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Differential expression of IFN-α, IL-12 and BAFF on renal immune cells and its relevance to disease activity and treatment responsiveness in patients with proliferative lupus nephritis

Aya Nawata ,[1,2] Shingo Nakayamada,[1] Satoshi Hisano,[1] Yusuke Miyazaki,[1] Tetsu Miyamoto,[3] Eisuke Shiba,[1] Masanori Hisaoka,[2] Yoshiya Tanaka [1]

To cite: Nawata A, Nakayamada S, Hisano S, *et al*. Differential expression of IFN-α, IL-12 and BAFF on renal immune cells and its relevance to disease activity and treatment responsiveness in patients with proliferative lupus nephritis. *Lupus Science & Medicine* 2023;**10**:e000962. doi:10.1136/lupus-2023-000962

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/lupus-2023-000962).

Received 1 June 2023
Accepted 13 June 2023

[1]First Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan
[2]Department of Pathology and Oncology, University of Occupational and Environmental Health, Kitakyushu, Japan
[3]Kidney Center, University of Occupational and Environmental Health Hospital, Kitakyushu, Japan

Correspondence to
Dr Yoshiya Tanaka; tanaka@med.uoeh-u.ac.jp

ABSTRACT

Objective Since molecularly targeted therapies are emerging for treating lupus nephritis (LN), this study aimed to assess the immunohistochemical findings of the cytokines in renal tissue and their pathological and clinical relevance in LN.

Methods Fifty patients with proliferative LN formed the case group; 5 with LN class II, IgA nephropathy and 10 with idiopathic haematuria were enrolled as controls. Immunohistochemical analysis for CD3, CD20, interferon (IFN)-α, interleukin (IL)-12/p40 and B-cell activating factor (BAFF) was performed by scoring the number of positive cells/area of the cortex. All immunohistochemical investigations were performed on formalin-fixed paraffin-embedded renal tissue. Proliferative LN cases were grouped by the dominant expression of IFN-α, IL-12/p40 and BAFF, and subsequently, clinicopathological features were compared.

Results Clinical data of patients with proliferative LN included urine protein creatinine ratio, 2.2 g/gCre; anti-double-stranded DNA antibody, 200.9 IU/mL; total complement activity (CH50), 21.9 U/mL and SLE Disease Activity Index, 19.8 points. Proliferative LN cases, including class III (n=18) and IV (n=32), were classified into three subgroups according to the immunohistochemical score based on the dominancy of IFN-α (n=17), IL-12 (n=16) and BAFF group (n=17) proteins. Hypocomplementaemia and glomerular endocapillary hypercellularity were significantly increased in the IFN-α group, whereas chronic lesions were significantly higher in the IL-12 group ($p<0.05$). The IFN-α group had a poorer renal prognosis in treatment response after 52 weeks.

Conclusions The immunohistochemistry (IHC) of IFN-α, IL-12 and BAFF for proliferative LN enabled grouping. Especially, the IFN-α and IL-12 groups showed different clinicopathological features and renal prognoses. The results indicated the possibility of stratifying cases according to the IHC of target molecules, which might lead to precision medicine.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Molecularly targeted therapies have emerged for the treatment of lupus nephritis (LN).

WHAT THIS STUDY ADDS

⇒ This study showed that predominant expression of interferon-α in LN renal tissue was related to renal activity and poor prognosis.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study suggests the possibility of precision medicine through the immunohistochemical analysis of renal tissue in proliferative LN.

INTRODUCTION

Lupus nephritis (LN) is a serious organ injury caused by SLE, which increases both its morbidity and mortality.[1] Most patients are administered glucocorticoids, which elevates the risk of infection, atherosclerosis and osteoporosis accompanied by immunosuppressants, including cyclophosphamide and mycophenolate mofetil, according to the recommendations of the American College of Rheumatology (ACR), EULAR and Kidney Disease Improving Global Outcomes.[2–4] The approval of new therapies for SLE, especially biologics such as belimumab and anifrolumab, is expected to improve its prognosis of organ involvement like rheumatoid arthritis.

Although B cells have been considered to serve a crucial role in SLE pathogenesis, clinical trials of B cell-targeted molecular therapy, including rituximab or epratuzumab, have failed to reveal significant differences. Recently, the three molecular targets of B-cell activating factor (BAFF), interferon (IFN)-α and interleukin (IL)-12, which are called bridging cytokines, have been highlighted.[5] Bridging cytokines, which are released by both macrophages and dendritic cells, play a pivotal role in harmonising between the

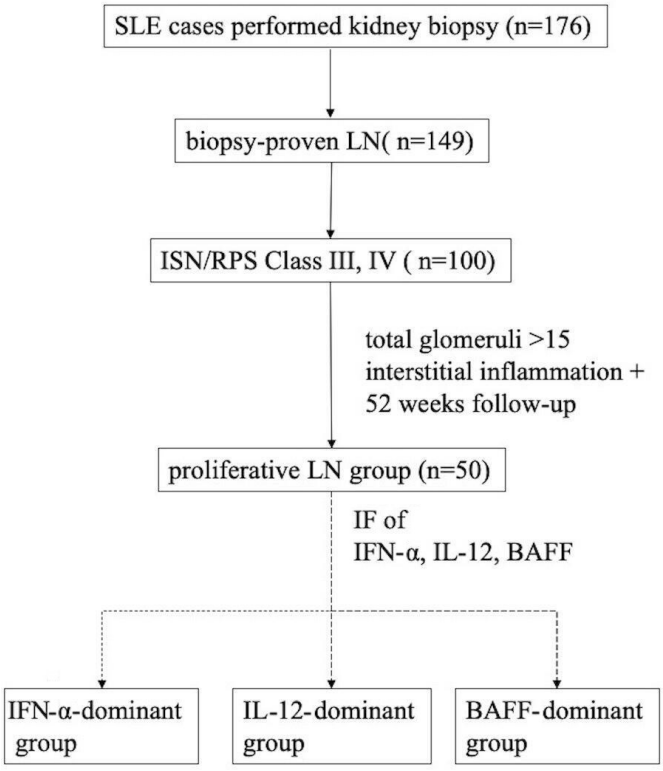

Figure 1 Study design. BAFF, B-cell activating factor; IF, immunofluorescent; IFN, interferon; IL, interleukin; ISN/RPS, International Society of Nephrology/Renal Pathology Society; LN, lupus nephritis.

innate immune system and acquired immune or autoimmune system.[6] BAFF, the target molecule of belimumab, is a B cell activating factor belonging to the tumour necrosis factor family.[6] BAFF inhibits B cell apoptosis and is associated with the differentiation of B cells into immunoglobulin-producing plasma cells.[7] The serum level of BAFF is increased in patients with SLE and is related to disease activity and treatment response.[7 8] Belimumab with standard therapy is highly effective in proliferative LN.[9] Moreover, a recent study indicated that the type I IFN pathway plays an important role in SLE pathogenesis. In SLE, IFN-α secreted by plasmacytoid dendritic cells is considered the most important cytokine as it activates T cells and promotes autoantibody release by B cells.[10] The serum concentration of IFN-α is related to disease activity or a flare-up.[11] Anifrolumab, which is a human monoclonal antibody to IFN-α receptor, has been newly approved for SLE treatment. A recent study reported that the IL-12-STAT4 pathway is important in patients with active SLE.[12]

SLE is a molecularly heterogeneous disease, and it is important, although difficult, to predict the response to the treatment of patients with SLE. There are only a few reports on precision medicine of SLE or LN. In psoriatic arthritis, the selection of biologics according to the peripheral blood T-lymphocytic phenotype in each patient exhibited remarkably higher efficacy than the standard therapy, indicative of the importance of precision medicine.[13] Kubo *et al* mentioned that patients with active SLE were classified into three groups in accordance with T-cell phenotypes and discusses the possibility of using phenotyping for precision medicine.[14] However, regarding precision medicine in LN, 'the inflammation site', indicating a renal biopsy specimen should be examined rather than the 'whole body' as in a peripheral blood sample. Renal biopsy is routinely performed when diagnosing LN; thus, using the remaining samples after diagnosis is less invasive for the patients. The histology of renal tissue in LN varies with patients, which might reflect the individual pathogenesis of LN. Our objective was to set the basis for future approaches in precision medicine in proliferative LN by targeting the above-mentioned three bridging cytokines and using renal biopsy specimens.

This study aimed to clarify whether the expression of molecular targets of biologics in renal tissue of LN was associated with disease activity, prognosis and pathological findings.

METHODS

Patients and study design

Patients who fulfilled the 2012 Systemic Lupus International Collaborating Clinics SLE classification criteria or the 2019 EULAR/ACR classification criteria for SLE were identified from the LOOPS registry, a registry of patients with SLE treated at the University of Occupational and Environmental Health (UOEH) hospital and affiliated hospitals.[15] Between 2011 and 2019, renal biopsy specimens were examined at the division of Surgical Pathology, UOEH hospital. Fifty patients with class III/IV LN were enrolled as the case group. All patients with LN underwent renal biopsy at the onset of LN. Patients who were treated with biologics such as rituximab or belimumab before biopsy were excluded. Five patients with class II LN and IgA glomerulonephritis with active glomerular lesions, and 10 with idiopathic haematuria were included as controls in the study. The idiopathic haematuria group exhibited minor glomerular abnormalities. All the biopsy specimens showed >15 glomeruli and tubulointerstitial inflammation. Figure 1 shows the study design. The following clinical and serological information were collected at the time of renal biopsy and 52 weeks after treatment: sex, age, duration of SLE, SLE Disease Activity Index (SLEDAI), urine protein creatinine ratio (UPCR), levels of blood urea nitrogen, serum creatinine, serum C3, C4 and total complement activity (CH50). The titres of ANA, anti-Sm, antiribonucleoprotein and anti-double-stranded DNA (anti-ds-DNA) antibodies and other antibodies were determined. The Safety of Estrogens in Lupus Erythematosus National Assessment-SLEDAI and the British Isles Lupus Assessment Group (BILAG) indices were used to measured general disease activity of SLE. The renal response after 52 weeks of treatment was assessed according to the recommendations of the ACR, Lupus Nephritis Assessment with Rituximab (LUNAR), Aspreva Lupus Management Study (ALMS)

and Belimumab in Subjects with SLE (BLISS)-LN studies and prednisolone dosage.[2 16–19]

Preparation of renal biopsy samples

Renal samples obtained using needle biopsy were fixed in 10% neutral-buffered formalin, subjected to dehydration, and embedded in paraffin. The formalin-fixed paraffin-embedded renal sections were stained with H&E, periodic acid-Schiff, Masson's trichrome and periodic acid-silver methenamine. Pathological diagnosis was made using light, immunofluorescent and electron microscopy. Fluorescein isothiocyanate-labelled rabbit antihuman IgG, IgA, IgM, C1q, C3 and fibrinogen (Dako, Copenhagen, Denmark) were used in immunofluorescent microscopy.

Histological re-evaluation of renal biopsy samples

The histopathological findings of LN were classified based on the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2018 revision classification of LN by two independent pathologists (AN and SH).[19] The number of glomeruli, percentage of glomeruli with both active lesions, including cellular/fibrocellular crescents, endocapillary hypercellularity, karyorrhexis, fibrinoid necrosis, wire-loop lesion or hyaline thrombi and chronic lesions such as global sclerosis, segmental sclerosis or adhesion, per total glomeruli were calculated. Regarding interstitium, the presence or absence of tubulitis and peritubular capillaritis and the cortical percentages of interstitial inflammation, interstitial fibrosis and tubular atrophy were included. Moreover, the modified National Institutes of Health (NIH) activity/chronicity index was evaluated and scored as previously described.[20]

Immunofluorescent staining of renal biopsy specimens

All the immunofluorescent staining investigations were performed on formalin-fixed paraffin-embedded renal specimens. Immunofluorescence staining of continuous sections was executed for each LN case and control case using the following antibodies: monoclonal antimouse antibodies against CD20 (clone L26, Dako, Glostrup, Denmark), CD3 (clone UCTH-1, Dako, Glostrup, Denmark), IFN-α (F-7, Santa Cruz Biotechnology; Dallas, USA) and IL-12 (clone I-1A4, BioRad, Hercules, USA), and polyclonal antirabbit antibody against BAFF (ab217329, Abcam, Cambridge, UK). Multiplex immunofluorescence staining was done using the Opal four-colour fluorescence immunohistochemistry (IHC) kit (Akoya Biosciences, Marlborough, Massachusetts, USA). The formalin-fixed paraffin-embedded renal tissue was sliced into 3 µm thick sections, deparaffinised and subjected to microwave treatment at 121°C for 10 min with tris-ethylenediamine tetraacetic acid buffer (pH, 9.0). The section was immersed in a blocking/antibody diluent for 10 min, followed by successive incubations with the primary antibodies for 1 hour at room temperature, followed by the secondary antibody (polymer HRP Ms+Rb) for 10 min and finally with an Opal fluorophore (Opal520, Opal570) for 10 min. The process of staining and antibody removal was repeated using a different Opal fluorophore. Finally, the section was stained with 4′,6-diamidino-2-phenylindole, a cover slip was mounted with Fluoromount (Diagnostic BioSystems; K024) and the slide was observed under a fluorescence microscope. Double staining for CD3 and CD20, that for IFN-α and IL-12 and single staining for BAFF were observed in each specimen.

Evaluation of immunofluorescence staining

For IHC analysis of the sections, the above-mentioned sections were digitalised using a virtual slide system (VS120, Olympus). The total number of cells positive for CD3, CD20, IFN-α, IL-12 and BAFF were counted, and the IHC score for each cytokine was assessed using the respective number of positive cells/mm^2 of the renal cortex. The mean number of cells positively stained for these molecules was estimated in the samples of patients with class III/IV LN and those of controls. The grouping was done by calculating Z-scores of the IHC scores of three cytokines (IFN-α, IL-12 and BAFF), and the patients were classified into the highest cytokine Z-score group.

Two independent pathologists (AN and SH) evaluated all the immunostaining sections in the absence of the clinical information. In cases of disagreement in assessment between both pathologists, a third pathologist (MH) made the consensus decision.

Statistical analysis

Data are shown as means±SD. The statistical analysis was evaluated by JMP software (V.10; SAS Institute, Cary, North Carolina, USA). Statistical comparison of categorical variables was performed using Fisher's exact or χ^2 test. Non-parametric Wilcoxon rank-sum test or Kruskal-Wallis test was used to compare differences between the mean values of defined patient groups. The Steel-Dwass test was used for a comparison between two of three groups. A value of $p<0.05$ was adopted to be statistically significant.

RESULTS

Clinicopathological characteristics of proliferative LN cases and control cases

Table 1 shows the baseline clinical characteristics of patients with proliferative LN (50), LN class II (5) and IgA-glomerulonephritis (GN) (5), and control (10) groups. No significant differences in sex, age and serum creatinine levels among these groups were observed.

Immunofluorescent evaluation of IFN-α, IL-12 and BAFF

Figure 2 shows the immunofluorescence assay results of renal expression of IFN-α, IL-12 and BAFF in the proliferative LN, LN class II, IgA and control groups. The patients with proliferative LN could be categorised into the INF-α-dominant, IL-12-dominant and BAFF-dominant groups according to the predominantly expressed biomarker in the biopsy specimens in our immunohistochemical study. The number of IFN-α-positive cells in the renal tissue were significantly higher in

Table 1 Baseline clinical characteristics and laboratory analysis of proliferative LN and control cases

	Proliferative LN group (n=50)	LN class II group (n=5)	IgA group (n=5)	Control group (n=10)	P value
Female (n, %)	43 (86.0)	5 (100)	4 (80)	6 (60.0)	0.1591
Age at biopsy (years)	43.5±16.9	34.8±8.7	32.4±10.5	40.5±18.0	0.4321
Duration of SLE (month)	111.2±131.4				
Diabetes mellitus (n, %)	6 (12.0)	0 (0)	0 (0)	0 (0)	0.2349
Hypertension (n, %)	10 (20.0)	0 (0)	0 (0)	1 (10.0)	0.4228
Antiphospholipid syndrome (n, %)	13 (26.0)	0 (0)	0 (0)	0 (0)	0.192
TTP or TMA (n, %)	2 (4.0)	0 (0)	0 (0)	0 (0)	0.6487
SLEDAI score	18.9±6.9	11.6±5.5			0.0310*
BILAG score	20.0±9.6	15.4±6.8			0.2590
White blood cells (/μL)	4530±2728	3480±672.3	5300±393.7	7250±2796.5	0.0076*
Lymphocyte (/μL)	718.1±594.2	758.6±554.1	1819±443.0	1739±362.8	<0.0001*
Haemoglobin (g/dL)	10.5±2.0	105±16	128±10	140±12	<0.0001*
Platelet (×10^4/μL)	37.2±66.2	18.7±10.8	25.6±5.5	30.2±13.8	0.1124
Serum albumin (g/dL)	2.8±0.8	3.2±0.9	4.1±0.3	4.2±0.5	<0.0001*
eGFR (mL/min/1.73 m^2)	75.8±30.0	109.0±26.0	80.6±19.0	80.5±27.5	0.1777
Serum BUN (mg/dL)	17.9±10.2	11.2±1.8	13.2±2.0	14.5±5.9	0.1210
Serum creatinine (mg/dL)	0.9±0.6	0.5±0.1	0.8±0.3	0.9±0.5	0.1157
ANA	1754±2489	2080±1899	8.0±17.9	4.0±12.7	0.0585
Anti-ds-DNA Ab (IU/mL)	200.9±183.0	140.7±166.7	0	0.7±1.3	0.0086*
Anti-Sm Ab (IU/mL)	79.9±180.9	140.6±258.5			0.3852
Anti-RNP Ab (IU/mL)	124.1±188.7	332.1±298.3			0.1746
Serum C3 (mg/dL)	45.7±21.6	53.4±26.2	104.2±22.7	107.5±32.9	<0.0001*
Serum C4 (mg/dL)	10.4±11.5	8.0±2.9	23.6±9.0	30.4±12.9	<0.0001*
Serum CH50 (U/mL)	22.0±14.4	24.4±13.9	56.4±5.5	51.2±11.8	<0.0001*
Serum IgG (mg/dL)	1868±780	3585±2836	1140±225	1287±563	0.0013*
UPCR (g/gCre)	2.2±2.4	0.2±0.1	0.5±0.3	0.5±0.4	0.0012*
Sedimentation of RBC (/HPF)	18.0±26.7	1.6±2.2	31.6±40.9	36.2±47.9	0.1378

Data are shown by mean±SD or n (%). P values were determined by the Wilcoxon rank-sum test, a Kruskal-Wallis test or χ^2 test.
*P<0.05.
Ab, antibody; BILAG, British Isles Lupus Assessment Group; BUN, blood urea nitrogen; ds-DNA, double-stranded DNA; eGFR, estimated glomerular filtration rate; HPF, high power field; LN, lupus nephritis; RBC, red blood cells; SLEDAI, SLE Disease Activity Index; TMA, thrombotic microangiopathy; TTP, thrombocytopenic thrombotic purpura; UPCR, urine protein creatinine ratio.

the IFN-α-dominant group than in the control group (p=0.0403). Significant infiltration of IL-12-positive cells was observed in the IL-12-dominant group than in the BAFF-dominant (p=0.0076), LN class II (p=0.0412), IgA (p=0.0188) or control groups (p=0.0009). BAFF-positive cells were significantly increased compared with the control group (p=0.0027). IFN-α was expressed in CD123-positive plasmacytoid dendritic cells and CD68-positive macrophages (online supplemental figure 1). IL-12-positive cells and BAFF were expressed mainly in CD68-positive macrophages.

Comparison of clinical characteristics among the IFN-α, IL-12 and BAFF groups

Table 2 shows the ISN/RPS classification of patients in each group and table 3 presents the clinical characteristics of patients at baseline and 52 weeks after therapy in these groups. Serum C3 and CH50 concentrations were significantly lower in the IFN-α-dominant group than in the IL-12-dominant group (C3: p=0.0298, CH50: p=0.0149). There was no significant difference in age, blood count, estimated glomerular filtration rate, anti-ds-DNA antibody titre, UPCR value, disease activity (SLEDAI and BILAG scores), the dosage of prednisolone and therapeutic drugs and other characteristics at 52 weeks.

Comparison of histological parameters among the IFN-α, IL-12 and BAFF groups

Table 4 shows the histological parameters of kidney biopsy in each group. As for glomerular lesions, the percentage of karyorrhexis was significantly higher in the IFN-α-dominant group than in the BAFF-dominant group (p=0.035). There were no significant differences in other glomerular or interstitial parameters. In the 2018

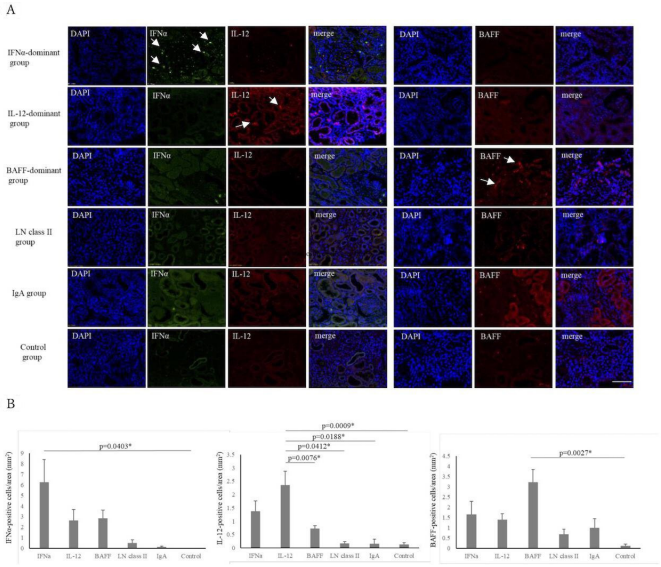

Figure 2 Immunofluorescence study of IFN-α, IL-12 and BAFF. (A) A representative immunofluorescence staining of human renal tissue for IFN-α, IL-12, BAFF and DAPI with composite multiplexed images in each group (scale bar: 50 µm). In the IFN-α-dominant group, IFN-α-positive cells increased (white arrow). IL-12-positive cells infiltrated in the IL-12-dominant group (white arrow), and BAFF-positive cells increased in the BAFF-dominant group (white arrow). (B) The graph shows the number of immunofluorescent positive cells/area of the cortex (mm^2) in the following groups of patients with proliferative LN: IFN-α-dominant group (n=17), IL-12-dominant group (n=16) and BAFF-dominant group (n=17), LN class II group (n=5), IgA-GN group (n=5) and control group (n=10). Mean±SD shown. *P<0.05. BAFF, B-cell activating factor; DAPI, 4′,6-diamidino-2-phenylindole; GN, glomerulonephritis; IFN, interferon; IL, interleukin; LN, lupus nephritis.

revision of the ISN/RPS classification of LN, the calculation of a modified NIH activity/chronicity score was recommended. Based on this scoring, the modified NIH total index was significantly higher in the IFN-α-dominant (p=0.0146) and IL-12-dominant groups (p=0.0166) than in the BAFF-dominant group. The IFN-α-dominant group also had higher scores for activity (p=0.0130), endocapillary hypercellularity (p=0.0357) and karyorrhexis (p=0.0410) than the BAFF-dominant group. The IL-12-dominant group showed a higher chronicity score (p=0.0393) and tubular atrophy score (p=0.0031) than the IFN-α-dominant group.

Immunofluorescent evaluation of CD3, CD20 and pathological findings

Figure 3 reveals CD3-positive and CD20-positive lymphocytes in the renal tissue and representative pathological features in each group. Infiltration of CD3-positive T lymphocytes and CD20-positive lymphocytes was observed in all groups, and there were no significant differences among these groups. Regarding pathological characteristics, the IFN-α-dominant group showed increased glomerular active lesions, including endocapillary hypercellularity; however, the IL-12-dominant group contained increased chronic lesions such as segmental sclerosis or tubular atrophy. The LN class II group exhibited mesangial cell hypercellularity. The IgA nephropathy group also showed active lesions, including fibrocellular crescent. The control group showed minor glomerular abnormalities.

Renal response at 52 weeks in the IFN-α-dominant, IL-12-dominant and BAFF-dominant groups

Figure 4 shows the comparison of renal response in IFN-α-dominant, IL-12-dominant and BAFF-dominant groups. According to the ACR and ALMS criteria, the

Table 2 ISN/RPS classification in the proliferative LN group

ISN/RPS classification (2003 and 2018 revision) (n, %)	Proliferative LN group (n=50)	IFN-α group (n=17)	IL-12 group (n=16)	BAFF group (n=17)
Class III	13 (26.0)	3 (17.7)	3 (17.7)	7 (41.2)
III (A)	3 (6.0)	1 (5.9)	0 (0)	2 (11.8)
III (A/C)	10 (20.0)	5 (29.4)	3 (18.8)	5 (29.4)
Class IV	26 (52.0)	12 (70.6)	8 (50.0)	6 (35.0)
IV-S (A)	9 (18.0)	5 (29.4)	2 (12.5)	2 (11.8)
IV-S (A/C)	10 (20.0)	3 (17.7)	4 (25.0)	3 (17.7)
IV-G (A)	1 (2.0)	1 (5.9)	0 (0)	0 (0)
IV-G (A/C)	6 (12.0)	3 (17.7)	2 (12.5)	1 (5.6)
Class III+V	5 (10.0)	0 (0)	2 (12.5)	3 (17.7)
III (A/C)+V	5 (10.0)	0 (0)	2 (12.5)	3 (17.7)
Class IV+V	6 (12.0)	2 (11.8)	3 (18.8)	1 (5.9)
IV-S (A/C)+V	4 (8.0)	1 (5.9)	2 (12.5)	1 (5.9)
IV-G (A/C)+V	2 (4.0)	1 (5.9)	1 (6.3)	0 (0)

A, active; BAFF, B-cell activating factor; C, chronic; IFN, interferon; IL, interleukin; ISN/RPS, the International Society of Nephrology/Renal Pathology Society; LN, lupus nephritis.

Table 3 Clinical characteristics of LN in the three groups at baseline and 52 weeks after therapy

	IFN-α group (n=17)	IL-12 group (n=16)	BAFF group (n=17)	P value
Baseline clinical characteristics				
Female (n, %)	12 (70.6)	12 (70.6)	17 (100)	0.0198*
White blood cell count (/μL)	4576±2434	5018±3577	4023±2087	0.7384
Lymphocyte count (/μL)	722±321	746±917	687±432	0.3928
Haemoglobin (g/dL)	10.5±1.8	10.5±1.9	10.5±2.3	0.9908
Platelet (×10^4/μL)	16.3±8.2	20.6±11.1	20.2±7.7	0.2956
Serum albumin (g/dL)	2.9±0.5	2.9±1.2	2.7±0.8	0.5753
Serum BUN (mg/dL)	20.5±13.0	18.6±7.8	14.6±8.5	0.0469*
Serum creatinine (mg/dL)	1.1±1.0	0.8±0.4	0.7±0.2	0.3772
eGFR (mL/min/1.73 m^2)	76.4±33.5	74.5±28.9	76.7±28.9	0.9703
Serum C3 (mg/dL)	37.5±15.1	56.7±29.4	43.5±14.0	0.1257
	(vs IL-12 p=0.0254*)			
Serum CH50 (U/mL)	16.0±11.4	30.0±16.4	20.5±12.3	0.0331*
	(vs IL-12 p=0.0124*)			
Serum IgG (mg/dL)	1899±737	1772±669	1926±942	0.8708
Anti-ds-DNA antibody (IU/mL)	250.1±160.4	199.3±220.0	153.3±162.8	0.1449
UPCR (g/gCre)	3.1±2.8	1.3±1.3	2.0±2.7	0.0968
Neuropsychiatric symptom (n, %)	3 (17.7)	0 (0)	5 (29.4)	0.0232*
SLEDAI score	19.6±7.5	15.9±5.9	21.0±6.5	0.0927
BILAG score	18.7±9.0	19.3±10.5	21.7±9.7	0.5088
Therapeutic drugs (remission induction therapy)				
PSL (mg/day)	51.2±8.9	51.9±14.1	52.1±10.2	0.6932
mPSL pulse (n, %)	4 (23.5)	1 (20.0)	5 (29.4)	0.3972
Intravenous cyclophosphamide (n, %)	6 (35.3)	6 (37.5)	9 (52.9)	0.5284
Mycofenorate mofetil (n, %)	8 (47.1)	5 (31.3)	4 (23.5)	0.5256
Rituximab (n, %)	2 (11.8)	1 (6.3)	3 (17.7)	0.6201
52 weeks after therapy				
White blood cell count (/μL)	6129±2636	6331±1626	6187±2636	0.6587
Lymphocyte count (/μL)	915±378	876±403	915±549	0.9455
Haemoglobin (g/dL)	11.7±1.3	12.4±1.8	12.2±1.4	0.6205
Platelet (×10^4/μL)	26.9±8.3	22.6±7.7	25.0±4.3	0.2796
Serum albumin (g/dL)	3.9±0.5	3.9±0.4	4.0±0.4	0.8334
Serum BUN (mg/dL)	19.8±19.8	16.4±6.1	13.5±4.8	0.5690
Serum creatinine (mg/dL)	0.9±0.8	0.7±0.2	0.8±0.3	0.9280
eGFR (mL/min/1.73 m^2)	82.4±38.0	76.3±28.2	78.2±25.2	0.4575
Serum CH50 (U/mL)	48.9±14.4	52.6±10.0	50.3±16.2	0.9240
Serum IgG (mg/dL)	920±331	1133±458	931±185	0.6920
Anti-ds-DNA Ab (IU/mL)	13.0±18.8	9.4±13.2	9.1±9.0	0.8868
UPCR (g/gCre)	0.33±0.56	0.05±0.10	0.26±0.78	0.4966
Sedimentation of RBC (/HPF)	3.4±5.4	2.8±6.4	2.1±5.1	0.5017
SLEDAI total score	2.5±3.0	2.3±4.2	1.1±1.9	0.2167
PSL dose (mg)	9.1±7.4	8.5±2.4	7.6±4.6	0.1864
Long-term prognosis (eGFR)	74.9±32.9	73.2±28.1	73.8±22.2	0.7019
(1 year~10 years)				

Data are shown by mean±SD or n (%). P values were determined by the Wilcoxon rank-sum test, a Kruskal-Wallis test, Steel-Dwass test or χ^2 test.
*P<0.05.
Ab, antibody; BAFF, B-cell activating factor; BILAG, British Isles Lupus Assessment Group; ds-DNA, double-stranded DNA; eGFR, estimated glomerular filtration rate; HPF, high power field; IFN, interferon; IL, interleukin; LN, lupus nephritis; mPSL, methyl prednisolone; RBC, red blood cells; SLEDAI, SLE Disease Activity Index; UPCR, urine protein creatinine ratio.

Table 4 Histological parameters of ISN/RPS classification of kidney biopsy in the three groups

	IFN-α group (n=17)	IL-12 group (n=16)	BAFF group (n=17)	P value
Glomerulus				
Class IV (n, %)	14 (82.4)	11 (68.8)	7 (41.2)	0.0371*
Class V positive (n, %)	2 (11.7)	5 (31.3)	4 (23.5)	0.3597
Total glomeruli (n)	43.0±13.0	38.0±20.0	40.3±18.3	0.339
Active lesion (%)	54.4±21.7	36.7±26.5	32.7±21.7	0.0292*
Cellular crescent (%)	5.1±6.0	5.8±9.9	1.8±2.6	0.3942
Fibrocellular crescent (%)	4.0±7.1	3.2±5.2	1.7±4.2	0.7327
Crescent positive (n, %)	11 (64.7)	10 (62.5)	9 (52.9)	0.759
Endocapillary hypercellularity (%)	41.3±21.0	31.4±27.2	24.6±18.3	0.0808
Karyorrhexis (%)	16.1±16.2 (vs BAFF p=0.0350*)	7.5±14.3	4.8±5.4	0.035*
Fibrinoid necrosis (%)	0.9±1.5	1.8±5.6	0.4±1.5	0.2368
Wire-loop lesion (%)	13.2±24.1	4.3±8.6	10.0±23.0	0.3326
Hyaline thrombi (%)	3.7±6.2	2.3±5.6	2.5±4.8	0.8578
Chronic lesion (%)	11.7±17.2	17.5±16.6	9.1±11.5	0.1210
Global sclerosis (%)	11.4±16.7	16.0±16.5	8.1±10.4	0.1626
Segmental sclerosis (%)	0.3±0.7	1.1±2.4	0.5±1.1	0.6926
Adhesion (%)	0.1±0.5	0	0	0.3789
Interstitium				
Tubulitis (n, %)	9 (52.9)	6 (37.5)	8 (47.1)	0.6694
ptc-its (n, %)	10 (58.8)	8 (30.8)	8 (47.1)	0.7753
Interstitial inflammation (% of the cortex)	13.8±15.6	21.6±15.6	9.4±7.0	0.1372
Interstitial fibrosis (% of the cortex)	7.4±7.5	10.3±9.6	5.0±3.5	0.1680
Tubular atrophy (% of the cortex)	8.8±11.4	15.0±13.7	7.1±9.7	0.1016
Modified NIH activity/chronicity index				
Modified NIH total index	9.9±2.9	9.9±2.5	7.0±3.3	0.0181*
	(vs BAFF p=0.0146*)	(vs BAFF p=0.0166*)		
Modified NIH activity index	7.7±2.6	6.4±2.6	5.1±2.4	0.0161*
	(vs BAFF p=0.0130*)			
Endocapillary hypercellularity score	2.2±0.8	1.8±0.9	1.5±0.6	0.0497*
	(vs BAFF p=0.0357*)			
Karyorrhexis score	1.2±0.8	0.7±0.7	0.6±0.5	0.0358*
	(vs BAFF p=0.0410*)			
Fibrinoid necrosis score	0.6±0.9	0.3±0.7	0.1±0.5	0.1619
Hyaline deposit score	0.9±1.1	0.4±0.7	0.8±0.9	0.3224
Celllular/Fibrocellular crescent score	1.5±1.3	1.8±1.4	1.0±1.0	0.2648
Interstitial inflammation score	1.5±1.1	1.4±0.7	1	0.0441*
Modified NIH chronicity index	2.2±1.3	3.5±1.4	2.5±1.5	0.0389*
		(vs IFN-α p=0.0393*)		
Global sclerosis score	0.8±0.9	1.1±0.6	0.9±0.6	0.3012
Fibrous crescent score	0	0	0	0
Tubular atrophy score	0.6±0.5	1.2±0.4	0.8±0.4	0.0054*
		vs IFN-α 0.0031*		
		BAFF 0.0245*		
Interstitial fibrosis score	0.8±0.7	1.1±0.5	0.8±0.5	0.1547

Data are shown by mean±SD or n (%). P values were determined by the Wilcoxon rank-sum test, a Kruskal-Wallis test, Steel-Dwass test or χ^2 test.
*P<0.05.
BAFF, B-cell activating factor; IFN, interferon; IL, interleukin; ISN/RPS, International Society of Nephrology/Renal Pathology Society; LN, lupus nephritis; NIH, National Institutes of Health.

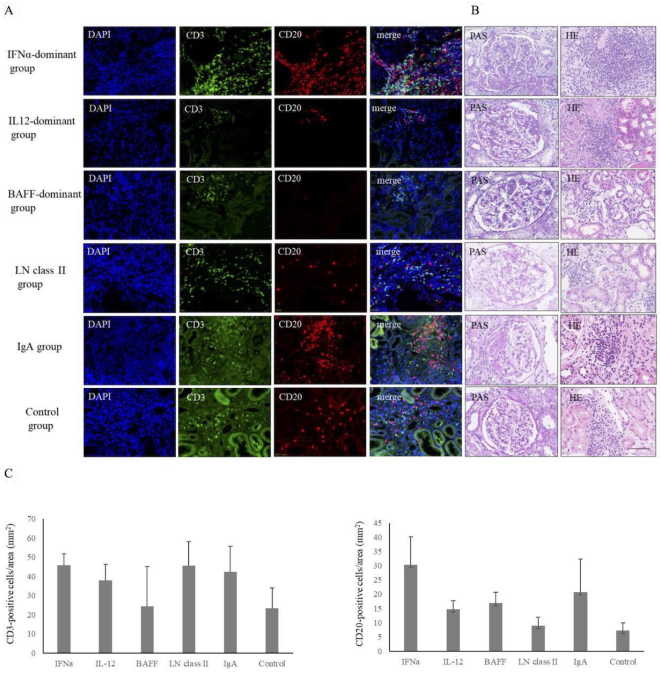

Figure 3 Immunofluorescence study of CD3 and CD20 and light microscopic images (PAS and H&E staining). (A) A representative immunofluorescence staining of human renal tissue for CD3, CD20 and DAPI with composite multiplexed images in each group (scale bar: 50 µm). All six groups show CD3-positive T lymphocytes and CD20-positive B lymphocytes in the kidney tissue. (B) A representative light microscopic image stained by PAS and H&E. The IFN-α-dominant group shows increased glomerular active lesions, including segmental endocapillary hypercellularity and global wire-loop lesions. The IL-12-dominant group shows segmental sclerosis and fibrous adhesion to the Bowman's capsule. The BAFF-dominant group shows segmental endocapillary hypercellularity. The LN class II group exhibits mesangial cell hypercellularity. The IgA group also shows fibrocellular crescent, and the control group exhibits minor glomerular abnormalities. (C) The number of immunofluorescent CD3-positive or CD20-positive cells/area of the cortex (mm^2) in the six groups are not significantly different. Mean±SD shown. BAFF, B-cell activating factor; DAPI, 4′,6-diamidino-2-phenylindole; IFN, interferon; IL, interleukin; LN, lupus nephritis; PAS, periodic acid-Schiff.

number of non-remission patients was significantly higher in the IFN-α-dominant group than in the BAFF-dominant group (p=0.0469and p=0.0393). The non-achievement ratio of <7.5 mg of PSL was significantly lower in the BAFF-dominant group than in the IL-12-dominant group (p=0.0494). There were no significant differences according to the LUNAR and BLISS-LN criteria, and the achievement of UPCR value of <0.8 g.

DISCUSSION

In this study, immunohistochemical expression of molecular targets of biologic agents in renal biopsy specimens of proliferative LN were likely associated with LN activity, prognosis and pathological findings. The IHC expression of IFN-α, IL-12 and BAFF was significantly higher in the

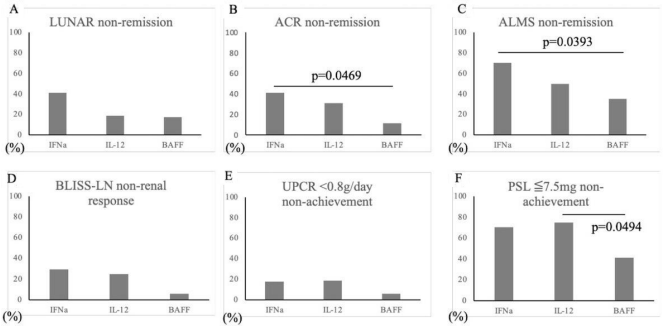

Figure 4 Non-remission rate comparison in the IFN-a, IL-12 and BAFF groups 52 weeks after treatment. Non-remission rate in LUNAR (A), ACR (B), ALMS (C), BLISS-LN (D) criteria and non-achievement of UPCR <0.8 g/day (E) and PSL ≤7.5 mg (F). ACR, American College of Rheumatology; ALMS, Aspreva Lupus Management Study; BAFF, B-cell activating factor; BLISS-LN, Belimumab in Subjects with SLE-Lupus Nephritis; IFN, interferon; IL, interleukin; LUNAR, Lupus Nephritis Assessment with Rituximab; PSL, prednisolone; UPCR, urine protein creatinine ratio.

proliferative LN group than in the IgA glomerulonephritis, LN class II or control group. The finding indicated that patients with proliferative LN had high levels of these cytokines in renal tissue. In this study, patients with proliferative LN could be classified into three subgroups (IFN-α-dominant group, IL-12-dominant group and BAFF-dominant group) using the IHC method. Moreover, this study demonstrated significant clinical or pathological characteristics of each group.

First, the IFN-α-dominant group showed a clinically low titre of C3 and CH50; pathologically high modified NIH activity, endocapillary hypercellularity and karyorrhexis index and poor prognoses at 52 weeks after treatment, suggesting that renal IFN-α was associated with LN activity. In addition, few studies have clearly identified IFN-α-positive cells in renal biopsy other than our study. Type I IFN, including IFN-α, is released by immature dendritic cells, binds to the IFN-α receptor, and activates antigen-presenting dendritic cells, and is thus related to SLE progression.[21] Mavragani *et al* reported high renal expression of IFN-α transcripts in patients with proliferative LN than in those with primary membranous nephropathy or healthy donors.[22] A recent single-cell RNA analysis in a study of kidney tissue demonstrated a significant upregulation of IFN response in all patients.[23] Consistent with these findings, our data confirmed the critical role of IFN-α response locally in renal tissue in patients with LN. Karyorrhexis is recognised as the presence of pyknotic, apoptotic and destroyed nuclei owing to neutrophil infiltration.[24 25] Neutrophils produce neutrophil extracellular traps, which induce plasmacytoid dendritic cell activation, IFN-α secretion and endothelial dysfunction in the SLE tissue.[26] These findings could explain why the IFN-α-dominant group was associated with high endocapillary hypercellularity and karyorrhexis index in this study. The type I IFN receptor-specific antibody, anifrolumab, was approved as a second biologic

for SLE. Unexpectedly, the phase II trial of anifrolumab in patients with LN did not meet primary end points, possibly because of the inadequate exposure of the anifrolumab-basic regimen group.[27] The anifrolumab-intensified regimen (IR) group achieved a complete renal response and sustained oral glucocorticoid reduction.[27] The study suggested that proliferative LN required sufficient exposure to the same dosage as that for the anifrolumab-IR group, warranting further investigation of the safety and efficacy of anifrolumab-IR; hence the phase III trial in proliferative LN has been ongoing. In our study, strong IFN-α expression in LN renal tissue was related to renal activity and poor prognosis. This result may imply that appropriate patient selection according to serum concentration or renal IFN-α expression might be helpful to achieve a renal response, and further studies are desirable.

Furthermore, the IL-12-dominant group exhibited a higher modified NIH chronicity index and higher tubular atrophy score, which indicate that IL-12 in renal tissue might be related to chronic lesion. IL-12, a pro-inflammatory cytokine, is produced by dendritic cells and macrophages, and helps differentiates T helper cells into T-helper type 1 cells.[28] The IL-12 and IL-23/T helper 17 axes play a vital role in SLE progression.[12 28 29] In the LN mouse model, high serum IL-12 exacerbated glomerular or interstitial inflammation.[30] As for the IHC study, Tucci *et al* reported that in IL-12/p70-positive mononuclear cells, higher serum or urinary IL-12 levels were seen in proliferative LN kidney tissue.[31] These results showed the possible relationship between IL-12 and LN activity. Anti-IL-12/IL-23 p40 antibody, named ustekinumab, had been anticipated as an effective biologic for SLE; nevertheless, the phase III trial of ustekinumab for patients with active SLE did not represent superiority over the placebo.[32] Our data showed increased pathological chronicity in the IL-12-dominant LN group; thus, suppressing serum IL-12 may not be valid in improving the activity of proliferative LN.

Furthermore, in our study, the BAFF-dominant group revealed lower modified NIH total index and activity index, fewer cases with class IV and better prognoses than the other groups. BAFF is located on dendritic cell and macrophage surfaces and secreted as soluble BAFF. By binding to the BAFF receptor, B-cell differentiation is induced by the transmembrane activator and cyclophilin ligand interactor and organ involvement of SLE is developed. The BAFF renal tissue expression in the previous study showed higher expression in proliferative NS than in class II LN, which is consistent with our results.[33] Belimumab is the first targeted biological treatment for SLE. The Belimumab International Study in LN (BLISS-LN study) showed that belimumab plus standard therapy was more effective than standard therapy alone, especially in proliferative LN.[17] Our data suggest that BAFF expression in renal tissue would appear in the earlier stages and lower LN activities, which is related to a good prognosis.

The renal histopathology in LN shows various morphological variations in the glomerular, interstitial and vessels of each individual, which might reflect individual pathogenesis and molecular heterogeneity in SLE. Precision medicine in SLE is challenging. Application of human immune profiling study results, including single cell analysis, omics analysis, transcriptome analysis and peripheral blood study with flow cytometry or mass cytometry, were attempted for precision medicine; however, SLE heterogeneity has made it difficult.[34] Itotagawa *et al* reported that patients with LN could be stratified by high serum BAFF and IFN-α bioactivities and showed an association between high BAFF and LN as well as high IFN and haematological or skin manifestation.[35] The IHC study of therapeutic target markers for precision medicine is technically simple and can be done at any pathology department with an immunofluorescent microscope. Belimumab and anifrolumab are now commercially available; thus, the application of biologics targeting highly expressed molecules, identified using IHC on renal biopsy tissue before treatment, should be considered in the future.

There are some limitations to our study. First, it was a retrospective and single-centre study and may have an unintentional selection bias. Second, we did not stain IFN-α, IL-12 and BAFF in the same specimen; if possible, triple staining of these three molecules in the same specimen should be done. Our findings need to be confirmed in a prospective study.

Despite the limitations mentioned above, this study suggested the possibility of precision medicine through an IHC study in renal tissue in proliferative LN. For instance, the use of anifrolumab might be favourably indicated in patients with LN showing predominant IFN-α expression, which relates to LN activity and poor prognosis. Accumulation of further evidence will contribute to clarifying the pathogenesis and development of LN.

Acknowledgements We would like to thank all members of our department of University of Occupational and Environmental Health for their helpful comments and general support. We are also grateful to Editage for editing the draft of this manuscript.

Contributors AN and SN contributed to the study design. AN conducted the experiments, analysis of the data and writing of the manuscript. SH, ES and MH contributed to analysing the data and reviewing the manuscript. TM contributed to performing kidney biopsy and analysis of the data. YT created the research concept and supervised the research, and reviewed the manuscript. AN is responsible for the overall content as the guarantor.

Funding This study was partially supported by a grant from the Kurozumi Medical Foundation.

Competing interests SN has received consulting fees, speaking fees and/or honoraria from Bristol-Myers, AstraZeneca, Pfizer, GlaxoSmithKline, Astellas, Asahi-kasei, Sanofi, AbbVie, Eisai, Chugai, Gilead and Boehringer Ingelheim and has received research grants from Mitsubishi-Tanabe. YT has received speaking fees and/or honoraria from Behringer-Ingelheim, Eli Lilly, AbbVie, Gilead, AstraZeneca, Bristol-Myers, Chugai, Daiichi-Sankyo, Eisai, Pfizer, Mitsubishi-Tanabe and GlaxoSmithKline, and has received research grants from Asahi-Kasei, AbbVie, Chugai, Eisai, Takeda, Daiichi-Sankyo and Behringer-Ingelheim.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval The study method was approved by the Human Ethics Review Committee of UOEH (IRB number: UOEHCRB21-093) and designed in line with the

Declaration of Helsinki and the Ethical Guidelines for Medical and Health Research Involving Human Subjects by the Ministry of Health, Labour and Welfare. Written informed consent was obtained from each patient.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository.

ORCID iDs
Aya Nawata http://orcid.org/0000-0002-7871-2425
Yoshiya Tanaka http://orcid.org/0000-0002-0807-7139

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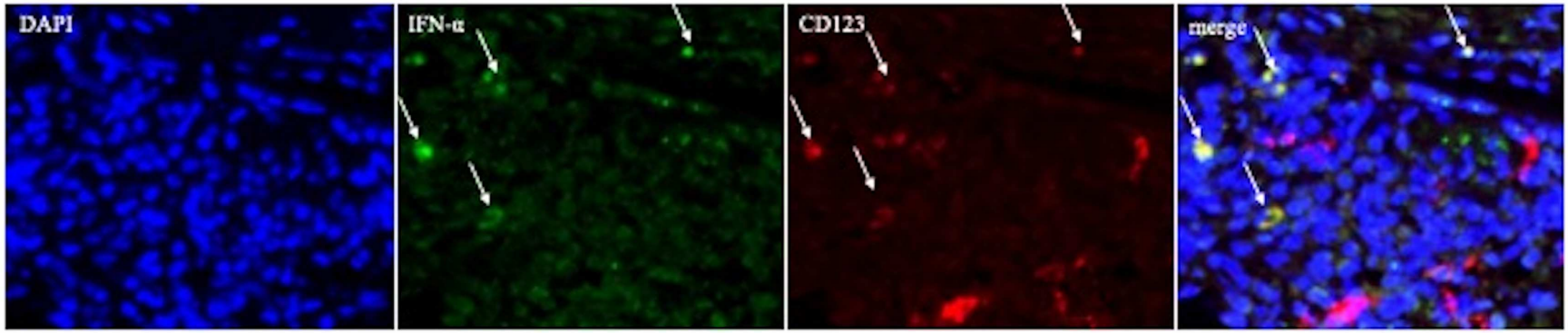

Supplement Figure 1:Immunofluorescent study of IFN-α and CD123

Supplementary text

Supplementary figure legends

Supplementary Figure 1. Immunofluorescent study of IFN-α and CD123
IFN-α positive cells are also positive for CD123, indicative of plasmacytoid dendritic cells.