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CXCR5/CXCL13 pathway, a key driver for migration of regulatory B10 cells, is defective in patients with rheumatoid arthritis

Claire Rempenault¹, Julie Mielle², Kristina Schreiber¹, Pierre Corbeau^{3,4}, Laurence Macia⁵, Bernard Combe¹, Jacques Morel¹, Claire Immediato Daïen^{1,2,*} and Rachel Audo^{1,2,*}

Abstract

Objectives. Chemokines (CKs) are key players of immune-cell homing and differentiation. CK receptors (CKRs) can be used to define T-cell functional subsets. We aimed to characterize the CKR profile of the regulatory B-cell subset B10⁺ cells and investigate the CKs involved in their migration and differentiation in healthy donors and patients with RA.

Methods. RNA sequencing and cytometry were used to compare CKR expression between B10⁺ and B10^{neg} cells. Migration of B10⁺ and B10^{neg} cells and IL-10 secretion of B cells in response to recombinant CKs or synovial fluid (SF) were assessed.

Results. CXCR5 was expressed at a higher level on the B10⁺ cell surface as compared with other B cells (referred to as B10^{neg} cells). In line with this, its ligand CXCL13 preferentially attracted B10⁺ cells over B10^{neg} cells. Interestingly, synovial fluid from RA patients contained high levels of CXCL13 and induced strong and preferential migration of B10⁺ cells. Besides its role in attracting B10⁺ cells, CXCL13 also promoted IL-10 secretion by B cells. In RA patients, the level of CXCR5 on B-cell surface was reduced. The preferential migration of RA B10⁺ cells toward CXCL13-rich SF was lost and CXCL13 stimulation triggered less IL-10 secretion than in healthy donors.

Conclusion. Our results identify that the CXCR5/CXCL13 axis is essential for B10⁺ cell biology but is defective in RA. Restoring the preferential migration of B10⁺ within the affected joints to better control inflammation may be part of the therapeutic approach for RA.

Key words: Regulatory B cells, chemokines, migration, IL-10, RA, inflammation

- CXCR5 level is highly increased on the regulatory B10 cell surface and its ligand CXCL13 preferentially attracts B10 cells.
- CXCL13 in rheumatoid arthritis (RA) inflammatory synovial fluid drives the preferential migration of B10 cells.
- In RA patients, CXCR5 level is decreased and the preferential migration of RA B10 cells toward CXCL13-rich synovial fluid is lost.

Introduction

It is now well established that some B cells can have anti-inflammatory and protective functions. These regulatory B cells (Bregs) are essential to maintain tolerance

and immune homeostasis, even if they represent <10% of circulating B cells in healthy individuals [1–4]. In humans, Bregs have been found dysregulated in several inflammatory diseases [1, 3–6]. Several subtypes of B cells have been suggested to be precursors of Bregs:

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CD5⁺ B cells, CD24^{hi}CD38^{hi} transitional B cells and CD24^{hi}CD27⁺ B cells [3, 5, 7–9]. However, we still lack phenotypic markers or transcription factors to clearly identify Bregs. As Breg functions are mainly mediated by the production of the anti-inflammatory cytokine IL-10, a functional definition based on their ability to produce IL-10 (B10⁺ cells) in response to Toll-like receptor activators (such as CpG, TLR9 activator) is commonly used [4, 5, 9]. Using this, we and others previously found decreased numbers of B10⁺ cells in peripheral blood of RA patients [5, 10]. Moreover, CpG-induced B10⁺ were inversely correlated with disease activity and autoantibody production [5]. In addition to their impaired number, impaired functions were also described in RA [3, 5, 11, 12]. Thus, these cells might represent a novel therapeutic target in RA to restore tolerance. Nevertheless, B10⁺ cells in humans await further characterization.

Chemokines (CKs) constitute a large family, which, together with adhesion receptors, orchestrate immune cell migration, in both homeostatic and inflammatory conditions. They drive cell homing and migration through their corresponding G protein-coupled receptors (CXC, CC, CX3C, XC Receptors) or host atypical CK receptor (ACKR1–4, CCRL2, PITPNM3, C5L2) [13]. Humans have ~50 CKs and 24 CK receptors (CKRs). Among them, CXCR5/CXCL13, CCR7/CCL19/CCL21 and CXCR4/CXCL12 receptor–ligand couples are the major regulators of circulating B-cell trafficking [14]. Because they control subset specific migration, CK expression profile is unique to a T cell subset and can serve as a phenotypic marker. Indeed, among effector memory T cells (considered CD4⁺/CCR7⁺), IFN- γ -secreting T helper 1 cells are phenotypically defined by the co-expression of CCR5 and CXCR3, whereas IL-4-secreting Th2 cells are phenotypically defined as CCR3⁺/CCR4⁺ and IL-17-secreting Th17 cells as CCR4⁺/CCR6⁺ [15, 16]. In addition to cell migration, CKs have also been involved in the differentiation of immune cells. Notably, CXCL9 and CXCL10 can drive Th1 responses *via* CXCR3 binding to naive T cells, whereas CXCL11 binding to CXCR3 stimulates the regulatory T-cell (Treg) response [17, 18] and CXCL12 drives CXCR4⁺CD4⁺ T cells into IL-10-producing Tregs (Tr1) [19]. CXCL12 also polarizes CXCR4⁺ macrophages into IL-10-secreting M2-like macrophages [20].

Little is known about the CKR expression on the B10⁺ and how, together with their corresponding chemokine, they drive B10⁺ cell migration. Indeed, decreased B10⁺ cell number in peripheral blood of RA patients could reflect either their recruitment to inflammatory sites or their decreased differentiation in the pathological context of RA. Further insight into CK and CKR role in B10⁺ cell biology would help target these cells. Therefore, we aimed here to characterize the CKR profile of B10⁺ cells and investigate which CKs drive their migration and differentiation, in both healthy controls (HC) and patients with RA.

Participants

Blood samples were obtained from patients with RA [21] or other inflammatory rheumatisms (OIRs; i.e. psoriatic arthritis or gout). HCs were healthy blood donors or patients seen for mild OA or mechanical pain. Synovial fluid (SF) samples were from RA or OIRs patients. Participants provided written informed consent to participate in the study in accordance with the 2013 Declaration of Helsinki and as approved by the Medical Ethics Committee of Nîmes hospital, France [CPP_2012-A00592-41 (NCT01642706) and CPP_2014-A01585-42 (NCT02615951)].

Cell activation

B cells, peripheral blood mononuclear cells (PBMCs) and SF mononuclear cells (SFMCs) were activated for 24 h with CpG (10 μ g/ml, ODN 2006, InvivoGen, Toulouse, France) [5, 11]. Of note, isolated B cells were cultivated on CD40L-coated plates for 24 h as previously described [5, 11]. To allow detection of IL-10 expression by FACS, phorbol 12-myristate 13-acetate (PMA) (0.1 μ g/ml, BioVision, Nanterre, France) and ionomycin (0.5 μ g/ml, BioVision) were added for the last 4 h of culture (PI). For intracellular staining of IL-10, Brefeldin A (GolgiPlug, BD Pharmingen, Le Pont de Claix, France) was also added in the last 2 h before staining for FACS analysis (PIB).

B10⁺ and B10^{neg} cell sorting

B10⁺ and B10^{neg} were sorted as previously described [11]. Briefly, B cells were freshly isolated from blood and cultivated 24 h with CpG, with addition of PI for the last 4 h of culture. B10⁺ cells were stained by using IL-10 secretion assay (Miltenyi Biotec, Paris, France) and CD19 antibody and isolated by cytometry (FACS Aria, BD Biosciences, Le Pont de Claix, France).

RNA sequencing (RNA-seq) and comparison of B10⁺ and B10^{neg} cells

A total of 1×10^5 to 2.5×10^5 B10⁺ cells were collected for TRIZOL mRNA isolation. mRNA was analysed by BGI Co. (Shenzhen, Guangdong, China) for library construction, sequencing and bioinformatics analysis. An analysis report was provided and is available on figshare repository

Analysis report B10⁺ RNAseq. All samples' gene expression and a complete list of differentially expressed genes analysis are also available on figshare B10⁺ vs B10^{neg} lists.

CR profile identification in B10⁺ and B10^{neg} cells

Freshly isolated B cells were activated with CpG (+PIB) and analysed by flow cytometry for extracellular staining of CKR and IL-10 expression detected by intracellular staining (more details and antibodies panels can be found

Results were expressed as percentage of CKR-positive cells (minus negative staining control) and as delta of mean fluorescence intensity (dMFI) calculated as MFI of CKR minus MFI of negative staining control (MFI_{CKR} of B subset—MFI_{Neg Ctl} of B subset). High variability of MFI range could be detected between experiments over the period of RA patients' recruitment. To compare CXCR5 expression on RA and HC B-cell subset, we therefore normalized to expressed MFI in comparison to B10^{neg} MFI of HC included in the same experiments [relative MFI= (MFI_{CXCR5} of B subset—MFI_{Neg Ctl} of B subset)/(MFI_{CXCR5} of HC B10^{neg}—HC B10^{neg} MFI_{Neg Ctl} of HC B10^{neg})].

Chemotaxis assay with B10⁺ and B10^{neg} cells

After cell sorting of B10⁺ and B10^{neg} cells, their migratory capacity toward recombinants chemokines (CXCL11, CXCL12, CCL19, CCL21, CCL22 and CXCL13) in comparison to RPMI-1% FCS +vehicle (basal migration) was evaluated [22]. We also tested migration toward SF from RA or OA patients (mixtures of SF samples from three different donors, diluted at 1/2 in RPMI-1% FCS) in comparison to RPMI-1% FCS. To inhibit CXCL13 present in RA SF, a CXCL13 antibody (AF801, R&D Systems, Minneapolis, Minnesota, USA, 10 µg/ml) was added. Results were expressed as fold-increased migration compared with basal migration.

B10 precursors analysis

For CKR profile analysis on B10 precursors or evaluation of their migration, B cells were stimulated for 24 h with or without CpG, in the absence of PMA/ionomycin that are massive and unphysiological activators (needed for B10⁺ detection by FACS), while CpG mimics bacterial DNA that activated TLR9 and mimics physiological conditions. Results obtained for B10 precursors (CD24^{hi}CD38^{hi}, CD24^{hi}CD27⁺ and CD5⁺ B cells) were expressed relative to CD24^{low} B cells subset.

Statistical analysis

Data are expressed as mean (s.d.). Wilcoxon's matched pairs signed rank test was used to compare paired data. Mann-Whitney test was used to compare independent data. Spearman test was used for correlation. All analyses

involved Graph Pad Prism 5 (San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Chemokine pathway enrichment in B10⁺ cells from HCs and RA patients

The gene expression profile of CpG-induced B10⁺ and B10^{neg} cells from five HCs and four RA patients was compared by RNA-seq (Fig. 1A). A total of 19413 genes were detected; 335 were differentially regulated in HC B10⁺ vs HC B10^{neg} cells (Fig. 1B). In RA patients, 188 differentially expressed genes (DEGs) were identified (Fig. 1C): 159 DEGs were shared by both RA patients and HCs, and 29 were specific to RA patients (Fig. 1D). Enriched gene ontology (GO) terms in DEG lists from HCs and RA patients were analysed to determine functions associated with B10⁺ cells. We found enrichment of genes belonging to CK pathways in HC B10⁺ cells (Fig. 1E), with fold enrichment >10 for CKR binding (fold enrichment: $\times 13.25$, $P = 1.33\text{e-}10$) but also for CK activity ($\times 15.3$, $P = 1.25\text{e-}10$) and for CXCR-CKR binding ($\times 18$, $P = 0.003$) and CCR-CKR binding ($\times 10.6$, $P = 0.003$). In RA patients, CK pathways were also among the top 20 most enriched pathways in B10⁺ cells but to a lesser extent than for HCs. The top 20 most enriched pathways contained only CK activity and CKR binding pathways, with fold enrichment $\times 12.3$ ($P = 0.003$) and $\times 9.25$ ($P = 0.0157$), respectively (Fig. 1F). All DEGs from CK pathways are shown

1. Despite being below the fold-change threshold, CXCR5, which has a key role in B-cell ontology, was also upregulated in HC B10⁺ cells ($P = 0.00089$)

CXCR5 is upregulated in B10⁺ vs B10^{neg} cells but is downregulated in RA patients compared with HCs

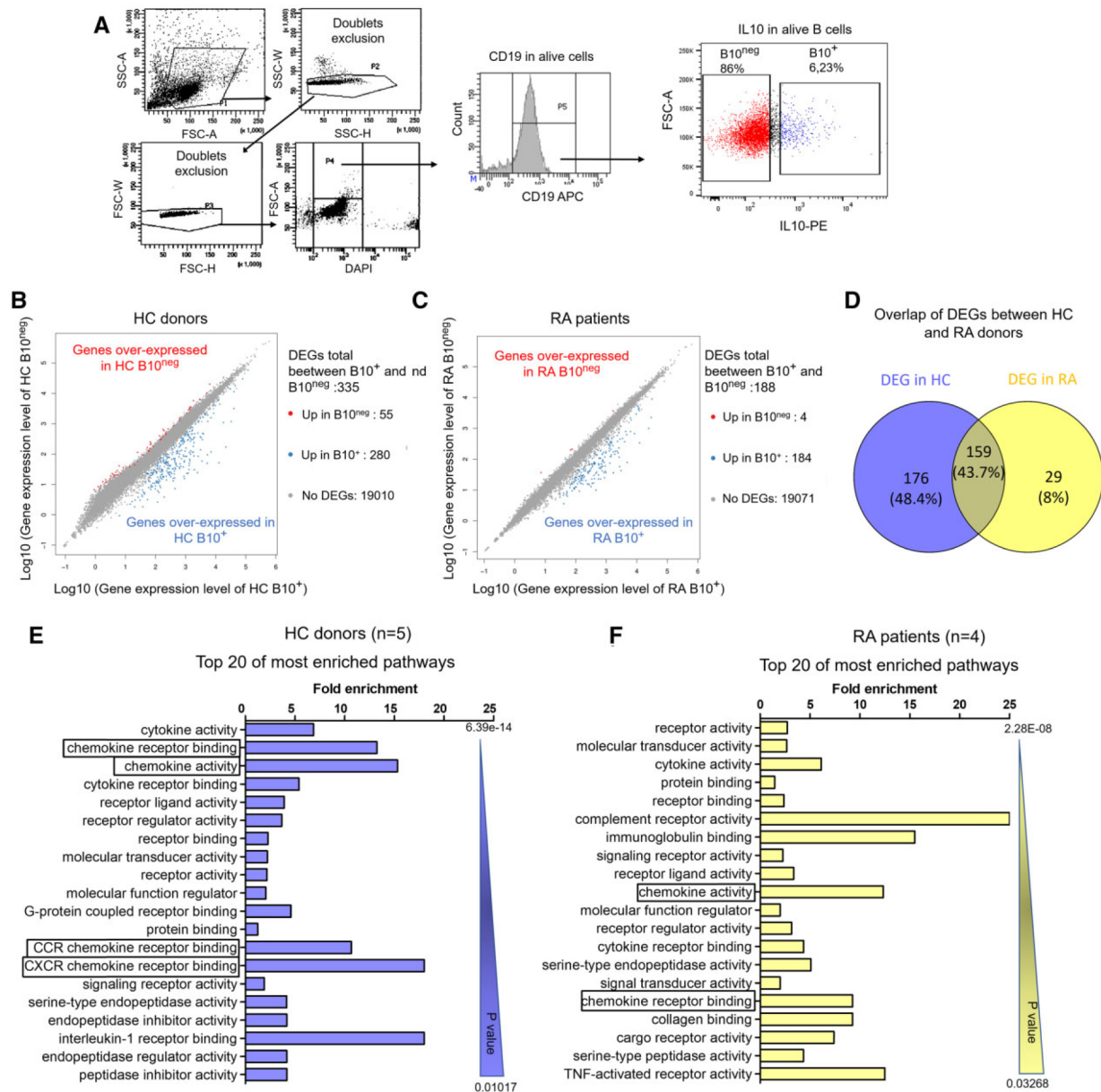
For cell surface analysis, we selected candidate CKRs that were identified from RNAseq

or CKRs crucial for B cell [13, 23, 24] or known to regulate anti-inflammatory cells [17–19, 25, 26]. CKR protein expression at the cell surface of CpG-treated B cells from HCs was evaluated by flow cytometry

Of note, TLR9 activation using CpG stimulation modified some CKR profiles as shown

In particular, CXCR5 was increased after CpG stimulation (Rheumatology online). We assessed whether these CKRs were differentially expressed in HC B10⁺ vs HC B10^{neg} cells. CXCR3, CXCR5, CXCR7, CCR4 and CCR5 were more frequently expressed (% positive cells) and at higher levels (delta MFI) on the surface of HC B10⁺

FIG. 1 Transcriptomic analysis of B10⁺ cells from healthy controls and patients with RA



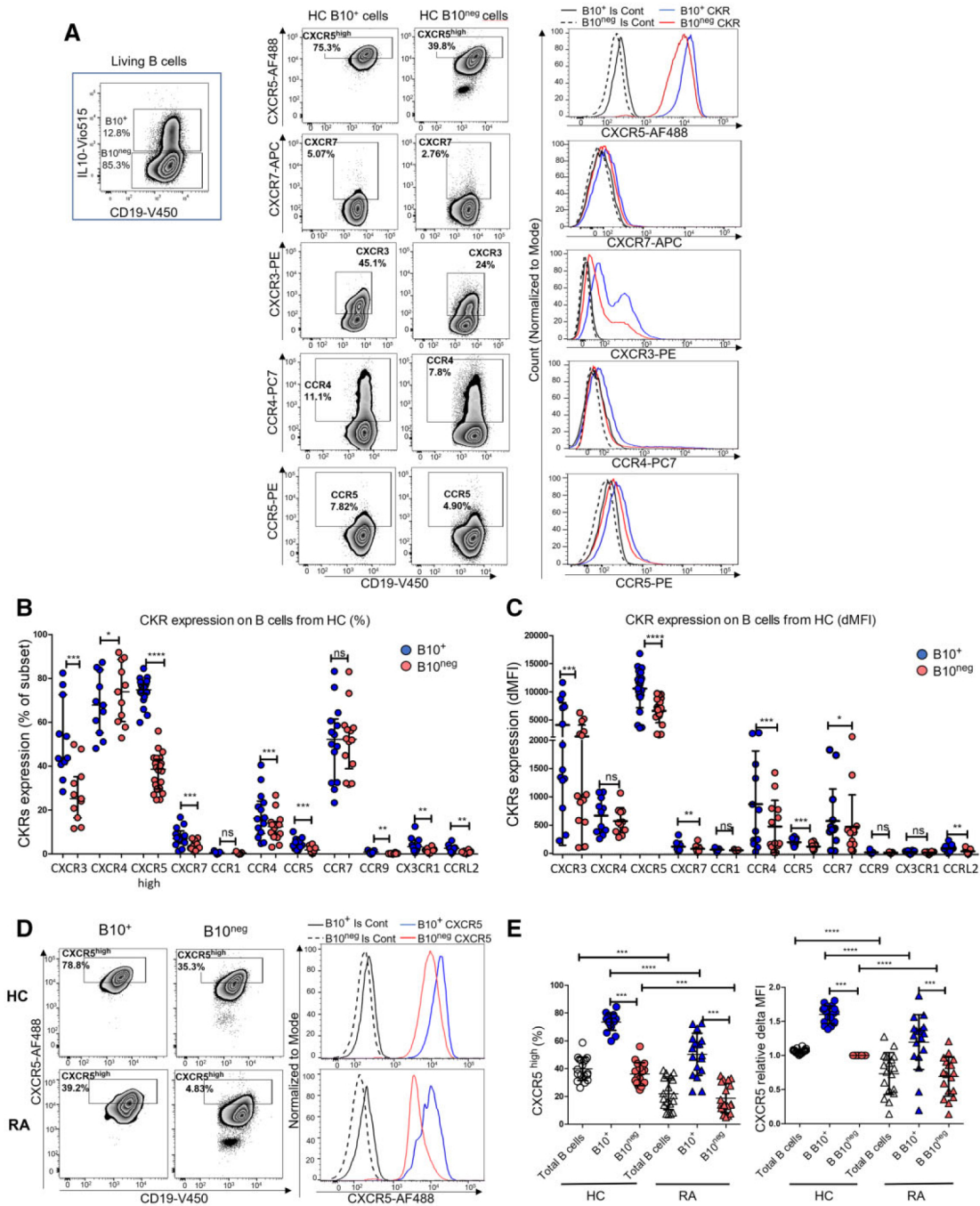
(A) Gating strategy for B10⁺ and B10^{neg} sorting. **(B, C)** DEGs between B10⁺ and B10^{neg} cells in HCs and RA patients. Scatter plot of DEGs is shown in HCs ($n=5$) **(B)** and RA patients ($n=4$) **(C)**. Axis represents log10-transformed gene expression in B10^{neg} (x-axis) and B10⁺ cells (y-axis); red=upregulated genes in B10^{neg} cells; blue=upregulated genes in B10⁺ cells; gray=non-DEGs. **(D)** Venn diagram shows B10⁺ cell DEGs from HCs and RA patients. **(E, F)** Gene ontology (GO) analysis of DEGs between B10⁺ and B10^{neg} cells in HCs and RA patients (y-axis=GO terms classified by P-value, x-axis=fold enrichment). DEGs: differentially expressed genes; HC: healthy controls.

than B10^{neg} cells (Fig. 2A-C). The most important difference was in CXCR5 expression: mean of 74.2 (5.2) % of B10⁺ cells but only 37.0 (8.0) % of B10^{neg} cells were CXCR5^{high} ($P < 0.0001$, $n=24$) (Fig. 2B). Of note, CXCR5 was also overexpressed on all B10 precursors (CD24^{hi}CD38^{hi}, CD24^{hi}CD27⁺ and CD5⁺ B cells) compared with CD24^{low} B cells

The cell-surface expression of CKRs was also assessed in B10⁺ and B10^{neg} cells from RA patients

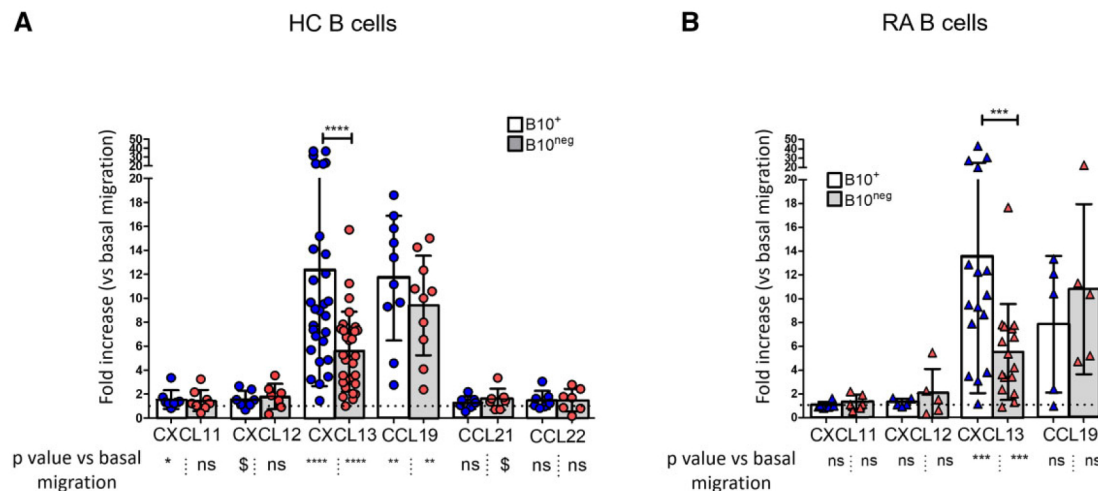
CXCR5 also showed differential expression in RA B10⁺ cells compared to RA B10^{neg} cells ($P = 0.0039$, $n=18$) (Fig. 2D-F). However, the proportion of CXCR5^{high} cells and MFI

Fig. 2 Comparison of chemokine receptor expression between B10⁺ and B10^{neg} cells



(A) Representative plots for CKR expression on HC B10⁺ cells and IL-10-negative B cells (B10^{neg}). **(B, C)** Proportion of cells positive for CKRs **(B)** and delta MFI **(C)** in B10⁺ vs B10^{neg} cells from HC ($n = 9-24$). **(D-F)** CXCR5 expression in HC and RA B10⁺ and B10^{neg} cells. Comparison of CXCR5^{high} percentage **(E)** and relative delta MFI **(F)** in B10⁺ and B10^{neg} cells from HCs ($n = 18$) and RA patients ($n = 18$). Statistical differences between B10⁺ and B10^{neg} within donor groups were evaluated by Wilcoxon's test and differences between HCs and RA patients by Mann-Whitney t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. CKR: chemokine receptor; HC: healthy control; MFI: mean fluorescence intensity.

Fig. 3 B10⁺ and B10^{neg} cell migration toward chemokines in healthy controls and RA patients



(A) Comparison of migration of HC B10⁺ and B10^{neg} cells toward CXCL11 ($n=8$), CXCL12 ($n=7$), CXCL13 ($n=31$), CCL19 ($n=10$), CCL21 ($n=7$) and CCL22 ($n=7$). **(B)** Comparison of migration of RA B10⁺ and B10^{neg} cells toward CXCL11 ($n=6$), CXCL12 ($n=5$), CXCL13 ($n=16$) or CCL19 ($n=5$). Results are expressed as fold increase compared with basal migration toward control media (RPMI 1%FCS+vehicle) and represented as mean (s.d.). Wilcoxon test was used to compare migration of B-cell subset toward chemokines vs basal migration and to compare B10⁺ vs B10^{neg} cell migration toward CKs. \$ $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. HC: healthy control.

for CXCR5 among B10⁺ or B10^{neg} cells were significantly lower in RA patients than HCs (Fig. 2E and F). These data were confirmed by using an antibody conjugated to another fluorochrome less prone to be affected by auto-fluorescence

B10⁺ cells preferentially migrate toward CXCL13, the only ligand of CXCR5

We then evaluated migration of sorted B10⁺ and B10^{neg} toward CXCL11 (CXCR3 and CXCR7 ligand), CXCL12 (CXCR7 and CXCR4 ligand), CXCL13 (CXCR5 ligand), CCL21 and CCL19 (CCR7 ligands) and CCL22 (CCR4 ligand). We sorted B10⁺ and B10^{neg} cells by FACS and performed chemotaxis assay toward recombinant CKs. Among all CKs tested, only CXCL13 and CCL19 induced a high and significant migration of B10⁺ and of B10^{neg} cells both from HCs (Fig. 3A) and RA patients (Fig. 3B). Importantly, CXCL13 attracted significantly more B10⁺ than B10^{neg} cells, in both HCs ($P < 0.0001$, $n=31$) and RA patients ($P = 0.0021$, $n=16$), whereas CCL19-induced migration did not significantly differ between B10⁺ and B10^{neg} ($P = 0.16$ for HCs and $P = 0.31$ for RA patients). CXCL13 induced similar migration of HC B10⁺ cell and RA B10⁺ cell ($P = 0.75$) and of RA B10^{neg} and HC B10^{neg} cells ($P = 0.99$). We also evaluated migration of B10 precursors stimulated or not by CpG

and found that in HC donors, after TLR9 activation, CXCL13-induced migration of CD24^{hi}CD38^{hi} B10⁺ precursors was slightly but significantly increased compared with CD24^{low} B cells ($P = 0.0078$, $n=9$)

Rheumatology online). In RA, migration of B10⁺ precursors and of CD24^{low} to CXCL13 were not statistically different ($P = 0.11$, $n=8$)

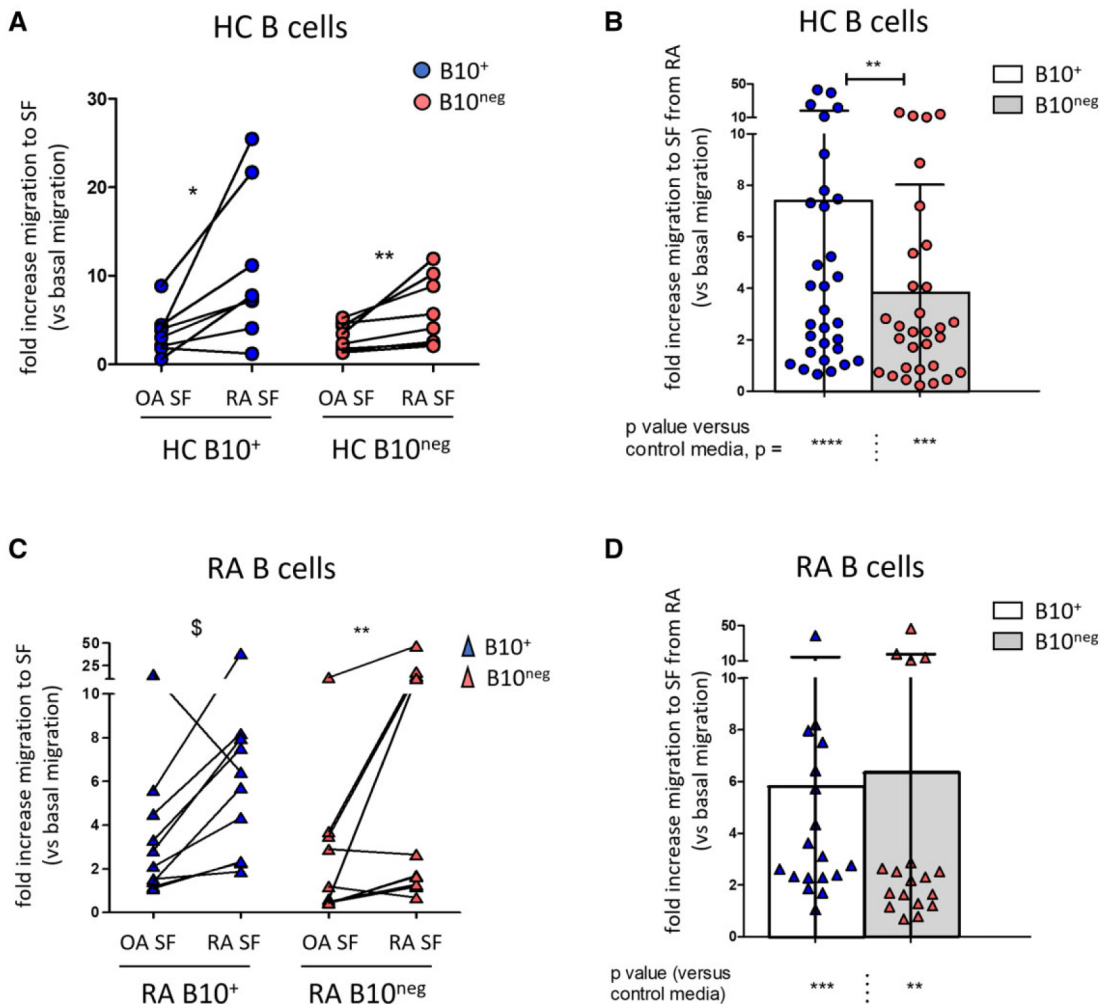
Of note, B10 precursors' repartition among total B cells did not differ between RA and controls in freshly isolated B cells neither after CpG stimulation

nor among B10⁺ and B10^{neg}

SF from RA patients induces a high and preferential migration of HC B10⁺ over B10^{neg} cells but a similar migration of RA B10⁺ and B10^{neg} cells

We then assessed the migration capacity of B10⁺ and B10^{neg} cells from HCs toward inflammatory SF from RA or toward SF from patients with non-inflammatory rheumatism (OA patients). Migration of HC B10⁺ cells was greater toward SF from RA than SF from OA patients ($P = 0.03$, $n=8$), as was migration of HC B10^{neg} cells (Fig. 4A). Importantly, although both HC B10⁺ and B10^{neg} cells were attracted, HC B10⁺ cells migrated significantly more than HC B10^{neg} cells toward RA SF ($P = 0.002$, $n=31$) (Fig. 4B). RA B10⁺ cell migration was also greater toward RA SF than OA SF, although not significant (Fig. 4C) ($P = 0.06$, $n=10$). In contrast to HC B10⁺ cells, RA B10⁺ cells did not significantly migrate more than RA B10^{neg} cells toward SF from RA patients ($P = 0.16$, $n=18$) (Fig. 4D). Of note, migration of RA B10⁺ and CTL B10⁺ cells toward SF from RA patients did not significantly differ ($P = 0.83$), nor that of RA B10^{neg} and CTL B10^{neg} cells ($P = 0.64$).

Fig. 4 Migration of healthy control and RA B10⁺ and B10^{neg} cells toward OA and RA synovial fluid (SF)



(A) Migration of HC B10⁺ and HC B10^{neg} cells toward SF from RA patients compared with SF from OA patients ($n=8$). (B) Comparison of HC B10⁺ vs HC B10^{neg} cell migration toward RA SF ($n=31$). (C) Migration of RA B10⁺ and RA B10^{neg} cells toward SF from RA patients compared with SF from OA patients ($n=10$). (D) Comparison of RA B10⁺ vs RA B10^{neg} cell migration toward RA SF ($n=18$). Results are expressed as fold increase compared with basal migration toward control media (RPMI 1%FCS) and represented as mean (s.d.). Wilcoxon test $\$P=0.06$, $*P<0.05$, $**P<0.01$. HC: healthy control.

CXCL13 in SF drives the preferential migration of HC B10⁺ cells but not RA B10⁺ cells

To decipher the preferential migration of HC B10⁺ cells toward RA SF, we measured the level of CXCL13 in SF from RA and OA patients used in migration assay. Mean CXCL13 level was significantly higher in RA SF than OA SF [1359.5(484.8) vs 93.6(103.3)pg/ml, $P=0.0005$] (Fig. 5A). Furthermore, HC B10⁺ cell migration toward RA SF was correlated with CXCL13 level ($r=0.6$, $P=0.03$, $n=14$) (Fig. 5B), confirmed in a second set of experiments ($r=0.8$, $P=0.02$, $n=8$).

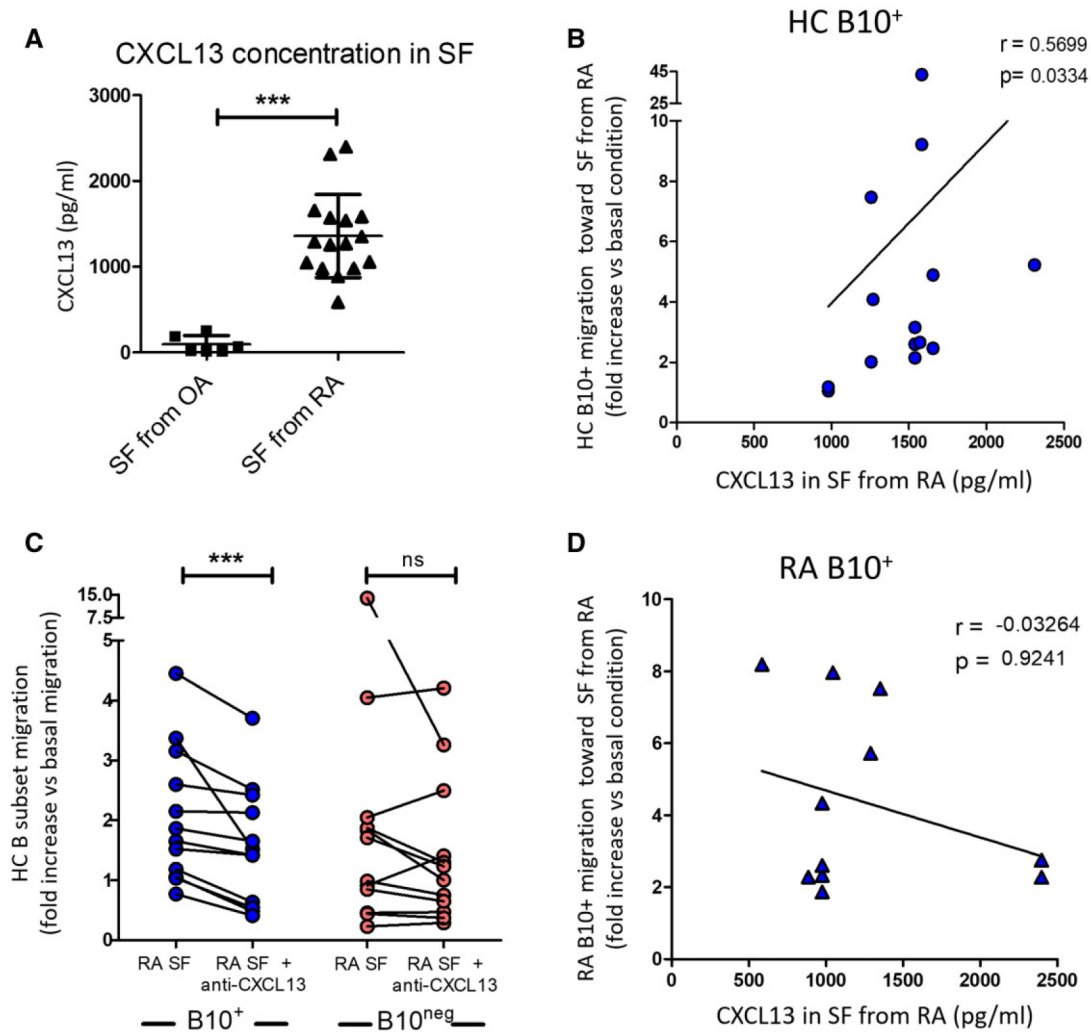
In contrast, HC B10^{neg} cell migration toward RA SF did not significantly and consistently correlate with CXCL13 level ($r=0.47$, $P=0.09$, $n=14$ and $r=0.3$, $P=0.43$, $n=8$). Moreover, the addition of a CXCL13-neutralizing antibody to RA SF

significantly reduced the migration of HC B10⁺ cells ($P=0.0005$, $n=11$), whereas the migration of B10^{neg} cells was not significantly affected ($P=0.23$) (Fig. 5C). Of note, isotype control antibody did not significantly impact B-cell migration. In contrast to what was observed in HCs, RA B10⁺ cell migration was not correlated with CXCL13 level in RA SF ($r=-0.03$, $P=0.9$, $n=11$) (Fig. 5D), which suggests a defect in the CXCL13/CXCR5 axis in B10⁺ cells from RA patients.

CXCL13 level is correlated with synovial B10⁺ cells and promotes IL-10 production

We next assessed B10⁺ cell frequency in SF from RA patients. Because SF from OA donors contained very few synovial fluid mononuclear cells (SFMCs), we used SF from patients with other inflammatory rheumatism

Fig. 5 Impact of CXCL13 in SF on migration of B10⁺ cells from healthy controls and RA patients

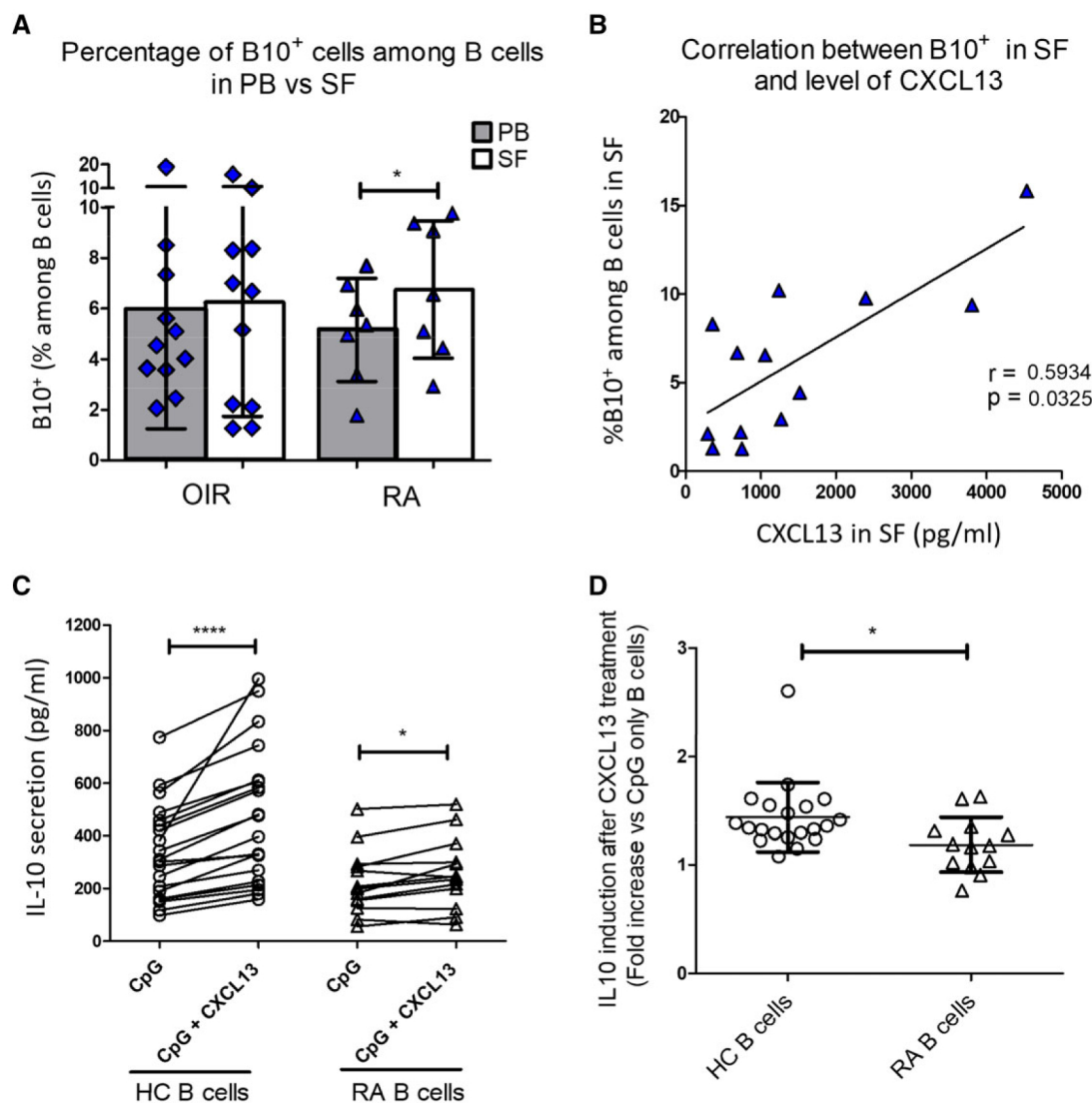


(A) CXCL13 level in SF from OA and RA patients by ELISA; Mann–Whitney test, * $P < 0.05$, ** $P < 0.01$ **(B)** Correlation between migration of HC B10⁺ cells toward SF from RA patients and CXCL13 level in SF; Spearman test. **(C)** Comparison of HC B10⁺ and B10^{neg} cell migration toward SF from RA patients with or without CXCL13 antibody ($n = 11$); Wilcoxon test, * $P < 0.05$, *** $P < 0.001$ **(D)** Correlation between migration of RA B10⁺ cells toward SF from RA patients and CXCL13 level measured in SF; Spearman test. HC: healthy control.

(OIRs) as a comparison group. We compared the proportion of B10⁺ cells (among B cells) in peripheral blood (PB) and SF. The proportion of B10⁺ cells among B cells was significantly higher in SF than PB from RA patients [6.7 (2.7) % vs 5.1 (2.0) %, $P = 0.04$] (Fig. 6A) but not from OIRs. We also found a correlation between B10⁺ cell frequency in SF and CXCL13 level (five SF from RA patients and eight SF from OIR patients) ($r = 0.59$, $P = 0.03$, $n = 13$) (Fig. 6B). Thus, circulating B10⁺ cells or B10⁺ precursors might be attracted to SF, or the micro-environment including CXCL13 may promote differentiation of B10⁺ cells. Nevertheless, we found a positive correlation between PB B10⁺ and SF B10⁺ ($r = 0.66$; $P = 0.01$, $n = 13$) which

does not support that migration into the joint mirrors B10 depletion in RA blood. Consequently, we hypothesized that micro-environment including CXCL13 may promote local differentiation of B10⁺ cells. To assess the impact of CXCL13 on B10⁺ cell induction, we cultured B cells with CpG and CXCL13 for 24 h and measured IL-10 by ELISA. In HCs, CXCL13 induced a significant increase in IL-10 secretion as compared with control media [1.44 (0.3)-fold increase, $P = 0.0002$, $n = 17$] (Fig. 6C). Of note, basal IL-10 secretion tended to be lower in RA than HC B cells ($P = 0.074$). In RA patients, CXCL13 induced a significant but minor IL-10 secretion [1.2 (0.2)-fold increase, $P = 0.005$, $n = 12$] (Fig. 6C). In accordance with lower expression of

Fig. 6 Local impact of CXCL13 on B cells (CD19+)



(**A**) Percentage of B10⁺ cells among B cells in PB vs B cells in SF from RA patients ($n=7$) and from OIR patients [$n=10$ (four psoriatic arthritis and six gout)]; Wilcoxon test. (**B**) Correlation between % of B10⁺ cells in SF and CXCL13 level in SF (five RA SF and eight OIR SF); Spearman correlation test. (**C**, **D**) IL-10 in supernatant of CXCL13-treated B cells in HCs ($n=20$, left) and RA patients ($n=13$, right). Comparison to control media (CpG alone) using Wilcoxon test. (**D**) Comparison of CXCL13-induced IL10 secretion between HC and RA B cells isolated from blood; Mann-Whitney test. * $P < 0.05$, **** $P < 0.000$. OIR: other inflammatory rheumatism; PB: peripheral blood...

CXCR5 expression on RA B cells (Fig. 2D, E), induction of IL-10 secretion was significantly lower in RA than HC B cells ($P = 0.014$) (Fig. 6D).

Discussion

Compared with regulatory T cells and macrophage subsets, Bregs are an emerging field and still need to be better functionally and phenotypically characterized. Transcriptomic analyses are precious tools to identify differentially expressed markers or pathways. In humans,

Van de Veen *et al.* perform transcriptomic comparison by microarrays of B10⁺ and B10^{neg} cells after 3 days of TLR9 stimulation with CpG [6], and Lin *et al.* sorted B10⁺ cells after 3 days of TLR9/BCR activation [27]. Because we previously showed that 24h of CpG stimulation allowed for optimal generation of B10⁺ cells with low mortality [5], we used this protocol to generate B10⁺ cells for transcriptional analysis. In our study, we found that CKs and their associated signalling pathways were among the most differentially expressed in B10⁺ cells as compared with B10^{neg} cells, which suggests that they are

crucial for B10⁺ cell biology and that CKR could be useful to identify B10⁺ cells. In the study from Van de Veen *et al.*, several genes from CK pathways were also differentially expressed in B10⁺ vs B10^{neg} cells, with the CKRs CXCR5 and ACKR2 respectively up- and down-regulated in B10⁺ vs B10^{neg} cells [6]. In a recent meta-analysis by Dubois *et al.* [28], the GO pathway enrichment analysis performed in transcriptomic studies of Bregs revealed one common enriched GO term related to 'leucocyte migration' [6, 27–29]. However, in previous studies, CKRs were not further investigated, and data on CKR expression in Bregs are lacking.

In our study, we therefore went a step further and assessed the expression of the candidates at the cell-surface level by flow cytometry. Although many CKRs were overexpressed on B10⁺ cells compared with B10^{neg} cells, we were unable to identify a CKR pattern that strictly defined B10⁺ cells. However, our CKR analysis revealed for the first time that CXCR5 was the most differentially expressed between B10⁺ and B10^{neg} cells. We further investigated CKR functions and found that, among all CKs tested, CXCL13 and CCL19 could induce a high migration of B10⁺ cells. More importantly, only CXCL13 was able to preferentially attract B10⁺ cells as compared with B10^{neg} from HC and RA patients. This finding is consistent with the higher expression of CXCR5 on B10⁺ than on B10^{neg} cells.

In the present work, consistent results between B10⁺ cells and B10⁺ precursors were found, with overexpression of CXCR5 for all B10⁺ precursors and an increased migration towards CXCL13 for CD24^{hi}CD38^{hi} compared with other B cells. However, the fact that CD24^{hi}CD27⁺ and CD5⁺ precursor subsets did not preferentially migrate towards CXCL13 suggests that not only CXCR5 expression but also other cellular receptor beyond the scope of this study (such as integrin, S1PR1, ACKR2) might impact CXCL13-induced migration [30–33].

CXCL13 is highly expressed on inflammatory sites including RA SF and RA synovial tissue [34–36]. We found that SF from RA patients induced a high and preferential migration of HC B10⁺ over B10^{neg} cells, and CXCL13 level in SF was correlated with HC B10⁺ migration toward RA SF. Therefore, our results reveal an important role for CXCL13 in driving regulatory B10⁺ cell migration toward the inflammatory site. However, they also suggest that this pathway is impaired in RA patients. First, CXCR5 level was decreased in RA B10⁺ vs HC B10⁺ cells, in line with a previous publication that described decreased expression of CXCR5 on freshly isolated B cells from RA patients [37]. Second, RA B10⁺ cells were not preferentially attracted toward RA SF. Because RA B cells express a lower level of CXCR5, the CXCR5–CXCL13 axis is likely no longer the major determinant of B10⁺ migration in RA. Synovial fluid from RA contains a high amount of CKs, that could participate in B-cell recruitment into the joint [36, 38]. The presence of other CKs in RA SF could therefore interfere with CXCL13-induced migration and the subsequent

preferential migration of B10⁺ cells from RA patients [38–40]. In addition, a recent study described that, in RA, CD24^{hi}CD27⁺ B cells convert into pathogenic cells and highly accumulated into RA SF [36]. The frequency of CD24^{hi}CD27⁺ B cells in RA SF was correlated with CXCL13 but also with CXCL12 and CCL19 level in SF [36]. Therefore, the defect of RA B10⁺ cells' preferential recruitment could also be explained by the CK-rich environment in RA SF, which could target CKRs that can be differentially expressed in RA patients vs HCs.

B10⁺ cells can be found in SF from patients with inflammatory rheumatism, including RA patients, and the frequency of B10⁺ cells was higher in SF than peripheral blood samples (our results and [41]). In addition, we found SF B10⁺ cells correlated with CXCL13 level measured in this SF, so CXCL13 may mediate B10⁺ cell precursor migration within inflammatory joint or a local differentiation of B10⁺ cells. Indeed, one hypothesis could be that B10 migrated from blood to the joint, and might therefore explain the decrease of B10 cells in the blood of RA patients, as already proposed for CD24^{hi}CD38^{hi} B cells and CD24^{hi}CD27⁺ B cells [3, 36]. However, in the present study, the decrease of B10⁺ cells frequency in the blood did not correlate with their frequency in SF, which does not support that migration into the joint mirrors B10 depletion in blood. This led us to investigate whether CXCL13 could also be involved in B10⁺ cells' differentiation. Indeed, CXCL13 increased IL-10 secretion in B cells stimulated with CpG in HC and in RA patients. Thus, the rate of B10⁺ cells found in RA SF might reflect B10⁺ cell migration but also local differentiation of B cells due to the micro-environment including CXCL13. However, in accordance with the lower level of CXCR5 on RA B cells, induction of IL-10 secretion was lower in RA than HC B cells.

Altogether, our results reveal a new role for CXCL13 in attracting and sustaining regulatory features of B10⁺ cells within the inflammatory sites. More importantly, we observed decreased expression of CXCR5 in RA, which was associated with altered migration of RA B10⁺ cells to RA SF and a decreased IL-10 secretion of B cells in response to CXCL13. Restoring the preferential migration of B10⁺ within the affected joints to better control inflammation may be part of the therapeutic approach for RA.

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