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Recruitment of CXCR3$^+$ T cells into injured tissues in adult IgA vasculitis patients correlates with disease activity☆

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ABSTRACT

Objectives: Adult immunoglobulin A vasculitis (IgAV) is an immune complex small vessel vasculitis. So far, the involvement of T cells in this pathology has been poorly studied. The aim of this study was to analyze T-cell homeostasis as well as cytokine and chemokine concentrations in the blood and tissues of IgAV patients.
Methods: T cells, cytokine and chemokine concentrations were analyzed in peripheral blood using flow cytometry and multiplex assays. T-cell infiltrates in the kidney and the skin were characterized by immunohistochemistry. This study prospectively included 44 adult patients with biopsy-proven IgAV and 24 age- and sex-matched healthy controls.
Results: We observed reduced proportions of circulating CXCR5-and CXCR3-expressing memory CD4 T cells at diagnosis but normal values at remission. The plasma levels of Th1-related cytokines (IL-12, IL-27 and IFNγ) and of the TFH-related cytokine, IL-21, were paradoxically not reduced in patients. We observed increased plasma concentrations of the CXCR5 ligand, CXCL13, and of the CXCR3 ligands, CXCL10/11, suggesting a potential relocation of the corresponding T cells into inflamed tissues. We then confirmed the recruitment of CXCR3-expressing T cells into the skin and kidneys. In the skin, T-cell infiltrates mainly co-localized with damaged dermal small vessels. Finally, patients with the largest kidney T-cell infiltrates were also those with the highest proteinuria.
Conclusion: Altogether, our results strongly suggest that, in IgAV patients, CXCL10/11 orchestrate the recruitment of CXCR3-expressing T cells in injured tissues, contributing to tissue damage and disease activity.

1. Introduction

Immunoglobulin A (IgA) vasculitis, previously known as Henoch–Schönlein purpura, is an immune complex small vessel vasculitis with IgA1-dominant immune deposits [1]. Whereas IgA vasculitis (IgAV) is the most common systemic vasculitis in childhood with an annual incidence of 3–26 per 100 000 children, its outcome is generally good with most children experiencing rapid remission [2]. In contrast, while the disease is rare in adults with an incidence of about 0.8–1.8 per 100 000 inhabitants [3], the prognosis of patients is much worse [4,5]. Indeed, as recently reported in a French cohort of 260 patients, adult IgAV frequently affects the skin, the gastrointestinal tract, the joints with arthralgia and/or arthritis, and the kidneys [6,7]. Gastrointestinal tract and renal involvements represent the main causes of morbidity and mortality in adults.

IgAV is an autoimmune disease with contributing genetic (polymorphisms located in the HLA class II region, HLA-DRB1) and environmental components such as mucosal infections [6,8]. Like in IgA Nephropathy (IgAN), circulating galactose-deficient IgA1, produced by plasma cells, constitutes a key auto-antigen leading to the production of

☆ Data from 44 patients included in a French Multicenter rospective Survey.

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autoantibodies and the subsequent formation of immune complexes [9,10]. In kidneys, circulating immune complex deposits are thought to induce mesangial cells to proliferate and to overproduce cytokines/chemokines [11–14]. In agreement, preformed immune complexes of human hypoglycosylated IgA1 and specific autoantibodies injected into SCID mice deposit on mesangial cells and induce their proliferation as well as proteinuria and hematuria [9]. Skin histological changes include leukocytoclastic vasculitis affecting dermal small vessels and characterized by nuclear debris from infiltrating neutrophils [6].

A bias of circulating T cells toward Type-1 (Th1) and Type-17 (Th17) helper T cells has been described in pediatric IgAV [15,16], but, so far, infiltration of T lymphocytes into injured tissues remains to be studied in both pediatric and adult IgAV. Here, we have evaluated the contribution of various T-cell subsets to adult IgAV with a particular emphasis on chemokine receptor expression. Accordingly, we have compared the proportions of CCR4-, CCR6-, CXCR3-and CXCR5-expressing T cells in the peripheral blood of 44 adult patients with IgAV and 24 healthy individuals by flow cytometry and concomitantly analyzed the plasma levels of 19 cytokines and chemokines. In addition, we have characterized T cells infiltrating the skin and kidneys on a small number of patients. Taken together, our present results point out CXCR3 and its ligands CXCL10/11 as key players in T-cell infiltration in tissues in adult IgAV patients. Such trapping of T cells in injured tissues might exacerbate tissue damage.

2. Materials and methods

2.1. Patients

IgAV patients were included in the prospective cohort "HSPrognosis" (Assitance Publique-Hôpitaux de Paris, APHP) [17]. This survey was approved by the "Comité Consultatif sur le Traitement de l'Information en matière de Recherche dans le domaine de la Santé, CCTIRS" (7th April 2011) and by the "Commission Nationale de l'Informatique et des Libertés, CNIL" (20th December 2011). All patients gave informed consent before inclusion in accordance with the Declaration of Helsinki. This multicentric survey was conducted in French Hospitals in departments of Internal Medicine, Nephrology and Dermatology. The inclusion/exclusion criteria have been exhaustively described previously [17]. Briefly, patients were included if they were > 18 years and presented IgA vasculitis. Patients were considered to have IgAV if they presented [1] purpura with or without involvement of such one organ among kidney, joint, or intestinal tract [2], histologically proven small vessels vasculitis with IgA deposits. Clinical and biologic data were recorded for each patient at the time of the initial evaluation and at the end of follow-up (12 months after inclusion), by the practitioners in charge of the patients with the use of a standardized form. Laboratory assessment included in particular the determination of serum creatinine level and an urinalysis to determine hematuria and the urine protein/creatinine ratio. Renal failure was defined as an eGFR < 60 mL/min/1.73 m^2, assessed with the Modified Diet in Renal Disease equation [18]. Proteinuria was defined as urine protein/creatinine > 0.5 g/g, and hematuria was defined as > 10 red cells/mm^3 in the urine considered as macroscopic if > 1500 red cells/mm^3. Remission of the disease 12 months after diagnosis was defined as an improvement in at least one-half of the baseline clinical manifestations, and in case of renal involvement as an improvement of proteinuria > 50% of the baseline value, disappearance or not of hematuria, and no decrease of the GFR greater than 20% from baseline. All others patients were classified as active. Table 1 shows the demographic and clinical characteristics of 44 adult patients with IgAV and 24 age and sex-matched Healthy Controls (HC). Mean age at diagnosis of the patient group was 51.7 and 52.1 years for HC with half patients (47%) and HC (54%) being male. Clinical manifestations included cutaneous involvement with purpura in all patients (100%), renal involvement in 30 patients (68%), arthralgia/arthritis in 22 patients (50%) and gastrointestinal involvement in 15 patients (34%). Forty percent of the patients showed renal failure (eGFR < 60 ml/min/1.73 m^2) at diagnosis. Patients with renal involvement displayed a 79 ± 8 mL/min/1.73 m^2 eGFR mean, a 1.31 ± 0.17 g/g proteinuria level median, and 83% presented hematuria at diagnosis. Levels of serum IgA were 4.51 ± 0.27 and 2.67 ± 0.19 g/L in patients and HC respectively. Half of the cohort (52%) but no HC presented elevated IgA levels at diagnosis (IgA > 3.5 g/L).

Table 1
Baseline clinical and biological characteristics of patients with IgA vasculitis and healthy controls.

Baseline characteristics	Patients n = 44	Controls n = 24	p
Epidemiological features			
Age at diagnosis, y, mean ± SEM	51.7 ± 2.7	52.1 ± 4.0	0.9308
Male, n (%)	21/44 (47)	13/24 (54)	0.8000
Clinical manifestations			
Skin involvement, n (%)	44/44 (100)		
Kidney involvement, n (%)	30/44 (68)		
Joints involvement, n (%)	22/44 (50)		
GI involvement, n (%)	15/44 (34)		
Biologic features			
Serum IgA level, g/L, mean ± SEM	4.51 ± 0.27	2.67 ± 0.19	0.0016
Elevated serum IgA (> 3.5 g/L), n (%)	24/41 (58)	0/10 (0)	0.0008
Serum creatinine level, µmol/L, mean ± SEM	103 ± 11		
eGFR, mL/min/1.73m^2, mean ± SEM	79 ± 8		
eGFR < 60 mL/min/1.73m^2, n (%)	12/30 (40)		
Hematuria, n (%)	25/30 (83)		
Proteinuria/creatinuria, g/g, mean ± SEM*	1.31 ± 0.17		
Proteinuria/creatinuria > 1 g/g, n (%)	13/30 (43)		

SEM = standard error; eGFR = estimated glomerular filtration rate,*among the 30 patients with renal involvement.

2.2. Samples

2.2.1. Isolation of PBMCs and plasma collection

Peripheral blood from patients and HC were collected on heparin. Peripheral blood mononuclear cells (PBMCs) were freshly purified on a Ficoll density gradient. Then, PBMCs were frozen in 10% dimethylsulfoxide (DMSO)/90% fetal calf serum (FCS). Plasma was collected, aliquoted and stored at −80 °C.

2.2.2. Tissue specimen collection

Skin (n = 13) and renal (n = 7) biopsies were retrospectively obtained from untreated patients recruited in the cohort. The skin specimens consisted of residual tissues from 5 mm punch biopsies obtained for clinical purposes. Tissue samples were embedded in paraffin for long-term storage according to standard clinical protocols. Once requested by the survey, 5-µm sections were available for immunohistochemistry (IHC) analysis. Skin and renal specimens from individual controls were respectively obtained from 3 healthy volunteers who underwent breast plastic surgery (n = 3) and from patients (n = 2) with diabetic nephropathy.

2.3. Fluorescence staining and flow cytometry

The following monoclonal antibodies were used in this study: for 11-color membrane flow cytometry analyses: CD3-allophycocyanin-H7 (clone SK7, dilution factor: 1/20); CD4-Pacific Blue (clone RPA-T4, 1/40); CD8-Brilliant Violet 650 (clone SK1, 1/50); CD25-PE-Cyanine7 (clone M-A251, 1/20); CD45RA-Brilliant Violet 711 (clone HI 100, 1/40), CCR6-Brilliant Violet 510 (clone 11A9, 1/10), CXCR3-PE-Cyanine

7 (clone 1C6, 1/10) and γδ TCR-PerCP-Cyanine5.5 (clone B1, 1/10) from BD Biosciences; CXCR5-Alexa 488 (clone J252D4, 1/40) from Biolegend; CD127-allophycocyanin (clone REA614, 1/20) from Miltenyi Biotec. In all experiments, the Live/Dead blue Dye (Invitrogen) was used to exclude dead cells.

Frozen PBMCs were incubated during 10 min in RPMI 1640 Glutamax (Gibco) supplemented with 10% FCS (Biochrom) warm up to 37 °C then washed in PBS. Cell suspensions were collected and dispensed into 96-well round-bottom microtiter plates (Greiner Bioscience; 2×10^6 cells/well). PBMCs were washed and incubated for 30 min at +4 °C with Live/Dead blue dye in PBS. Five percent (vol/vol) heat-inactivated human AB serum (Abcys) was added for an extra 15 min at +4 °C. Next, cells were labeled for 30 min at +4 °C with antibodies diluted in PBS with 5% FCS (Biochrom) and 0.1% NaN_3 (Sigma-Aldrich) PBS. Cells were then washed, fixed with 0.5% paraformaldehyde, and events acquired using BD Fortessa flow cytometer (BD Biosciences). List-mode data files were analyzed using Diva software (BD Biosciences). Data acquisition was performed on the Cochin Cytometry and Immunobiology (CYBIO) facility.

2.4. Analysis of cytokine and chemokine plasma concentrations

A multiplex cytokine immunoassay panel was used to quantify plasma concentrations of CCL17, CCL20, CCL22, CXL10, CXCL11, GM-CSF, IFN-γ, IL-1β, IL-4, IL-6, IL-12p70, IL-17A, IL-21, IL-22, IL-23, IL-27, IL-33, and TNF-α, with a 1:2 dilution (U-PLEX Biomarker Group 1 Assays; MesoScale Discovery, Rockville, Maryland, USA). A standard curve was generated for each set of reagents. The minimum and maximum detection limits depend on the cytokines and chemokines, all of them were in the detection range. Quantification was performed using Workbench4.0 (MesoScale Discovery). Plasma CXCL13 concentration was analyzed by ELISA (Quantikine ELISA Kit, R&D Systems, Minneapolis, USA) with a 1:3 dilution (detection limit = 7.8 pg/ml) according to the manufacturers' instructions. Results were expressed as mean concentration of duplicates (pg/ml).

2.5. Immunohistochemistry

Antigen retrieval was performed on serial tissue sections (5 μm) before 20 min of incubation with primary antibodies (CD4 (clone1F6, 1/40, mouse, EDTA pH9) from Novocastra, CD8 (1/200, rabbit, EDTA pH9) from Abcam or CXCR3 (clone 1C6, 1/50, mouse, citrate pH6) from BD Biosciences). Following incubation with primary antibodies, all tissue sections were washed in PBS and incubated with secondary antibody: anti-rabbit IgG (25 μg/ml) or anti-mouse IgG (10 μg/ml) from Leica Biosystems (Bond Polymer Refine Detection) for 8 min. Image acquisition was performed on a Perkin Elmer Multilabel Lamina Slide Scanner in the brightfield scan mode. Data acquisition was performed on the Cochin Histology (HISTIM) facility. Quantitative analysis was conducted using inForm® Cell Analysis. Data are expressed as percentage of infiltration that is the ratio of the area of the signal on the total surface of the tissue analyzed.

2.6. Statistics

Data are expressed as mean ± SEM. Statistical comparisons between categories were done using the Mann-Withney test for unpaired samples and the Wilcoxon test for paired samples. Values of $p < 0.05$ were considered to be statistically significant. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Generation of graphs and statistical analysis were performed using Graph Pad 6. Correlation between two parameters was tested using the Spearman's rank test. P-values < 0.05 were considered statistically significant.

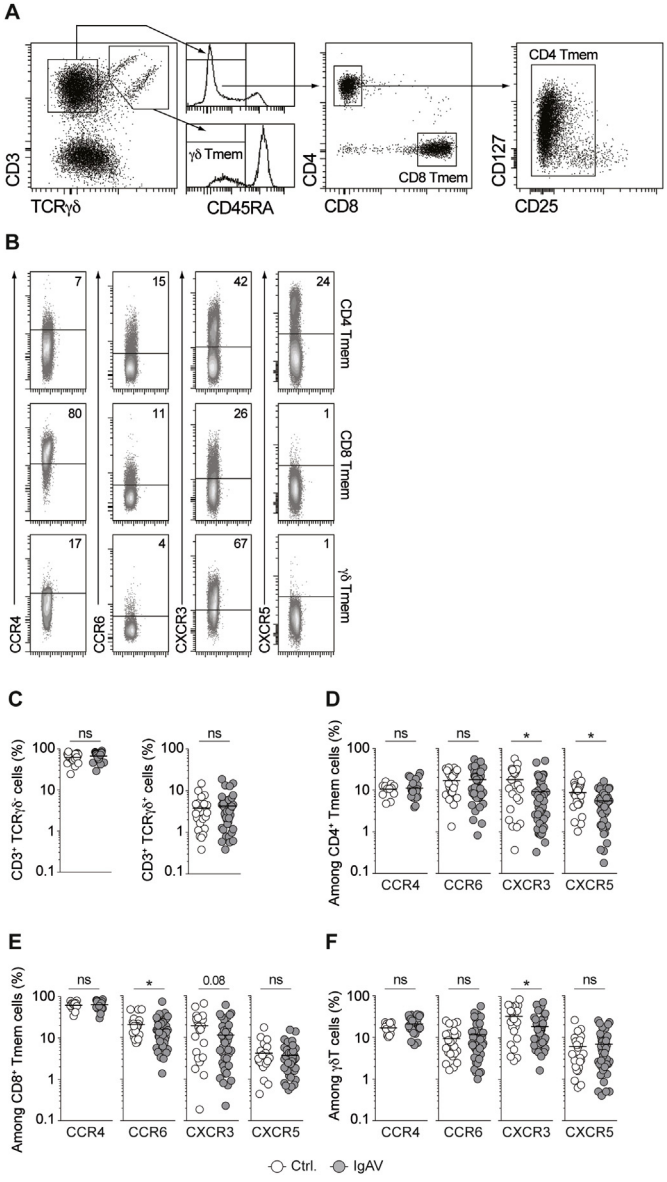

Fig. 1. Analysis of circulating T cells in the blood of IgAV patients at diagnosis. A/Gating strategy used to define CD4, CD8 and γδ Tmem cells. **B**/The expression of CCR4, CCR6, CXCR3 and CXCR5 chemokine receptors is shown for gated CD4, CD8 and γδ Tmem cells of a representative healthy control (HC). **C**/Percentages of $CD3^+$ $TCR\gamma\delta^-$ and $CD3^+$ $TCR\gamma\delta^+$ cells among living cells in the blood of IgAV patients and HC. **D-F**/Percentages of circulating $CCR4^+$, $CCR6^+$, $CXCR3^+$ and $CXCR5^+$ cells among CD4 (**D**), CD8 (**E**) and γδ (**F**) Tmem cells. Each dot represents an individual patient (grey) or HC (white). Values of $p < 0.05$ were considered significant (Mann-Withney).

3. Results

3.1. Decreased proportions of CXCR3-and CXCR5-expressing memory CD4 T cells in the blood of IgAV patients at diagnosis

To characterize circulating memory T cells, we carried out 11-color flow cytometric analyses (Fig. 1A and B). Memory CD4 T cells (CD4 Tmem) were defined as $CD3^+$ $TCR\gamma\delta^-$ $CD45RA^-$ $CD4^+$ $CD8^-$ cells. Activated regulatory T cells ($CD127^-$ $CD25^{hi}$) were excluded from the analysis of CD4 Tmem cells. Memory CD8 T cells (CD8 Tmem) were defined as $CD3^+$ TCR $\gamma\delta^-$ $CD45RA^-$ $CD4^-$ $CD8^+$ cells. Memory γδ T cells (γδ Tmem) were defined as $CD3^+$ TCR $\gamma\delta^+$ $CD45RA^-$ cells (Fig. 1A). These memory T-cell subsets were analyzed for the expression

of chemokine receptors (Fig. 1B). As previously described, 4 main cell-subsets can be identified within the CD4 Tmem-cell compartment according to the expression of CCR4, CCR6, CXCR3 and CXCR5 respectively corresponding to Th2, Th17, Th1 and TFH cells [19]. The gating strategy used to separate these various subpopulations is shown for a representative healthy donor (Fig. 1B). Naive CD4 T cells ($CD45RA^+$) do not express these 4 chemokine receptors [20]. The non-expression of CCR4, CCR6, CXCR3 and CXCR5 by naive CD4 T cells allowed us to define the expression positivity threshold for these chemokine receptors.

To highlight a potential imbalance in circulating Th cells in IgAV, we first studied the PBMCs of 24 healthy volunteers (or "controls", HC) and 44 patients with untreated IgAV at diagnosis. The proportions of $CD3^+$ $TCR\gamma\delta^-$ and $CD3^+$ $TCR\gamma\delta^+$ cells among living cells were identical in IgAV patients and HC (67.8% ± 1.8% versus 62.8% ± 3.0% and 4.3% ± 0.6% versus 3.8% ± 0.7, respectively) (Fig. 1C). We then compared the expression of the chemokine receptors CCR4, CCR6, CXCR3 and CXCR5 on circulating CD4, CD8 and γδ Tmem cells from HC and IgAV patients (Fig. 1D–F).

The proportions of circulating $CXCR3^+$ cells among CD4 and γδ Tmem cells were significantly reduced in IgAV patients compared to HC (9.8% ± 1.8% versus 17.7% ± 3.1%, p = 0.02 and 19.7% ± 2.7% versus 33.1% ± 4.7, p = 0.03, respectively) (Fig. 1D and F). The proportion of circulating $CXCR3^+$ cells among CD8 Tmem cells also tended to decrease in IgAV patients when compared to HC (12.3% ± 2.1% versus 19.1% ± 3.5%, p = 0.08) (Fig. 1E). By contrast, the proportions of $CCR4^+$ cells among all studied blood Tmem-cell subsets and of $CCR6^+$ cells in circulating CD4 and γδ Tmem cells were not statistically different between IgAV patients and HC (Fig. 1D–F). Interestingly, the proportion of CXCR5-expressing cells among CD4 Tmem cells was also significantly lower in IgAV patients than in HC (6.5% ± 0.9% versus 9.3% ± 1.1%, p = 0.02; Fig. 1D). Of note, the proportions of $CXCR3^+$ cells and of $CXCR5^+$ cells among CD4 Tmem cells did not differ between patients with or without renal, articular, or gastrointestinal involvement (Supplementary Fig. 1A and B). The proportion of circulating $CXCR5^+$ cells among CD4 Tmem cells only tended to be even lower in IgAV patients developing renal, articular and gastrointestinal manifestations than in the other IgAV patients (Score 0–2) without reaching significance (Supplementary Fig. 1C and D).

3.2. IgAV remission is associated with increased proportions of circulating CXCR3-and CXCR5-expressing memory CD4 T cells

We then compared among 26 patients of the 44 included in the cohort the composition of T-cell populations in PBMCs of IgAV patients at diagnosis and 12 months after diagnosis. Sixteen patients were in remission and 10 were still active after 12 months. Interestingly, the proportions of $CXCR3^+$ cells among CD4 and CD8 Tmem cells as well as among γδ Tmem cells increased in patients in remission 12 months after diagnosis when compared to the values observed at diagnosis (mean of the difference: 15.33% ± 4.7%, 13.6% ± 4.4% and 20.6% ± 6.7, respectively) (Fig. 2A). Similarly, the percentage of CXCR5-expressing cells among CD4 Tmem cells increased in patients in remission (mean of the difference: 5.0% ± 1.3%; Fig. 2B). Interestingly, such increases were not observed in patients who were still diagnosed as developing an active IgAV 12 months after the initial diagnosis (Fig. 2C and D). It should be noted that the proportions of $CXCR3^+$ cells among CD4, CD8 and γδ Tmem cells and of $CXCR5^+$ cells among CD4 Tmem cells were similar at diagnosis between patients who have achieved or not remission in the next 12 months (Supplementary Fig. 2). The comparison of T-cell subsets in the blood of patients before and after treatment supports our hypothesis that IgAV was accompanied by a selective decrease of circulating CXCR3+ and CXCR5+ CD4 Tmem cells probably reflecting their relocation into non-lymphoid inflamed tissues.

3.3. Plasma levels of Th1- and TFH-related cytokines are paradoxically not decreased in IgAV patients

We first hypothesized that the decrease of blood $CXCR3^+$ and $CXCR5^+$ CD4 Tmem cells in patients with IgAV reflect a defect in Th1 and TFH responses. Accordingly, we measured the concentration of 13 cytokines in the plasma of IgA-V patients and HC. Among the 5 tested pro-inflammatory cytokines tested, only IL-6 and TNF-α plasma levels were significantly increased in IgA-V patients when compared to HC (2.0 pg/ml ± 0.5 versus 0.4 pg/ml ± 0.1, p = 0.02 and 2.0 pg/ml ± 0.5 versus 1.0 pg/ml ± 0.1, p = 0.01, respectively) (Fig. 3A). Consistent with comparable proportions of circulating CCR4-and CCR6-expressing CD4 Tmem cells in IgAV patients and HC, plasma levels of Th2- and Th17-related cytokines did not differ between the 2 groups (Fig. 3B). Surprisingly, whereas the percentages of CXCR3-expressing cells among CD4, CD8 and γδ Tmem cells and of $CXCR5^+$ cells among CD4 Tmem cells were lower in IgAV patients than in HC, the plasma levels of Th1-related cytokines (IL-12, IL-27 and IFNγ) and of the TFH-related cytokine, IL-21, were not reduced in patients when compared to controls (Fig. 3B). Interestingly, IFNγ plasma level tended to increase in patients with renal and gastrointestinal manifestations and was significantly higher in IgAV patients exhibiting at the same time renal, articular and gastrointestinal manifestations (Score 3) than in the other patients (Score 0–2; Supplementary Fig. 3A and B).

3.4. Plasma levels of CXCR3 and CXCR5 ligands are increased in IgAV patients

We then hypothesized that, in patients with active IgAV, $CXCR3^+$ and $CXCR5^+$ T cells might be recruited into secondary lymphoid organs and/or inflamed non-lymphoid tissues in response to their specific ligands. CXCR3 binds three interferon (IFN)-γ-inducible ligands, CXCL9 (monokine induced by gamma-interferon), CXCL10 (interferon-induced protein-10) and CXCL11 (interferon-inducible T-cell alpha chemoattractant) whereas the unique ligand of human CXCR5 is CXCL13 [21].

We thus measured the plasma concentrations of CXCL10, CXL11 and CXCL13 but also of CCL17 and CCL22 (ligands of CCR4) and of CCL20 (ligand of CCR6) in IgAV patients and HC. (Fig. 4). In contrast to comparable CCL17, CCL22 and CCL20 plasma levels in IgAV patients and HC, those of CXCL10 and CXCL11 were significantly higher in IgAV patients than in HC (263.5 pg/ml ± 74.6 versus 100.2 pg/ml ± 21.3, p = 0.014 and 250.0 pg/ml ± 47.7 versus 98.9 pg/ml ± 41.9, p = 0.006, respectively). Of note, the concentration of CXCL13 in the plasma was also significantly increased in patients when compared to controls (140.7 pg/ml ± 9.2 versus 79.6 pg/ml ± 13.6, p = 0.0012). Interestingly, the plasma concentrations of CXCL10 and CXCL11 tended to increase in patients with gastrointestinal involvement (Supplementary Fig. 4A and B) and such increases were not far from significance when IgAV patients developing at the same time renal, articular and gastrointestinal manifestations (Score 3) were compared to the other IgAV patients (Score 0–2; Supplementary Fig. 1C and D).

3.5. CXCR3-expressing T cells infiltrate the skin and kidneys of IgAV patients

Increased plasma concentrations of CXCL10 and CXCL11 in IgAV patients might be due to a higher production of these chemokines in injured tissues. Release of these chemokines may then lead to the recruitment of Tmem cells expressing the corresponding chemokine receptor (CXCR3) into these tissues. To address this point, we performed histological analysis of 2 tissues frequently targeted in IgAV, the skin and kidneys. Specifically, we characterized T-cell infiltrates in these tissues by staining sections with anti-CD4, anti-CD8 or anti-CXCR3 antibodies (Fig. 5). By contrast to HC, in nearly all tested skin samples from IgAV patients, large infiltrates of $CD4^+$ cells, $CD8^+$ cells and $CXCR3^+$ cells were detected (Fig. 5A and B). Accordingly, the areas

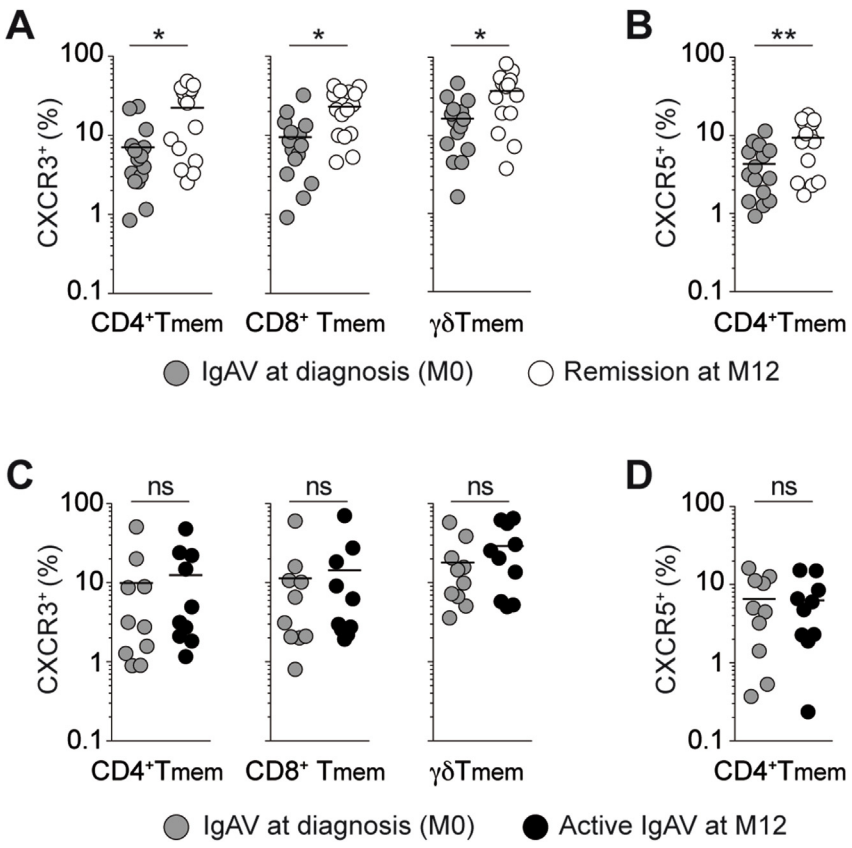

Fig. 2. Follow-up of IgAV patients reveals that remission is associated with an increase in the proportions of circulating CXCR3-and CXCR5-expressing memory T cells. Percentages of CXCR3$^+$ and CXCR5$^+$ T cells were estimated in the blood of IgAV patients at diagnosis and 12 months after diagnosis. Results were analyzed according to the clinical status of the disease, active versus remission, at that time. **A-B**/Percentages of circulating CXCR3$^+$ cells among CD4, CD8 and γδ Tmem cells (**A**) and of circulating CXCR5$^+$ cells among CD4 Tmem cells (**B**) of patients in remission. **C-D**/Percentages of circulating CXCR3$^+$ cells among CD4, CD8 and γδ Tmem cells (**C**) and of circulating CXCR5$^+$ cells among CD4 Tmem cells (**D**) of patients with active disease. Each dot represents an individual patient at diagnosis (grey) and after 12 months: patients in remission 12 months after diagnosis are represented by white dots and patients still with active disease 12 months after diagnosis by black dots. Values of $p < 0.05$ were considered significant (Wilcoxon).

corresponding to these cells were significantly larger in the skin of IgAV patients than in the skin from HC (Fig. 5B). Interestingly, T-cell infiltrates mainly co-localizes with damaged dermal small vessels (Fig. 5A). In the kidneys, the results were more heterogeneous with only part of IgAV patients showing convincing infiltrates of CD4$^+$, CD8$^+$ and CXCR3$^+$ cells, mostly limited to the renal interstitium (Fig. 5C and D). Interestingly, IgAV patients with the largest T-cell infiltrates in kidneys were also those with the highest proteinuria (Fig. 5E).

4. Discussion

The pathological mechanisms leading to IgAV remain largely unknown. The main data come from studies of IgA nephropathy (IgAN), which can be in fact considered as one of the localized consequences of IgAV. Aberrant deposition of hypoglycosylated IgA1 and complement activation are thought to contribute to IgAN and IgAV [9,17,22]. However, precise mechanisms leading to vasculitis in elected tissues remain to be studied. Deposits of immune complexes including IgA and infiltration of neutrophils have been observed in the skin of IgAV patients, but, so far, no study has highlighted the presence of T-cell infiltrates in the tissues classically injured in the course of IgAV.

Our work highlight for the first time infiltrates of CXCR3$^+$ T cells in 2 tissues classically injured in the course of IgAV, the skin and kidneys. As in most studies in humans, the main limitation of this survey is that we cannot fully demonstrate the involvement of CXCR3$^+$ T cells in the physiopathogenesis of the disease. In addition, there is currently no robust mouse model in which we could have tested this hypothesis. It would have been indeed interesting to inhibit the CXCL10-11/CXCR3 axis at the onset of the disease and to analyze then its severity. Finally, it could have been better to work with fresh, unfrozen blood samples but, obviously, this was barely feasible due to the rarity of adult IgAV. However, we think that our results are robust because PBMCs from healthy controls and patients were all processed and frozen with the same protocol, in the same center (Bichat Hospital, Paris, France). The main strength of this work is that we had the opportunity to study many patients with regard to the rarity of the disease. Moreover, for some patients, we had the chance to be able to study injured tissues in addition to their PBMCs. Indeed, this opportunity has allowed us to show that what can be observed in the blood is not a reflection of what happens in tissues.

Chemokine receptors and their ligands are critical regulators of leukocyte trafficking and immune functions [23]. In particular, the chemokine receptor CXCR3 and its ligands play a key role in promoting the recruitment of lymphocytes into inflamed tissues. Absent from naïve T cells, CXCR3 expression is up-regulated following T-cell activation. CXCR3 has 3 ligands preferentially induced by type II/I IFN: CXCL9 (monokine induced by gamma-interferon), CXCL10 (interferon-induced protein-10) and CXCL11 (interferon-inducible T-cell alpha chemoattractant) [21]. T-bet, the transcription factor whose expression characterize Th1 CD4 T cells and cytotoxic CD8 T cells, directly transactivates CXCR3 expression [21]. Consequently, CXCR3 expression is mainly associated with these αβ T-cell subsets [19,24,25]. In addition to these cells, CXCR3 is also highly expressed by other lymphocyte subsets such as γδ T cells [26]. CXCR3 has been implicated in many autoimmune and inflammatory diseases including coeliac disease, rheumatoid arthritis, autoimmune thyroiditis, type 1 diabetes mellitus and inflammatory bowel disease [27–31]. In transgenic mice, the overexpression of CXCL10 in the pancreas induces a rapid recruitment of effector CD4 and CD8 T cells and accelerates the progression of type I diabetes [32]. Our data showed, for the first time, the presence of infiltrating CXCR3$^+$ CD4 and CD8 T cells in the kidneys and the skin of IgAV patients. These infiltrates might explain the decreased proportions of CXCR3$^+$ cells among Tmem cells in the blood of IgAV patients at diagnosis and their increase in remission of the disease. Interestingly, CXCL10 and CXCL11 plasma levels were significantly higher in IgAV patients than in HC. In IgAV patients, the skin and kidneys may represent an important source of CXCL10 and CXCL11. In various tissues,

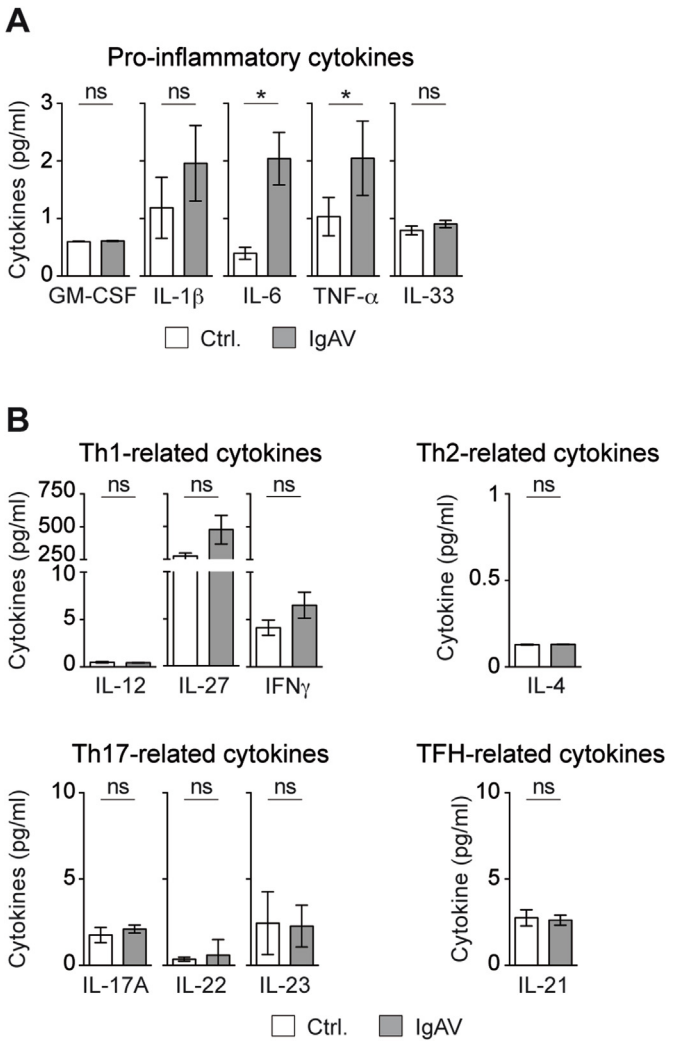

Fig. 3. Analysis of cytokine concentrations in the plasma of IgAV patients and HC. The concentrations of 13 cytokines were measured in the plasma of IgAV patients and HC. **A**/Concentrations of pro-inflammatory cytokines. **B**/ Concentrations of Th1, Th2, Th17 and TFH-related cytokines. White bars correspond to HC and grey bars to IgAV patients. Values of $p < 0.05$ were considered significant (Mann-Withney).

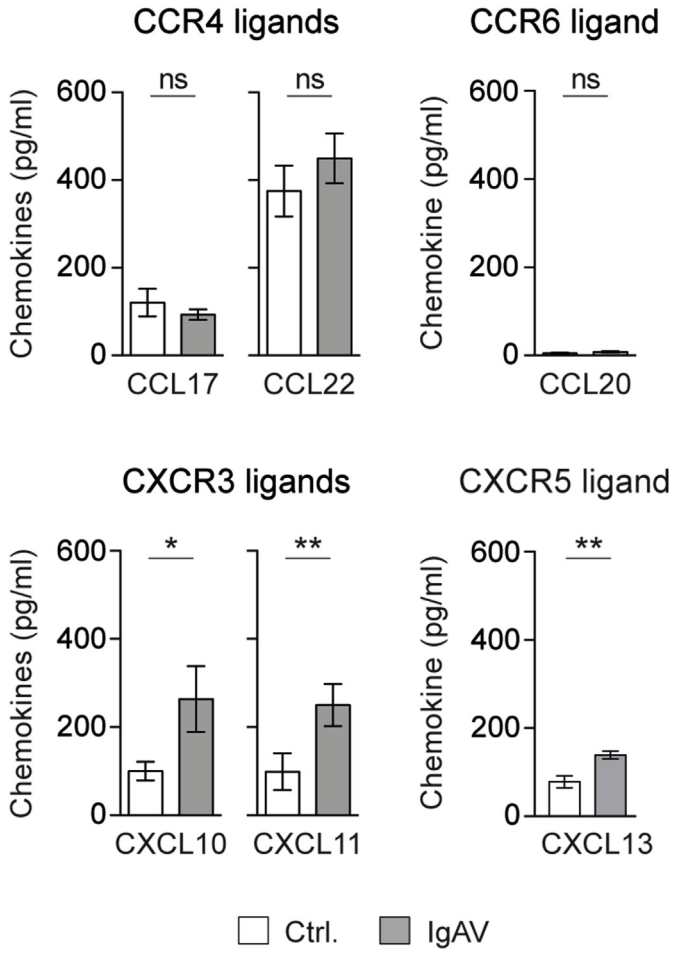

Fig. 4. Analysis of chemokine concentrations in the plasma of IgAV patients and HC. The concentrations of ligands of CCR4 (CCL17 and CCL22), CCR6 (CCL20), CXCR3 (CXCL10, CXL11) and CXCR5 (CXCL13) were measured in the plasma of IgAV patients and HC. White bars correspond to HC and grey bars to IgAV patients. Values of $p < 0.05$ were considered significant (Mann-Withney).

CXCL10 can be produced by different cells, including T cells, NK cells, macrophages, dendritic cells, fibroblasts, and endothelial cells [33]. Moreover, CXCL10 can be secreted by mesangial cell lines after stimulation with proinflammatory cytokines (especially γ-interferon) or lipopolysaccharide *in vitro* [34]. We thus hypothesize that endothelial cells in the skin and mesangial cells in the kidney might overproduce CXCL10 and CXCL11 when activated by immune complexes.

Retention of activated Th1 cells in the skin and kidneys may participate to tissue damages. In the skin, infiltrates of T cells surround injured dermal capillaries. We have not been able to directly correlate the extent of T-cell infiltration in the dermis with the severity of the disease, as purpura was reported in patient clinical records only as present or absent. In kidneys, infiltration of $CD4^+$, $CD8^+$ and $CXCR3^+$ cells was mostly restricted to the renal interstitium. Interestingly, the extent of T-cell infiltration in the kidneys correlates with the severity of the disease. Indeed, patients with the largest T-cell infiltrates are also those exhibiting the highest proteinuria. In agreement with our data, in human glomerulonephritis, including IgAN and lupus nephropathy, it has been shown that the number of $CXCR3^+$ cells, mainly interstitial T cells, correlated with renal function, proteinuria, and the percentage of sclerosed glomeruli [35].

During IgAN and IgAV, while most studies have focused on IgA1 themselves, very few have assessed the involvement of T cells in the regulation of IgA1-producing plasma cells. IgA1 are preferentially produced in response to protein antigens through a T-cell dependent mechanism involving the formation of germinal centers [36]. Follicular helper T cells (TFH) play a crucial role in the generation of plasma cells in germinal centers [37]. TFH cells are defined by their expression of CXCR5 which, in response to its ligand (CXCL13), allows these cells to localize in the B-cell zone of germinal centers. However, recent studies have revealed the existence of $CXCR5^+$ TFH cells within the peripheral blood where they represent 15–20% of circulating memory CD4 T cells in physiological settings. Increase in circulating TFH cells is considered as a reliable biomarker for the severity of various autoimmune diseases, in particular in systemic lupus erythematosus [38]. In contrast, we have rather observed a decreased percentage of $CXCR5^+$ cells among CD4 Tmem cells in the blood of IgAV patients at diagnosis and restored proportions during remission. Plasma level of the CXCR5 ligand, CXCL13, was also increased in IgAV patients. We thus hypothesize that, in IgAV patients, TFH cells may be trapped in secondary lymphoid organs in which they participate to IgA production. Our data on TFH are not concordant with a previous study of TFH cells in IgAV in children [39]. Indeed, in this study, the frequency of circulating TFH cells in children with IgAV was significantly higher than that observed in HC. Differences in the generation/circulation of TFH cells with age may

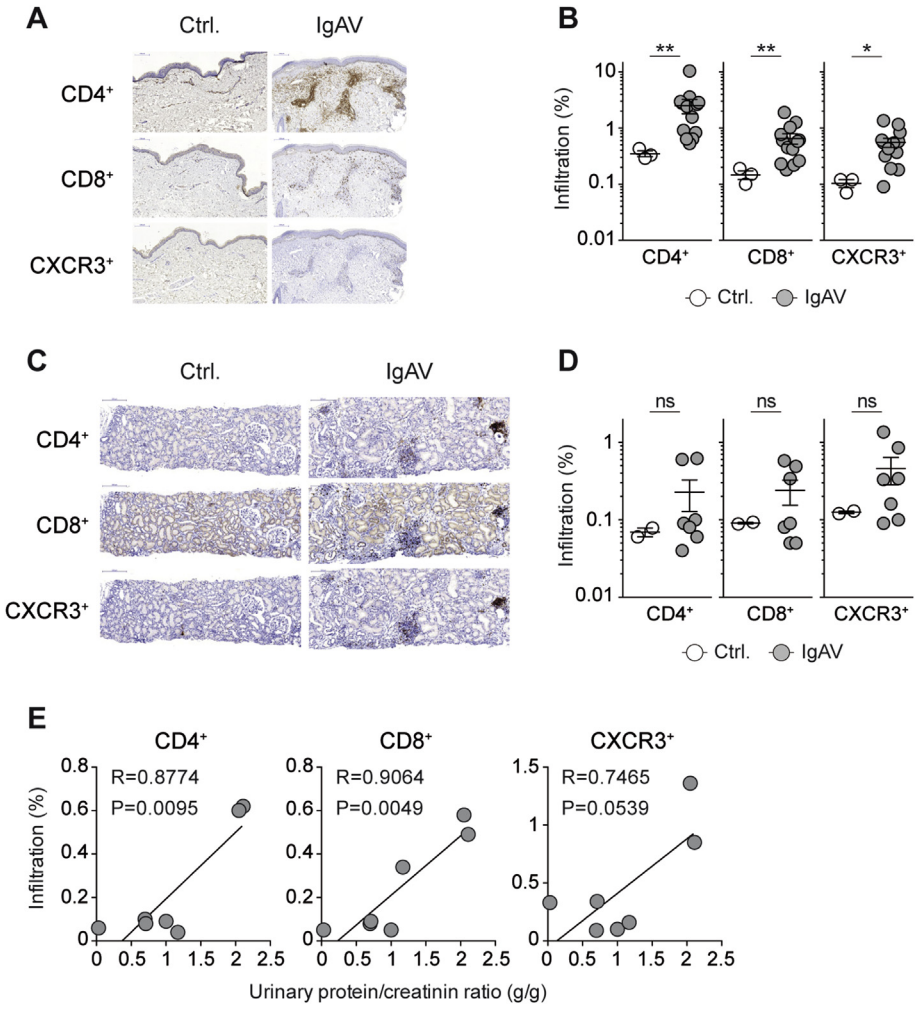

Fig. 5. Analysis of non-lymphoid tissues reveals T-cell infiltration in the skin and kidneys of IgAV patients. Histological analyzes of the skin and kidneys of IgAV patients and HC were performed. Sections were stained with anti-CD4, anti-CD8 or anti-CXCR3 antibodies. **A**/Representative skin sections showing $CD4^+$, $CD8^+$ and $CXCR3^+$ cells in HC (left) and IgAV patients (right). **B**/Quantification of $CD4^+$, $CD8^+$ and $CXCR3^+$ cell infiltrates in the skin of HC and IgAV patients. **C**/Representative kidney sections showing $CD4^+$, $CD8^+$ and $CXCR3^+$ cells in HC (left) and IgAV patients (right). **D**/Quantification of $CD4^+$, $CD8^+$ and $CXCR3^+$ cell infiltrates in the kidneys of HC and IgAV patients. **B, D**/Each dot represents an individual HC (white) or IgAV patient (grey). Values of $p < 0.05$ were considered significant (Mann-Withney). **E**/Correlations between the extent of $CD4^+$, $CD8^+$ and $CXCR3^+$ cell infiltrates in kidneys and proteinuria (Pearson).

explain this discrepancy.

To the best of our knowledge, this study provides the first demonstration of the presence of $CXCR3^+$ T-cell infiltrates in the skin and kidney of IgAV patients which correlates, in kidneys, with the severity of the disease and could thus represent a new pathological mechanism involved in IgA Vasculitis.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jaut.2019.01.012.

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