skin and joint inflammation (59). The gut–joint axis is of particular interest, as alterations in the gut microbiome can influence the systemic immune response through mechanisms such as the activation of innate lymphoid cells and Th17 cells, which play a central role in the inflammation seen in PsA (60). Furthermore, microbial infections, particularly those involving the gastrointestinal tract, have been linked to PsA flare-ups, suggesting that microbial pathogens can act as environmental triggers for disease onset or exacerbation (61). Additionally, the use of antibiotics, which can disrupt the gut microbiome, has been associated with an increased risk of developing PsA. The growing evidence of microbiome involvement suggests that modulating the gut microbiota may present novel therapeutic strategies for PsA, with probiotics or dietary interventions being potential avenues for reducing inflammation and

improving disease outcomes (62).

1.3.2 The immune system in psoriatic arthritis: focusing on T cell subsets

The autoimmune nature of PsA is underpinned by an activation cascade that begins with the innate immune system, involving antigen-presenting cells (APC), neutrophils, and macrophages, which release pro-inflammatory cytokines that set the stage for chronic inflammation (63). This innate activation leads to the recruitment and activation of T cells, where the adaptive immune response plays a central role in perpetuating and targeting inflammation specifically to the skin, joints, and entheses (64).

Distinct T cells subpopulations contribute to both cutaneous and joint inflammation. $CD8^+$ T cells have emerged as central players in both the initiation and progression of PsA (65,66). During the formation of psoriatic plaques, these cells, particularly those expressing the CCR4 receptor, expand clonally within the skin. Recent studies have identified a unique capability in these skin-specific, autoantigen-reactive T cells to migrate from the skin into the bloodstream and then target the synovial fluid (SF) of affected joints (67). Upon entering the joint environment, these $CD8^+$ T cells undergo a phenotypic shift, losing CCR4 expression while acquiring CXCR3, a receptor that reflects a more differentiated and cytotoxic effector phenotype (Tc) (68). This shift seems to be influenced by the inflammatory environment within the joint, which is enriched in CXCL10, a chemokine known to bind to CXCR3 (69).
In addition to $CD8^+$ T cells, $CD4^+$ T cells, especially Th1 and Th17, play a crucial role in amplifying inflammation in PsA. These $CD4^+$ T cells, activated in psoriatic plaques, can produce IL-17, a cytokine integral to inflammation in both the skin and joints (70). Evidence suggests that some of these $CD4^+$ T cells

found in the SF may have originated from psoriatic lesions in the skin, highlighting a potential link between cutaneous and joint inflammation in PsA. While γδ T cells are an unconventional subset of lymphocytes less prominent than other T cells in PsA, they do play an important role in psoriatic inflammation (71). Present in the skin, γδ T cells can produce IL-17, thus amplifying the local inflammatory response (72). Although their precise role in PsA remains unclear, these cells may be particularly relevant to early stages of joint inflammation. Meanwhile, tissue-resident memory T cells (TRM) represent another key component of the cutaneous immune response in PsA (73). Both $CD4^+$ and $CD8^+$ TRM cells remain in the epidermis following infection or inflammation and are primed for rapid immune responses upon antigen re-exposure. In PsA, $CD8^+$ TRM cells, particularly those with a Tc17 phenotype, are implicated in providing immune memory that may contribute to the recurrent nature of psoriatic lesions (74).

In contrast, Treg, which are typically involved in dampening immune responses and maintaining tolerance, appear to be dysregulated in PsA, potentially failing to control the activity of pro-inflammatory Th cells and thus allowing inflammation to persist (75).

Thus, T cells demonstrate a complex and dynamic involvement in PsA pathogenesis. $CD8^+$ T cells likely drive joint inflammation through their migratory behavior from skin to synovium, while $CD4^+$ T cells amplify this response. Although γδ T cells and TRM cells play supporting roles, they contribute in distinct ways to the chronic inflammation observed in PsA.

Th17

The role of IL-17 and Th17 in the pathogenesis of PsA has garnered significant attention, as these elements contribute substantially to the inflammatory

processes and joint damage observed in the disease (76). Th17 cells are uniquely characterized by their ability to produce IL-17, a potent pro-inflammatory cytokine (77). Under specific conditions, such as the presence of cytokines like IL-6, IL-23, and transforming growth factor (TGF)-β, naive T cells differentiate into Th17 cells (78). In PsA, these cells migrate to the affected joints and accumulate within the SF, where they play a central role in driving local inflammation. Though the precise conditions within the joint are complex, studies have shown that Th17 cells, upon stimulation, are capable of producing IL-17 (79), supporting their involvement in the inflammatory milieu of PsA (80).

IL-17 binds to its receptor, which is expressed on a variety of cell types, including synovial fibroblasts, chondrocytes, and osteoblasts. This binding triggers a cascade of intracellular signals that culminate in the production of other pro-inflammatory cytokines, chemokines, and matrix metalloproteinases (81). Specifically, IL-17A induces synovial fibroblasts to produce cytokines such as IL-6, IL-8, and MMP-3, which contribute to the ongoing inflammation and damage to cartilage in the affected joints (82). Furthermore, IL-17A promotes bone erosion by upregulating the expression of RANKL, a key molecule involved in osteoclast differentiation and bone resorption. This combination of cartilage destruction and bone remodeling is a hallmark of PsA, underscoring the pivotal role of IL-17A in the disease's pathophysiology (83). While IL-17A is primarily responsible for initiating tissue injury, IL-17F appears to be more involved in sustaining and driving the chronicization of the inflammatory response. Although IL-17F signaling responses are generally weaker than those of IL-17A, the two cytokines act synergistically, amplifying the inflammatory cascade (84).

In addition to the direct effects of IL-17A, the cytokine IL-23 is integral to the

differentiation and activation of Th17 cells (85). Genetic studies have identified polymorphisms in the IL23R gene that are associated with an increased susceptibility to PsA, highlighting the importance of IL-23 in the disease's development (86,87). IL-23, produced by immune cells such as macrophages and DC, acts to promote the survival, proliferation, and continued activation of Th17 cells (88). The presence of IL-23 is thus critical for maintaining the Th17-driven inflammation that characterizes PsA (89). Interestingly, the enthesial tissue in PsA can produce IL-17 even without the typical IL-23 stimulation, indicating its importance in the disease's pathogenesis (90).

Th9

Th9 cells promote inflammation in both intestinal and synovial environments and are characterized by the production of IL-9, a pleiotropic cytokine with pro-inflammatory activity (91). IL-9 exerts its effects through binding with the IL-9R, which is expressed on various immune and non-immune cells, including effector T cells, B cells, mast cells, epithelial cells, and smooth muscle cells.

Th9 expansion at multiple tissue levels is a key feature of PsA (92), that is driven by several cytokines, such as TGF-β, IL-4 and Thymic Stromal Lymphopoietin (TSLP), produced by epithelial and stromal cells. Moreover, the transcriptional machinery involved in Th9 development and IL-9 gene locus activation include the critical transcription factor PU.1, as well as, Interferon Regulatory Factor-4 (IRF-4) and B cell Activating Transcription Factor-like (BATF) (93). Several other signals, such as IL-25, IL-33, type I interferons and IL-1β modulate IL-9 production in Th9 cells by activating the NF-κB transcription factor and inducing STAT-1 and IRF-1 expression (94). Finally, interactions between T cells and APC, mediated by TCR/MHC II,

CD28/CD80, OX40/OX40L, and NOTCH ligands like DLL and Jagged, influence Th9 differentiation, highlighting the complexity of signals required for optimal Th9 polarization (95).

IL-9 stands out as a specific marker of intestinal inflammation in PsA, distinguishing it from other inflammatory diseases such as ankylosing spondylitis (AS) and Crohn's disease (96). A particularly high IL-9 expression has been observed in the ileum of PsA patients, produced by both inflammatory cells and Paneth cells. These Paneth cells, in addition to generating IL-9, express IL-9R as well, suggesting the presence of an autocrine circuit that amplifies the inflammatory response (97). IL-9 produced by Paneth cells may also contribute to the gut microbiota alterations often observed in PsA patients, establishing a link between dysbiosis and immune responses. Notably, the expression of the $\alpha 4\beta 7$ integrin, an intestinal homing marker, on Th9 cells isolated from both synovium and peripheral blood suggests that Th9 cells activated in the gut may migrate to other inflammatory sites, such as the joints, thereby contributing to PsA pathogenesis (98).

In PsA, IL-9 also plays a significant role in activating $\gamma\delta$ T cells. Specifically, the IL-9/IL-9R interaction appears to be a primary driver of $\gamma\delta$ T cell activation in PsA, with a stronger influence than the IL-23/IL-23R interaction (98).

To further stress the potential role for these cells in maintaining systemic inflammation is important to underline that the proportion of circulating Th9 cells correlates with disease activity and decreases following treatment with anti-TNF agents or ustekinumab (98).

Treg

Treg exert immunosuppressive functions and are crucial for maintaining immune homeostasis and preventing autoimmunity. Their primary

mechanism of action involves the secretion of cytokines such as IL-10 and TGF-β, which suppress the activity of other immune cells (99). However, in individuals with psoriasis, Treg cells exhibit several abnormalities, including reduced expression of CD39 and CD74, increased expression of IL-6Rα, and diminished suppressive capacity. Additionally, they display a greater tendency to differentiate into IL-17-producing cells (6).

These alterations contribute to an imbalance between pro-inflammatory and anti-inflammatory responses, creating a microenvironment conducive to the chronic inflammation characteristic of psoriasis (100). IL-23 appears to play a key role in Treg dysfunction (101). This cytokine can reduce the expression of Foxp3, the essential transcription factor for Treg development and function, and promote their differentiation into Th17 cells, further amplifying inflammation. The balance between the expression of Foxp3 and RORγt, the transcription factor guiding Th17 differentiation, is critical in determining whether Treg cells maintain their immunomodulatory profile or adopt a pro-inflammatory role (102).

Although the available data on Treg cells PsA is more limited, some studies suggest that patients with PsA have a lower number of Treg cells in peripheral blood compared to HC, and this number negatively correlates with disease activity (103). Furthermore, Treg cells in the inflammatory microenvironment of PsA-affected joints exhibit distinct characteristics from circulating Treg. In this context, Treg cells may downregulate Foxp3, assume a phenotype and functions similar to effector T cells, and begin producing pro-inflammatory cytokines, including IL-17. They also show increased expression of inhibitory immune receptors such as CTLA-4 and TIGIT, as well as high levels of CD161, RORγt, and ICOS (103). These alterations contribute to the maintenance of joint inflammation in PsA.

1.4 Clinical manifestations

PsA presents with a diverse range of clinical manifestations, reflecting the heterogeneity of the disease, and can be divided into articular and extra-articular features (104).

1.4.1 Articular manifestations

Articular involvement is typically characterized by five major subtypes, as described by Moll and Wright, each with distinct patterns of joint involvement (2). The oligoarticular subtype affects fewer than five joints, typically in an asymmetric distribution, and is often less severe, affecting larger joints such as the knees and elbows. In contrast, the polyarticular subtype involves five or more joints, with a symmetric pattern that may resemble RA (105). This form of PsA can lead to joint deformities and functional impairments (106). The distal subtype, which targets the distal interphalangeal (DIP) joints of the hands and feet, usually occurs in combination with other subtypes and is frequently associated with nail changes, including pitting and onycholysis (107,108). Although the arthritis mutilans subtype is rare, it is a severe and destructive form, leading to rapid bone resorption, joint deformity, and "telescoping" fingers or flail digits (109). The axial subtype, primarily affects the spine and sacroiliac joints, causing inflammation, stiffness, and, over time, spinal fusion, contributing to a significant loss of mobility (110). Enthesitis, affects 30-50% of PsA patients, most commonly in the plantar fascia, Achilles tendon, patellar tendons and common extensor tendon at the lateral epicondyle of the elbow (111). This can lead to chronic pain and disability if not managed effectively (112). Dactylitis, or "sausage digits," is observed in 40-50% of PsA patients and is characterized by swelling of entire fingers or

toes, often accompanied by pain, redness, and warmth in its acute form (113,114). Chronic dactylitis, however, is marked by persistent swelling without acute inflammation, and it is often associated with more severe disease, including bone erosion and new bone formation (115).

1.4.2 Extra-articular manifestations

Beyond the articular symptoms, extra-articular manifestations also play a significant role in the disease course and can provide important diagnostic clues (116). Psoriatic skin lesions are the hallmark of PsA and are often seen in areas such as the scalp, elbows, knees, and lower back, though they can also appear in more unusual locations like the umbilical area and natal cleft. These lesions, which may precede or coincide with joint symptoms, are essential in differentiating PsA from other forms of arthritis (117). Nail involvement, including nail pitting, onycholysis, and hyperkeratosis, is another prominent extra-articular feature, present in up to 50% of PsA patients (118). Nail changes are frequently seen in conjunction with distal joint involvement and are considered a key early sign of PsA (119). Furthermore, PsA patients are at an increased risk for developing inflammatory bowel disease (IBD), particularly Crohn's disease and ulcerative colitis (120). This association is believed to stem from shared immunological mechanisms, particularly involving the IL-23/IL-17 axis, which plays a central role in both PsA and IBD (121). Uveitis, particularly anterior uveitis, affects 10-30% of PsA patients, especially those with the axial subtype (122). This can present with eye pain, redness, and blurred vision, and if left untreated, can lead to vision loss (123). Moreover, PsA is associated with an increased risk of cardiovascular disease, including atherosclerosis and myocardial infarction, likely due to the chronic systemic inflammation that characterizes the disease (124). Finally, patients with PsA

are also more likely to develop metabolic syndrome, with higher rates of obesity, hypertension, and dyslipidaemia (125). These metabolic abnormalities are linked to the inflammatory process in PsA and contribute to a higher risk of comorbid conditions.

The wide range of symptoms, coupled with the potential for disease progression and complications, underscores the importance of a comprehensive clinical assessment in managing PsA (126).

1.5 Diagnosis and classification

The diagnosis of PsA is primarily clinical and relies on the recognition of characteristic patterns of joint involvement, skin manifestations, and extra-articular features, supported by imaging studies (127). PsA commonly presents with oligoarticular involvement at onset, often asymmetric, although it can evolve to a polyarticular, symmetric form over time. It typically affects the DIP, especially in the hands, which distinguishes it from RA, where proximal joints are more commonly involved, and from osteoarthritis (OA), where DIP involvement is not associated with inflammatory changes but with bony osteophytes. The presence of dactylitis, enthesitis and nail changes further support the diagnosis (128). Spinal involvement in PsA, particularly sacroiliitis and syndesmophytes, distinguishes it from RA, where spinal disease is less frequent. A careful assessment of family history of psoriasis, presence of psoriatic skin lesions, and extra-articular manifestations such as IBD or uveitis is essential. Differential diagnosis must consider conditions with overlapping clinical features (129). RA can be distinguished by its symmetric joint distribution and the involvement of proximal joints, sparing the DIP. Gout and pseudogout may mimic the acute joint involvement seen in PsA, especially monoarthritis affecting the toes, but these conditions typically

present with crystal-induced inflammation, which can be confirmed through SF analysis or ultrasound study. The asymmetric spinal involvement in PsA distinguishes it from AS, where the disease typically presents earlier in life and is more severe with symmetric sacroiliac involvement (130). Reactive arthritis shares some similarities with PsA, especially in the context of skin and joint involvement, but the pathogenesis and clinical features are distinct. The skin lesions in subacute cutaneous lupus can resemble psoriasis but are not accompanied by the joint manifestations and specific patterns of enthesitis and dactylitis seen in PsA (131). Ultimately, diagnosing PsA requires a comprehensive evaluation, including clinical assessment, imaging, and consideration of family and personal history, with an emphasis on identifying the characteristic features that differentiate it from other forms of inflammatory arthritis (132).

Diagnostic tests for PsA include negative results for rheumatoid factor and anti–cyclic citrullinated peptide antibodies in 95% of cases, and when positive, clinical and imaging features must be used to differentiate it from RA. HLA-B27 positivity occurs in approximately 25% of patients with PsA (133). Inflammatory markers, such as C-reactive protein and erythrocyte sedimentation rate, are elevated in only 40% of patients (134).

Imaging plays a crucial role in diagnosing PsA, with radiographs showing characteristic bone loss with eccentric erosions, joint-space narrowing, and new bone formation in the form of periostitis, bony ankylosis, and enthesophytes. In the axial skeleton, unilateral sacroiliitis and bulky, vertical syndesmophytes are more common in PsA compared to the bilateral sacroiliac involvement and paramarginal syndesmophytes observed in AS. Magnetic resonance imaging (MRI) may reveal focal erosions, synovitis, and bone marrow edema, especially at entheses (135). Bone marrow edema is best

observed on T2-weighted, fat-suppressed, short-tau inversion recovery (STIR) sequences (136). Moreover, Power Doppler (PD) ultrasound is useful for detecting synovitis, enhanced blood flow, tenosynovitis, enthesitis, and early erosive disease (137).

The classification of PsA has evolved over time, with several criteria developed to aid in its diagnosis. One of the earliest attempts to standardize the classification was the Moll and Wright criteria (1973), which identified five major clinical subtypes of PsA: oligoarticular, polyarticular, distal, arthritis mutilans, and axial (2).

Other historical criteria have also contributed significantly to the classification of PsA, such as the European Spondyloarthropathy Study Group (ESSG) and the American College of Rheumatology (ACR) criteria. Both sets were found less specific than following criteria and have been largely replaced by more recent ones (3,138,139).

Specifically, the CASPAR criteria, introduced in 2006, refined the classification further by integrating both clinical features and laboratory results (3). It includes major criteria such as psoriasis, nail involvement, dactylitis, and enthesitis, with supporting evidence of peripheral arthritis or spinal involvement. A patient is classified as having PsA if they have at least three of the following: (1) current psoriasis, (2) nail dystrophy, (3) dactylitis, (4) history of psoriasis, (5) negative rheumatoid factor, and (6) clinical or radiographic evidence of bone erosion or new bone formation. The CASPAR criteria has shown a high sensitivity and specificity for PsA, making it a widely used tool in clinical practice (140). In addition to the CASPAR criteria, the Assessment of SpondyloArthritis International Society (ASAS) criteria for peripheral SpA, are also important for identifying cases of PsA, particularly when peripheral joint involvement is predominant. The ASAS criteria focus on key features of

spondyloarthropathy, such as arthritis, enthesitis, dactylitis, a history of inflammatory back pain, and family history of SpA. In this framework, PsA is categorized when there is arthritis in the lower limbs, enthesitis at typical sites, dactylitis, and extra-articular manifestations like psoriasis or IBD. The presence of HLA-B27 positivity can further support the diagnosis of peripheral SpA (141). However, the ASAS criteria are used to assess a broader spectrum of peripheral SpA, and while they are helpful, they are often used in conjunction with the CASPAR criteria for more specific diagnosis of PsA.

Each of these criteria sets helps in distinguishing PsA from other inflammatory arthritis forms, particularly RA, OA, gout, pseudogout, and other spondyloarthropathies being fundamental in both clinical settings and research.

1.6 Therapeutics

The treatment of PsA is multifaceted, aimed at controlling inflammation, preventing joint damage, and improving quality of life. The approach to treatment is driven by the domains involved in each individual patient's clinical manifestations (142). These domains can include peripheral arthritis, axial disease, enthesitis, dactylitis, and skin involvement, and the therapy should be tailored to target the specific features present (143).

1.6.1 Treatment options for psoriatic arthritis

The treatment strategy generally follows a stepwise model, beginning with non-biological therapies before progressing to biological agents for more severe or refractory disease (144). Non-biological drugs, primarily NSAIDs, are commonly used to manage pain and inflammation in mild cases. These agents are effective in reducing joint pain and stiffness, particularly in the early

stages of PsA or in patients with predominant axial or enthesial involvement. In addition to NSAIDs, conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), such as methotrexate, sulfasalazine, and leflunomide, are often used to modify the course of the disease and prevent long-term damage (145). Methotrexate is the most commonly prescribed csDMARD for PsA, particularly in cases with significant peripheral arthritis, although its effectiveness in treating skin psoriasis is limited. Sulfasalazine is another option, particularly for patients with predominantly axial disease or spondylitis, while leflunomide is used in cases of polyarthritis or dactylitis. However, these agents are not universally effective for all patients, and some may require biologic agents for more targeted therapy (146).

The introduction of biological therapies has revolutionized the treatment of PsA, particularly for patients with moderate to severe disease or those who have failed conventional treatments (147). TNF-α inhibitors, such as etanercept, infliximab, adalimumab, and certolizumab, are widely used as first-line biologic agents. These drugs are highly effective in controlling both the joint and skin manifestations of PsA by targeting the inflammatory cytokine TNF-α, which plays a central role in the pathogenesis of the disease. IL12/23 inhibitors, such as ustekinumab, guselkumab and risankizumab and IL-17 inhibitors, including secukinumab and ixekizumab, are also commonly employed, offering benefits in patients with both peripheral and axial disease, as well as psoriasis (148). These agents target key cytokines involved in the immune dysregulation seen in PsA, helping to control the overactive immune response. Additionally, JAK inhibitors, such as tofacitinib and upadacitinib, represent a newer class of systemic therapy that targets intracellular signaling pathways, offering an alternative treatment for patients with PsA who have not responded to biologics (149).

Finally, apremilast, an oral phosphodiesterase 4 (PDE4) inhibitor may be used in patients with mild to moderate disease or those who are not candidates for biologic therapies. By inhibiting PDE4, apremilast increases intracellular cyclic adenosine monophosphate (cAMP) levels, leading to a reduction in pro-inflammatory cytokine production. It offers a convenient oral alternative to biologics with a relatively favourable safety profile (150).

When choosing a treatment plan, it is essential to consider the comorbidities of each patient, as conditions like cardiovascular disease, obesity, diabetes, and IBD can affect both the course of PsA and the choice of therapy. For example, some biologics, such as TNF inhibitors, may increase the risk of infections and malignancies, which requires careful monitoring, particularly in patients with a history of infections or cancer. Additionally, the presence of psoriasis and its severity should guide therapy, as some treatments are more effective for skin manifestations, while others may focus more on joint disease (151). The choice of biologic therapy depends on the specific clinical features of PsA, the patient's comorbidities, and individual preferences (152). Overall, a personalized, domain-driven approach is critical to managing PsA effectively, with the goal of achieving disease remission, preventing long-term joint damage, and improving the overall well-being of the patient (153).

1.6.2 Unmet needs in psoriatic arthritis treatment

Despite significant advances in the treatment of PsA, several unmet needs remain, particularly with regard to treatment failure and the development of difficult-to-treat diseases (154). While biologic therapies have revolutionized the management of moderate to severe PsA, a considerable proportion of patients still experience inadequate response or disease flares over time. The rate of treatment failure in PsA varies across different therapies, with up to 40-

50% of patients failing to achieve sustained disease control on initial biologics. Moreover, patients often tend to lose response to treatment after several lines of therapy, with diminishing efficacy observed as the disease progresses and the number of treatment options increases (155). This issue is particularly notable in patients with high disease burden, multi-domain involvement, or those with long-standing disease. Furthermore, some individuals develop difficult-to-treat PsA characterized by persistent joint inflammation, enthesitis, dactylitis, and psoriasis despite adequate use of biologic therapies. These patients are often at risk for functional impairment and progressive joint damage, making it imperative to explore novel therapeutic targets and combination therapies that address the underlying immune dysregulation more effectively (156). Moreover, the development of refractory PsA raises concerns about the long-term sustainability of current biologic treatments and emphasizes the need for ongoing research to identify predictive biomarkers for treatment response and to optimize treatment algorithms. Addressing these unmet needs remains a critical challenge in improving outcomes for patients with PsA (157).

To overcome these challenges, it is essential to discover new axes of intervention that can be targeted in PsA therapy. Understanding the immune pathways and biological mechanisms that drive the disease is crucial to developing innovative treatments that go beyond the current options. One promising approach could be the alteration of the balance between inflammatory and anti-inflammatory pathways, a strategy inspired by the use of checkpoint inhibitors in oncology. By modulating these pathways, it may be possible to restore immune homeostasis and better control the pathological inflammation seen in PsA. Additionally, achieving a deeper phenotypization of patients is pivotal for addressing the heterogeneity of PsA, which currently

complicates treatment strategies and results in the treatment ceiling effect, where even the best therapies fail to provide complete and sustained disease control for all patients (158,159). By identifying distinct clinical and immunological subtypes of PsA, clinicians may be able to tailor therapies more effectively to individual patients, ensuring that treatment strategies target the underlying causes of the disease more precisely (160). The ultimate goal is to expand the therapeutic armamentarium, enabling more patients to achieve long-term remission and to break through the current limitations of treatment, thereby improving PsA management.

CHAPTER 2
GITR/GITR LIGAND AXIS

2.1 GITR and GITR ligand axis overview

The glucocorticoid-induced tumor necrosis factor receptor-related gene (GITR or TNFRSF18) is part of the TNF receptor superfamily (TNFRSF) and encodes for a type 1 membrane receptor of molecular weight 34−40 kDa (161,162). The cytoplasmic portion of the receptor has good homology with other TNFRSF family receptors such as 4-1BB, OX40, CD40 and CD27, all acting as co-activating molecules in different cells and tissues (co-stimulatory TNFR subfamily) (163).

GITR functions as a co-stimulatory molecule, impacting the activity of several immune cell populations, including T lymphocytes, NK cells, and APC (164). This modulation makes GITR pivotal in both adaptive and innate immunity, particularly within inflammatory and autoimmune processes. GITR expression is highly regulated and closely tied to the activation status of immune cells (165). In resting states, T lymphocytes (both $CD4^+$ and $CD8^+$) exhibit low GITR expression levels, which rapidly increase upon antigenic stimulation. The upregulation of GITR peaks around 2-3 days after stimulation, acting as a clear marker of T cell activation (166). This transient rise in GITR levels is significant for understanding the dynamics of immune responses, as it suggests that GITR expression mirrors the initiation and expansion phases of the immune response.

The binding of GITR to its ligand, GITRL, triggers a co-stimulatory signal that profoundly impacts effector T cell functionality. This activation cascade promotes the proliferation of effector T cells, enabling a robust immune response against pathogens (167). Additionally, GITR signaling facilitates the release of pro-inflammatory cytokines such as IL-2 and IFN-γ, which are instrumental in pathogen clearance (168,169). Notably, GITR activation also enhances resistance to apoptosis, supporting the longevity and persistence of

effector T cells within an inflammatory milieu (170). These properties underscore the role of GITR as a crucial amplifier of the immune response, enhancing T cell-mediated immunity. The expression of GITR is notably elevated on Treg, a key immunosuppressive T cell subset responsible for maintaining immune tolerance and preventing excessive inflammation (171). The GITR/GITRL interaction in Treg is complex, with short-term GITR stimulation potentially diminishing Treg suppressive functions, thus allowing a more active immune response from effector T cells (172). In contrast, prolonged GITR activation may favour the proliferation and expansion of Treg, ultimately promoting immune suppression (167). This duality suggests that GITR signaling could be context-dependent, with distinct roles in either amplifying or suppressing immune responses based on the duration and nature of the stimulus (173,174).

GITRL, the ligand for GITR, is primarily expressed on APC, including macrophages, B cells, and DC. Through binding to GITR on T cells, GITRL can initiate distinct intracellular pathways in APC that contribute to immune regulation. For example, GITRL activation on macrophages drives the production of pro-inflammatory factors, which enhances immune responses (175,176). On DC, however, GITRL activation may exert immunoregulatory effects, leading to reduced IL-12 production and promoting immune tolerance (177,178). In endothelial cells, GITRL expression rises during inflammation, facilitating leukocyte migration to inflamed tissues (179). These findings illustrate the multifaceted role of GITRL in modulating immune responses across cell types and tissue environments.

The intricate biological functions of GITR and GITRL have attracted interest as potential therapeutic targets, particularly in the fields of oncology, inflammatory diseases, and autoimmunity (180). Anti-GITR agonist

antibodies, such as DTA-1, have shown promise in murine cancer models, where they enhance the activation of $CD8^+$ T cells and inhibit Treg function, thereby exerting antitumor effects (181,182). Additionally, GITR-Fc fusion proteins, designed to block the interaction between GITR and GITRL, have demonstrated anti-inflammatory effects in preclinical models (183). This capacity to either promote or suppress immune responses through GITR/GITRL modulation offers significant potential for therapeutic applications in conditions characterized by dysregulated immunity. This nuanced understanding of GITR/GITRL biology highlights its relevance in the pathogenesis of immune-mediated diseases and its emerging role as a target in immunomodulatory therapies.

2.2 GITR/GITR ligand in autoimmunity

The GITR/GITRL system has been shown to play a significant role in autoimmune diseases where inflammation is a key pathophysiological component. In RA, GITR activation correlates with disease severity, and experimental models suggest that GITR ablation can protect against arthritis development by reducing leukocyte extravasation and inhibiting conventional $CD4^+$ and $CD8^+$ T cell activation (184). In Sjögren's syndrome, elevated serum GITRL levels are linked to increased disease activity, as measured by the ESSDAI, as well as to systemic manifestations such as leukopenia, thrombocytopenia, and pulmonary involvement (185). High GITRL levels also correlate with autoantibodies, including rheumatoid factor and anti-SSA antibodies, which are central markers of Sjögren's syndrome. Additionally, the GITRL/GITR interaction is thought to promote a Th17-driven immune response that intensifies inflammation and tissue damage, especially within the salivary glands (186). Experimental studies support this by showing that

blocking GITRL in animal models of Sjögren's syndrome reduces inflammation, lowers autoantibody levels, and suppresses the Th17 response (187).

In experimental colitis, a model of human inflammatory bowel disease, GITR ablation has similarly demonstrated protective effects by decreasing mucosal Th1 responses, thus reducing disease severity (188,189). Findings align with evidence from type 1 diabetes models, where GITR triggering exacerbates disease progression by enhancing pathogenic T cell activity (190). In autoimmune thyroiditis, high GITRL levels have been associated with increased presence of Th17 cells, suggesting that GITRL may amplify inflammation by promoting Th17-mediated responses central to thyroid gland damage in conditions like Hashimoto's thyroiditis (191). Likewise, in experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis, GITR activation worsens disease outcomes, implicating the GITR/GITRL axis in neuroinflammatory processes (192). These observations suggest that, while GITR inhibition may provide anti-inflammatory effects by limiting effector T cell activity, long-term GITR suppression could also reduce Treg populations, as GITR signaling is crucial for Treg expansion.

This balance underscores the need for a deeper insight into GITR/GITRL functions and eventual manipulation to restore immune homeostasis.

2.3 GITR / GITR ligand axis in inflammatory arthritis

The GITR/GITRL pathway has been identified as a crucial mediator in arthritis pathogenesis (193), particularly through its effects on the regulation and activation of pathogenic Th17 cells (194). Evidence from collagen-induced arthritis (CIA) models demonstrates elevated GITRL expression in DC within draining lymph nodes and in joint tissue, mirroring elevated serum levels in

patients with RA that correlate with IL-17 levels and DAS28 disease activity scores. Notably, recombinant GITRL administration exacerbates arthritis in CIA mice, with earlier onset, increased severity, and greater joint damage (195). In SpA, pathogenic Th17 cells, expressing both IL-17 and IFNγ, are marked by GITR and OX40 co-expression, and are enriched within inflamed SF. A recent single-cell transcriptomic analysis, which included TCR profiling and protein expression using CITE-seq, revealed pathogenic Th17 cells with polyfunctional profiles (IL-17A$^+$IFNγ$^+$) in patients with AS, expressing GPR65 and KLRB1 alongside GITR and OX40 surface markers. These Th17 cells demonstrated plasticity, a property arising from the instability of RORγ-positive feedback loops, which is modulated by the inflammatory cytokine environment, driving pathogenicity (196).

In AS, the dual blockade of GITR and OX40 successfully reduced pathogenic Th17 cells in murine models, correlating with decreases in clinical parameters such as ASDAS and C-reactive protein. These pathogenic Th17 cells, associated with inflammatory markers and ultrasonographic scores, highlight the importance of these costimulatory molecules in sustaining Th17-driven inflammation (196).

In parallel, Th17 responses in AS are commonly targeted by anti–IL-17 biologics like ixekizumab and secukinumab, which have proven effective but carry risks, including fungal infections and worsening intestinal inflammation, thereby limiting their applicability (197). Targeting GITR and OX40 instead of IL-17A could mitigate these risks, selectively depleting pathogenic Th17 cells while sparing IL-17A-associated innate immunity. Beyond AS, the GITR/OX40 axis also promotes Th17 activity in other inflammatory models (198); GITRL enhanced Th17 responses and RORγt expression in arthritis, while OX40 activation sustained Th17 responses in models of uveitis (199), underscoring a

broader applicability for targeting this pathway across Th17-mediated diseases. Elevated GITRL and OX40L levels in AS and RA patients indicate that dual inhibition of these ligands may provide therapeutic advantages by selectively reducing pathogenic Th17 cell responses (200).

CHAPTER 3
EXPERIMENTAL STUDY

3.1 Hypothesis

The inflammatory process characterizing PsA is mainly driven by IL-23/IL-17 axis and IL-9 overexpression, in presence of Th17 and Th9 expansion. Recently, a correlation between IL-9 and GITR, whose ligand (GITRL) is expressed on APC, was described. Specifically, the activation of GITR/GITRL promotes Th9 and Th17 differentiation and alters Treg functions fueling inflammation.

Given the involvement of Th9 and Th17 cells in PsA and the pro-inflammatory role of GITR, we hypothesized that GITR/GITRL interaction may play a crucial role in the pathogenesis of PsA by modulating the differentiation and functional responses of Th17, Th9, and Treg cells. Specifically, the upregulation of GITR/GITRL at multiple tissue level in PsA patients may contribute to the expansion of Th17 and Th9 cells while impairing Treg function, thereby driving the chronic systemic inflammatory process in PsA.

3.2 Objectives

1. Investigate the expression levels of GITR and GITRL on peripheral blood mononuclear cells isolated from PsA patients, with a focus on Th17, Th9, and Treg subsets.

2. Analyze the expression of GITR and GITRL in key sites of inflammation in PsA, including synovial tissue, synovial fluid, and ileal mucosa samples.

3. Examine the impact of GITR stimulation on the proliferation and functional status of T cells derived from peripheral blood and synovial fluid in PsA patients.

3.3 Materials and methods

3.3.1 Patients

Twenty-one patients consecutively referred to the Rheumatology Unit of the Policlinico "Paolo Giaccone" University Hospital, Palermo, Italy, were recruited for this study.

All patients, with a sex ratio M/F of 11/20, mean age ± standard deviation (SD) 50.4 ± 12,6 years, fulfilled the 2006 CASPAR criteria (3), presented active disease, defined by a Disease Activity in PSoriatic Arthritis (DAPSA) score > 14, and were naïve to disease modifying antirheumatic drug (DMARDs) (Table 1). Patients were previously treated with a stable dose of NSAIDs.

Sixteen HC and 4 OA (OA) patients were also enrolled.

Table 1. Clinical characteristics of patients and controls

	PsA (*n* = 21)	**OA** (*n* = 4)	**HC** (*n* = 16)
Age mean, years (range)	50.4 (32-70)	58.5 (45-77)	47.2 (31-60)
Female sex, *n* (%)	9 (42.8)	2 (50%)	6 (37.5)
Disease duration, months (range)	84 (6-240)	-	-
CRP mg/l, mean (range)	10.2 (4-25.2)	2.3 (0-4.3)	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: OA; PsA: psoriatic arthritis

The study was approved by local Ethical Committee of the University of Palermo and complied with the dictates of the Declaration of Helsinki (registration number 04/2019). Informed consent was obtained from each patient and each control.

3.3.2 Isolation of peripheral blood mononuclear cells and synovial fluid cells

Blood samples and SF samples were collected. peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) were isolated by discontinuous density gradient centrifugation on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden), washed with RPMI 1640 medium (Euroclone, MI, Italy) and counted in Neubauer chamber diluting cells with Trypan Blue 0.01%. Cell viability was always > 95%. PBMC and SFMC obtained from every 2 PsA patients and from 2 HC were pooled to improve cells availability.

3.3.3 In vitro functional assay

PBMC of patients and controls, and SFMC of PsA patients were cultured in 24-well flat-bottomed plates at the density of 1 x 10^6 in different conditions: i) RPMI 1640 medium (Euroclone, MI, Italy) supplemented with 10% fetal bovine serum (FBS), L-glutamine (Euroclone, MI, Italy) and antibiotics (Euroclone, MI, Italy), ii) complete RPMI medium plus T Cell TransAct (T cell activation via CD3 and CD28) (Miltenyi Biotec), for 48 hours at 37° C and 5% of CO2, in the presence of 10 mg/ml of monensin.

After incubation, cells were collected, washed, and cultured again. All cells were stimulated with the human recombinant protein TNFSF18 (Abnova) for 48 hours at 37° C and 5% of CO2 plus 10 mg/ml of monensin. PBMC obtained from every 2 PsA patients were pooled to improve cells availability.

Due to the reduced number of cells in the SF, SFMC from OA patients were *ex vivo* stained.

3.3.4 Flow cytometric analysis

After incubation, cells were harvested and washed with phosphate-buffered saline (PBS) (Euroclone). Cells were stained with Fixable Viability Dye (Biolegend, San Diego, CA) and washed with Macs Rinsing solution (Miltenyi Biotec) with 2% of FBS (Euroclone) to identify live cells. Afterward, cells were stained with conjugated monoclonal antibodies (mAbs): APC-Vio 770 anti-human CD45 (REAfinity - Miltenyi Biotec); FITC anti-human CD3 (Miltenyi Biotec), PE anti-human GITR (REAfinity - Miltenyi Biotec), PerCp-Vio 700 anti-human CD4 (REAfinity -Miltenyi Biotec), Pe-Vio 615 anti-human CD8 (REAfinity Miltenyi Biotec), APC anti-human Foxp3 (REAfinity - Miltenyi Biotec), Pe-Vio 770 anti-human CD25 (REAfinity - Miltenyi Biotec), PerCP/Cyanine5.5 anti-human IL-9 (clone MH9A4, Biolegend), Pe-Vio 770 anti-human CD4 (REAfinity -Miltenyi Biotec), Pe-Vio 615 anti-human IL-17, VioGreen anti-human CD3, FITC anti-human CD14, PE anti-human CD11c (clone Bu15, Biolegend), PerCp anti-human GITRL (R&D Systems, Minneapolis, Canada), Pe-Vio 770 anti-human HLA-DR, APC anti human CD19 (REAfinity -Miltenyi Biotec), Alexa Fluor®405 anti-human $\alpha 4\beta 7$ (Biolegend, San Diego, CA).

The same antibodies listed above were also used to label SFMC from OA patients.

Cells were acquired on FACSAria II flow cytometer (BD Biosciences, CA, USA). At least 100.000 cells (events) were acquired for each sample. Data were analyzed with FlowJo software (version 10.5.3 Treestar Inc., Ashland, OR, USA).

3.3.5 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 University Hospital biobank. The sections were treated to remove paraffin. Antigens were unmasked after rehydration using Dako Target Retrieval Solution (Glostrup, Denmark; pH 9.0), as directed by the manufacturer. Then, all sections were incubated with rabbit polyclonal TNFSF18 antibody (Cat# BS-2456R, Bioss). The staining was processed by secondary staining with goat anti-rabbit FITC (Cat# A10523, Invitrogen) for 1 hour and 30 minutes. After secondary staining, the sections were stained with monoclonal mouse anti-human CD19 (M7296, Dako) and mouse anti-human CD11c (Cat# MA1-35070, Invitrogen), respectively. Sections stained with mouse anti-human CD68 (Cat# MA1-80133, Invitrogen) were previously permeabilized with 0.1% Triton X-100 for 10 minutes. Primary antibodies were diluted in PBS in a concentration according to the manufacturer's guideline, containing 3% bovine serum albumin and 0.05% Tween20 (PBS/BSA 0.05 TW20) and incubated overnight at 4 °C. Afterward, the sections were incubated for 1 hour and 30 minutes with rabbit anti-mouse Alexa Fluor®555 (Cat#A21427, Invitrogen) diluted 1:200 with PBS/BSA 0.05 TW20. Finally, nuclei were counterstained with Hoechst 33342 (Cat. H1399, Invitrogen) 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.

3.3.6 RNA isolation and quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR)

RNA was extracted from peripheral blood mononuclear cells (PBMC) of both patients and controls using the Nucleospin miRNA Kit (Macherey-Nagel, Düren, Germany). Following RNA quantification with a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, NC, USA), reverse transcription was carried out using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with TaqMan reagents to measure the expression levels of IL-10 (Hs00465632_CE) and FOXP3 (Hs00305859_CE). The PCR reactions were conducted on the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Results were presented as the relative expression of IL-10 and FOXP3, normalized to the housekeeping gene GAPDH (Hs02758991_g1), using the fold of induction (FOI) method. Group comparisons were made using the Mann-Whitney test, a non-parametric statistical approach.

3.4 Statistical Analysis

All data were analyzed using GraphPad Prism version 8.0.1 (GraphPad). Statistical analysis was performed using T-test and ANOVA; p values < 0.05 were considered significant.

3.5 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 of PsA patients compared with HC after *in vitro* stimulation for 48 hours with anti-CD3-CD28 mAb (Figure 1A). Particularly, GITR expression was increased among $CD4^+$ T cells (Figure 1B). Th17 showed an enhanced GITR expression statistically significant after stimulation, while GITR expression in Th9 did not change between PsA and HC (Figure 1C).

Assessing GITRL expression, $CD45^+$ cells of PsA patients showed an increased expression of GITRL compared with HC, in absence of any stimulation (Figure 1D). Specifically, cytofluorimetric analysis detected an up-regulation of GITRL on $CD14^+$, $CD19^+$ and $CD11c^+$ cells in PsA vs HC, statistically significant in the first two cell subsets (Figure 1E).

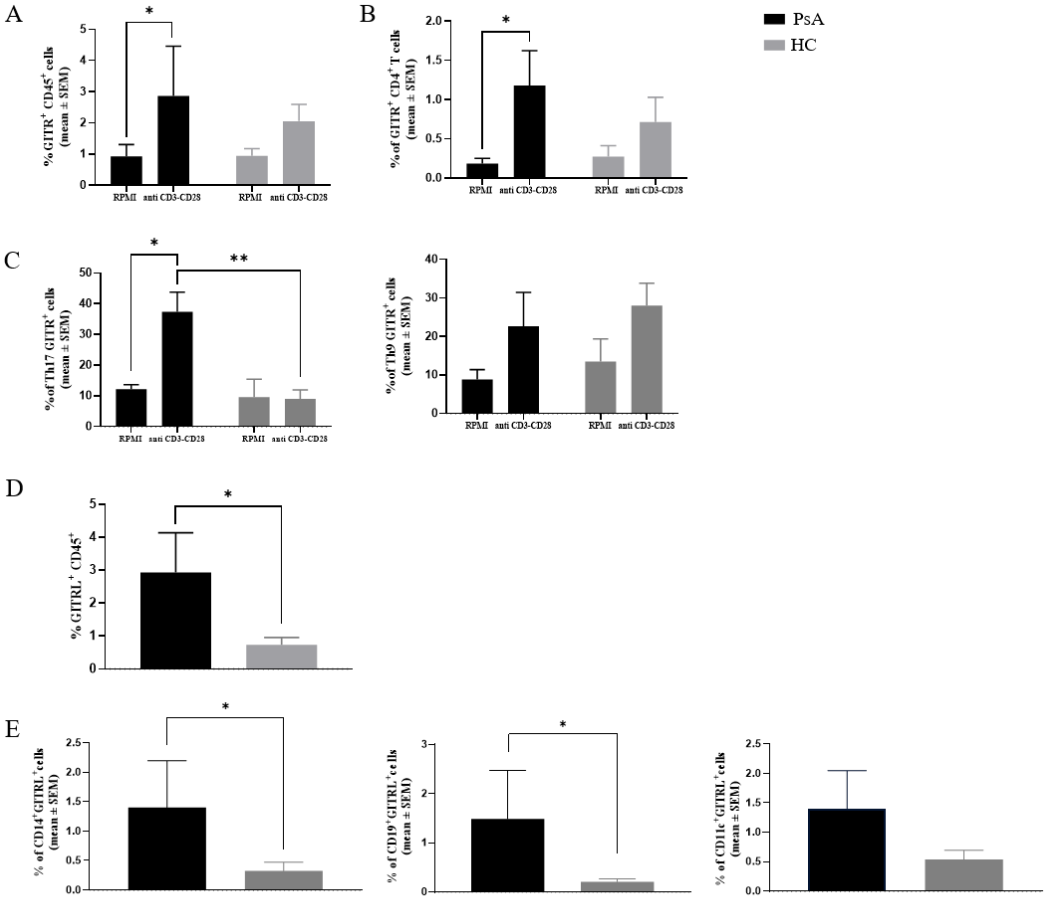

Figure 1: Expression of GITR and GITRL among PBMC in PsA patients and HC

Expression of GITR by $CD45^+$ cells (A); by $CD4^+$ T cells (B); by Th17 cells (C left part) and Th9 cells (C right part).

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 Tumor Necrosis Factor-related receptor; GITRL:

GITR ligand; HC: healthy control; PBMC: peripheral blood mononuclear cells; PsA: Psoriatic Arthritis; Th: T helper.

The frequency of cell subsets and the expression levels of human HLA-DR, constitutionally expressed on $CD14^+$, $CD11c^+$ and $CD19^+$ cells were not different between the two groups (Figure 2).

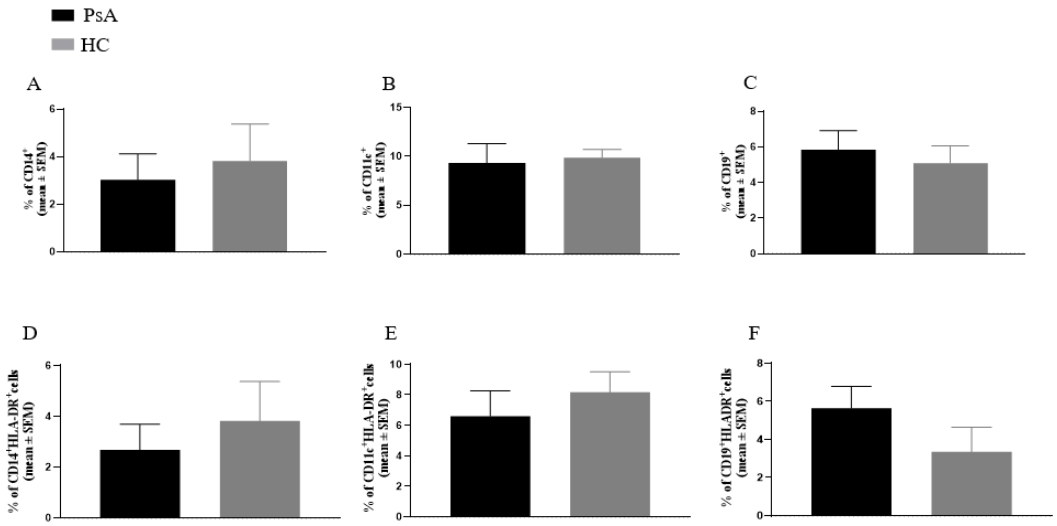

Figure 2: Peripheral frequency of APC and HLA-DR expression in PsA patients and HC

Peripheral frequency of $CD14^+$ cells (A), $CD11c^+$ cells (B) and $CD19^+$ cells (C). *Ex vivo* HLA-DR expression by (D) $CD14^+$ cells, (E) $CD11c^+$ cells, (F) $CD19^+$ cells.

APC: antigen-presenting cells; HC: healthy control; HLA-DR: human leukocyte antigen-DR; PsA: Psoriatic Arthritis.

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

GITR and GITRL expressions were also evaluated in PsA target sites.

An increased GITR expression was detected on Th cells, especially Th17 and Th9, in SF of PsA patients vs OA patients (Figure 3A-C), together with an increase in GITRL expression on CD45$^+$ cells in PsA SF (Figure 3D). Among APC, GITRL was more expressed by CD14$^+$ cells in PsA SF (Figure 3E).

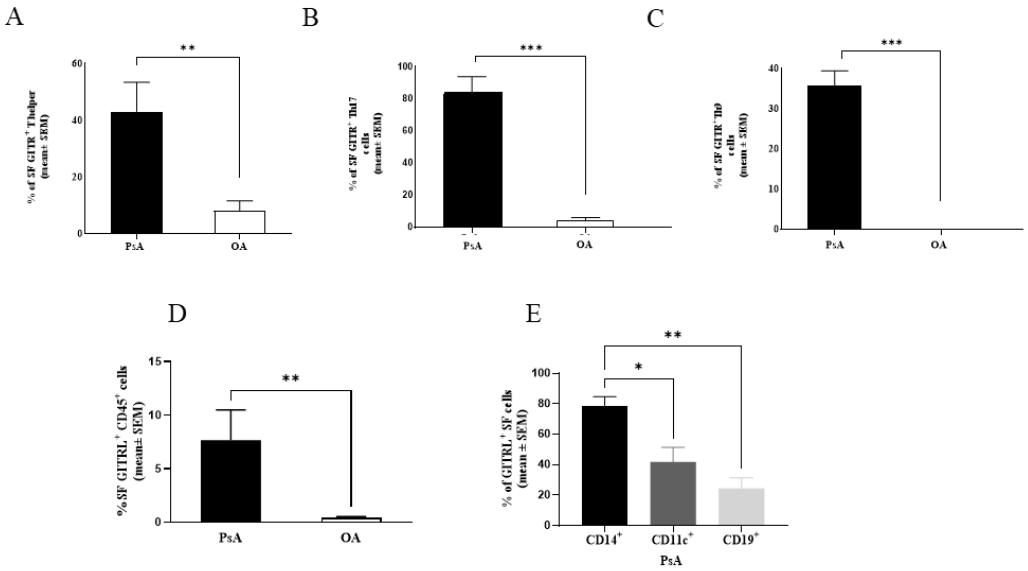

Figure 3: GITR and GITRL expression by SF cells of PsA and OA patients

Expression of GITR by Th cells (A), Th9 cells (B) and Th17 cells (C); the expression of GITR was assessed after 48 hours of cell incubation with RPMI (complete medium) and with anti CD3-CD28 activation beads for PsA samples and *ex vivo* for OA subjects.

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 Tumor Necrosis Factor-related receptor; GITRL: GITR ligand; OA: Osteoarthrtitis; PsA: Psoriatic Arthritis; SF: synovial fluid, Th: T helper.

Immunofluorescence on PsA synovial and ileal samples revealed an overexpression of both GITRL and GITR in PsA samples vs controls (Figure 4A, B). The expression of $\alpha 4\beta 7$, 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 (Figure 4C).

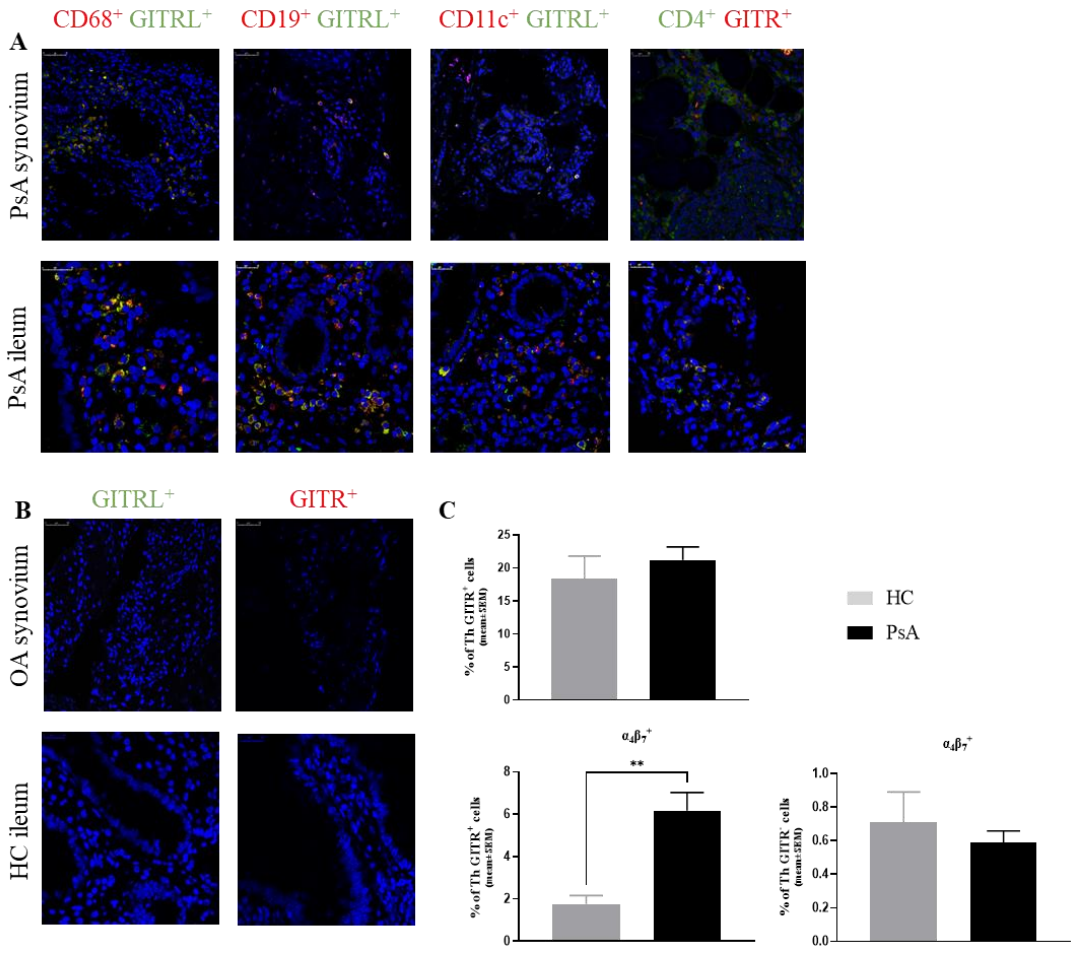

Figure 4: GITRL and GITR evaluation on synovium and ileum of PsA patients and controls and α4β7 expression among Th GITR$^+$ and GITR$^-$ cells in PB

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 Tumor Necrosis Factor-related receptor; GITRL: GITR ligand; HC: healthy control; OA: Osteoarthritis; PB: peripheral blood; PsA: Psoriatic Arthritis; Th: T helper.

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 evaluated the in vitro effect of GITR and GITRL interaction among CD4$^+$ T cell subsets. Therefore, PBMC from PsA patients were stimulated with the recombinant GITR agonist for 48 hours. Afterwards, the percentages of Th9 and Th17 cells were assessed by flow cytometry analysis. In vitro stimulation with recombinant GITR agonist for 48 hours resulted in an increased expansion of PsA-derived Th9 and Th17 cells in presence of anti-

CD3-CD28 stimulation beads, statistically significant compared with HC (Figure 5A, 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 vs OA (Figure 5C, D).

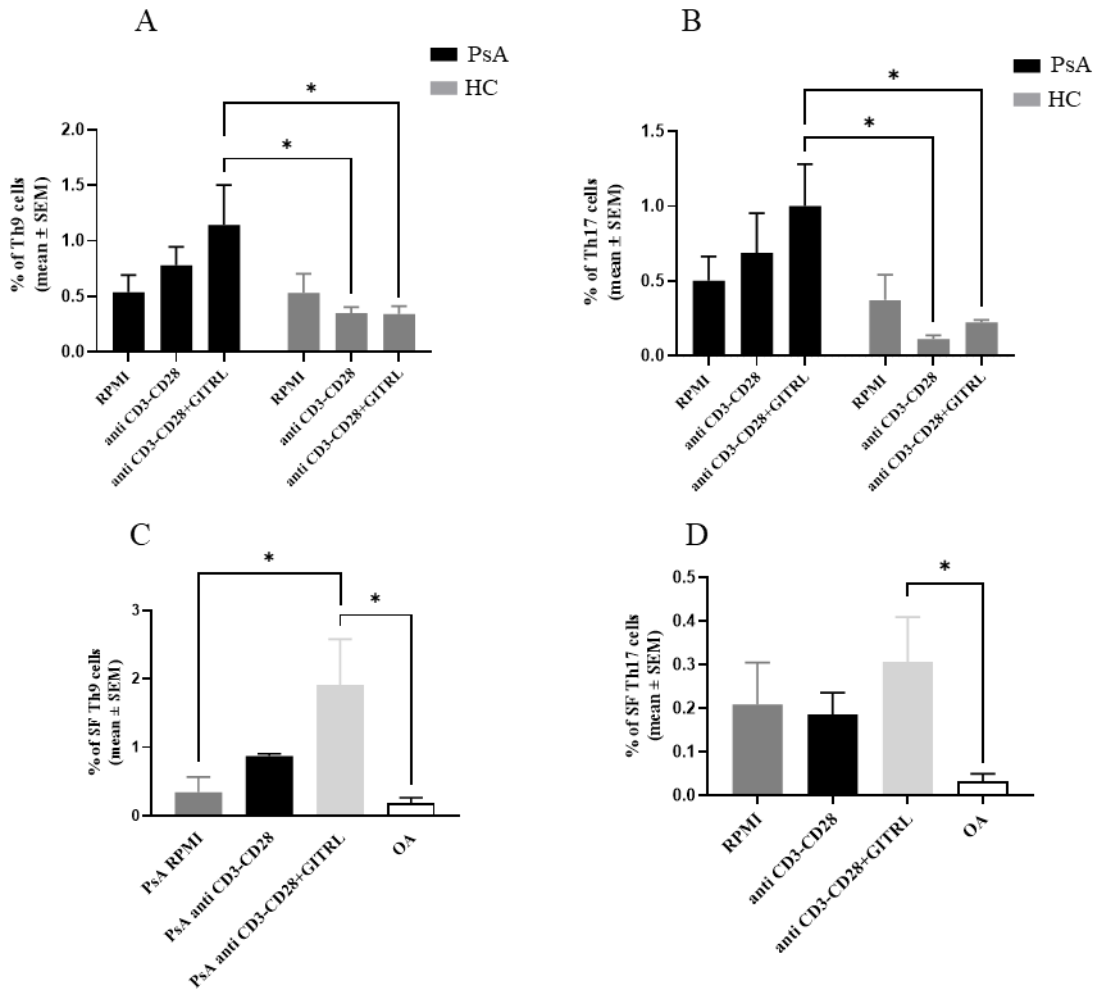

Figure 5: Effects of recombinant GITRL on Th9 and Th17 cells frequency from PB and SF

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 Tumor Necrosis Factor-related receptor; GITRL: GITR ligand; HC: healthy control; OA: Osteoarthritis; PB: peripheral; PsA: Psoriatic Arthritis; SF: synovial fluid; Th: T helper.

GITRL up-regulation inhibits the immunosuppressive functions of Treg in PsA patients

Treg cells were expanded in PsA samples compared with HC after stimulation with anti-CD3 CD28 mAb. No differences were found after specific stimulation with GITRL in PsA samples (Figure 6A).

The in vitro suppression assay was performed to determine Treg suppressive capacity. The expressions of FOXP3 and IL-10 were found to be reduced in the GITR agonist-stimulated Treg of PsA patients compared with HC (Figure 6B). Moreover, no differences in Treg frequency after GITRL stimulation were found in SF as well (Figure 6C).

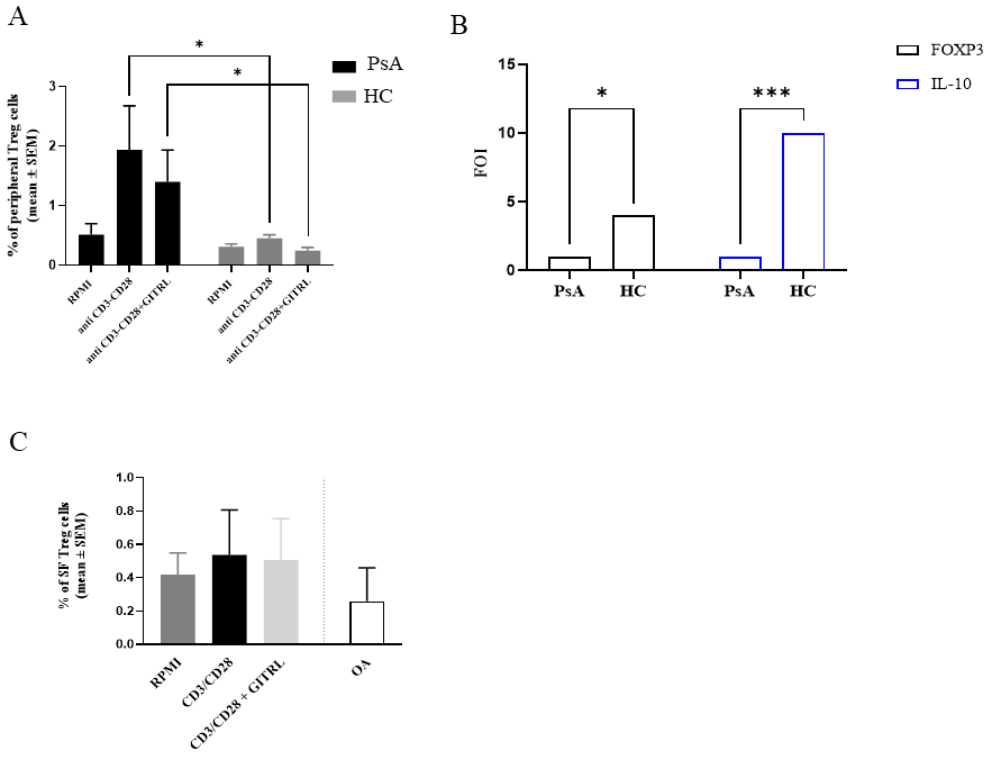

Figure 6: Effects of recombinant GITRL on Treg frequency from PB and SF

Percentage of Treg in PsA patients and HC in RPMI (complete medium) alone, anti CD3-CD28 activation beads alone and anti CD3-CD28 with GITRL (A).

mRNA quantification of FOXP3 and IL-10 after stimulation with GITRL in Treg from PsA patients and HC by qRT-PCR (B).

Frequency of Treg of PsA and OA patients from SF (C).

*p<0.05

GITR: Glucocorticoid-induced Tumor Necrosis Factor-related receptor; GITRL: GITR ligand; HC: healthy control; IL-10: interleukin-10; OA: Osteoarthritis; PB: peripheral blood; PsA: Psoriatic Arthritis; qRT-PCR: quantitative real-time reverse transcription–polymerase chain reaction; SF: synovial fluid; Treg: T regulatory.

DISCUSSION

The proinflammatory function of GITR activation in autoimmune diseases has recently been highlighted, suggesting a dual mechanism through the co-stimulatory action on T effector cells coupled with the immunosuppressive effect on Treg cells (201). GITR stimulation can promote the differentiation and proliferation of Th17 and Th9 (202), crucial cell subsets in PsA. Moreover, the administration of recombinant GITRL exacerbated the progression of arthritis in CIA mice, confirming the role of the GITR/GITRL axis in determining joint inflammation (170).

In the present thesis, the first evidence for the role of GITR/GITRL interaction in the immunopathogenesis of PsA is provided. 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 vs HC.

In light of a growing body of evidence accounting for the systemic nature of PsA, in which the inflammatory response involves several structures, GITR/GITRL expression in multiple target tissues was assessed. 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, favoring the interaction between APC and T cells that can then recirculate and reach target sites of inflammation (203). This hypothesis is further supported by the expression of $\alpha 4\beta 7$, marker of intestinal homing, on the peripheral $GITR^+$ Th cells in PsA. Considering the up-regulation of GITR/GITRL on PBMC and the paramount relevance of Th9 and Th17 cells in PsA, one of the main aim of this research was to assess whether GITR/GITRL interaction could contribute in driving the expansion of Th9 and Th17 cells in PsA.

After addition of the recombinant GITR agonist, an increased expansion of peripheral Th9 and Th17 cells was detected. The present findings are in line with previous data on the strong Th9 polarization as the predominant immunological feature in PsA (203) and the robust data on Th17 expansion in PsA inflammatory sites (204).

Recently, expression of GITR together with OX40 was found on the surface of pathogenic Th17 cells in SF from active AS and the authors demonstrated that the simultaneous blockade of GITR and OX40 suppressed clinical arthritis in the murine model of SpA (205). These findings shed light on the complexity of GITR/GITRL activation that may require a double signal via OX40 to exert its functions; such mechanism may represent a future point to address in order to better understand the aberrant immune responses evidenced in PsA.

The effect of GITR/GITRL axis in driving joint inflammation goes beyond Th and seems to act also through the modulation of Treg cells. Specifically, although the addition of GITR agonist did not result in any change in Treg frequency, the mRNA expression of IL-10 and FOXP3 was reduced in Treg after stimulation, suggesting a potential decrease in Treg immunosuppressive function. In this regard, conflicting data have been described on the effect of GITR stimulation on Treg proliferation, depending on the experimental model, the culture conditions, the intensity of GITR activation and the agonist used to activate it (189,206,207). However, taken together, the presented data let suppose that GITR/GITRL may contribute to alter the balance between Treg and Th cells shaping a strong proinflammatory milieu in PsA through the impairment of regulatory functions and activation of pathogenic cell subsets. Indeed, GITR agonist combined with checkpoint inhibitors were demonstrated able to promote T effector functions and hinder the suppression

of Treg cells in cancer immunotherapy (208) strengthening the described observations.

Certainly, the study presents some limitations, such as the small sample size and the absence of a second evaluation point after treatment. So, future goals include increasing the number of patients enrolled, evaluating the effect of therapy on the expression of GITR and GITRL and studying the downstream mechanisms underlying the modulation of this axis on Th9 and Th17 expansion.

In conclusion, the reported data define a novel role of GITR/GITRL in promoting Th9 and Th17 expansion during PsA joint inflammation and pave the way for exploring whether manipulation of this pathway may be useful in the treatment of inflammatory joint disease.

CONCLUSION

- GITR/GITRL axis may effectively play a role in PsA immunopathogenesis. Specifically, the present study provides the first evidence of GITR/GITRL interactions in PsA, showing increased GITR expression on peripheral $CD4^+$ T cells and GITRL expression on APC in PsA patients compared to HC.

- GITR/GITRL concur to the systemic nature of PsA inflammation. Enhanced GITR/GITRL expression was observed in PsA synovial fluid, synovium, and ileum, supporting the gut-joint axis hypothesis in PsA development, where immune cells activated in the gut may target distant sites of inflammation.

- Th9 and Th17 cell expansion can be related to GITR activation in PsA. GITR/GITRL interactions drive the expansion of Th9 and Th17 cells, as shown by the increased peripheral expansion of these cells following recombinant GITR agonist treatment on cells obtained from both peripheral blood and synovial fluid of patients.

- Modulation of Treg in PsA may be related to GITR. GITR activation leads to a reduction in IL-10 and FOXP3 expression in Treg, suggesting impairment of their immunosuppressive function, potentially favouring a pro-inflammatory environment in PsA.

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