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SHORT COMMUNICATION

Involvement of activated cytotoxic T lymphocytes and natural killer cells in Henoch–Schönlein purpura nephritis

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Abstract

Objectives. Immunoglobulin A vasculitis/Henoch–Schönlein purpura (IgAV/HSP) is a major cause of vasculitis in children. It is often accompanied by nephritis (HSPN) and could progress to chronic kidney disease. Galactose-deficient IgA_1 was recently reported to be involved in the pathogenesis of HSPN, for which immunosuppressive drugs are considered key treatment. However, the involvement of immune cells in the development of HSPN remains unclear. **Methods.** We compared gene expressions of peripheral blood mononuclear cells (PBMCs) among healthy controls (n = 10), IgAV/HSP patients (n = 21) and HSPN patients (n = 8), which required nephritis development within 3 months of IgAV/HSP onset. Immunohistochemistry analysis and flow cytometry were performed to assess renal biopsy specimens and PBMCs, respectively. Serum CX3CL1 levels were measured by ELISA. **Results.** *GNLY* and *GZMB* expressions increased in HSPN patients, consistent with increased number of glomerular granulysin- and/or granzyme B-positive cells demonstrated by immunohistochemistry analysis. Additionally, circulating cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells were activated with the up-regulated surface expressions of human leucocyte antigen DR (HLA-DR) and CX3CR1 in HSPN patients with severe proteinuria. Renal biopsies demonstrated increased number of $CD8^+$ cells and/or $CD56^+$ cells and up-regulated expression of glomerular CX3CL1, a specific ligand for CX3CR1, along with increased serum CX3CL1 level. **Conclusion.** Activated CTLs and NK cells play roles in the development of nephritis in IgAV/HSP patients and can be used as novel biomarkers for HSPN.

Keywords: IgA vasculitis, Henoch–Schönlein purpura nephritis, CX3CL1/fractalkine, HLA-DR, biomarker

INTRODUCTION

Henoch–Schönlein purpura (HSP), also known as immunoglobulin A vasculitis (IgAV), is a major cause of systemic vasculitis in children. IgAV/HSP is often accompanied by Henoch–Schönlein purpura nephritis (HSPN), which can manifest as haematuria, proteinuria, nephritic and/or nephrotic syndrome. Although approximately 80% of patients with haematuria alone had normal renal function during long-term follow-up,[1] more than half of the patients who exhibited both nephritic and nephrotic syndromes at onset developed chronic kidney disease (CKD).[2] Currently, the optimal treatment for HSPN remains controversial and its pathogenesis remains to be clarified. Several studies have indicated that galactose-deficient IgA_1 is involved in the pathogenesis of HSPN and IgA nephropathy (IgAN), both of which are characterised by predominant glomerular IgA deposition and mesangial proliferative changes.[3,4] It has been suggested that the glomerular deposition of circulating immune complexes initiates inflammatory responses and glomerular injury.[2,5] Aberrant immune responses to the kidney are implicated in the development of HSPN, but the role of immune cells remains unclear. It has been previously reported that IgAV/HSP was frequently associated with recent or simultaneous infections,[2] and monocytes and T cells often accumulated in the glomeruli of HSPN patients.[6] A genome-wide association study demonstrated that human leucocyte antigen (HLA) class II region is the major susceptibility locus for IgA vasculitis,[7] whereas other studies have implicated HLA class I region in HSPN.[8,9] In addition, immunosuppressive drugs such as cyclophosphamide, cyclosporin A and mycophenolate mofetil, which specifically target lymphocytes, were reported to be effective on HSPN.[10-12] Therefore, we investigated the involvement of immune cells, especially lymphocytes, in the development of HSPN.

RESULTS

GNLY and GZMB expressions increased in peripheral blood mononuclear cells (PBMCs) in HSPN patients

To evaluate the involvement of immune cells in the development of nephritis in IgAV/HSP patients, we performed microarray analyses using PBMCs from IgAV/HSP ($n = 2$) and HSPN ($n = 2$) patients under 15 years of age, which required nephritis development within 3 months of IgAV/HSP onset. HSPN was defined as the development of haematuria or proteinuria consecutively at least twice. Among the immune cell-related genes, *KLRF1*, *GNLY* and *GZMB* expression levels had markedly increased in HSPN patients compared with IgAV/HSP patients (Figure 1a and b and Supplementary table 1). Furthermore, functionally related gene groups were compared through Gene Ontology (GO) classifications from GO Consortium. Up-regulated genes exhibiting 1.5-fold increased expressions in HSPN compared with IgAV/HSP were selected for analyses. GO functional category analyses demonstrated that a significantly larger number of defence response (GO:0006952) genes were up-regulated in HSPN patients (Supplementary table 2). *GNLY* and *GZMB*, both known to be highly expressed in cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, were among the up-regulated genes in the defence response category (Supplementary table 3). Granulysin and granzyme B, encoded by *GNLY* and *GZMB*, respectively, play key roles in the cytotoxic effect. We hypothesised that the cytotoxic effect induces renal inflammation, leading to nephritis in IgAV/HSP patients. To validate the microarray analysis results, we conducted quantitative reverse transcription–PCR (RT-PCR) to analyse the expressions of *GNLY* and *GZMB* in PBMCs from healthy controls ($n = 10$), IgAV/HSP patients ($n = 21$) and HSPN patients ($n = 8$) under 15 years of age. Our results confirmed that *GNLY* and *GZMB* expressions were up-regulated in HSPN patients (Figure 1c).

Circulating CTLs and NK cells were activated in HSPN patients accompanied by nephrotic-range proteinuria

Because granulysin and granzyme B are highly expressed in activated CTLs and NK cells, we then evaluated the activation status of circulating CTLs and NK cells in one HSPN patient with nephritic and nephrotic syndromes (HSPN#1), three HSPN patients with nephrotic-range proteinuria [urine protein–creatinine ratio (UPCR) >2 g gCr^{-1}] (HSPN#2–4), one HSPN patient with non-nephrotic-range proteinuria and macrohaematuria (HSPN#5), and three IgAV/HSP patients (HSP#1–3) (Supplementary table 4). HSPN#1 was a 5-year-old

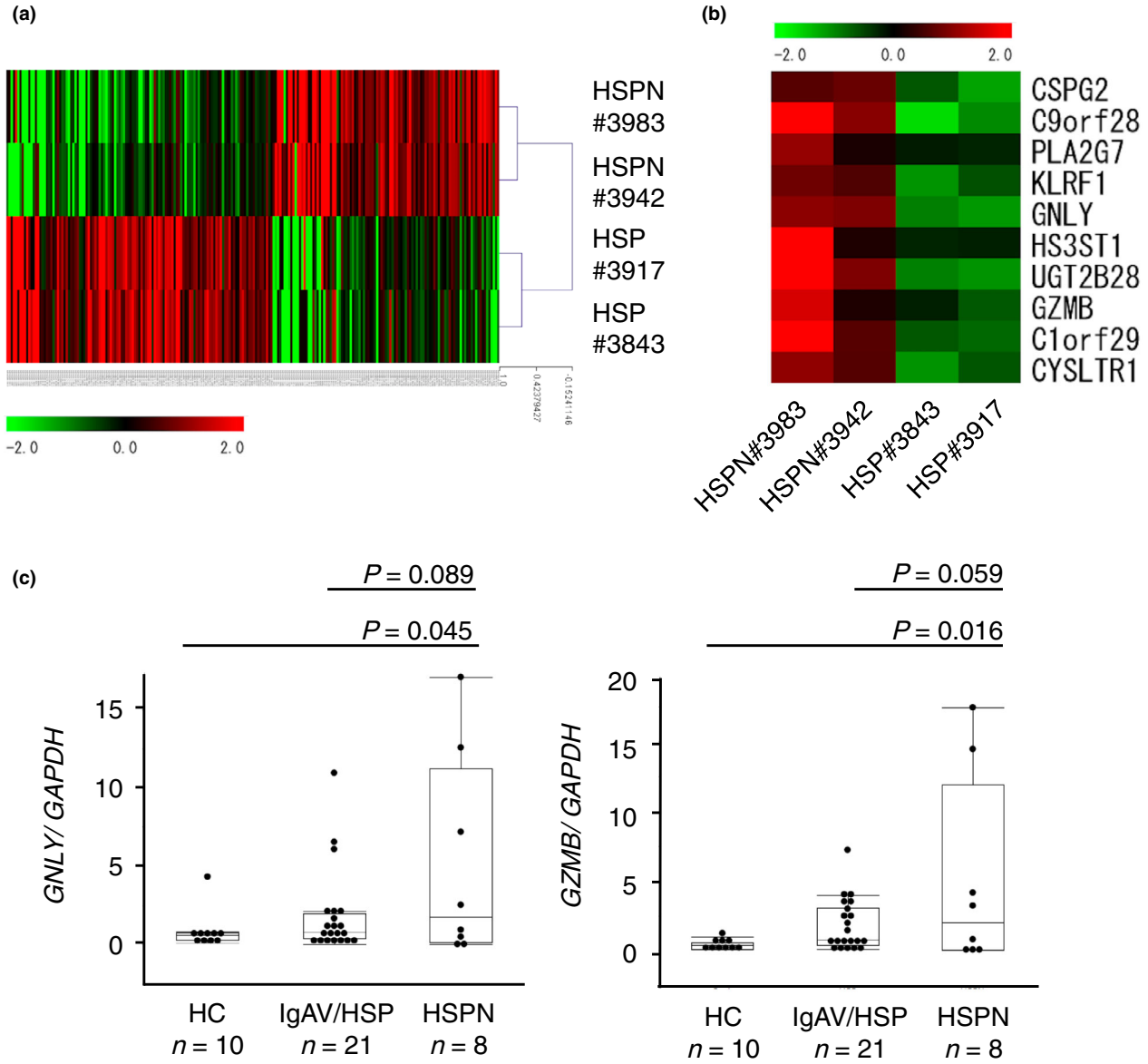

Figure 1. Gene expression of *GNLY* and *GZMB* was up-regulated in peripheral blood mononuclear cells (PBMCs) of Henoch–Schönlein purpura nephritis (HSPN) patients. **(a)** Clustering diagram of genes and heat map of gene expression were generated using MeV. PBMCs from HSPN (#3983 and #3942) and IgAV/HSP (#3843 and #3917) patients were analysed. Rows represent the samples, and columns represent the genes. Colours indicate the distance from the median of each row. Red and green represent high and low, respectively. **(b)** A heat map of 10 most up-regulated genes, with a fold change greater than 1.5-fold in PBMCs from HSPN (#3983 and #3942) than in those from IgAV/HSP (#3843 and #3917) patients. Rows represent the genes, and columns represent the samples. **(c)** Quantitative RT-PCR of *GNLY* and *GZMB* in PBMCs from healthy controls (n = 10), IgAV/HSP patients (n = 21) and HSPN patients (n = 8). One-way ANOVA combined with the Tukey–Kramer honestly significant difference test was used for comparison of different groups. Data are from a single experiment.

boy with palpable purpura, abdominal pain, proteinuria, haematuria and oedema on admission, which accompanied nephritic and nephrotic syndromes. Renal biopsy identified International Study of Kidney Disease in Children (ISKDC) grade IIIa HSPN. The immunosuppressive treatment resulted in a gradual decrease in the UPCR (Figure 2a). The activation of circulating CTLs and NK cells was monitored by measuring the surface expression level of HLA-DR using flow cytometry (Figure 2b). CTLs and NK cells were activated during the acute stage, with the

activation diminishing during the convalescent stage (Figure 2a and b). Similarly, in HSPN#2, HSPN#3 and HSPN#4, CTLs and/or NK cells were activated during the acute stage with heavy proteinuria, instead of the convalescent stage without proteinuria (Figure 2c and d and Supplementary figure 1). In sharp contrast to the HSPN patients with nephrotic-range proteinuria, neither circulating CTLs nor NK cells were activated in HSP#1–3 (Supplementary figure 2a–c), or in HSPN#5 without nephrotic-range proteinuria (Supplementary figure 2d).

Expression of granulysin and granzyme B and the number of CTLs and NK cells were increased in the glomeruli of HSPN patients

The renal biopsy specimens of HSPN#1–4 were stained with anti-granulysin and anti-granzyme B antibodies. Glomerular granulysin and granzyme B expressions were up-regulated in HSPN#1–4 compared with healthy controls (Figure 3). To examine the involvement of CTLs and NK cells in nephritis, renal biopsy specimens were stained with anti-CD8 and anti-CD56 antibodies. Our results indicated that the number of glomerular $CD8^+$ cells and/or $CD56^+$ cells increased in HSPN#1–4 compared with healthy controls (Figure 3).

Glomerular CX3CL1 expression and surface expression of CX3CR1 on CTLs and NK cells were highly expressed in HSPN patients

To elucidate the mechanism underlying the localisation of CTLs and NK cells to glomeruli in HSPN patients, we assessed glomerular chemokine expression levels because several chemokines were proposed to direct mononuclear cell infiltration in acute renal inflammation.[13] In addition to its chemotactic function, CX3CL1/fractalkine triggers the adhesion of a subset of mononuclear cells expressing CX3CR1, a specific CX3CL1 receptor, to endothelial cells under flow conditions.[14,15] Furthermore, glomerular CX3CL1 expression increased in IgAN patients,[16] and in a crescent glomerulonephritis rat model.[17] Therefore, we analysed glomerular CX3CL1 expression through immunohistochemistry and identified increased CX3CL1 expression in all HSPN (Figure 4a–d) and IgAN (Supplementary figure 3a–d and Supplementary table 5) patients. Additionally, CX3CR1 surface expression was up-regulated on circulating CTLs and NK cells during the acute stage in HSPN patients (Figure 4e and f). Moreover, serum CX3CL1 levels during the acute stage were higher than those during the convalescent stage (Figure 4g).

DISCUSSION

We found in this study that circulating CTLs and NK cells were activated and the numbers of CTLs and NK cells in the glomeruli were increased in HSPN patients with nephrotic-range proteinuria. In addition, CX3CR1 surface expressions on circulating CTLs and NK cells and CX3CL1 expression in the glomeruli were highly expressed. These findings indicate that activated circulating CTLs and NK cells play a key role in the development of nephritis in IgAV/HSP patients.

The results show that circulating CTLs and NK cells were activated and that the number of glomerular CTL and NK cell was increased, along with up-regulation of granulysin and granzyme B expressions in HSPN patients with nephrotic-range proteinuria. Consistent with our results, a recent study demonstrated that the expression level of T-bet, which was reported to induce the development of Th1 cells, was significantly higher in the urinary sediment of HSPN patients than in that of the healthy controls; this also correlates with the obtained UPCR.[18] Additionally, the renal biopsy specimens of HSPN patients exhibited a high number of a subset of T cells.[6] By contrast, although CTLs and NK cells were found in the glomeruli in IgAN patients, granulysin or granzyme B expression did not increase (Supplementary figure 3a–d), and circulating CTLs and NK cells were not activated (Supplementary figure 3e–h). Taken together, these data indicate that activated CTLs and NK cells in the blood circulation and glomeruli play roles in the development of nephritis in IgAV/HSP patients, but not in IgAN patients.

We demonstrated that glomerular and serum CX3CL1 expressions increased during the acute stage in HSPN patients and that CX3CR1 surface expression was up-regulated in activated circulating CTLs and NK cells in HSPN patients. A recent study reported that focal adhesion and cell adhesion molecules were enriched in urine proteome in HSPN patients compared with healthy controls.[19] Furthermore, CX3CR1 was highly expressed on the cell surface of infiltrating T cells in inflammatory kidney diseases.[20]

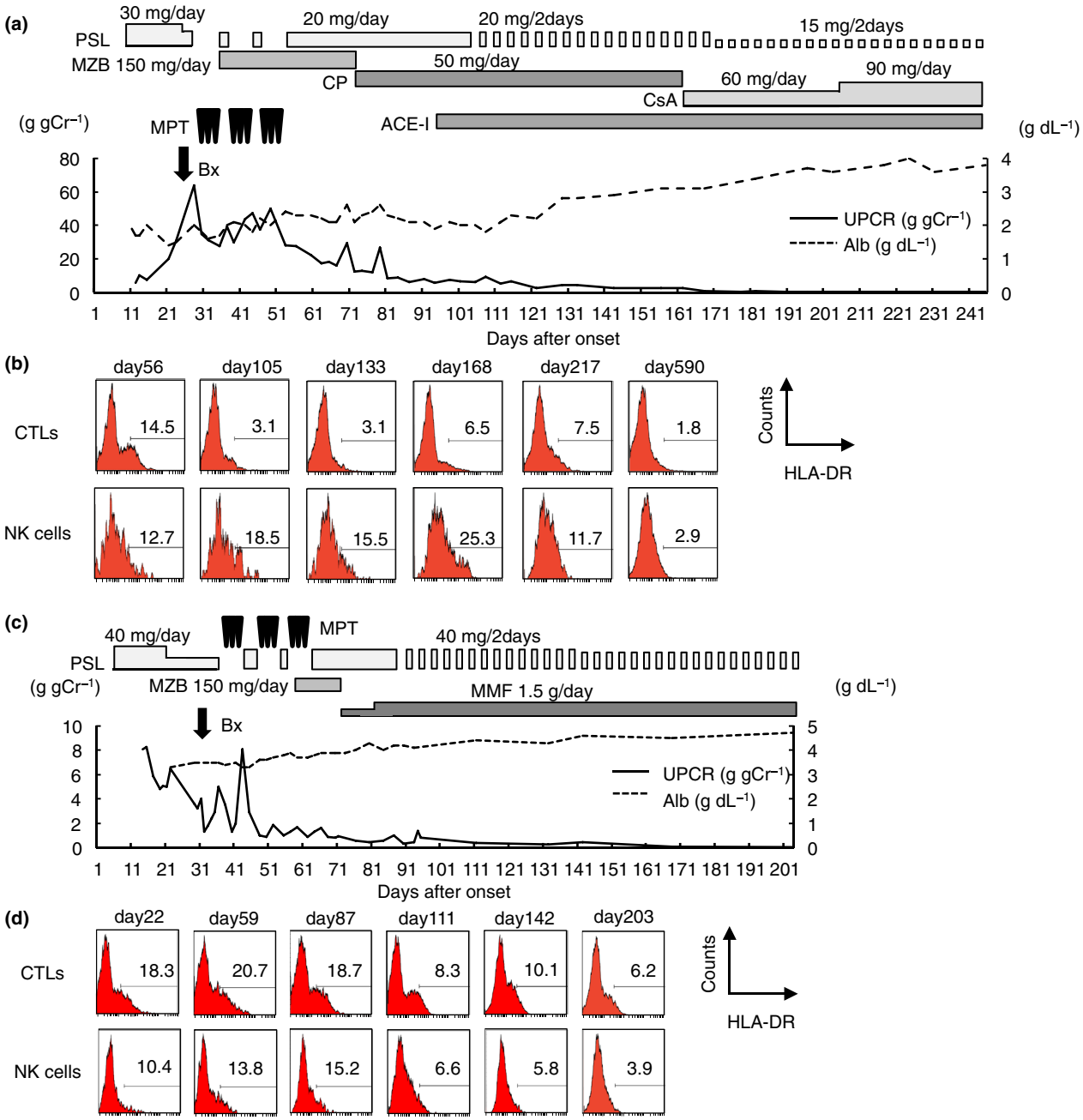

Figure 2. Activation of circulating cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells during the acute stage of HSPN. **(a)** Clinical course and treatment of HSPN#1. UPCR, urine protein–creatinine ratio; PSL, prednisolone; MPT, methylprednisolone pulse therapy; MZB, mizoribine; CP, cyclophosphamide; CsA, cyclosporine A; Bx, renal biopsy. **(b)** The frequency of circulating HLA-DR$^+$ CTLs and NK cells in HSPN#1 on the indicated day after onset. Numbers in the figures indicate the frequency as a percentage of total cells. **(c)** Clinical course and treatment of HSPN#2. **(d)** The frequency of circulating HLA-DR$^+$ CTLs and NK cells in HSPN#2. MMF, mycophenolate mofetil. Data are from a single experiment for each patient.

Collectively, these data suggest that activated CTL and NK cells expressing CX3CR1 migrate to the glomeruli where CX3CL1 expression is up-regulated, thereby damaging the endovascular endothelium and leading to nephritis in IgAV/HSP patients.

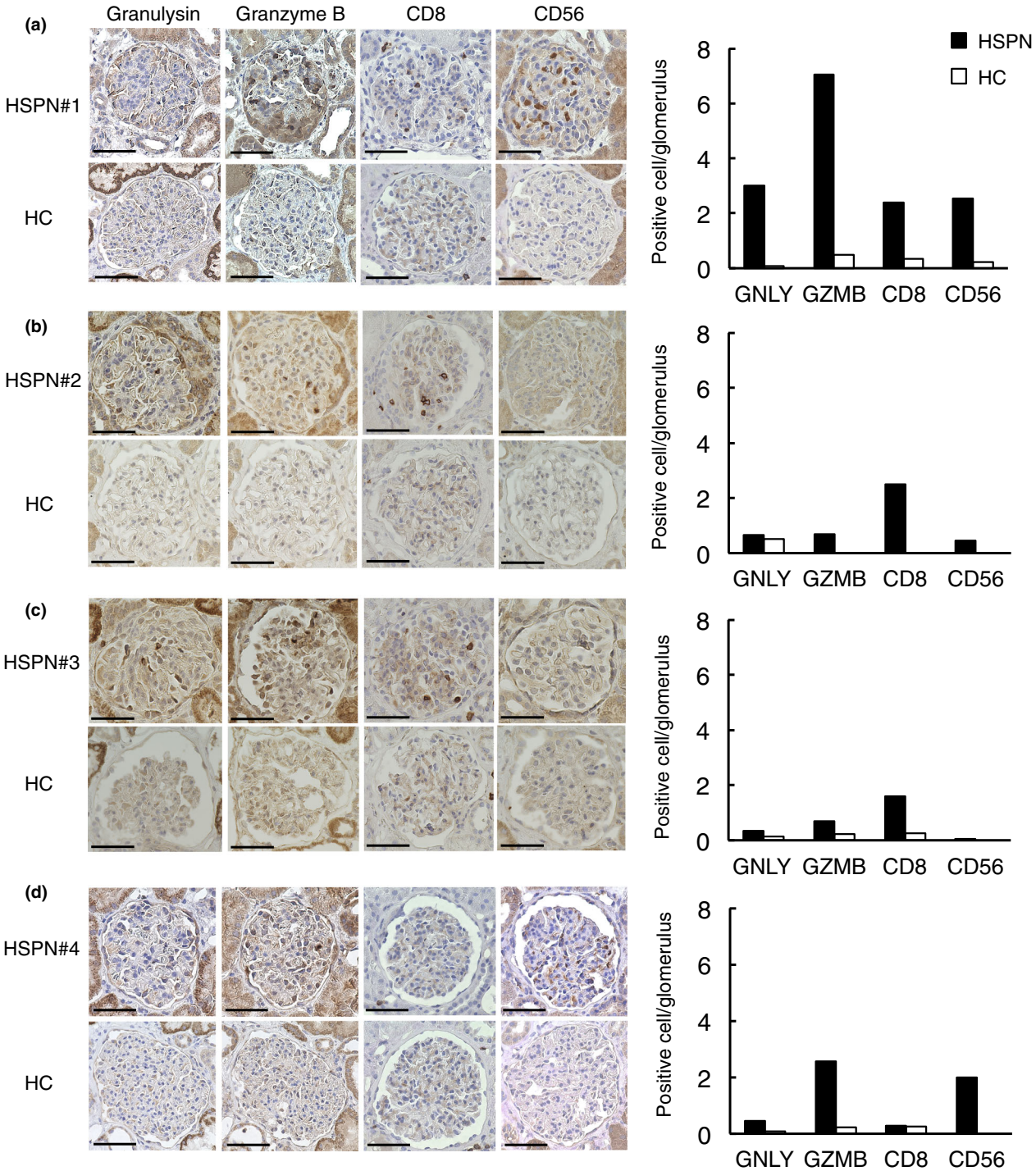

Figure 3. Recruitment of CTLs and NK cells in the glomeruli of HSPN patients. **(a–d)** Immunohistochemical staining of renal biopsy specimens from HSPN#1–4 in the upper panels and from healthy controls in the lower panels with anti-granulysin, anti-granzyme B, anti-CD8 and anti-CD56 antibodies. The scale bar is 50 μm. Quantification of stained cells in the glomeruli of HSPN patients and healthy controls is shown in the right graphs. HC, healthy control. Data are from a single experiment.

In the present study, the gene expression levels of *GNLY* and *GZMB* in PBMCs and the protein amount of granulysin and granzyme B in the glomeruli were up-regulated in HSPN patients. A previous study reported that vascular permeability was significantly reduced in granzyme B-deficient

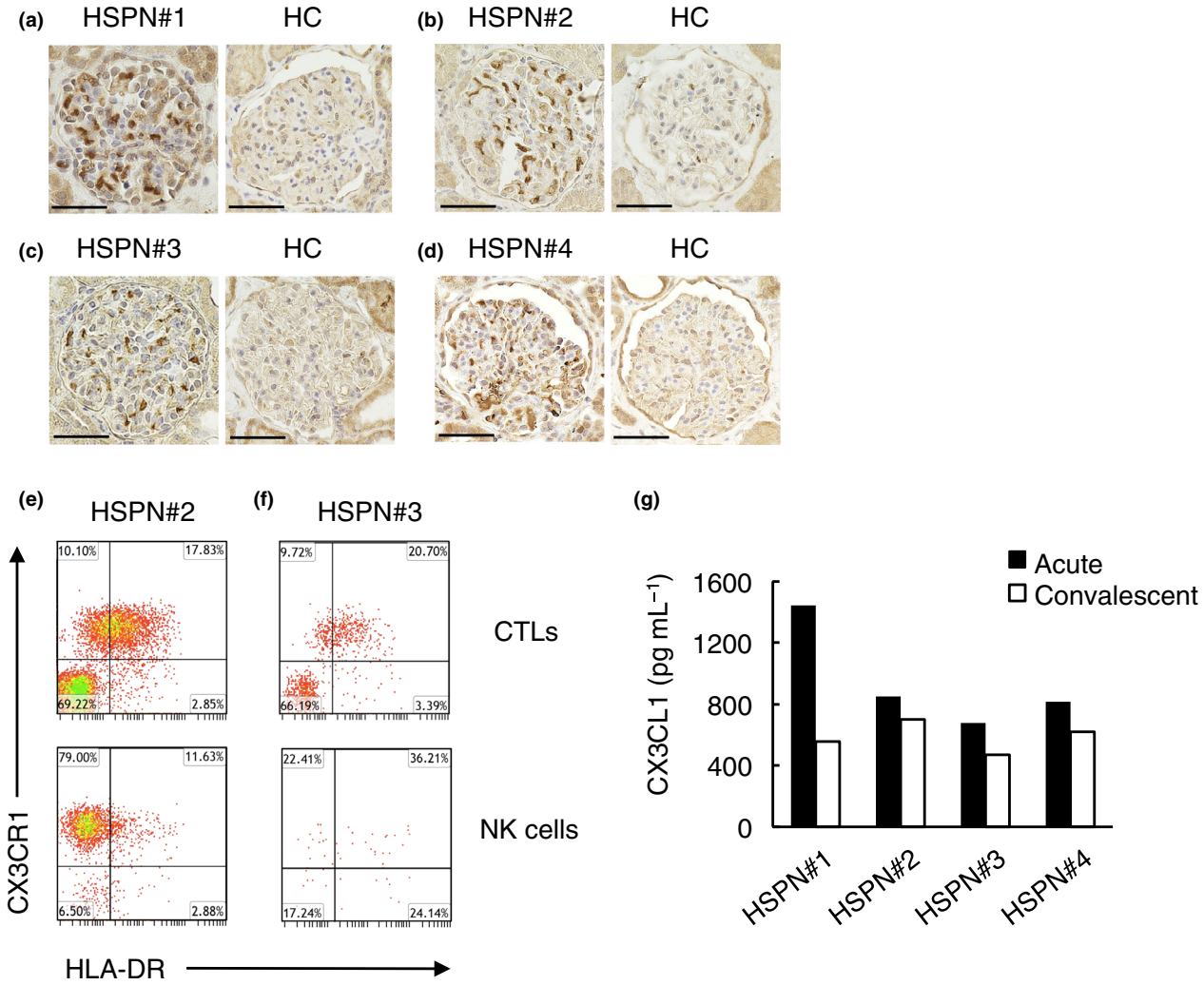

Figure 4. CX3CL1 expression in the glomeruli and surface expression of CX3CR1 on CTLs and NK cells of HSPN patients. **(a–d)** Immunohistochemical staining of renal biopsy specimens from HSPN#1–4 and from healthy controls with anti-CX3CL1 antibody. The scale bar is 50 μm. **(e** and **f)** Surface expression of CX3CR1 on CTLs (upper panels) and NK cells (lower panels) in HSPN#2 **(e)** and HSPN#3 **(f)** during the acute stage. **(g)** Serum concentration of CX3CL1 in HSPN#1–4 during the acute and convalescent stages. Data are from a single experiment.

mice compared with wild-type controls, indicating that granzyme B induces vascular permeability.[21] Granulysin and granzyme B in CTLs and NK cells are known to have cytotoxic effect to induce cell death.[22,23] In particular, HLA-DR$^+$ NK cells showed intense degranulation effect.[24] Furthermore, it was demonstrated that NK cells exert cytotoxic activity towards human glomerular endothelial cells *in vitro*.[25] On the basis of these results, we reason that activated CTLs and NK cells express granulysin and granzyme B, which cause vascular permeability and cell death, and destroy the integrity of the blood vessels in the kidney, leading to proteinuria and haematuria.

Several reports have demonstrated that early initiation of HSPN treatment is important for preventing the progression to CKD.[10,26] Correspondingly, HSPN patients with nephritic or nephrotic syndrome at onset are more likely to develop renal failure.[2] Therefore, a novel biomarker is needed to detect severe proteinuria at an early stage following onset. Previous reports indicated that serum pentraxin 3 or urinary pro-inflammatory cytokines can be used to differentiate patients who initially had nephritis from those who did not.[27,28] In this study, the activation of circulating CTLs and NK cells, monitored by surface HLA-DR expression, was detected in HSPN patients even before

proteinuria reached the nephrotic range (Supplementary figure 1a and b). This suggests that HLA-DR expressions on the cell surface of circulating CTLs and NK cells are novel biomarkers for predicting the development of nephritis, particularly the occurrence of nephrotic-range proteinuria, as well as indicators for treatment during the early stage. There are several limitations in the current study, including the small number of enrolled patients and lack of flow cytometry data during the very early stage following onset. Therefore, further studies are needed to increase the generalisability and clinical applications of our findings.

METHODS

Study approval

All patients provided written informed consent for the study. This study was approved by the Institutional Review Board of Kyushu University (#217, #2019-606).

PBMC samples for microarray and quantitative RT-PCR

Peripheral blood mononuclear cells were collected within 7 days after onset of IgAV/HSP. HSPN was defined as the development of haematuria or proteinuria consecutively at least twice within 3 months after onset of IgAV/HSP.

RNA extraction, amplification, labelling and cDNA synthesis

Total RNA was extracted using Isogen reagents (Nippon Gene, Tokyo, Japan). For microarray analysis of PBMCs, linear amplification of RNA was carried out using an Amino Allyl Message Amp aRNA Kit (Ambion, Austin, TX, USA). Briefly, double-stranded complementary DNA (cDNA) was synthesised from total RNA using an oligo-dT primer with a T7 RNA polymerase promoter sequence added to the 3′ end. Then, *in vitro* transcription was performed in the presence of amino allyl UTP to produce multiple copies of complementary RNA (cRNA) labelled by amino allyl. Amino allyl-labelled cRNA was purified, and then, 15 μg of cRNA was incubated with N-hydroxy succinimide esters of Cy3 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for cRNA from the patients or Cy5 (Amersham Pharmacia Biotech) for that from healthy controls according to the protocol of Hitachi Software Engineering (Yokohama, Japan).

Microarray analysis

Microarray analysis of PBMCs from IgAV/HSP ($n = 2$) and HSPN ($n = 2$) patients was performed using AceGene Human Oligo Chip 30K (Hitachi Software Engineering, Tokyo, Japan), which contains approximately 30 000 genes. Expression levels of each gene in PBMCs were determined by comparison with those in the standard sample. The arrays were scanned using FLA-8000 (Fuji Photo Film, Tokyo, Japan), and signals were converted to numerical values using ArrayVision (Amersham Biosciences, Piscataway, NJ, USA). The Cy5/Cy3 ratio of all spots on the DNA microarray was normalised using the method of LOWESS. Data from two HSPN patients were compared with those from two IgAV/HSP patients. A heat map was generated using MeV, and the genes were sorted using hierarchical clustering.[29] Colours indicate the distance from the median of each row. Red and green represent high and low, respectively. The Pearson correlation was used for distance metric and 'average linkage clustering' for linkage. We established the following criteria for differentially expressed genes: up-regulated genes (ratio $\geq$ 1.5-fold) and down-regulated genes (ratio $\leq$ 0.66). Data were analysed using GeneSpring v.14.9 (Agilent Technologies, Santa Clara, CA). DAVID (v.6.8) functional annotation bioinformatics microarray analysis was used for the analysis of GO biological process functional category.[30]

Quantitative real-time PCR

Quantitative PCR of *GNLY* and *GZMB* in PBMCs from healthy controls ($n = 10$), IgAV/HSP ($n = 21$) patients and HSPN ($n = 8$) patients was performed. The Assay-on-Demand Gene Expression assay reagents Hs.00246266_m1 (*GNLY*) and Hs.00188051_m1 (*GZMB*) (Applied Biosystems, Foster City, CA, USA) were used as primers and probes. Pre-developed TaqMan Assay Reagent Human *GAPDH* (Applied Biosystems) was used as an internal control. The TaqMan assay was performed according to the manufacturer's instructions. In brief, the PCR primer set and TaqMan probe for each target gene were added into the TaqMan Universal PCR Master Mix (Applied Biosystems) to a final volume of 25 μL. The PCR condition was as follows: 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of amplification at 95°C for 15 s and 60°C for 1 min. PCR amplification and detection of the PCR products were performed using the ABI PRISM 7700 Sequence Detector (Applied Biosystems). Gene expression levels were calculated by normalising the expression level of each gene of interest against that of the internal control *GAPDH*.

Immunohistochemistry staining

Formalin-fixed, paraffin-embedded tissue specimens from patients and healthy donors for kidney transplantation were sectioned into 3-μm-thick slices and placed on coated slides. Regarding antigen retrieval, slides were heated in a microwave oven (700 W) for 15 min with an antigen retrieval solution at pH 9.0 (Nichirei Biosciences, Tokyo, Japan). Tissue sections were stained with anti-granulysin (F-9, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-granzyme B (ab4059, Abcam, Cambridge, UK), anti-CD8 (EP1150Y, Abcam), anti-CD56 (123C3, Invitrogen, Carlsbad, CA, USA) and anti-CX3CL1 (ab25088, Abcam) antibodies. Antigen–antibody complexes were visualised with Histofine Simple Stain MAX PO (Nichirei Biosciences).

Surface staining

Whole blood cells were stained with anti-CD3 (UCHT1, Beckman Coulter, Miami, FL, USA), anti-CD8 (B9.11, Beckman Coulter), anti-CD56 (N901, Beckman Coulter; HCD56, BioLegend, San Diego, CA, USA), anti-HLA-DR (Immu-357, Beckman Coulter) and anti-CX3CR1 (K0124E1, BioLegend) antibodies. Surface markers were monitored using EC800 Cell Analyzer (Sony Corporation, Tokyo, Japan). The gating strategy for flow cytometry is available in Supplementary figure 4.

Cytokine assay

The concentration of CX3CL1 in the serum was measured using human CX3CL1 ELISA kit (R&D Systems Inc., Minneapolis, MN, USA).

Accession number

Raw microarray data were submitted to the Gene Expression Omnibus database (accession number GSE149333).

Statistical analyses

In microarray analyses, Fisher's exact test was performed to detect significantly over-represented GO categories and to characterise the enrichment of specific pathways as functionally related gene groups. Regarding quantitative real-time PCR results, differences among the three categories were determined by one-way ANOVA combined with the Tukey-Kramer honestly significant difference test. Differences were considered statistically significant when the *P*-value was < 0.05.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

TI and TH designed the study; TI, KU and TT conducted the experiments; TI, KN and TT analysed the data; YK provided the reagents; and TI and SO wrote the manuscript. All authors contributed to the drafting of this manuscript.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.