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Identification of alternatively activated macrophages in new-onset paediatric and adult immunoglobulin A nephropathy: potential role in mesangial matrix expansion

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Identification of alternatively activated macrophages in new-onset paediatric and adult immunoglobulin A nephropathy: potential role in mesangial matrix expansion

Aims: New onset of the clinical symptoms of immunoglobulin A (IgA) nephropathy (IgAN) manifests with proliferative glomerular lesions in children, whereas adults exhibit mesangial matrix expansion and interstitial fibrosis. Alternatively, activated (M2) macrophages have been implicated in promoting tissue fibrosis in some settings. Therefore, the aim of this study was to investigate whether M2 macrophages are present in new-onset IgAN and if they are related to pathological differences between paediatric and adult disease.

Methods and results: Biopsy specimens from paediatric (<10 years, $n = 14$; >12 years, $n = 15$) and adult ($n = 27$) IgAN showed a significant infiltrate of $CD68^+$ macrophages. M2 macrophages, identified by CD163 or CD204 expression, were detected in glomeruli and the interstitium, being more prominent in adults versus young children. $CD163^+$ and $CD204^+$ macrophages were present in areas of fibrosis containing myofibroblasts, and double staining showed that $CD163^+$ cells produced the profibrotic molecule, connective tissue growth factor. In young children, total $CD68^+$ macrophages, but not M2 macrophages, correlated with glomerular hypercellularity. In contrast, in adults and older children, mesangial matrix expansion correlated with M2 macrophages but not with the total $CD68^+$ macrophage infiltrate.

Conclusions: Alternatively activated M2 macrophages are present in new-onset paediatric and adult IgAN, and this population may promote the development of fibrotic lesions.

Keywords: CD163, CD204, M2, SOCS1, SOCS3

Abbreviations: CTGF, connective tissue growth factor; ESRD, end-stage renal disease; FITC, flourescein isothiocyanate; HRP, horseradish peroidase; IF, immunofluorescence; IgA, immunoglobulin A; IgAN, IgA nephropathy; NTN, nephrotoxic serum nephritis; PAP, peroxidase-conjugated mouse antiperoxidase; SMA, smooth muscle actin; SOCS, suppressor of cytokine signalling; TBMD, thin basement membrane disease; TRITC, tetramethyl-rhodamine isothiocyanate

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Introduction

Immunoglobulin A (IgA) nephropathy (IgAN) is the most common form of glomerulonephritis worldwide, and most commonly affects people in their teens and 20s. Approximately 30–40% of patients eventually progress to end-stage renal disease (ESRD).[1–3] There are distinct pathological differences evident at the onset of IgAN in children versus adults. Mesangial enlargement in paediatric IgAN is due mainly to mesangial hypercellularity, whereas mesangial enlargement in new-onset adult IgAN is due mainly to mesangial matrix expansion.[4] Interstitial fibrosis is also more prominent in adult-onset IgAN,[4] indicating a more fibrotic pathology in adult versus paediatric IgAN. However, the mechanisms underlying the differences between these pathologies remain to be elucidated.

Macrophage infiltration within the glomerular and interstitial compartments is a common feature in most forms of glomerulonephritis, including IgAN.[5–8] Macrophage infiltration is also evident at the onset of IgAN and correlates with proteinuria and interstitial damage.[4] However, macrophages can cause damage and promote tissue repair and thus could have distinct roles in the onset of IgAN. Proinflammatory M1-type macrophages are often associated with tissue damage, whereas alternatively activated M2-type macrophages are associated usually with tissue repair.[9,10] The presence of proinflammatory M1-type macrophages has been described in IgAN based upon macrophage production of tumour necrosis factor (TNF)-α and interleukin (IL)-1β,[11] and by the presence of activated $CD169^+$ and $MRP8/14^+$ macrophages.[7,12] However, a potential role for M2-type macrophages in IgAN, or indeed in other forms of human glomerulonephritis, has not been examined.

The aim of this study was to investigate whether M2-type macrophages are present at the onset of IgAN and whether such cells may explain the underlying differences in pathology in paediatric and adult disease. The term 'new-onset' IgAN is used in this paper to describe individuals in which the clinical symptoms of biopsy proven IgAN have been detected within approximately 2 years of their last normal urinalysis.

Materials and methods

PATIENTS

A total of 56 biopsy diagnosed childhood and adult IgAN patients undergoing diagnostic renal biopsy were examined at the Departments of Pediatrics or Medicine, Niigata University Medical and Dental Hospital from 2000 to 2008. The same criteria were used in the decision to biopsy paediatric and adult patients: (i) continuous haematuria and proteinuria of approximately 0.3 g/day or (ii) continuous macrohaematuria and proteinuria. An additional criterion for inclusion in the study was biopsy proven IgAN identified within 2 years of the first detection of urine abnormalities and no treatment during this period. Patients were divided into three groups by age at biopsy, with clinical parameters as shown in Table 1. All patients gave informed consent for the use of renal biopsy tissue, in excess of that required for diagnostic purposes, to be used for research purposes. The diagnosis of IgAN was based upon the demonstration by direct immunofluorescence of IgA as the dominant or codominant immunoglobulin in a predominantly mesangial distribution and the lack of clinical or serological evidence for systemic lupus erythematosus, vasculitis or Henoch–Schönlein purpura.

All patients underwent their renal biopsy before commencing any treatment. Controls of 'near normal' renal tissue used biopsy specimens from paediatric patients with thin basement membrane disease (TBMD): TBMD aged <10 years (range 7.0–9.7 years; 9.0 ± 0.9, $n = 4$) and aged <12 years (range 13.0–15.3 years; 14.1 ± 1.5, $n = 4$); or adult biopsy specimens taken prior to transplantation of kidney from healthy donors aged 35–43 (39.5 ± 3.4, $n = 4$). Serum creatinine (sCr), creatinine clearance (Ccr), haematuria and 24-h protein excretion were determined at the time of biopsy with assays performed by the Department of Biochemistry, Niigata University Medical and Dental Hospital. To evaluate the degree of haematuria, 10 ml of fresh urine was centrifuged at 500 *g* for 5 min and examined by microscope at high-power, and then scored between 0 and 4 according to the number of red blood cells in the sediment as follows: 0 (0–5), 1 (6–20), 2 (21–50), 3 (51–100) and 4 (>100).

QUANTIFICATION OF HISTOLOGICAL DAMAGE IN RENAL BIOPSIES

For light microscopy, renal specimens were taken by standard needle biopsy methods, fixed in Carnoy's solution and embedded in paraffin, and then 2-μm sections were stained with periodic acid-Schiff (PAS) and periodic acid-methenamine silver. The specimens were reviewed and analysed by an independent histopathologist who was blinded to both clinical data and the quantification of macrophage accumulation.

Sections were analysed for glomerular cellularity, glomerular matrix expansion and tubulointerstitial fibrosis and/or tubular cell atrophy. The morphometric analysis utilized SigmaScan Pro version 5.0 (Systat

Table 1. Comparison of clinical parameters

	Children (<10 years)	Children (>12 years)	Adults (26–35 years)
Number of patients	14	15	27
Gender (male:female)	8:6	6:9	11:16
Age at discovery (years) (range)	7.1 ± 1.2 (5.0–9.5)	12.8 ± 1.7 (10.3–15.0)	30.2 ± 2.8 (26.0–34.9)
Age at biopsy (range)	7.9 ± 1.2 (5.7–9.8)	14.1 ± 1.1 (12.5–15.9)	30.9 ± 2.9 (26.1–35.0)
Time from last normal urine examination to biopsy (years)	1.6 ± 1.0	2.2 ± 1.2	2.0 ± 1.5
Time from discovery to biopsy (years)	0.8 ± 0.8	1.3 ± 1.2	0.8 ± 0.6
Proteinuria (g) (1.73 m^2/day)	1.2 ± 1.3	0.9 ± 0.4	0.8 ± 0.4
Haematuria (score)	2.9 ± 1.3	3.0 ± 1.0	2.8 ± 1.3
GFR (ml/min/1.73m^2)	107.4 ± 17.3	121.1 ± 14.1	104.3 ± 28.2
Number of patients with past history of hypertension and/or hyper lipidemia	0	0	5*

Data are shown as mean ± 1 standard deviation.
GFR, Glomerular filtration rate.
*$P < 0.05$ compared with all children.

Software Inc., Point Richmond, CA, USA) and consisted of the following steps: (i) capturing PAS-stained glomerular cross-sections; (ii) tracing the outline of the glomerular tuft to obtain the total glomerular area; and (iii) the mesangial area was measured in an automated fashion based on selection of the red/pink component of the PAS stain (omitting blue nuclei) and expression as a percentage of the total glomerular area to provide a measure of mesangial matrix accumulation. The mesangial area including mesangial matrix and nuclei was also measured. The number of nuclei in the mesangial, endocapillary and extracapillary areas in each glomerulus was counted at the same time and expressed as mean number of glomerular nuclei per square millimetre. All cell counts were restricted to the glomerular tuft, with cells in crescents excluded. All glomeruli (≥12 in all cases) were examined in each patient and the average glomerular damage score calculated. Globally sclerosed glomeruli were not included in the analysis. The degree of interstitial fibrosis was scored between 0 and 4 according to the area of the tubulointerstitium demonstrating fibrosis and/or tubular atrophy as follows: 0 (none), 1 (0–25%), 2 (26–50%), 3 (51–75%) and 4 (>76%). At least 12 consecutive high-power fields, covering the entire cortex, were evaluated for each patient and expressed as mean ± standard deviation (SD).

ANTIBODIES

Fluorescein isothiocyanate (FITC)-conjugated rabbit antihuman IgG, IgA, IgM, C3c, C4, C1q and fibrinogen sera (Dako, Glostrup, Denmark) were used for routine immunofluorescence (IF) staining for determination of the diagnosis. Primary antibodies used in this study were: 10D6, antihuman CD163 (mouse IgG1; Visionbiosystems, Benton Lane, UK); SRA-E5, antihuman CD204 (macrophage scavenger receptor A, mouse IgG1; Trans Genic Inc., Kobe, Japan); Y1/82A, anti-CD68 labels human monocyte and macrophages (mouse IgG2b; BD Biosciences Pharmingen, San Diego, CA, USA); 1A4, antihuman smooth muscle actin (α-SMA; mouse IgG2a; Dako); 2154–60, antihuman connective tissue growth factor (CTGF; mouse IgM; Acris Antibodies, Hiddenhausen, Germany); rabbit polyclonal antibodies to suppressor of cytokine

signalling (SOCS) 1 (Abcam, Tokyo, Japan), SOCS3 (Abcam) and human type I collagen (Cosmo Bio, Tokyo, Japan). Another anti-CD163 antibody (chicken IgY; BMA Biomedical, Augst, Swizerland) was used for double-labelling staining in the IF study. Secondary antibodies used were: FITC-conjugated goat antimouse IgG1 (Southern Biotechnology Associates, Birmingham, AL, USA), FITC-conjugated goat antimouse IgG2a (Southern Biotechnology Associates); tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated goat antimouse IgG1 (Southern Biotechnology Associates), TRITC-conjugated goat antimouse IgG2b (Southern Biotechnology Associates), FITC-conjugated rabbit antichicken IgY (AnaSpec, San Jose, CA, USA), FITC-conjugated goat antimouse IgM (Chemicon, Temecula, CA, USA) and TRITC-conjugated swine antirabbit immunoglobulins (Dako). Other secondary detection antibodies included horseradish peroxidase (HRP)-conjugated goat antimouse IgG (Dako) peroxidase-conjugated mouse antiperoxidase complexes (PAP; Dako).

IMMUNOHISTOCHEMISTRY

Detection of macrophages and α-SMA in 2-μm sections fixed in Carnoy's solution used a three-layer PAP method. Sections were dewaxed, autoclaved for 20 min in 0.1 M sodium citrate buffer pH 6.0 (pH 9.0 for anti-CD204 and anti-α-SMA) (Target Retrieval Solution; Dako), washed in phosphate-buffered saline (PBS), and then blocked in 10% normal goat serum, 10% fetal calf serum (FCS) and 1% bovine serum albumin (BSA) in PBS for 30 min. Primary antibodies diluted in 10% normal human serum and 1% BSA in PBS were added to sections overnight at 4°C. Sections were then washed three times in PBS, endogenous peroxidase inactivated in 0.3% H_2O_2 in methanol for 20 min and washed in PBS. After washing in PBS, sections were incubated with HRP-conjugated secondary antibodies followed by PAP complexes for 45 min each at room temperature. After washing in PBS, sections were developed with the diaminobenzidine substrate (Dako) to produce a brown or blue colour using TrueBlue Substrate (KPL, Gaithersburg, MD, USA).

The number of cells stained for CD68, CD163 or CD204 antigens were counted in at least 12 glomeruli per patient. Interstitial cells stained for CD68, CD163 or CD204 antigens were counted in at least nine consecutive high-power fields (×400). For two-colour immunostaining, sections were stained with CD163 or CD204, treated with autoclave heating to prevent antibody cross-reactivity,[13] and then stained with α-SMA antibody with a different colour development. The interstitial area stained by α-SMA was analysed by image analysis and expressed as a percentage of the interstitial area (omitting glomeruli).

IMMUNOFLUORESCENCE

Tissue samples for immunofluorescence studies were snap-frozen in precooled *n*-hexane and stored at −70°C. Glomerular deposition of IgG, IgA, IgM, C3c, C4, C1q and fibrinogen was assessed in 3-μm frozen sections by direct immunofluorescence. The intensity of immunofluorescence staining was evaluated on a semiquantitative scale as follows: 0 (negative), 1 (weak positive), 2 (strong positive without halation) and 3 (strong positive with halation). Macrophages were detected by indirect immunofluorescence using monoclonal antibodies described above.

To examine whether cells stained for CD163, CD204, SOCS1 or SOCS3 also expressed the CD68 antigen (a pan macrophage marker), two-colour immunofluorescence studies were performed on frozen tissue sections using 10D6, antihuman CD163 (mouse IgG1), SRA-E5, antihuman CD204 (macrophage scavenger receptor A, mouse IgG1), anti-SOCS1 (rabbit polyclonal) or anti-SOCS3 (rabbit polyclonal) and Y1/82A (anti-CD68; mouse IgG2b) as primary antibodies followed by incubation of sections with FITC-conjugated goat antimouse IgG1 or FITC-conjugated antirabbit Igs, and TRITC-conjugated goat antimouse IgG2b. Finally, to access whether $CD163^+$ cells and $CD204^+$ cells were identical, anti-CD163 polyclonal antibody and SRA-E5 were used as primary antibodies.

To identify CTGF expression and its localization, double staining with anti-CTGF (mouse IgM) and anti-CD163 (mouse IgG1) or anti-type I collagen (rabbit polyclonal) as primary antibodies followed by FITC-conjugated antimouse IgM and TRITC-conjugated antimouse IgG1 or TRITC-conjugated antirabbit immunoglobulins, respectively was performed.

STATISTICAL ANALYSES

Comparisons were made between two groups by Mann–Whitney test (GraphPad 5.0, San Diego, CA, USA) or Fisher's exact test. One-way analysis of variance (ANOVA) with *post-hoc* analysis using the Turkey multiple comparison test was used for comparison of three or more groups. Data are shown as the mean ± 1 SD. Correlation analysis for parametric data used Pearson's single-correlation coefficient, while non-parametric data were analysed using Spearman's correlation coefficient. Multiple linear regression was used to assess the independent association between proteinuria and the number of $CD68^+$ glomerular cells

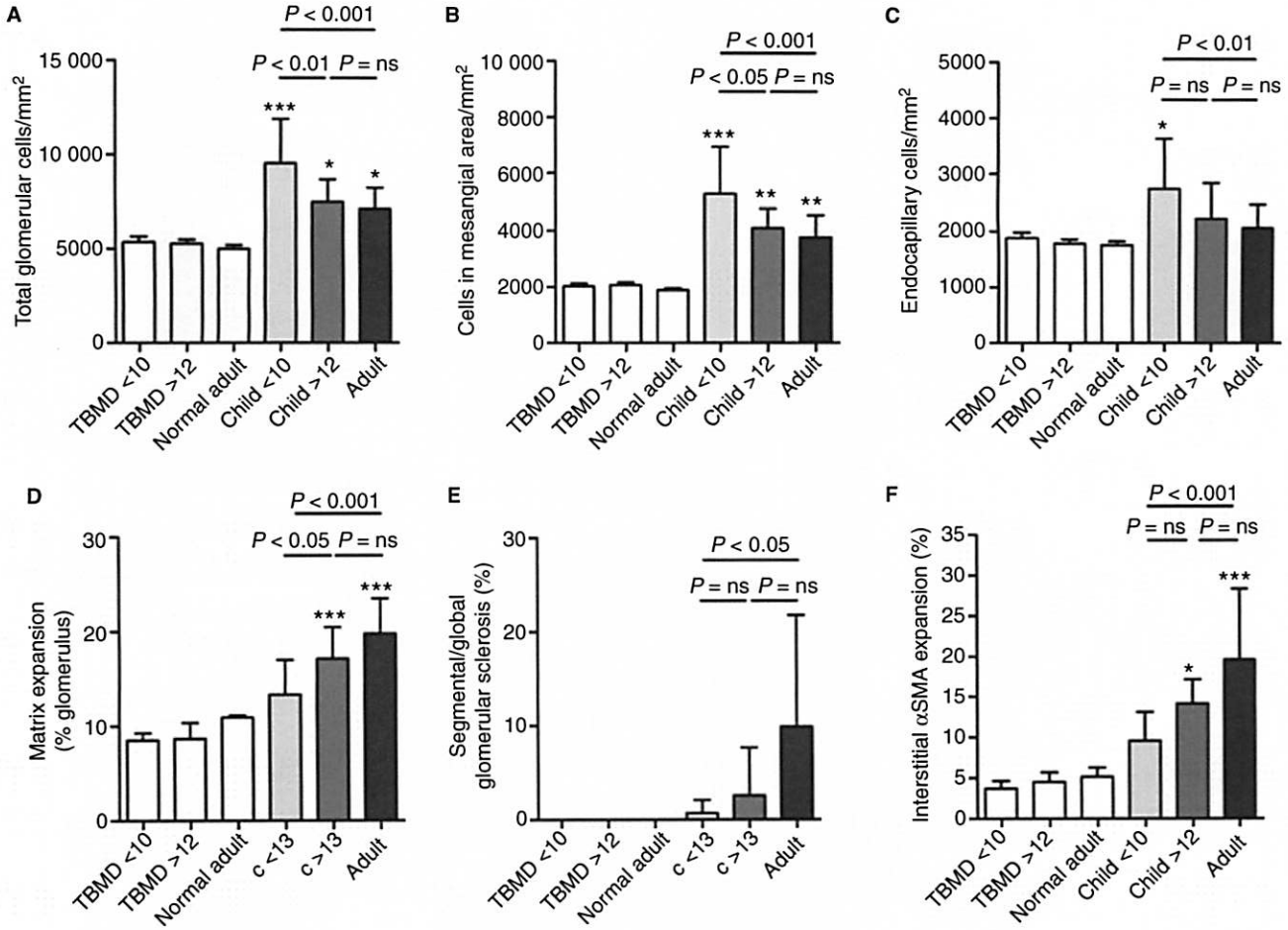


Figure 1. Summary of histological findings in early-onset immunoglobulin A nephropathy (IgAN) in children <10 years, children >12 years and adults in comparison to paediatric thin basement membrane disease (TBMD) and normal adult kidney controls [nephrotoxic serum nephritis (NTN)]. A, glomerular cellularity (total nuclei within the glomerular tuft). B, cells in the mesangial area. C, endocapillary cellularity. D, glomerular mesangial expansion. E, segmental and global glomerulosclerosis. F, percentage of α-smooth muscle actin (SMA) staining area in the cortex. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus age-matched controls.

while controlling for other histological features known to be an important factor in determining outcome including cells in the mesangial area, endocapillary and extracapillary cell numbers and the extent of segmental or global glomerulosclerosis. Where appropriate, skewed data were log-transformed. The assumptions underlying multiple linear regression were checked. We declared a finding to be statistically significant if the two-sided *P*-value was <0.05. All analyses were conducted on Intercooled Stata version 10.1 (Statacorp, College Station, TX, USA).

Results

CLINICAL PARAMETERS

Most childhood cases of IgAN were detected via the annual urinary protein/haematuria screen in schoolchildren, while the other childhood cases were detected by chance urinary investigation. To assess the histological difference between young children and children close to adolescence, childhood cases were selected from two different age groups: <10 years and more than 12 years of age (Table 1). Patients in the adult IgAN group were selected from employees who have annual medical examinations at their workplaces, thus allowing detection of recent-onset disease. All the adults also underwent renal biopsy about 1 year after detection of urine abnormalities, providing a comparable period between detection and biopsy among each group (Table 1). No paediatric patients presented with hypertension at biopsy or had a history of hypertension and/or hyperlipidaemia, while five patients in the adult group had histories of hypertension and/or hyperlipidaemia ($P < 0.05$ by Fisher's exact test) (Table 1).

RENAL HISTOLOGY

Glomerular hypercellularity was the most common finding in biopsies of younger paediatric IgAN, which was increased significantly compared to adult IgAN (Figures 1A and 2A). This was due to increased cells in the mesangium and endocapillary cells (Figure 1B,C). In contrast, glomerular matrix expansion was the most common finding in adult IgAN, with matrix expansion and segmental glomerulosclerosis being increased significantly compared to younger paediatric IgAN (Figures 1D,E and 2B). Interstitial fibrosis, as determined by the area of α-SMA staining, was significantly greater in adult versus younger paediatric patients (Figure 1F). The older paediatric group showed glomerular lesions more akin to that of the adult IgAN group, with glomerular hypercellularity and matrix expansion significantly different compared to the younger paediatric group but not different to the adult group (Figure 1). The degree of interstitial fibrosis in the older paediatric group was intermediate between that of the younger paediatric and adult groups (Figure 1F). No difference in glomerular deposition of IgA, IgM, IgG, C1q, C3c or fibrinogen was seen between the patient groups (Table 2).

In the younger paediatric group, proteinuria correlated with glomerular hypercellularity and mesangial cellularity, but not with endocapillary or extracapillary cellularity, matrix expansion or segmental/global glomerulosclerosis. In contrast, proteinuria correlated with mesangial matrix expansion and segmental/global glomerulosclerosis but not glomerular hypercellularity or mesangial cellularity in the adult group (Table 3). In the older paediatric group, proteinuria correlated with both glomerular cellularity and mesangial matrix expansion. The degree of interstitial fibrosis correlated with proteinuria in all three groups (Table 3).

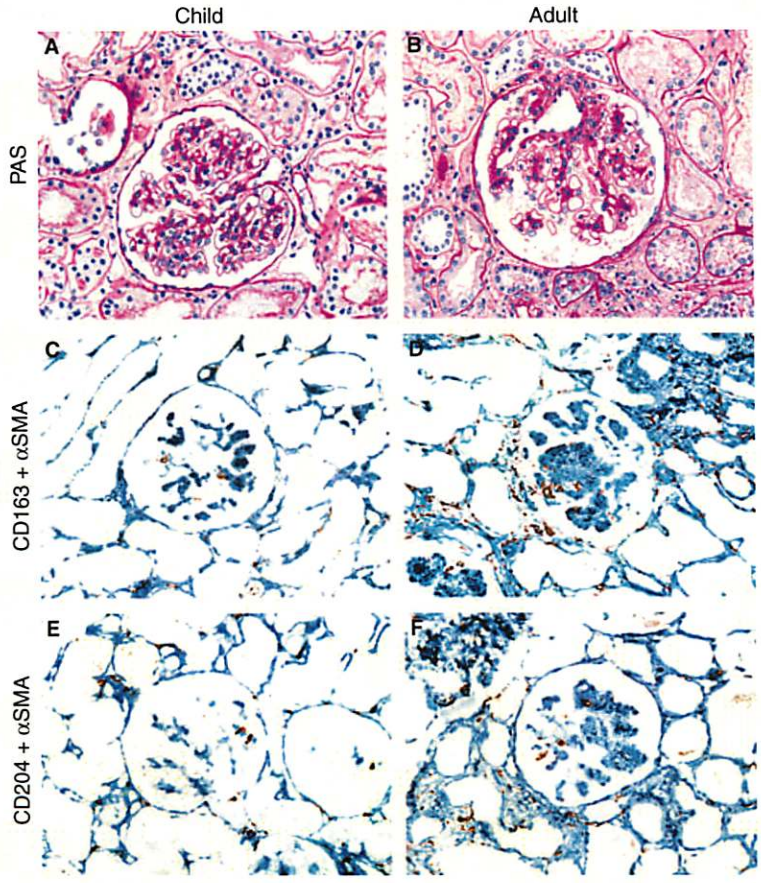


Figure 2. Photomicrographs of biopsy specimens in the young (<10 years) paediatric group (A, C, E) and adult (B, D, F) immunoglobulin A nephropathy (IgAN). A, B, periodic acid-Schiff staining. C, D, two-colour immunostaining showing $CD163^+$ cells (brown) in areas with α-smooth muscle actin (SMA) + myofibroblasts (α-SMA, blue). E, F, two-colour immunostaining for $CD204^+$ cells (brown) in areas with α-SMA + myofibroblasts (blue).

Table 2. Immunofluorescence findings

		IgG	IgA	IgM	C1q	C3c	Fibrinogen
Children <10 years (n = 14)	Intensity score (frequency, %)	0.3 ± 0.5 (28.6)	2.6 ± 0.4 (100)	1.1 ± 0.4 (100)	0.07 ± 0.27 (7.1)	1.6 ± 0.5 (100)	0.4 ± 0.5 (42.9)
Children >12 years (n = 15)	Intensity score (frequency, %)	0.3 ± 0.5 (33.3)	2.8 ± 0.4 (100)	1.0 ± 0.6 (80.0)	0.07 ± 0.35 (13.3)	1.6 ± 0.5 (100)	0.8 ± 0.5 (73.3)
Adult (n = 27)	Intensity score (frequency, %)	0.3 ± 0.5 (25.9)	2.7 ± 0.4 (100)	0.8 ± 0.6 (74.1)	0.04 ± 0.19 (3.7)	1.2 ± 0.6 (92.6)	0.8 ± 0.6 (77.8)

Data are shown as mean ± 1 standard deviation.
Ig, Immunoglobulin.

Table 3. Correlation between histological findings and proteinuria

	Proteinuria (g) (1.73 m^2/day)		
	Children (<10 years)	Children (>12 years)	Adults
Glomerular cellularity	0.56*	0.62*	NS
Cellularity in mesangial area	0.61*	0.65**	NS
Endocapillary cellularity	NS	NS	NS
Extracapillary cellularity	NS	NS	NS
Matrix expansion	NS	0.57*	0.54**
Segmental/global glomerulosclerosis	NS	NS	0.70***
Interstitial fibrosis	0.74***	0.78***	0.55**

Data were analysed using Pearson's single correlation coefficient (R).
NS, Not significant.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

ALTERNATIVELY ACTIVATED MACROPHAGES IN IGAN

First, we assessed the total macrophage infiltrate using the CD68 marker. Glomerular and interstitial accumulation of $CD68^+$ macrophages was evident in all IgAN groups compared to age-matched control renal tissue, being significantly greater in adult versus the young paediatric group (Figure 3A,B). To detect alternatively activated macrophages, we used two well-characterized M2 markers: the macrophage haemoglobin scavenger receptor CD163[9,14] and the class A macrophage scavenger receptor CD204.[9,15] A significant glomerular and interstitial infiltrate of $CD163^+$ cells was seen in the older paediatric and adult IgAN groups, while the young paediatric group showed a reduced number of $CD163^+$ cells compared to the older paediatric group, although this difference did not reach statistical significance (Figure 3C,D). Similarly, a significant glomerular and interstitial infiltrate of $CD204^+$ cells was seen in adult IgAN with a reduced infiltrate seen in the older and younger paediatric IgAN groups (Figure 3E,F).

Two-colour immunofluorescence confirmed that $CD163^+$ cells and $CD204^+$ cells are macrophage subsets based upon double staining with CD68 (Figure 4A–F). Analysis of double staining found that 29% of glomerular $CD68^+$ macrophages expressed CD163 in the younger paediatric group, and this proportion was elevated significantly to 65% in the adult group (Figure 3G). A similar finding was evident in the proportion of glomerular $CD68^+$ macrophages expressing CD204, being increased significantly in older versus younger children, and increased further in adults (Figure 3H). In contrast, virtually all interstitial $CD68^+$ macrophages expressed both CD163 and CD204 antigens across all patient groups (Figure 3G,H). Double staining showed that virtually all interstitial $CD163^+$ cells also expressed CD204 (Figure 4G–I); however, distinct populations of glomerular cells expressed CD163 and CD204, either alone or in combination.

LOCALIZATION OF ALTERNATIVELY ACTIVATED MACROPHAGES IN AREAS OF INTERSTITIAL FIBROSIS

$CD163^+$ and $CD204^+$ macrophages were present in areas of glomerular and interstitial fibrosis identified by prominent α-SMA^+ myofibroblast accumulation, increased deposition of collagen type I and strong staining for the profibrotic factor, connective tissue growth factor (CTGF) (Figures 2 and 4). Furthermore, double staining identified CTGF production by $CD163^+$ macrophages in areas of fibrosis (Figure 4M–O).

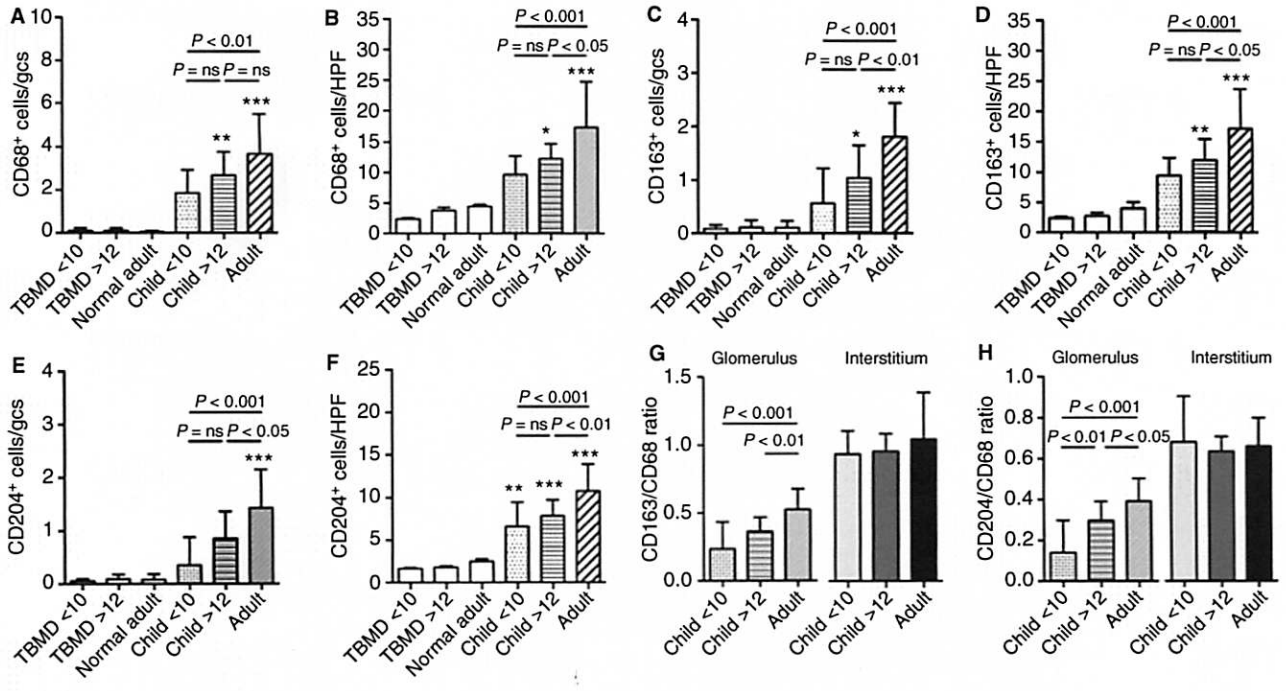


Figure 3. Quantification of macrophage accumulation in early onset immunoglobulin A nephropathy (IgAN) in children <10 years, children >12 years and adults in comparison to paediatric thin basement membrane disease (TBMD) and normal adult kidney controls. Total $CD68^+$ macrophages in: (A) glomeruli and (B) interstitium. $CD163^+$ macrophages in (C) glomeruli and (D) interstitium. $CD204^+$ macrophages in (E) glomeruli and (F) interstitium. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus age-matched controls. G, the ratio of $CD163^+$ cells to $CD68^+$ cells in glomeruli and the interstititum. H, the ratio of $CD204^+$ cells to $CD68^+$ cells in glomeruli and the interstititum.

EXAMINATION OF OTHER M1/M2 MARKERS

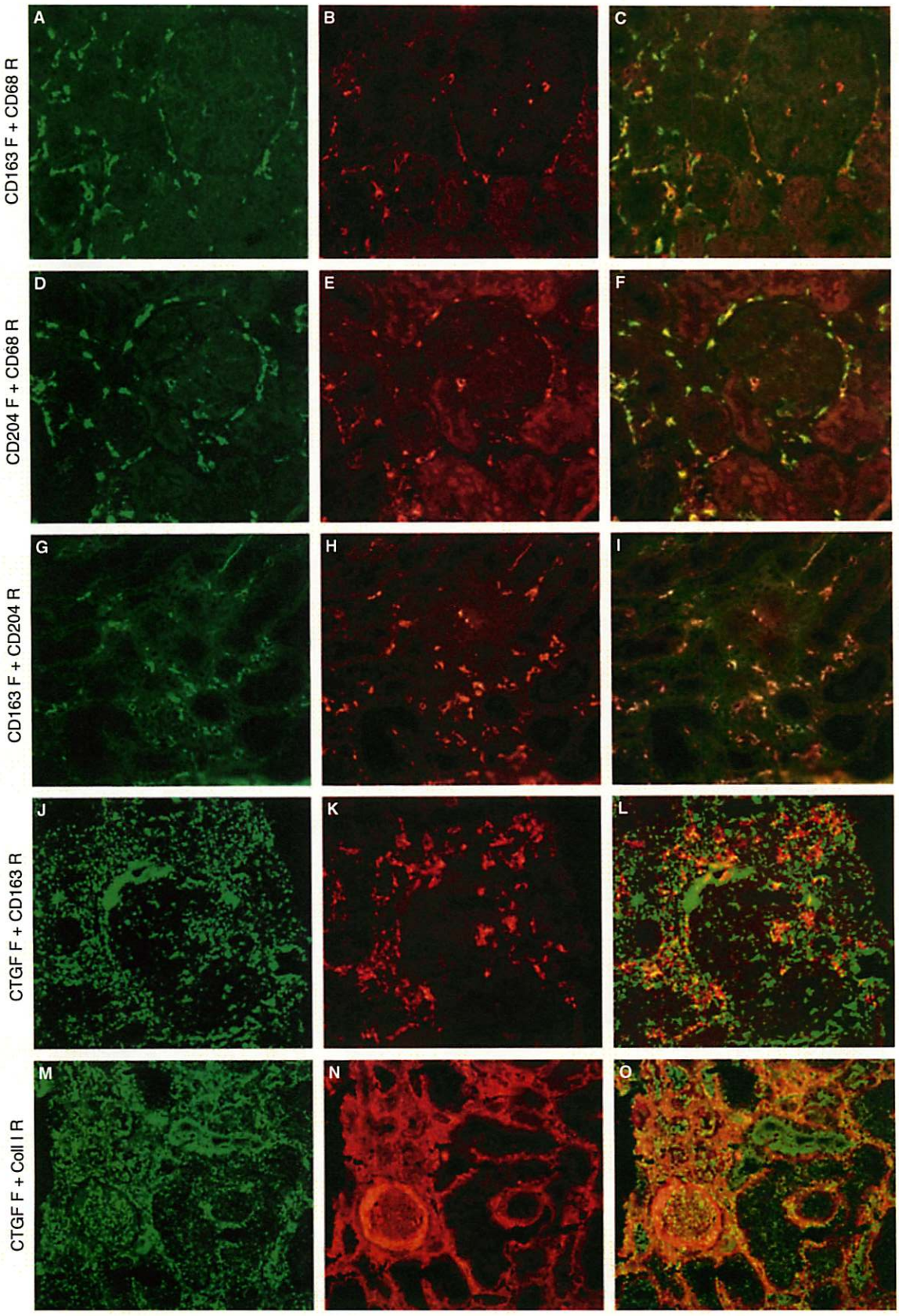
A recent study in a rat model of glomerulonephritis identified SOCS1 and SOCS3 as markers of M2 and M1 macrophages, respectively.[16] Therefore, we examined SOCS1 and SOCS3 expression in IgAN. Double immunofluorescence identified SOCS1 expression by glomerular cells which coexpressed CD68 (Figure 5A–C); overall 86.7% (76.3–98.2%) of $CD68^+$ macrophages expressed SOCS1. In contrast, no glomerular cells and only a small number of interstitial cells, were stained for SOCS3 and no SOCS3/CD68 double staining was seen (Figure 5D–F).

CORRELATION OF MACROPHAGE POPULATIONS WITH CLINICAL PARAMETERS AND RENAL HISTOLOGY

Total glomerular $CD68^+$ macrophages correlated with the degree of proteinuria in all IgAN groups (Table 4). Total glomerular $CD68^+$ macrophages correlated with glomerular cellularity, cells in the mesangial area and endocapillary cellularity in both paediatric groups but not in adult IgAN, whereas $CD68^+$ macrophages correlated with glomerular matrix and segmental/global glomerulosclerosis in adult but not paediatric groups (Table 4). $CD163^+$ macrophages correlated with proteinuria in all groups, and correlated with glomerular matrix and segmental/global glomerulosclerosis in the adult and older paediatric group, but failed to correlate with glomerular matrix or segmental/global glomerulosclerosis in the younger paediatric group and did not correlate with glomerular cellularity in any of the groups (Table 4). Glomerular $CD204^+$ macrophages showed the same correlation results as $CD163^+$ macrophages, except for failing to correlate with proteinuria in the younger paediatric group and correlating with glomerular cellularity and cells in the mesangial area in the older paediatric group (Table 4). The interstitial $CD68^+$, $CD163^+$ and $CD204^+$ macrophage populations correlated with proteinuria and interstitial fibrosis in all the IgAN groups (data not shown). Finally, multivariant analysis of all three IgAN groups showed that glomerular $CD68^+$ macrophages, cells in the mesangial area and segmental/global glomerulosclerosis correlated independently with proteinuria (Table 5).

Discussion

It is well established that macrophage infiltration is a common feature of IgAN which correlates with the severity of proteinuria and histological damage.[5–8]


A
B
C
CD163 F + CD68 R
D
E
F
CD204 F + CD68 R
G
H
I
CD163 F + CD204 R
J
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CTGF F + CD163 R
M
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CTGF F + Coll I R

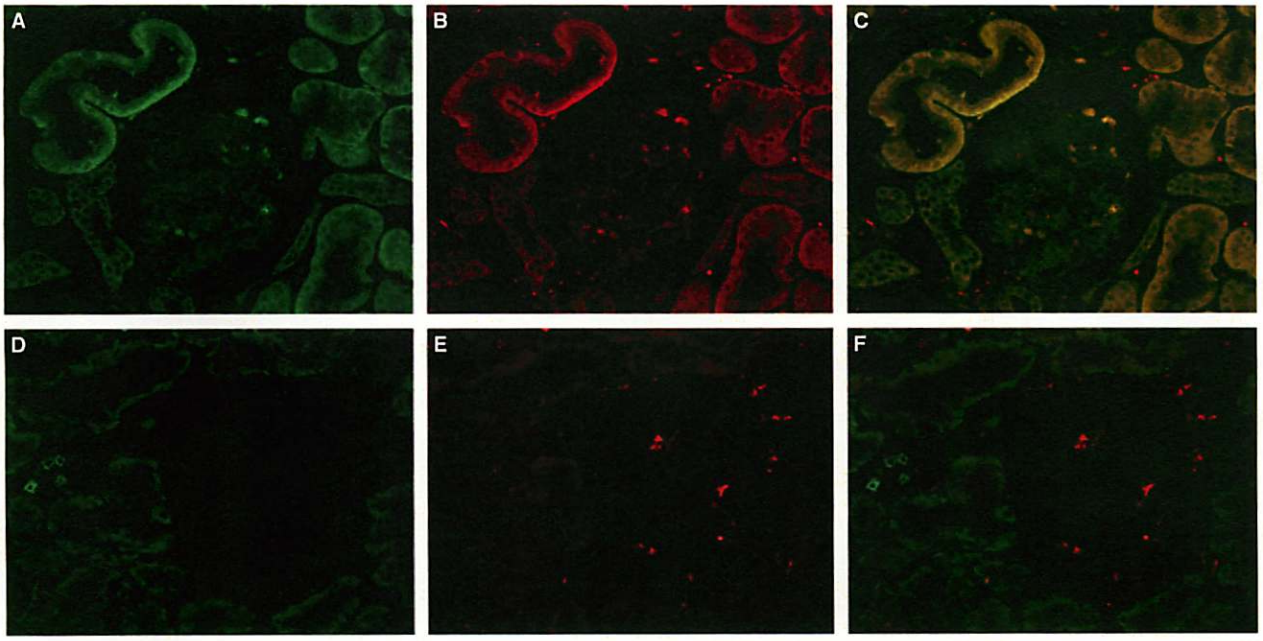

Figure 5. Photomicrographs of two-colour immunofluorescence (IF) staining in adult early-onset immunoglobulin A nephropathy (IgAN). A–C, dual IF staining shows the presence of suppressor of cytokine signalling (SOCS)1^+ cells within a glomerulus (green) and that these cells coexpress CD68 (red). D–F, dual IF staining shows only occasional $SOCS3^+$ cells (green) in the interstitium and are absent from the glomerulus. $SOCS3^+$ cells do not coexpress CD68 (red).

The present study has demonstrated that there is a substantial population of alternatively activated macrophages in IgAN based upon expression of CD163 and CD204 antigens.

Glomerular macrophages showed substantial heterogeneity with $CD163^+$ cells accounting for 29% of glomerular macrophages in the younger paediatric group and 65% of glomerular macrophages in adult IgAN. In contrast, virtually all interstitial macrophages exhibited CD163 and CD204 staining, indicating a predominant M2 phenotype in this compartment. Several lines of evidence argue that M2-type macrophages promote renal fibrosis in early-onset IgAN and the relative proportion of M2-type cells in the macrophage infiltrate may be an important factor in determining whether early-onset IgAN manifests as a cellular lesion or as a more fibrotic lesion. First, the $CD163^+$ and $CD204^+$ macrophage subsets correlated with glomerular matrix expansion and segmental/global glomerulosclerosis in the patient groups where these histological changes were more prominent (older paediatric and adult), but $CD163^+$ and $CD204^+$ macrophages failed to correlate with glomerular cellularity in the patient group where this histological change was prominent (younger paediatric). This was a particularly striking result in the older paediatric group, as the total $CD68^+$ macrophage population failed to correlate with glomerular matrix expansion. In addition, the $CD163^+$ and $CD204^+$ macrophage subsets correlated with the degree of interstitial fibrosis in all patient groups. Secondly, $CD163^+$ and $CD204^+$ macrophages were localized in areas of renal fibrosis, which was more clearly evident with double staining for α-SMA^+ myofibroblasts and collagen type I deposition. Thirdly, double immunofluorescence staining showed that $CD163^+$ macrophages produce CTGF within areas of renal fibrosis. This is consistent with our recent *in vitro* studies showing CTGF production by $CD163^+$ alternatively activated macrophages (Y. Ikezumi, unpublished observations).

Figure 4. Photomicrographs of two-colour immunofluorescence (IF) staining in adult early-onset immunoglobulin A nephropathy (IgAN). A–C, dual IF staining shows that most interstitial $CD163^+$ cells, but only a subset of glomerular $CD163^+$ cells (green), coexpress CD68 (red). D–F, similarly, most interstitial $CD204^+$ cells and some glomerular $CD204^+$ cells (green) coexpress CD68 (red). G–I, almost all interstitial $CD163^+$ cells (green) coexpress CD204 (red). J–L, dual IF staining shows the presence of connective tissue growth factor (CTGF) (green) in many cells and in the extracellular matrix in an area with many $CD163^+$ macrophages (red), with coexpression of CTGF in many $CD163^+$ cells seen in the merged field. M–O, dual IF staining shows deposition of collagen type I (green) in an area with marked CTGF staining.

Table 4. Correlation of leucocyte accumulation with proteinuria and histological findings

	Cells per glomerular cross-section		
	$CD68^+$	$CD163^+$	$CD204^+$
Proteinuria			
Children (<10 years)	0.71***	0.67*	NS
Children (>12 years)	0.79***	0.77***	0.74**
Adults	0.69***	0.73***	0.61***
Glomerular cellularity			
Children (<10 years)	0.86***	NS	NS
Children (>12 years)	0.66**	NS	0.56*
Adults	NS	NS	NS
Cellularity in mesangial area			
Children (<10 years)	0.78**	NS	NS
Children (>12 years)	0.60*	NS	0.53*
Adults	NS	NS	NS
Endocapillary cellularity			
Children (<10 years)	0.80**	NS	NS
Children (>12 years)	0.62*	NS	NS
Adults	NS	NS	NS
Extracapillary cellularity			
Children (<10 years)	NS	NS	NS
Children (>12 years)	NS	NS	NS
Adults	−0.39*	NS	NS
Glomerular matrix			
Children (<10 years)	NS	NS	NS
Children (>12 years)	NS	0.63*	0.58***
Adults	0.53**	0.68***	0.55**
Segmental/global glomerulosclerosis			
Children (<10 years)	NS	NS	NS
Children (>12 years)	NS	0.61*	NS
Adults	0.44*	0.50**	0.39*

Data were analysed using Pearson's single correlation coefficient (*R*).
NS, Not significant.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Understanding the role of alternatively activated macrophages in disease pathogenesis is only just beginning. Most studies of alternatively activated macrophages have used simple *in vitro* systems, the results of which are difficult to extrapolate to complex diseases. However, there is limited evidence to support the notion that M2-type macrophages can promote fibrosis. Alternatively activated macrophages produce a number of profibrotic factors including transforming growth factor (TGF)-β1, platelet-derived growth factor (PDGF)-B and fibronectin.[9,10,17] Coculture of M2 macrophages with fibroblasts promotes fibroblast proliferation and collagen synthesis.[18] Similarly, IL-4 and IL-13, which induce macrophage alternative activation, also promote pathological fibrosis in a number of diseases including pulmonary fibrosis and systemic sclerosis – situations associated with prominent macrophage infiltration,[19,20] although whether these alternatively activated macrophages promote tissue fibrosis directly in these situations has yet to be established.

We used SOCS1 as an additional marker of alternatively activated macrophages. A recent study has demonstrated that SOCS1 is induced in cultured macrophages by IL-4, whereas combined IFN-γ plus lipopolysaccharide (LPS) induces M1 markers but not SOCS1.[16] In addition, the ability of IL-4 or IL-10 to suppress renal injury in a rat model of nephrotoxic serum nephritis (NTN) was associated with an increase in the proportion of macrophages expressing SOCS1.[16] Our finding that SOCS1 is expressed by glomerular $CD68^+$ macrophages is further evidence of alternatively activated macrophages in IgAN. However, it should be pointed out that SOCS1 expression in fibroblasts is thought to suppress collagen gene expression and have an antifibrotic role.[21,22] SOCS3 has been described as a marker of M1 proinflammatory macrophages in rat NTN,[16] and other studies have described the presence of proinflammatory macrophages in IgAN.[4,7,11,12] We were therefore surprised that very few $SOCS3^+$ cells, and no $SOCS3^+$ macrophages, were detected in IgAN; however, this may reflect differences between the aggressive renal injury in rat NTN and the milder lesions seen in early onset IgAN, or a species difference.

A second important aspect of this study is the further examination of the different pathologies seen with early-onset IgAN in children versus adults. The current study stratified early-onset paediatric IgAN into a young prepubescent group (<10 years) and an older postpubescent group (>12 years). A clear difference was evident between the younger and older paediatric groups, with the younger group showing glomerular hypercellularity in the absence of a significant increase in glomerular matrix expansion, whereas the older paediatric group had reduced cellularity and increased glomerular matrix expansion and segmental/global glomerulosclerosis. Furthermore, the older paediatric

Table 5. Multivariant analysis of macrophages and pathological features with proteinuria

Log (proteinuria)	β Coefficient	95% confidence interval
Log (glomerular $CD68^+$ cells)	0.199501**	0.079752 to 0.31925
Cells in mesangial area	0.000202***	0.000097 to 0.000308
Endocapillary cells	−0.000014	−0.000214 to 0.000186
Extracellular cells	0.010713	−0.000278 to 0.00031
Segmental/global glomerulosclerosis	0.010713*	0.000442 to 0.020985

Number of observations = 56 (all patient groups combined).
Overall $R^2 = 0.56$.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

group was not significantly different to the adult IgAN group in terms of glomerular cellularity or matrix expansion. This difference was also evident in correlation analysis in which proteinuria in the younger paediatric group correlated with glomerular cellularity but not matrix expansion, whereas proteinuria in the older paediatric and adult IgAN groups correlated with glomerular matrix expansion and not with glomerular cellularity.

In conclusion, this study has identified the presence of alternatively activated macrophages in the glomerulus and interstitum in early onset of IgAN. These M2 macrophages correlate with glomerular matrix expansion but not with glomerular hypercellularity. Overall, these findings strengthen the argument that early-onset IgAN follows a different course in adult and postpubertal children compared to that in prepubescent children, with a potential role for M2 macrophages in promoting glomerular matrix expansion.

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