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Kinase activity profiling reveals contribution of G-protein signaling modulator 2 deficiency to impaired regulatory T cell migration in rheumatoid arthritis

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ABSTRACT

The ability of regulatory T (T_{reg}) cells to migrate into inflammatory sites is reduced in autoimmune diseases, including rheumatoid arthritis (RA). The reasons for impaired T_{reg} cell migration remain largely unknown. We performed multiplex human kinase activity arrays to explore possible differences in the post-translational phosphorylation status of kinase related proteins that could account for altered T_{reg} cell migration in RA. Results were verified by migration assays and Western blot analysis of $CD4^+$ T cells from RA patients and from mice with collagen type II induced arthritis. Kinome profiling of $CD4^+$ T cells from RA patients revealed significantly altered post-translational phosphorylation of kinase related proteins, including G-protein-signaling modulator 2 (GPSM2), protein tyrosine kinase 6 (PTK6) and vitronectin precursor (VTNC). These proteins have not been associated with RA until now. We found that GPSM2 expression is reduced in $CD4^+$ T cells from RA patients and is significantly downregulated in experimental autoimmune arthritis following immunization of mice with collagen type II. Interestingly, GPSM2 acts as a promoter of T_{reg} cell migration in healthy individuals. Treatment of RA patients with interleukin-6 receptor (IL-6R) blocking antibodies restores GPSM2 expression, thereby improving T_{reg} cell migration. Our study highlights the potential of multiplex kinase activity arrays as a tool for the identification of RA-related proteins which could serve as targets for novel treatments.

1. Introduction

Regulatory T (T_{reg}) cells have the potential to suppress inflammation and restrain autoimmunity. Interleukin-2 induced expansion of T_{reg} cell populations and adoptive transfer of T_{reg} cells improve rheumatic autoimmune diseases, including rheumatoid arthritis (RA) [1,2]. Recently, we reported that efficient treatment with conventional or biological disease-modifying antirheumatic drugs (DMARDs) significantly increases the frequency of T_{reg} cells in the peripheral blood of patients with RA [3]. However, the ability of T_{reg} cells from patients with RA to migrate into inflamed joints is limited. Results from studies investigating the suppressive capacity of T_{reg} cells from RA patients are controversial [3–5]. A recent meta-analysis has shown that T_{reg} cell frequencies are lower in RA patients with high disease activity as compared to patients in remission [4]. This association suggests that high T_{reg} cell numbers are beneficial for the course of disease. A better

Abbreviations: RA, rheumatoid arthritis; PTK, protein tyrosine kinase; STK, serine/threonine kinase; IL-6, interleukin-6; T_{reg}, regulatory T cells; GPSM2, G-protein signaling modulator 2; PTK6, protein tyrosine kinase 6; VTNC, vitronectin precursor.

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understanding of the mechanisms regulating T_{reg} cell migration into the synovial fluid may help to develop new targeted therapies for RA.

T_{reg} cells were initially described as $CD4^+$ T cells with high CD25 expression or as $CD4^+CD25^{high}Foxp3^+$ T cells [6]. Murine T_{reg} cells are well defined by FoxP3 expression in $CD4^+$ T cells. However, in humans this definition applies not only to T_{reg} cells as a transient FoxP3 up-regulation can also be observed in activated human effector $CD4^+$ T cells. Therefore, human T_{reg} cells need to be characterized by assessment of their *in vitro* suppressive capacity and/or the demethylation of the *FOXP3* TSDR (Treg cell-specific demethylated region) in addition to their phenotype [7]. Importantly, the majority of functional $FoxP3^+$ T_{reg} cells is found within the $CD4^+CD25^{high}CD127^{low/-}$ cell population [8]. This population is therefore often used to identify T_{reg} cells in humans [9–11]. The balance between T_{reg} cells and Th17 cells is regulated by IL-6, which contributes to the pathogenesis of RA by promoting Th17 cell differentiation, enhancing antibody production by B cells and by the recruitment of neutrophils in inflamed tissues [12–15]. Remarkably, T_{reg} cells can suppress Th17 cells by inhibiting IL-6 production in monocytes [16]. Due to its central role in systemic inflammation, IL-6 is an important target for the treatment of cytokine release syndromes and autoimmune diseases [17]. Inhibition of IL-6 ameliorates experimental autoimmune arthritis and is an efficient treatment for RA and other rheumatic diseases, including juvenile idiopathic arthritis, adult-onset Still's disease, giant cell arteritis and Takayasu arteritis [15,18–21]. Importantly, the IL-6 signaling pathway can not only be activated by membrane-bound IL-6R (mIL-6R; *cis*-signaling), but also by soluble IL-6R (sIL-6R; *trans*-signaling) [22]. Both, mIL-6R and sIL-6R are targeted by the monoclonal antibodies sarilumab and tocilizumab, the latter of which is approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of cytokine release syndromes in patients receiving chimeric antigen receptor (CAR) engineered T cells [15,23]. Recently, tocilizumab has also been used as an experimental treatment in patients with severe COVID-19 (SARS-CoV-2 infections) suffering from cytokine release syndromes [24,25]. In RA, significantly increased levels of IL-6 are found in the peripheral blood as well as in the synovial fluid of inflamed joints from patients with active diseases [26]. Here, we explored how inhibition of IL-6 affects the migratory function of T_{reg} cells in RA. Moreover, we analyzed the kinase activity profile of $CD4^+$ T cells to identify potential mechanisms involved in T_{reg} cell migration.

2. Material and methods

2.1. Patients

Peripheral blood samples form patients with RA visiting the outpatient clinic of the Department of Rheumatology and Clinical Immunology at the University Hospital Cologne were included in this study. Blood samples from randomly selected patients with ankylosing spondylitis (AS), systemic lupus erythematosus (SLE), or psoriatic arthritis (PsA) were used as controls. In addition, age and sex matched healthy individuals served as controls. Patients with RA fulfilled the 2010 ACR/EULAR classification criteria and were either untreated or treated with IL-6R inhibitors sarilumab or tocilizumab [27]. Patients with AS, SLE or PsA fulfilled the modified New York AS criteria, the SLICC criteria or the CASPAR criteria, respectively [28–30]. Written informed consent was obtained from the patients before blood was drawn in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of the University Hospital Cologne (no. 13–091).

2.2. Human T cell isolation

Primary human lymphocytes were isolated from the peripheral blood of patients with RA, AS, SLE, PsA, and healthy individuals by Pancoll® denity gradient centrifugation (PAN™-Biotech GmbH, Aidenbach, Germany). $CD4^+$ T cells were purified by negative selection using the $CD4^+$ T cell isolation kit and the QuadroMACS device (all Miltenyi Biotec, Bergisch Gladbach, Germany). The purity was verified by flow cytometry and was at least 96%. Viable cells were counted using the automated cell counter CellCountess (Life Technologies GmbH, Darmstadt, Germany).

2.3. Multiplex human kinase activity array

Serine/Threonine and Tyrosine kinase activity was analyzed on a PamChip® with the Pamstation®12 (PamGene, 's-Hertogenbosch, Netherlands). PamChip® contains 4 arrays with 144 target peptides that can be phosphorylated by kinases [31]. $CD4^+$ T cells from untreated patients with active RA as well as patients treated with the IL-6R inhibitors tocilizumab or sarilumab were lysed in M-PER buffer (ThermoFisher Scientific, Carlsbad, U.S) containing inhibitor cocktails (Halt Phosphatase Inhibitor Cocktail and Halt Protease Inhibitor Cocktail EDTA free 1/100, Pierce). $CD4^+$ T cells from patients with AS, SLE or PsA and healthy individuals served as controls. Total soluble protein lysates were loaded on the PamChip® and phosphorylation activity was tagged by a FITC conjugated antibody and captured with a computer-controlled CCD camera. Data were analyzed using the BioNavigator software (PamGene, 's-Hertogenbosch, Netherlands). The signal intensity of each individual peptide spot was log2 transformed and displayed as heat maps. Network visualization was performed using STRING© [32]. Significant differences were calculated using the mean S100-QC log transformed value of each group and ANOVA. BioNavigator software was used for calculating statistics.

2.4. Migration assay

$CD4^+$ T cells were cultured for 48 h and stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (1 μg/ml) antibodies. After 48 h, cells were starved for 2 h in x-vivo 15 media without supplements (Lonza, Cologne, Germany) and treated with specific GPSM2 blocking antibodies (Novus Biologicals, Centennial, U.S.). Simultaneously, 6.5 mm Transwells® with 5 μM pore size (Corning™ Incorporated Costar, New York, U.S.) were equilibrated for 2 h in x-vivo 15 media without supplements. The medium from the equilibration step was replaced by medium supplemented with the chemokine CCL20 (BioLegend Inc., San Diego, USA) and cells were seeded to the Transwell® and incubated under cell culture conditions (5% CO_2, 37 °C) for 4 h. Migrated cells were stained for the expression of CD25, CD127 and FoxP3 using fluorescently labelled antibodies (all BioLegend Inc., San Diego, USA) and counted for 60 s using the Gallios 10/3 flow cytometer (Beckman Coulter, Krefeld, Germany). The suppressive capacity of $CD4^+CD25^{hi}CD127^{low}FoxP3^+$ T_{reg} cells was verified by classical T_{reg} cell suppression assays as described previously [33].

2.5. Collagen induced arthritis mouse model (CIA)

All animal procedures included in this study were approved by the local authorities and animal protection committee (LANUV NRW, 81-02.04.2018.A161) and were performed in accordance with FELASA recommendations. The collagen induced arthritis mouse model was performed as described previously by Brand et al. (2007) [34]. Eight to twelve weeks old female mice from the DBA1/J strain were injected with an emulsion of collagen and Complete Freund's Adjuvant (CFA) containing a final concentration of 0.5 mg/ml M. *tuberculosis* on day 0. On day 21 mice were injected with an emulsion of collagen and Incomplete Freund's Adjuvant (IFA). Incidence of arthritis was around 80%. No animals were excluded from the study. Mice were sacrificed when reaching an arthritis score between 12 and 16. $CD4^+$ T cells were harvested from the spleens using the $CD4^+$ T cell isolation kit and the QuadroMACS device (all Miltenyi Biotec, Bergisch Gladbach, Germany).

2.6. Micro-CT imaging

Samples were measured using the micro-CT scanner "LaTheta" LCT-100A (Aloka Co., Ltd.; Tokyo, Japan). The micro-focus x-ray tube has a focal spot size of 50 mm and was operated at 35 kV with a constant current of 1 mA. Scans were acquired with a transaxial field-of-view of 30 mm and a rotation time of 18s. The objects were scanned with a minimum spacing of 30 mm. Images were reconstructed with a 480 × 480 matrix leading to an in-plane pixel resolution of 62.5 μm. For visualization, images were processed using Horos ® (version 3.3.6, Horosproject.org, Nimble Co LLC d/b/a Purview in Annapolis, MD USA).

2.7. Statistics

Statistical analysis was performed using the BioNavigator Software (Pamgene) and SPSS. Significant differences in the human kinase activity array were determined using the BioNavigator Software and were calculated using the mean S100-QC log transformed value of each group and one-way ANOVA. Significance of results including multiple parameters were analyzed by one-way ANOVA and are presented as the mean ± SEM. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. GPSM2 phosphorylation is decreased in RA and restored by IL6Ri

We used an established multiplex human kinase activity array [35–39] to compare kinase activities in healthy subjects, RA patients and patients with other rheumatic autoimmune diseases. Peptide substrate phosphorylation was analyzed in $CD4^+$ T cells from patients with active RA, ankylosing spondylitis (AS), psoriatic arthritis (PsA), or systemic lupus erythematosus (SLE). The patients' characteristics are summarized in Table 1. Analysis of peptide phosphorylation revealed significant differences between the serine/threonine kinase (STK) activity signatures of $CD4^+$ T cells from RA patients, healthy individuals and SLE patients (Fig. 1A). The STK activity signatures in AS and PsA have more in common with the RA signature than with the signatures in SLE patients or healthy individuals. To explore the influence of IL-6R blockade on kinase activity, we analyzed peptide substrate phosphorylation in $CD4^+$ T cells from RA patients treated with IL-6R inhibiting antibodies. Interestingly, the pattern of phosphorylation in $CD4^+$ T cells from patients receiving IL-6R inhibiting antibodies is very similar compared to healthy controls, showing that *in vivo* IL-6R inhibition restores the level of kinase activity in $CD4^+$ T cells from RA patients (Fig. 1B). A summary of peptide substrates with significantly different phosphorylation status in untreated RA patients compared to healthy individuals and to patients treated with IL-6R inhibitors is shown in Supplementary Table 1. The proteins and kinases linked to the peptide substrates may represent possible candidates for new targeted therapies. Our analysis identified G-protein signaling modulator 2 (GPSM2; $p < 0.000001$), protein tyrosine kinase 6 (PTK6; $p < 0.00001$) and vitronectin precursor (VTNC; $p < 0.00001$) as top candidates which have not been previously related to RA pathogenesis. Phosphorylation of GPSM2 is significantly downregulated in untreated RA patients as well as in AS, PsA and SLE patients. Treatment with IL-6R inhibiting antibodies restores GPSM2 phosphorylation in $CD4^+$ T cells from RA patients (Fig. 1B). Remarkably, GPSM2 is significantly downregulated on protein level in $CD4^+$ T cells from untreated RA patients as compared to healthy individuals and RA patients receiving treatment with IL-6R inhibiting antibodies (Fig. 1C and D).

3.2. Multiplex kinase activity arrays reveal altered phosphorylation of NF-κB pathway members

In addition to the identification of GPSM2, PTK6 and VTNC as potentially RA-related proteins, the results of our multiplex kinase activity arrays support previous data showing a possible implication of the NF-κB signaling pathway in RA [40]. Phosphorylation of cAMP response element-binding protein (CREB1; $p < 0.0001$) and the nuclear factor NF-kappa-B p105 subunit (NFKB1; $p < 0.001$) are altered in patients with active RA (Fig. 1B). Moreover, treatment of RA patients with an IL-6R inhibitor increases the phosphorylation of NF-κB subunit c-REL proto-oncogene (c-REL; $p < 0.05$). In addition to STK activity profiles, the protein tyrosine kinase (PTK) activity profiles of $CD4^+$ T cells show a clear difference between patients with active RA and healthy controls (Supplementary Fig. 1A). In accordance with previous publications showing that the NF-κB signaling pathway is involved in RA pathogenesis, the NF-κB associated peptide Ras GTPase-activating protein (RASA1) is significantly more phosphorylated in RA patients compared to healthy individuals ($p < 0.05$) (Supplementary Fig. 1B) [40]. Interestingly, beta-type platelet-derived growth factor receptor (PGFRB) is also more phosphorylated in RA. This is remarkable as disturbed platelet function has been reported in RA patients and is expected to contribute to the pathogenesis of RA [41].

3.3. GPSM2 is downregulated following immunization with collagen type II in experimental autoimmune arthritis

The aim of this study was to identify new potential mechanisms accounting for impaired T_{reg} cell migration in RA. Previous studies have linked GPSM2 to cell migration in malignant cells [42,43]. We therefore investigated the potential role of GPSM2 in T_{reg} cell migration. First, we wanted to know if loss of GPSM2 can be triggered *in vivo* by induction of autoimmune arthritis. To study the influence of disease onset on GPSM2 expression, we injected DBA1/J mice with an emulsion of collagen type II and complete Freund's adjuvant in an animal model of collagen

Table 1
Patients' characteristics. Blood samples for multiplex kinase assay were obtained from untreated patients with active rheumatoid arthritis (RA ut) and compared to RA patients treated with IL-6R inhibiting antibodies (IL6Ri) as well as patients with AS, PsA and SLE. Healthy individuals (HC) served as controls. Data are presented as mean values. n.a. = not applicable. RF = rheumatoid factor. ACPA = anti-citrullinated protein antibodies. * = treated with tocilizumab.

	Sample size	disease activity	CRP [mg/l]	ESR [mm/h]	RF + [%]	ACPA + [%]	years since first diagnosis	number of treatments	age	sex [female %]
RA ut	6	active disease (DAS28-CRP = 5.3)	25.2	32	70	79	7	4.3	64 ± 16	66.7
IL6Ri*	7	remission (DAS28-CRP = 2.1)	2.8	11	74	85	8	5.2	62 ± 11	14.3
AS	4	high disease activity (ASDAS = 2.8)	12.5	28	n.a.	n.a.	12	3.7	53 ± 9	25
PsA	4	high disease activity (DAPSA = 31)	21.7	21	n.a.	n.a.	9	4.1	48 ± 4	0
SLE	4	Active disease (SLEDAI-2K = 7	23.1	33	n.a.	n.a.	6	2	57 ± 14	100
HC	5	n.a.	2.1	8	n.a.	n.a.	n.a.	n.a.	55 ± 13	20

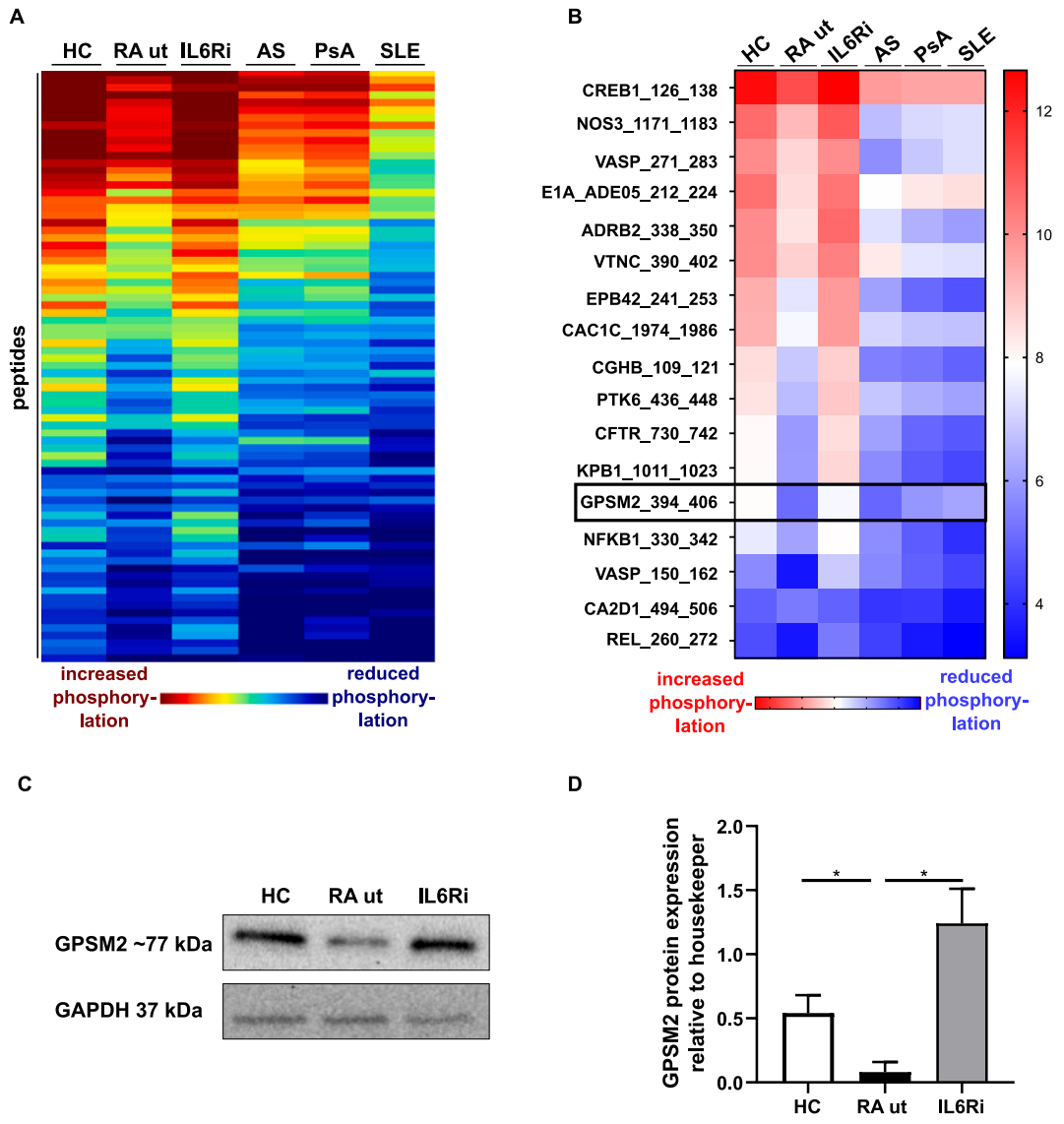

Fig. 1. Multiplex human kinase activity analysis reveals significant differences between GPSM2 phosphorylation in $CD4^+$ from RA patients and healthy individuals. (A) Mean value of raw data of serine/threonine kinase activity in $CD4^+$ T cells presented as a heat map of log-transformed fluorescence signals upon substrate serine/threonine phosphorylation. $CD4^+$ T cells from healthy controls (HC, n = 5), untreated patients with active rheumatoid arthritis (RA ut, n = 6), patients with RA receiving treatment with IL-6R inhibitors (IL6Ri, n = 7) and patients with AS (n = 4), PsA (n = 4) or SLE (n = 4) were analyzed. **(B)** Kinome profiling reveals a distinct kinase activity signature which is reversed by treatment with IL-6R inhibitors. Peptides with significantly different phosphorylation levels between HC, RA ut and IL6Ri. Peptide of interest (GPSM2) is highlighted (box). **(C)** Representative example of GPSM2 (~77 kDa) Western blot analysis of $CD4^+$ T cells from healthy controls, untreated RA patients and patients receiving IL-6R inhibitors. **(D)** Quantification of GPSM2 expression relative to housekeeper from Western blot analysis (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; data are presented as mean ± SEM; significant differences were determined by one-way ANOVA using BioNavigator software.

induced arthritis (CIA) (Fig. 2A). Four weeks after first signs of swelling of the joints, we observed a complete loss of GPSM2 expression in $CD4^+$ T cells from CIA mice as compared to the control group. Loss of GPSM2 expression in CIA mice was associated with increased paw size but not with radiographical signs of bone erosions (Fig. 2B and C, Supplementary Fig. S3).

3.4. *GPSM2 promotes T_{reg} cell migration*

In a next step, we measured the percentage of $CD25^{hi}CD127^{low}FoxP3^+$ T_{reg} cells among $CD4^+$ T cells migrating towards CCL20 in a migration assay to evaluate the impact of GPSM2 downregulation on T_{reg} cell migration (Fig. 3A–C). In healthy individuals, GPSM2 specific antibodies significantly inhibit T_{reg} cell migration whereas blockade of GPSM2 has no influence on the migratory capacity of T_{reg} cells from patients with active, untreated RA (Fig. 4A). In contrast, T_{reg} cell migration is higher in IL6Ri treated patients compared to untreated patients and is inhibited by GPSM2 specific antibodies. Importantly, our results show that T_{reg} cells from patients treated with IL-6R inhibiting antibodies migrate significantly better than T_{reg} cells from untreated patients or T_{reg} cells from healthy individuals (Fig. 4A). The migratory behavior of total $CD4^+$ T cells from patients treated with IL-6R inhibiting antibodies is shown in Supplementary Fig. S4. Specific blockade of GPSM2 has no significant influence on total $CD4^+$ T cell migration (Fig. 4B). In contrast to the observation, that IL6Ri treatment improves T_{reg} cell migration in RA, *in vitro* incubation with the IL6Ri tocilizumab had no influence on the migratory capacity of T_{reg} cells (Fig. 4C and D). Therefore, long-term inhibition of IL6R signaling seems to be required for efficient restoration of GPSM2 phosphorylation and expression levels.

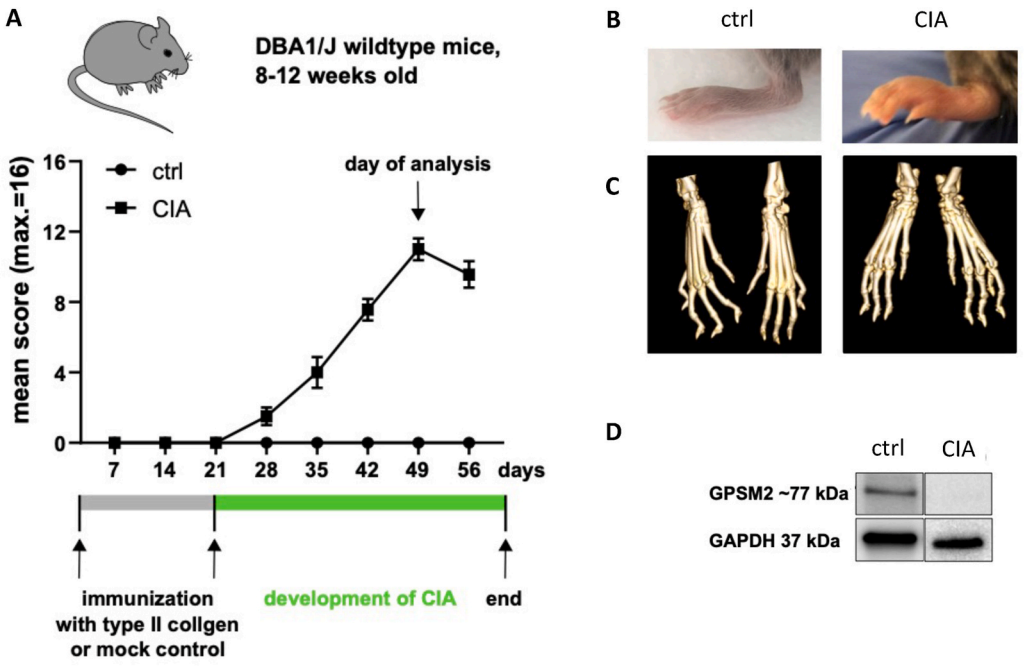

Fig. 2. GPSM2 is downregulated in the collagen-induced arthritis mouse model. (A) Experimental set up of CIA mouse model in DBA1/J strain (n = 5 per group). Mean inflammation score of control and collagen induced arthritis mice is shown, maximum score is 16. **(B)** Representative example of inflammation of the paws and **(C)** microCT imaging of the paws in CIA mice and control mice at day 49. **(D)** Western blot analysis of GPSM2 in $CD4^+$ T cells from the spleen of CIA mice and control mice.

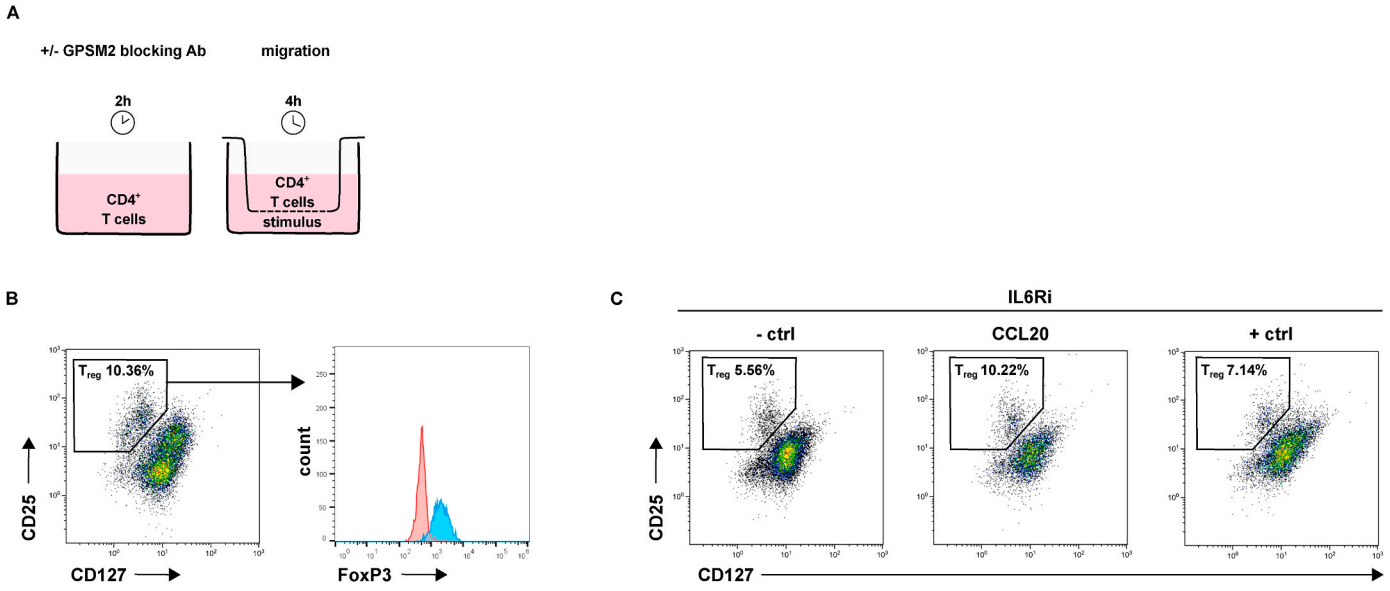

Fig. 3. Gating strategy of regulatory T cells in T cell migration assay. (A) Schematic view of the $CD4^+$ T cell migration assay. T cells were let rest for 2 h or treated with GPSM2 blocking antibody. T cells migrated towards the chemokine CCL20 or x-vivo™ media with or without supplements serving as positive and negative controls (+ctrl/- ctrl). $CD4^+$ T cells were allowed to migrate through a Transwell® for 4 h. Migrated cells were counted and stained for CD25, CD127 and FoxP3. **(B)** Representative example FoxP3 positive T_{reg} cells. **(C)** Representative example of T_{reg} cells among the population of migrated cells (lower chamber in **A**).

3.5. *IL6Ri modifies NF-κB signaling pathway in RA*

The results of our multiplex human kinase activity analysis confirm a possible role of NF-κB in the pathogenesis of RA [40]. NF-κB pathway members have been shown to play an essential role in cell migration [44]. In addition to GPSM2 deficiency, alterations of the NF-κB signaling pathway may therefore account for the reduced migratory function of T_{reg} cells in untreated patients. A protein network analysis based on peptides with significantly different phosphorylation status in RA and healthy controls shows the interactions between c-REL, NFKB1 and CREB1 (Fig. 5A). We analyzed the expression of the NF-κB pathway members NFKB1, c-REL, CREB1 and RASA1 in $CD4^+$ T cells from healthy controls, RA patients treated with IL-6R inhibiting antibodies and untreated patients by qRT-PCR and found significant changes on mRNA expression levels (Fig. 5B–E). In accordance with data from our kinome analysis showing less activity of NFKB1 in RA, the expression of NFKB1 is significantly decreased on mRNA level in RA patients compared to healthy individuals (Fig. 5B). Moreover, IL-6R inhibition upregulates mRNA expression of c-REL (Fig. 5C). This is in line with our results obtained from kinome profiling which show increased phosphorylation of c-REL in patients treated with an IL-6R inhibitor. CREB1 is not differentially expressed on mRNA level (Fig. 5D). Finally, RASA1 mRNA is expressed on a similar level in healthy controls and in patients with active RA and is decreased by IL-6R inhibition (Fig. 5E). In addition, we

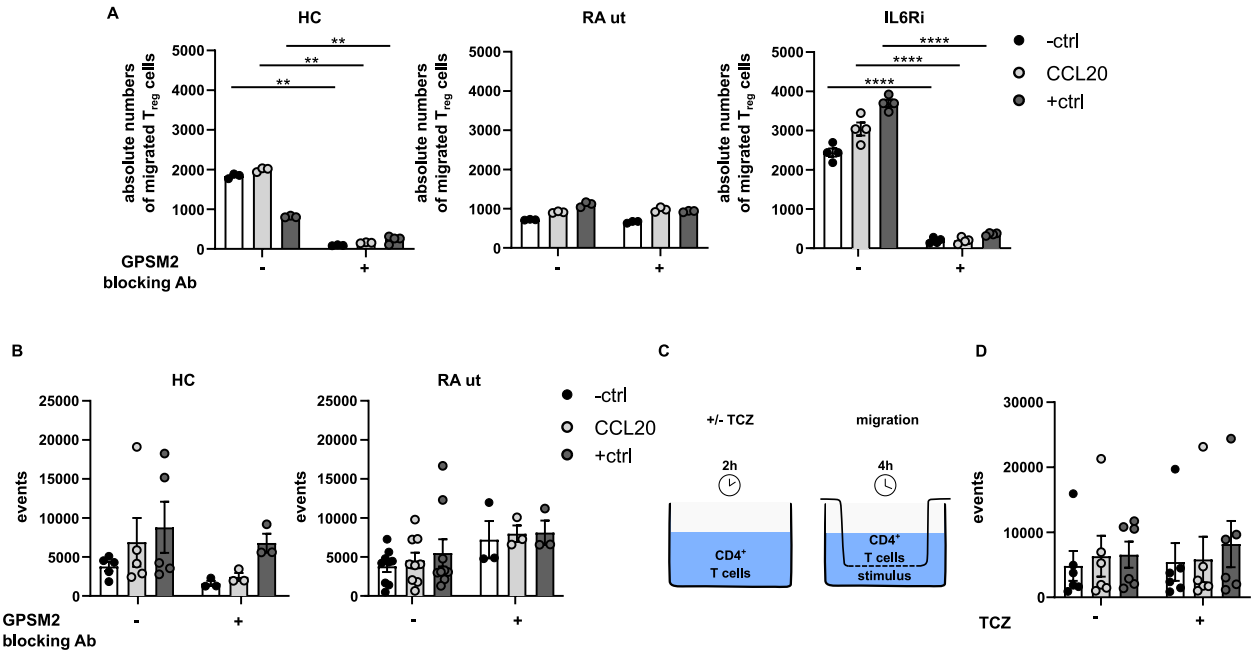

Fig. 4. Functional role of GPSM2 as a promotor of T_{reg} cell migration. (A) Absolut numbers of migrated regulatory T cells from healthy individuals (HC n = 3), patients with untreated active rheumatoid arthritis (RA ut n = 3) and, RA patients receiving treatment with IL-6R inhibitors (IL6Ri n = 4). T cells were either let rest for 2 h or treated with GPSM2 blocking antibody. Absolute numbers of migrated T_{reg} cells were measured using flow cytometry. **(B)** Migration of $CD4^+$ T cells from untreated RA patients (RA ut n = 9) was analyzed by flow cytometry. Healthy individuals served as controls (HC n = 5). $CD4^+$ T cells were either let rest for 2 h or treated with a GPSM2 blocking antibody (HC n = 3, RA ut n = 3, IL6Ri n = 4). **(C)** Schematic view of the $CD4^+$ T cell migration assay. T cells were treated for 2 h with tocilizumab. T cells migrated towards the chemokine CCL20 or x-vivo™ media with or without supplements serving as positive and negative controls (+ctrl/- ctrl). **(D)** $CD4^+$ T cells were treated *in vitro* with tocilizumab and were allowed to migrate through a Transwell® for 4 h. Migrated cells were analyzed by flow cytometry (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; data are presented as mean ± SEM; significant differences were determined using one-way ANOVA.

analyzed the expression of the NF-κB inhibitor α (IκBα, *NFKBIA*) and found higher levels in patients with active RA and a reduction by IL-6R inhibition (Fig. 5F). It has been shown that IL-6 induces hyperactivation of the STAT3 pathway in peripheral $CD4^+$ T cells from patients with active RA, thereby subsequently desensitizing the IL-6 response in T cells [45]. We therefore studied the influence of IL-6R inhibition on STAT3 expression. As expected, the elevated levels of STAT3 mRNA found in untreated RA patients are significantly reduced by IL-6R inhibition (Fig. 5G). Moreover, IL6R inhibition also reduces STAT1 mRNA expression (Supplementary Fig. 2). Our data confirm previous reports showing that the phosphorylation of STAT3 is significantly reduced by IL-6R inhibition (Fig. 5H and I) [46]. However, STAT3 protein expression is not altered by IL-6R blocking antibodies (Fig. 5J).

3.6. CXCR2 and CCR6 are upregulated by IL6Ri

To identify additional possible effects of IL6R inhibition on T_{reg} cell migration that are not related to GPSM2, we analyzed the expression of the chemokine receptors CXCR2 and CCR6 on $CD4^+$ T cells by flow cytometry and by qRT-PCR (Fig. 6A–G). $CD4^+$ T cells from patients with active RA express significantly less CXCR2 as compared to healthy controls (3.2 ± 1.1% vs. 15.4 ± 3.4%; $p = 0.0101$) whereas IL-6R inhibitors restore CXCR2 expression on protein level (13.0 ± 2.4%) and on mRNA level. Similar to previous studies on other proteins, we found a discrepancy between CXCR2 expression on protein level and on mRNA level [47]. The upregulation on mRNA level could represent a possible counterregulatory mechanism used by the cell to correct CXCR2 deficiency. CCR6 is upregulated by IL-6R inhibition on protein level (40.2 ± 9.7% vs. 14.3 ± 2.4%; $p = 0.0261$) and downregulated on mRNA level (Fig. 6D and E).

4. Discussion

Kinase activity profiling revealed altered activity of the NF-κB signaling pathway which has been previously linked to RA pathogenesis. Furthermore, it helped us to identify new potentially RA-related proteins, including GPSM2. This protein belongs to a group of proteins that modulate activation of G proteins which transduce extracellular signals into integrated cellular responses and is also known as Leu-Gly-Asn repeat-enriched protein (LGN) [48]. The C-terminal part of GPSM2 consists of four GoLoco motifs, which are involved in guanine nucleotide exchange and the *N*-terminal part contains 10 copies of leu-gly-asn (LGN) repeats [48]. We observed a significant reduction of GPSM2 phosphorylation in $CD4^+$ T cells from patients with active RA. Interestingly, *GPSM2* is part of a transcriptional regulatory network including *HLA-DRB1* [49]. This is remarkable, as *DRB1*04* alleles are the most important genetic risk factors for susceptibility to RA [50]. Moreover, downregulation of GPSM2 reduces migration of pancreatic and hepatocellular cancer cells, suggesting that GPSM2 is involved in cell migration [42,43]. Our findings reveal an important role of GPSM2 as a promotor of cell migration in T_{reg} cells, but not in other $CD4^+$ T cell subsets from healthy individuals. The migratory function of T_{reg} cells from healthy individuals can be significantly reduced by specific blockade of GPSM2. However, T_{reg} cells can still migrate in the absence of GPSM2, indicating that its function is redundant with that of other molecules. Remarkably, other members of the chemotactic G protein-coupled receptor family have been linked to T cell migration, including Leukotriene B4 (LTB4) receptor 1 (BLT1) [51,52]. In $CD4^+$ T cells from the peripheral blood of patients with active RA, GPSM2 expression is low and can be restored by IL6Ri treatment. Importantly, this is not the only mechanism by which IL6R blockade promotes T_{reg} cell migration in RA: IL6R inhibition drives T_{reg} cell differentiation in general and inhibition facilitates T_{reg} cell migration by modulating NF-κB signaling pathway activity. We found significant differences in

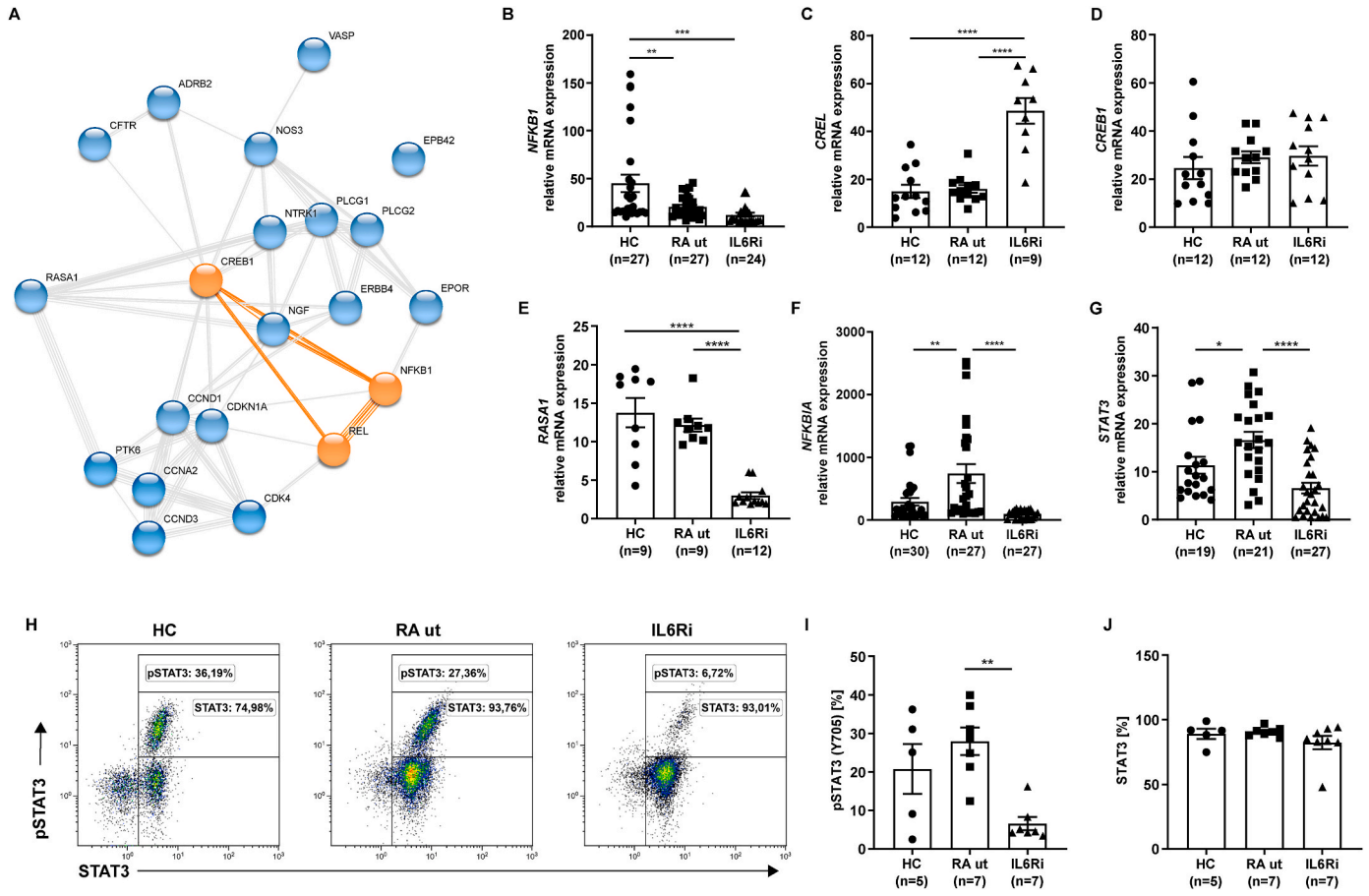

Fig. 5. IL-6R blockade modifies the NF-κB and STAT3 signaling pathways in CD4$^+$ T cells. (A) STRING© protein Network analysis based on peptides presented in Fig. 1B. NF-κB pathway related proteins are shown in orange. **(B–G)** Relative mRNA expression was assessed by qRT-PCR. **(B)** Relative mRNA expression of *NFKB1* (HC n = 27, RA ut n = 27, IL6Ri n = 24), **(C)** *CREL* (HC n = 12, RA ut n = 12, IL6Ri n = 9), **(D)** *CREB1* (HC n = 12, RA ut n = 12, IL6Ri n = 12), **(E)** *RASA1* (HC n = 9, RA ut n = 9, IL6Ri n = 12), **(F)** *NFKBIA* (HC n = 30, RA ut n = 27, IL6Ri n = 27), and **(G)** *STAT3* (HC n = 19, RA ut n = 21, IL6Ri n = 27). **(H–J)** Protein expression was assessed by flow cytometry. **(H)** Representative example of STAT3 and pSTAT3 (Y705) expression in CD4$^+$ T cells from healthy controls, untreated RA patients, and RA patients receiving IL-6R inhibitors. **(I)** Expression of pSTAT3 (Y705) in CD4$^+$ T cells (HC n = 5, RA ut n = 7, IL6Ri n = 7). **(J)** Total STAT3 expression in CD4$^+$ T cells (HC n = 5, RA ut n = 7, IL6Ri n = 7). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; data are presented as mean ± SEM; significant differences were determined using one-way ANOVA.

the activation status of NF-κB1, c-Rel and CREB1 in untreated RA as compared to patients treated with IL6Ri. These transcription factors are related to the NF-κB signaling pathway which consists of several dimeric transcription factors. The pathway is composed of members of the Rel family of DNA-binding proteins comprising p65 (RelA), RelB, c-Rel, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) [53]. CREB1 is a direct inhibitor of the NF-κB signalling pathway [54]. The altered activation status of different components of the pathway confirms previous results obtained from other studies showing an implication of NF-κB in RA pathogenesis [55]. In addition, our data reveal that the activation levels of NF-κB pathway members are restored by IL-6R inhibition. Importantly, our results show that T_{reg} cells from patients treated with IL-6Ri migrate significantly better than T_{reg} cells from untreated patients or T_{reg} cells from healthy individuals. This observation is in line with previous reports showing that tocilizumab increases T_{reg} cell numbers in the synovial fluid of RA patients as compared to the peripheral blood [4].

In patients with active RA, a specific kinase activity signature of CD4$^+$ T cells is found which has high similarity with the signature in PsA or AS patients, but not SLE patients. The similarity between RA, PsA and AS signatures reflects the similarity between the pathogenesis of these autoimmune diseases as compared to SLE. Excessive IL-6 signaling seems to be the main driver of kinase activity alterations in RA, as inhibition of IL-6 almost completely restores the kinase activity signature. These results underpin the central role of IL-6 in RA pathogenesis. In addition to factors which are already known to play a role in the development of RA, such as NK-κB, our kinome profile analysis identified several other potential new candidates for targeted therapy of RA. Proteins with significant changes in peptide phosphorylation following IL6Ri treatment include protein tyrosine kinase 6 (PTK6) and vitronectin precursor (VTNC) ($p \leqq 0.00001$). PTK6 is a member of a kinase family that is evolutionarily related to the SRC family of tyrosine kinases [56]. PTK6 is expressed in epithelia and is related to cell differentiation and cell cycle exit [56]. It has not been linked to RA, so far. VTNC is the precursor of vitronectin, a cell adhesion factor which is expressed in the synovial tissue of patients with RA [57]. Animal studies are needed to confirm a possible role of these candidates in RA pathogenesis and to evaluate their potential to serve as targets for the treatment of RA.

Noteworthy, some of the identified proteins with altered phosphorylation status in RA patients are related to the development and function of platelets or other hematopoietic cells. This can be explained by the fact the IL-6 stimulates thrombopoiesis through thrombopoietin induction and inhibits erythropoiesis by modulation of the erythropoietin axis [58,59]. Our findings demonstrate that IL-6 inhibition corrects the altered activation status in RA patients and are in line with previous studies showing that inflammatory anemia improves after treatment with tocilizumab [60]. In conclusion, kinome profiling represents a possible tool to identify molecules and signaling pathways which could

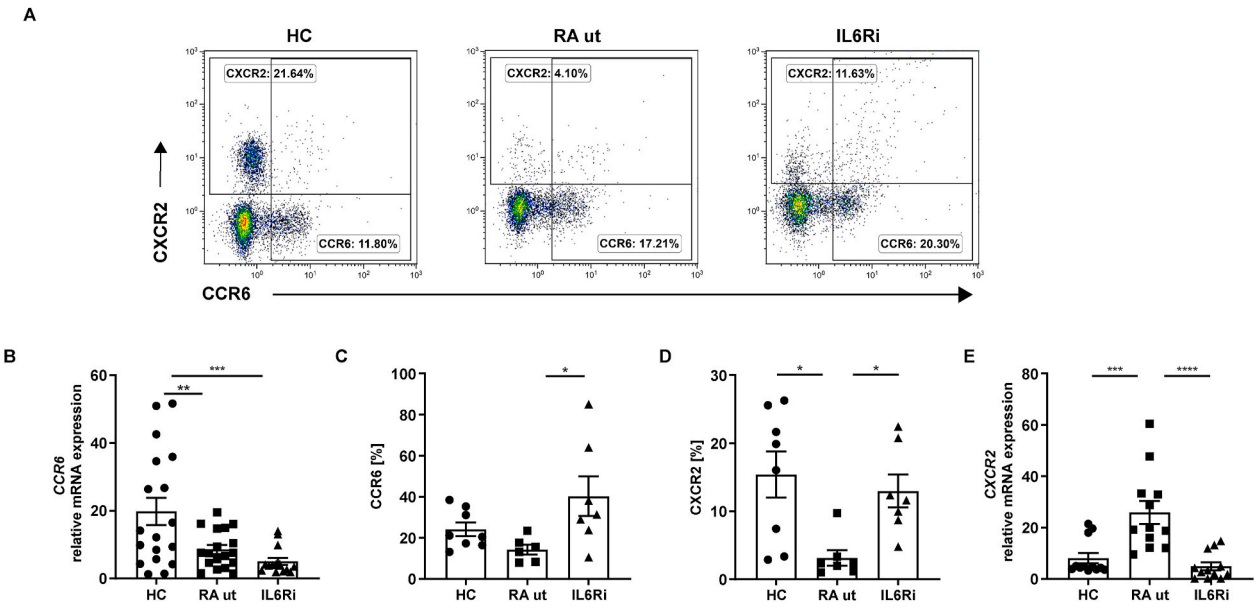

Fig. 6. The chemokine receptors CXCR2 and CCR6 are upregulated by treatment with IL-6R inhibitors. (A–C) Representative example of CXCR2 and CCR6 expression in $CD4^+$ T cells from healthy controls **(A)**, untreated RA patients **(B)**, and RA patients receiving IL-6R inhibitors **(C)** analyzed by flow cytometry. **(D)** Relative mRNA expression of *CCR6* was assessed using qRT-PCR (HC n = 18, RA ut n = 18, IL6Ri n = 15). **(E)** CCR6 protein expression in $CD4^+$ T cells analyzed by flow cytometry (HC n = 8, RA ut n = 6, IL6Ri n = 7). **(F)** CXCR2 protein expression in $CD4^+$ T cells analyzed by flow cytometry (HC n = 8, RA ut n = 7, IL6Ri n = 6). **(G)** Relative mRNA expression of *CXCR2* was assessed using qRT-PCR (HC n = 12, RA ut n = 12, IL6Ri n = 12). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; data are presented as mean ± SEM; significant differences were determined using one-way ANOVA.

be used as new targets for the treatment of autoimmune diseases.

5. Conclusions

GPSM2 deficiency contributes to impaired T_{reg} cell migration in RA and induction of GPSM2 restores the migratory function of T_{reg} cells. This study highlights the potential of multiplex kinase activity arrays as a tool to for the identification of proteins implicated in RA pathogenesis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2021.102726.

Author contributions

AM, CPP, TS and DMK made substantial contributions to the study concept and design. AM, VGN, SY, RLE, VB, SJB, LR, AN and HG made substantial contributions to the acquisition of the data. AM, VGN, SY, RLE, VB, SJB, LR, AN, TS, HG, CPP, and DMK drafted the article or revised it critically for important intellectual content. All authors reviewed the draft and approved the submission of the manuscript. Anja Meyer, Shuaifeng Yan, Viktoria Golumba-Nagy, Ruth L. Esser, Verena Barbarino, Stuart J. Blakemore, Lisa Rusyn, Anastasia Nikiforov, Tamina Seeger-Nukpezah, Holger Grüll, Christian P. Pallasch, and David M. Kofler.

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