Erythrocytosis associated with IgA nephropathy

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Summary

Background Erythrocytosis is a hematological disorder usually related to hematopoietic stem cell somatic mutations. However, unexplained erythrocytosis remains frequent. In this study, we evaluated the involvement of IgA1, a regulator of erythropoiesis also implicated in IgA nephropathy (IgAN) pathophysiology, in unexplained polycythemia/erythrocytosis (PE) of IgAN patients.

Methods IgAN-PE patients' serum was collected, analyzed and used to study IgA1 effect on proliferation and differentiation of erythroid progenitors. Hematological parameters of transgenic mice for human alpha1 heavy chain were studied. Multicentric observational cohorts of chronic kidney disease (CKD) patients, including both native kidney diseases and renal transplants, were studied to analyze patient hemoglobin levels.

Findings We retrospectively identified 6 patients with IgAN and unexplained PE. In large CKD cohorts, IgAN was associated with PE in 3.5% of patients ($p<0.001$ compared to other nephropathies). IgAN was an independent factor associated with higher hemoglobin levels (13.1g/dL vs 12.2 g/dL, $p=0.01$). During post-transplant anemia, anemia recovery was faster in IgAN patients. Elevated polymeric/monomeric IgA1 ratio as well as high Gd-IgA1 rate were observed in circulating IgA1 of the 6 IgAN-PE patients as compared with control or IgAN patients without PE. IgA1 from these patients increased the sensitivity of erythroid progenitors to Epo. In mice, we also observed an elevation of hematocrit in alpha1 knock-in mice compared to wild type controls.

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Interpretation These data identify a new etiology of erythrocytosis and demonstrate the role of pIgA1 in human erythropoiesis. This syndrome of IgA-related erythrocytosis should be investigated in case of unexplained erythrocytosis and renal disease.

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Keywords: IgA; IgA nephropathy; Erythrocytosis; Polycythemia

Research in context

Evidence before this study

Erythropoiesis is a tightly regulated mechanism allowing the production of red blood cells to supply oxygen to the tissues. When some of the regulation mechanisms are not fully functional, it can lead to anemia (decreased hemoglobin levels) or erythrocytosis (increased hemoglobin levels).

Erythrocytosis is a hematological disorder characterized by hemoglobin elevation usually related, in adults, to hematopoietic stem cell somatic mutations. However, some patients do not present known specific mutations like JAK2, and other etiologies of erythrocytosis remain to be identified. Interestingly, we previously reported that polymeric IgA1 (antibodies that usually control mucosal infections) are able to stimulate erythropoiesis in vitro and in vivo in IgA1 humanized mice. Thus, we hypothesized that polymeric IgA could also induce erythrocytosis in human.

Added value of this study

Here, we describe 6 patients presenting erythrocytosis associated with IgA nephropathy (IgAN), a renal disease known to be associated with higher polymeric and/or galactosylation deficiency (Gd) IgA1 rate. Circulating IgA1 were frequently polymeric and/or Gd-IgA1. Moreover, these IgA1 increased the sensitivity of erythroid progenitors to Epo in vitro. Lastly, in large chronic kidney disease cohorts, IgAN was an independent factor associated to higher hemoglobin levels, and higher risk of developing erythrocytosis.

Implication of all the available evidences

This study reveals that polymeric IgA1 might be a regulator of erythropoiesis in humans, and that dysregulation of polymeric/monomeric IgA1 ratio or Gd-IgA1 rate can drive erythrocytosis.

Given the frequent elevation of pIgA1 in IgAN patients, we propose that IgAN should be evoked in case of unexplained erythrocytosis.

Introduction

Erythropoiesis is a tightly regulated process that adapts the production of mature erythrocytes to supply organs with oxygen. This process relies essentially on the level of circulating erythropoietin (Epo) which acts on erythroid progenitors/precursors to regulate their survival, proliferation and, to a lesser extent, differentiation.[1,2]

Overproduction of erythrocytes can lead to polycythemia/erythrocytosis (PE).[3] Somatic mutations occurring in hematopoietic progenitors (*e.g.* $JAK2^{V617F}$) and, rarely, other mutations that induce hypersensitivity to Epo, lead to dysregulated erythropoiesis and are the most frequent etiologies of PE. Other causes include inappropriate production of Epo linked to kidney tumor, hypoxia or abnormal response to hypoxia. However, in numerous cases no etiology can be identified.[4]

We previously reported that polymeric IgA1 (pIgA1) interact with transferrin receptor 1 (TfR1/CD71) expressed in erythroid progenitors. Expression of human IgA1 or treatment of wild-type mice with pIgA1 accelerated recovery from acute anemia. Mechanistically, *in vitro*, pIgA1/TfR1 interaction increases the sensitivity of erythroid progenitors/precursors to Epo,[5,6] by inducing activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways. Given the role of pIgA1 in erythropoiesis regulation in these *in vivo* and *in vitro* models, we hypothesized that pIgA1 could also be involved in regulation of erythropoiesis in human. To test this hypothesis, we took advantage of patients with a pathology related to increased pIgA1 levels, i.e IgA nephropathy (IgAN).

IgA nephropathy (IgAN) is the most common primary glomerulonephritis. The pathogenesis of the disease includes the aberrant galactosylation of pIgA1 in the hinge region, which results in elevated serum levels of galactose-deficient polymeric IgA1 (Gd-pIgA1). These antibodies are recognized by glycan-specific IgA and IgG autoantibodies, leading to the formation of immune complexes of IgG-pIgA1. For unclear reasons, the kidney is the main target of these immune

complexes. The glomerular lesions in IgAN are related to the deposition of these pathogenic pIgA1 immune complexes in kidney mesangium.[7,8] These deposits induce the proliferation of mesangial cells, increased synthesis and deposition of extracellular matrix and variable infiltration of inflammatory cells, leading to glomerulosclerosis, tubulointerstitial fibrosis and kidney failure. Although the role of different receptors for pIgA1 in mesangial cells is still evoked, several studies have identified the TfR1 as a key receptor for binding pIgA1-containing immune complexes.[7,8] The impact of the interaction pIgA1/TfR1 in other cells/tissues in IgAN patients is still unknown.

In the present study, we identified 6 patients with IgAN and unexplained PE, despite extensive explorations. We demonstrate that pIgA1 and Gd-IgA1 were elevated in these patients, and that circulating IgA1 increase erythroid progenitor sensitivity to Epo, explaining the pathophysiology of this syndrome. Further, in agreement to our hypothesis, in large cohorts of patients with various chronic kidney disease (CKD) etiologies, IgAN was an independent factor associated with higher steady-state Hb level and anemia recovery after kidney transplantation, suggesting that pIgA1 is a regulator of erythropoiesis in human.

Methods

Patients and cohort analysis

Human subjects. Patients with biopsy-proven IgAN and unexplained polycythemia/erythrocytosis according to biological WHO criteria[3] (IgAN-PE) were identified by retrospective analysis of medical records from 2 tertiary nephrology centers in Paris (Necker, Tenon). Serum and DNA extracted from PBMC were obtained after written informed consent and local ethics committee approval. Control sera were from IgAN patients with normal hematocrit (HCT), and healthy volunteers. Circulating Epo and soluble TfR1 (sTfR1, Bio-Techne 2474-TR-050) were quantified by enzyme-linked immunosorbent assay (ELISA) and immunoturbidimetric methods, respectively. Estimated glomerular filtration rate (eGFR) was calculated according to CKD-Epi formula.[9]

Cohorts. Two multicentric prospective cohorts of CKD patients were studied, (i) the NephroTest study, that enrolled 1993 adult patients with iterative mGFR and written informed consent,[10] (ii) the DIVAT (Données Informatisées et VAlidées en Transplantation) cohort, which includes 2600 renal transplant patients from 2005 to 2015.[11] From the NephroTest study, 696 patients with biopsy-proven glomerulonephritis (GN) were selected to limit heterogeneity of the cohort that frequently includes patients with no histological diagnosis. We also excluded patients with autoimmune diseases who receive immunosuppressive drugs known to reduce Hb levels.

Cellular assays. Human CD34+ cord blood cells were cultured on methylcellulose (Methocult SF H4236, stemcell technologies, Vancouver, Canada) with interleukin-3 (IL-3), IL-6, SCF and Epo (0.05 or 1 U/mL) in the presence of sera from IgAN-PE or IgAN patients or healthy volunteers. Depletion of IgA1 was performed as previously described.[6] Briefly, sera were passed through a Jaccalin column to catch IgA1. We obtained IgA1-depleted serum on one hand, and purified IgA1 after elution of the column on the other hand. BFU-E–derived colonies in the presence of depleted and non-depleted sera were counted at day 14.

Binding assays. UT 7 cell line were a gift from Dr Patrick Mayeux, at Cochin institute.[12] Briefly, UT7 cells (0.25×10^6) were pre-incubated with 1 mg/mL of human IgG for 30 min on ice to block IgG receptors. IgA1 binding was examined by FACS by an indirect immunofluorescence assay, using a biotinylated anti human IgA1 (Southern Biotech, ref 2052-08), followed by a streptavidin-APC (BD pharmigen ref 554067).[13] For inhibition studies, cells were pre-incubated with sTfR1 (Bio-Techne, 2474-TR-050). Data were analyzed with FlowJo software (FlowJo LLC, Ashland, Oregon)

Biochemical assays. Quantification of IgA in serum was performed using Architect c16000® analyzer (Abbott) with an immunoturbidimetric method. Immunobloting for monomeric and polymeric IgA1 was performed as described.[14] Briefly, 1 μg plasma IgA or purified IgA mAbs were prepared without reducing agent and loaded on 10% polyacrylamide gels. Proteins were transferred to PVDF membranes. The human α-chain was detected using a purified mouse anti human IgA1/IgA2 (BD Pharmingen ref 555886) followed by an HRP-conjugated donkey anti mouse secondary antibody (Jackson immuno, ref 715-035-150).

Quantification of serum galactose-deficient IgA1 was evaluated by ELISA (Gd-IgA1 KM 55 kit, IBL-Japan), following manufacturer's instructions.

Mice housing and experiments

Alpha1 knock-in transgenic mice were previously described.[6] The α1KI mice feature insertion of the human Cα1 gene downstream of the endogenous JH region in the IgH locus, replacing IgM expression by IgA with human Cα1–constant regions. These animals were bred into a BALB/c background and compared with wild-type animals from similar backgrounds. Mice

were fed ad libitum and housed at constant ambient temperature in a 12-h light, 12-h dark cycle. Animal procedures were approved by the "Services Vétérinaires de la Préfecture de Police de Paris", by the "Ministère de l'Enseignement Supérieur de la Recherche et de l'Innovation" and by the ethical committee of the Université de Paris. Hematocrit was measured in mice using a MS9-5 (MS Laboratory). The Epo concentration was measured by ELISA (Quantikine IVD, R&D system) following the manufacturer's recommendations.

Reagent validation

Primary human CD34+ cord blood cells and UT7 cell line were previously validated (RRID). Cell line and antibody validation references are provided in Supplemental Table 1.

Statistical analysis

For *in vitro* experiments, statistical analyses were performed with GraphPad Prism Software version 5.0. Data are expressed as mean ± SEM of *n* determinations unless noted otherwise. Mann-Whitney test was used to compare two groups. Differences were considered significant at a P value <0.05 (*), <0.01 (**) or < 0.001 (***).

For statistical analysis of clinical cohorts, we used the Kruskal-Wallis test for quantitative co-variables, the Pearson's chi-square test for qualitative co-variables. We performed a multivariate linear regression with a bidirectional stepwise selection of covariates. The initial model included all the covariates associated with Hb level (P< 0.05) in a bivariate analysis. Analyses were performed using R Statistical software version 3.3.2.

Ethics

Written informed consent was obtained from each patient and controls. Ethic committee approval was obtained from the LabEx GR-Ex (N° DC-2016-2618, Imagine Institute, Paris, France).

Role of funding source

The funders had no role in study design, data collection, data analyses, interpretation, or writing of report.

Results

Case reports

Patient 1. A 20-year-old man was referred for microscopic hematuria and hypertension. Serum creatinine was 50µmol/L, without proteinuria. He presented no past medical history. Kidney biopsy revealed mild proliferative mesangial glomerulonephritis, with mesangial IgA and C3 deposits, confirming IgAN. Hemoglobin (Hb) level was 16.3 g/dL, and hematocrit (HCT) was 48%, but the criteria for PE were not reached. During follow-up, angiotensin receptor blocker (ARB) treatment was introduced to control new onset proteinuria (1g/day), and Hb levels progressively increase (17,5g/dL). At the age of 64, despite renal failure occurrence with a serum creatinine at 162µM (measured GFR (mGFR) of 37.8 mL/min/1.73m²) and a stable proteinuria of 0.7g/day, the patient developed PE (Hb 18.6g/dL, HCT 55.5%) with normal leukocyte and platelet counts and no splenomegaly.

Patient 2. A 63-year-old man with no past medical history was referred for microscopic hematuria, hypertension, proteinuria (4g/day), and a serum creatinine 230µmol/L (estimated (e)GFR of 25 mL/min/1.73m²). Kidney biopsy revealed mesangial glomerulonephritis with mesangial thickening but without proliferation or segmental lesions. Interstitial fibrosis was observed in approximately 20% of the biopsy. Immunofluorescence (IF) confirmed IgAN. Hb level was 12.9 g/dL and HCT 37.9%. Angiotensin conversion enzyme inhibitor (ACEi), ARB and thiazide treatments were introduced. A progressive increase of Hb levels occurred, and at the age of 74, he developed PE with Hb17.8g/dL and HCT 54.6%. Leukocyte and platelet counts were normal. No splenomegaly was evidenced. Renal function was altered with mGFR 28mL/min/1.73m². After 12 years of follow-up, serum creatinine was 171µM and proteinuria 0.2g/day.

Patient 3. A 26-year-old man was referred for microscopic hematuria and proteinuria (2.3g/day), with normal renal function (serum creatinine 88µmol/L, eGFR 91ml/min/1.73m²). Kidney biopsy revealed mesangial glomerulonephritis without proliferation. IF confirmed IgAN. Hb was 15.1 g/dL and HCT 45%. ACEi (but no diuretic) treatment was started with significant reduction of proteinuria. At the age of 69, while renal function started to be altered with mGFR 59mL/min/1.73m2, he developed PE (Hb18.5 g/dL, HCT55%). Leukocyte and platelet counts were normal. After 44 years of follow-up, serum creatinine was 128µM and proteinuria 0.8g/day.

Description of 3 other patients is available in supplementary information.

The 6 IgAN patients presented here (Table 1) developed a late-onset PE during follow-up, while paradoxically their mGFR decreased. Physical examinations did not show any evidence for myeloproliferative disease (e.g. no splenomegaly) or cerebellum hemangioblastoma. Renal and hepatic ultrasounds were normal, as well as chest X-ray. All patients had no history of smoking, normal levels of Hb oxygen

Patient #	age at polycythemia diagnosis	maximum hematocrit (%)	maximum hemoglobin (g/dL)	measured eGFR (mL/min/1.73m2)	proteinuria (g/24h)	EPO level (mUI/L)	pulsed oxygen saturation (%)	Ferritin (µg/L)	transferrin saturation coefficient (%)	sTfR (mg/L)	ACEi or ARB
1	64	55.5	18.6	43	0.7	9.9	98	102	23	3.8	ARB
2	74	54.6	17.8	28	0.2	8.4	96	61	22	3.23	ACEi and ARB
3	69	55.0	18.5	59	0.7	8.4	96	181	25	6.53	ACEi
4	45	54.3	18	83	0.4	5.8	97	236	23	3.77	ARB
5	61	56.4	18.4	53	0.2	6.8	98	201	22	2.2	ARB
6	26	59	18.5	27	1.1	6.8	97	153	24	4.42	ARB

Table 1: **Clinical, demographical and biological data from the six patients with IgAN-PE.**
Normal measurements: Epo: 1.4<N<13.7 mIU/L; sTfR: 1.9<N<4.4 mg/L. Abbreviations: IgAN: IgA nephropathy; GFR: glomerular filtration rate; Epo: erythropoietin, ACEi: angiotensin conversion enzyme inhibitor; ARB: angiotensin receptor blocker.

saturation, and normal Epo levels without JAK2 mutation. No patient developed myeloproliferative neoplasms or thrombotic complications. No red cell mass studies were available. The patients did not receive immunosuppressive therapy during the course of their renal disease. IgAN patients frequently have increased Gd-IgA1 and polymeric/monomeric IgA1 (pIgA1/mIgA1) ratio,[15] and pIgA1 induce a hypersensitivity to Epo of erythroid progenitors through activation of TfR1 in mice.[6] We thus hypothesized that pIgA1 from IgAN patients could stimulate erythropoiesis in humans.

Cohort study

We first compared PE prevalence and Hb levels in large cohorts of CKD patients. In the Nephrotest study,[10] a large cohort study of patients with CKD of different origins, we delineated 4 patient groups based on CKD etiology that included IgAN, other glomerulonephritis (GN), polycystic kidney disease (PKD), and diabetes nephropathy (DN) (Table S2). There was a higher prevalence of PE in IgAN patients according to WHO criteria,[3] since 6 out of 171 IgAN patients (3.5%) presented with PE compared with 2/117 PKD, 0/179 other GN and 0/229 DN patients (p=0.004). Overall, Hb level was higher in IgAN patients than in patients with PKD, diabetes and other GN (p <0.0001). The multivariate analysis including age, sex, mGFR, Epo or iron supplementation as covariates showed that IgAN is an independent factor associated with higher Hb levels (p=0.01) (Table S3 and Figure. 1a). Circulating Epo levels were similar in IgAN-PE to that of other CKD patients (Supplemental Fig. S1). Collectively, these data support a role for IgA1 in erythropoiesis in humans.

We then compared the Hb recovery status of IgAN *versus* other CKD patients following kidney transplantation since anemia occurs in the early post-renal transplant period[16] and pIgA accelerate recovery from anemia in mice.[6] In the DIVAT post-transplant cohort Hb levels at 3 different time points (Month (M) 0, day of renal transplantation, M3 and M12 post-transplantation) were available for 2600 patients including 271 IgAN (10.4%), 447 PKD (17.2%) and 248 DN (9.5%) patients. At M0, only male gender and PKD were associated with Hb level by multivariate analysis. The mean change of Hb level at M3 and M12 compared to M0 was higher in IgAN compared to DN and other groups (ΔHb M3-M0=0.87, -0.06 and 0.22g/dL respectively; ΔHb M12-M0=1.36, 0.55 and 0.71g/dL, respectively) (Figure. 1b). Moreover, twelve months after transplantation, IgAN patients presented higher Hb level compared with other patients (13.1 vs 12.7g/dL, p=0.001). Taken together, these data demonstrate that Hb recovery after transplantation is faster in IgAN patients.

Patient study

Given these epidemiological data, we further explored at the cellular level whether IgAN-PE observed in the 6

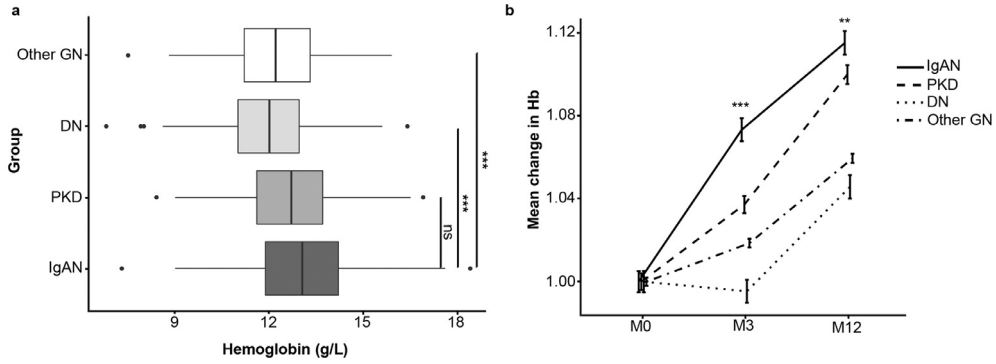

Figure 1. CKD cohort analysis. (a) Box plot of Hb level in Nephrotest cohort. Abbreviations: IgAN: IgA nephropathy; PKD: polycystic kidney disease; DN: diabetic nephropathy; GN: glomerulonephritis. *** $p<0.001$ for the comparison of Hb level between IgAN patients and other GN patients (b) Mean change in Hb from M0, to M3 and M12 after renal transplantation in DIVAT cohort. ** $p<0.01$, *** $p<0.001$; Quantifications are shown for IgAN compared to other GN patients, for ΔHb between M3 and M0, and M12 and M0

patients presented in case reports involved pIgA1. Since we noted that Epo levels were similar in IgAN patients and controls (Supplemental Fig. S1) despite elevation of hematocrit in IgAN patients, we studied a mouse model of IgAN which overexpresses human pIgA1, the α1KI mice.[6] Similar to IgAN patients, we observed an elevation of hematocrit in these mice, with no difference in Epo levels (Supplemental Fig. S2 and S3). These results suggested that elevated circulating IgA1 could amplify erythropoiesis *in vivo* with no significant impact in Epo levels, as observed in IgAN patients. To further explore the disease mechanisms in the 6 IgAN patients with PE, we then studied the role of IgA1 in these patients. To this aim, we plated $CD34^+$ cord blood cells with serum from IgAN-PE patients, or control individuals (IgAN without PE with eGFR 26-86 mL/mn/1.73m² and normal ferritin levels, and healthy controls), in semisolid medium with a cytokine cocktail allowing erythroid progenitor proliferation, survival and differentiation. At suboptimal Epo concentration (0.05 U/mL), the number of erythroid burst-forming unit (BFU-E)-derived colonies was increased in the presence of IgAN-PE *versus* control or IgAN serum (Figure. 2a). Removal of IgA1 from IgAN-PE serum decreased BFU-E-derived colony number to that observed with serum from healthy volunteers, while adding back the corresponding IgA1 to depleted IgAN-PE serum restored this number to its initial values (Figure. 2a). We then sought to understand the peculiarities of IgA from IgAN-PE patients that could explain these data. Serum IgA concentration showed no significant difference between IgAN-PE, IgAN and controls despite a trend towards a higher concentration for IgAN and IgAN-PE in this limited cohort (Figure. 2b). Immunoblot analysis of whole serum for total IgA (IgA1 and IgA2) did not show a higher pIgA/mIgA ratio (Figure. 2c). However, when serum IgA1 was first Jacalin-purified before immunoblot analysis, we observed an increased pIgA1/mIgA1 ratio in IgAN-PE serum (Figure. 2d). Moreover, quantification of galactose-deficient IgA1 (Gd-IgA1) by KM55 mAb based ELISA in serum from IgAN-PE, IgAN patients and healthy controls showed an elevation of Gd-IgA1 in IgAN-PE patients (Figure. 2e).

We previously reported the role of pIgA1-TfR1 interaction in erythropoiesis stimulation in transgenic mice.[6] To test the hypothesis that a similar mechanism is involved in our present human study, we examined IgA1 binding to TfR1 on UT7 cells (a human erythroid cell line expressing TfR1) as compared to IgA1 from healthy volunteers and from IgAN patients without PE. These experiments showed that IgA1 purified from IgAN-PE serum bound UT7 cells to a higher extent than IgA1 from IgAN patients without PE and healthy volunteers (Fig. S4). Moreover, preincubation of IgA1 with soluble TfR1 inhibited this binding, suggesting that it was TfR1-dependent. Although the low number of samples precludes to reach definitive conclusions, these results suggest that IgA1-TfR1 interaction may be involved in erythropoiesis of IgAN-PE patients.

Overall, in line with previous data in mice,[6] these results demonstrate that IgA1 from IgAN-PE patients mediate hypersensitivity to Epo of erythroid progenitor cells.

Discussion

Here we describe a new etiology of human PE in a subpopulation of IgAN patients related to an IgA1-dependent hypersensitivity to Epo of erythroid progenitors. This syndrome appears among IgAN patients with the highest pIgA1/mIgA1 ratio, and was suspected in 3.5% IgAN patients. In support for the role of pIgA1 in erythropoiesis stimulation, epidemiological data showed slightly elevated Hb levels in IgAN patients.

In a large multicentric cohort of CKD patients, we report that IgAN is associated with a higher frequency

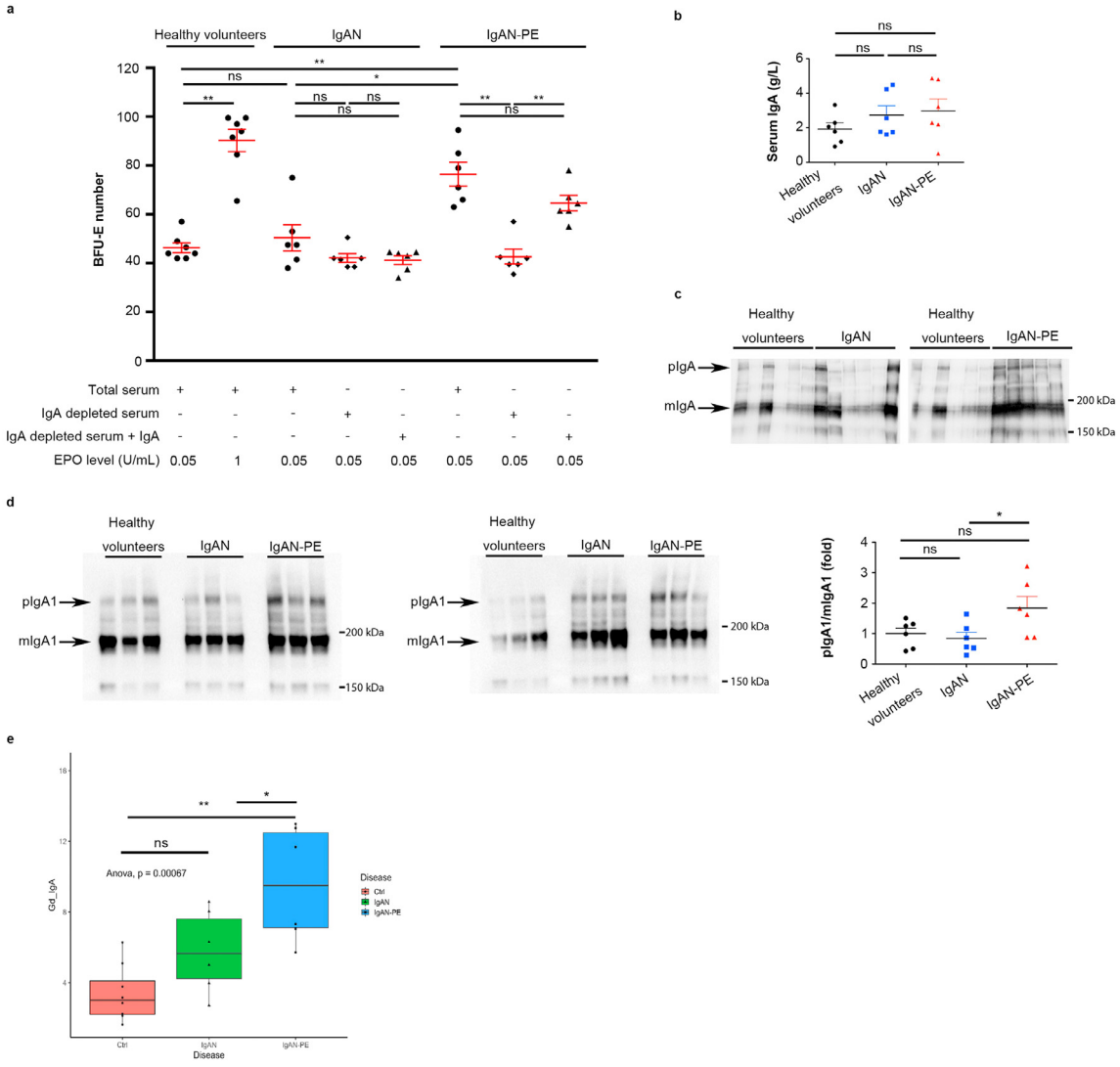

Figure 2. In vitro analysis of IgAN-PE serum (a) BFU-E-derived colonies from human CD34+ cord blood progenitors. The methylcellulose assay was performed with IL-3, IL-6, SCF and suboptimal or saturating concentrations of Epo (0.05 and 1 U/mL) in the presence of sera from healthy volunteers, IgAN or IgAN-PE patients. IgA1 depletion was performed by a jacalin purification protocol (black diamonds). Purified IgA1 were added back at the concentration of 0.2 mg ml-1 (0.6µM) (black triangles). All data are mean ± SEM, * $p<0.05$; ** $p<0.01$. (b) Quantification of IgA in serum. (c) The presence of IgA in serum was analyzed by immunoblotting with mouse monoclonal anti-human IgA1/IgA2 (BD Pharmingen) followed by anti-mouse secondary antibody coupled to horseradish peroxidase (Sigma-Aldrich). (d) The ratio of polymeric to monomeric IgA1 (1µL loaded in SDS-PAGE gels) was evaluated by immunoblotting of purified IgA1 with anti-IgA1 heavy chain antibody under non-reducing conditions. Individual samples (n=6 per condition) were loaded in two independent gels (* $p<0.05$). (e) Level of serum galactose deficient IgA1 (Gd-IgA1) was assessed by KM 55 ELISA (IBL) in healthy controls (Ctrl), IgAN without PE and IgAN-PE patients. ANOVA followed by t-test, *$p<0.05$, **$p<0.001$

of PE and, overall, with a higher Hb level than other kidney diseases. Notably, this effect was independent from other factors modulating erythropoiesis such as age, sex, or GFR. Although the Hb level was only slightly increased in IgAN patients, these epidemiological data revealed that erythropoiesis is stimulated in these patients in steady state conditions. Moreover, we show in a cohort of kidney transplant patients that IgAN is associated with a faster post-transplant anemia recovery. This suggests that the stimulated erythropoiesis occurring in IgAN patients is beneficial when these patients undergo renal transplantation. Besides, these analyses in transplant patients underscore that immunosuppression is not sufficient to inhibit the IgA-related

erythropoiesis stimulation after transplantation (similarly to recurrence of IgA deposits observed in 20-25% cases[17]). Since increased levels of pIgA1 immune complexes are observed in IgAN,[18] these data support in human the role of pIgA1 previously observed in three humanized mouse models of anemia which showed that pIgA1 may control erythropoiesis and allow rapid anemia recovery.[6]

Indeed, we have previously shown in a detailed mechanistic study that human pIgA1 drive hematocrit elevation via pIgA1 binding to TfR1. In this model, deficiency of the J chain (leading to an absence of pIgA1) significantly reduces hematocrit elevation.[6]

Interestingly, we previously observed in vitro that both IgA1 polymerization and IgA1 hypogalactosylation modulate IgA1 binding to TfR1 in cellular models.[5] Considering the role of Gd-IgA1 in IgAN pathophysiology,[7] we evaluated Gd-IgA1 in patients with IgAN-PE and controls. We observed that Gd-IgA1 were elevated in patients with IgAN-PE, compared to IgAN without PE and healthy controls (Figure. 2e). This result suggests that patients with IgAN-PE have markers of dysregulated IgAN galactosylation. Overall, our results show that IgAN-PE patients have elevated serum polymeric IgA1 (Figure. 2d) and Gd-IgA1 (Figure. 2e) that support increased BFU-E number in vitro (Figure. 2a).

Whether Gd-IgA1 were preferentially in monomeric or polymeric form was not technically evaluable, given the lack of enough residual volume of serum samples needed to perform HPLC studies in this human study. Thus, while pIgA1 plays a major role in erythropoiesis amplification in vivo through TfR1 interaction, the impact of another, non-exclusive mechanism of erythropoiesis stimulation involving Gd-IgA1 in humanized mice and humans remains to be studied. In particular, whether it is the Gd-pIgA1 within pIgA1 that is the main factor responsible for IgA1 involvement in IgAN-PE will need full attention in a future dedicated study.

The production of Hb physiologic levels is a tightly regulated process which could depend of several factors, including age, renal function, or iron status. Patients with IgAN and PKD could have fewer comorbidities than patients with other causes of GN, since they usually present with an early, mainly kidney-specific disease. However, our multivariate analyses suggest that the highest Hb levels observed in IgAN are not dependent of associated factors such as age, mGFR or iron status. Moreover, regarding IgAN-PE patients, we developed in vitro studies to better describe the pathophysiology of this syndrome, which clearly demonstrate the role of IgA1 in erythropoiesis activation.

Our study demonstrates in humans the role of IgA1 in unexplained cases of IgAN-PE. To our knowledge, only one case of IgAN with unexplained PE has been previously reported.[19] However, this new pathophysiological mechanism is probably not restricted to IgAN patients, since elevated pIgA1/mIgA1 and Gd-IgA1 ratio has been also described during chronic liver disease or IgA myeloma.[20] Moreover, in familial forms of IgAN, increased pIgA1/mIgA1 ratio has been observed in both patients and unaffected relatives.[21] Therefore, our report suggests that the potential role of IgA1 should be considered in sporadic and familial forms of unexplained polycythemia[4] with no JAK2 mutations, normal/subnormal Epo levels and no obvious secondary polycythemia etiology. Unfortunately, analysis of pIgA1/mIgA1 ratio and serum Gd-IgA1 is not available in routine practice, calling for further studies to develop a simple diagnosis test allowing to identify PE cases, which may be dependent on this mechanism. In the meantime, renal explorations of patients with unexplained PE, including kidney ultrasound, renal function evaluation, and urinalysis with search for proteinuria and haematuria should be proposed, followed, in cases of high suspicion of IgAN, by kidney biopsy.

Interestingly, Epo levels were similar in IgAN-PE and other CKD patients (Supplemental Fig. S1), as previously observed in some patients with JAK2 mutation and "normal" Epo level.[22] Regulation of erythropoiesis in CKD is poorly understood. Actually, Epo levels are not correlated with Hb in non-anemic CKD patients, and may be regulated by other factors frequent in CKD, such as iron deficiency, obesity or inflammation.[23] The exact regulation of Epo levels in IgAN-PE patients remains to be studied. However, we anticipate that these normal Epo levels could participate to erythropoiesis stimulation in IgAN patients, since pIgA1 increases the sensitivity to Epo-dependent stimulation of erythropoiesis.

Most IgAN patients are currently treated with angiotensin converting enzyme inhibitors (ACEi) or angiotensin II receptor blockers (ARB).[24] Actually, these drugs have a major role in inhibiting renin angiotensin system (RAS) activation in IgAN, leading to reduction of intraglomerular pressure, proteinuria, and CKD progression.[25] Interestingly, RAS activation has a positive effect on erythropoiesis.[26] Moreover, RAS inhibition may even control Epo-dependent polycythemia.[26] Consequently, we can hypothesize that IgA1-amplified erythropoiesis stimulation might be attenuated by RAS inhibition in IgAN patients. As shown in our case reports, 6 patients developed erythrocytosis while they were treated with RAS blockers. This suggests that this syndrome could be even more pronounced in the absence of RAS blockers. In addition, the limiting effect of RAS blockers on erythrocytosis may explain why IgAN-PE was mainly observed in patients with long-term diagnosis of IgAN, since the effect of RAS inhibition may decline after long-term blockade.[27] In fact, aldosterone breakthrough (e.g., paradoxical elevation of aldosterone levels after long-term RAS inhibition) has been largely documented in chronic kidney disease patients.[28] Alternatively, we cannot rule out that other additional hematological factors associated with age

(such as acquisition of somatic clonal hematopoiesis) may impact the evolution of polycythemia in our patients.[29] Lastly, we cannot exclude a recruitment bias in patients with advanced CKD and elevated levels of Hb, a very unusual event in CKD patients. This bias is limited by our epidemiological study showing that 3.5% of patients with IgAN in the Nephrotest study have PE according to WHO criteria.

This study has several limitations. We have shown the role of IgA1 only in patients with PE, and not in the whole cohort of IgAN patients. Moreover, in IgAN patients, we did not study the molecular consequences and the signaling pathways induced by the interaction of IgA1 with TfR1. However, our in vitro data shows the impact of IgA1 on the number of erythroid burst-forming unit (BFU-E)-derived colonies, which is in line with our epidemiological observations.

Finally, this work confirms our previous report showing that IgA1 is a regulator of erythropoiesis. In support, IgAN patients presented higher Hb levels and better Hb recovery after transplantation than other CKD etiologies. Further *in vitro* studies will be necessary to decipher the molecular mechanisms involved in IgA1 promotion of a hypersensitivity to Epo.

Contributors

CC, SC, KB, MD, KEK, ICM and OH designed the study, collected the data, verified the data and performed *in vitro* experiments. CC and AN performed the statistical analysis. MBLS, GF, MF, FV, AH, BK, LM, ER, TTM, FF, NC, TNK, SM, CL, RCM, collected the data. CC, SC, MB, OH, KEK and ICM drafted the manuscript and made the figures. All authors approved the final version of the manuscript.

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Declaration of interests

No conflict of interest to disclose

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Data sharing statement

Data are available upon reasonable request

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2021.103785.

References

1 Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med. 10 mars* 2005;**352**(10). 1011-23.
2 Camaschella C. Iron-deficiency anemia. *N Engl J Med* 2015;**372**(19). 7 mai1832-43.
3 Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood* 2016;**127**(20). 19 mai2391-405.
4 Camps C, Petousi N, Bento C, Cario H, Copley RR, McMullin MF, et al. Gene panel sequencing improves the diagnostic work-up of patients with idiopathic erythrocytosis and identifies new mutations. *Haematologica* 2016;**101**(11). nov1306-18.
5 Moura IC, Arcos-Fajardo M, Sadaka C, Leroy V, Benhamou M, Novak J, et al. Glycosylation and size of IgA1 are essential for interaction with mesangial transferrin receptor in IgA nephropathy. *J Am Soc Nephrol JASN. mars* 2004;**15**(3). 622-34.
6 Coulon S, Dussiot M, Grapton D, Maciel TT, Wang PHM, Callens C, et al. Polymeric IgA1 controls erythroblast proliferation and accelerates erythropoiesis recovery in anemia. *Nat Med* 2011;**17**(11). 23 oct1456-65.
7 Lai KN, Tang SCW, Schena FP, Novak J, Tomino Y, Fogo AB, et al. IgA nephropathy. *Nat Rev Dis Primer* 2016;**11**(2):16001.
8 Robert T, Berthelot L, Cambier A, Rondeau E, Monteiro RC. Molecular Insights into the Pathogenesis of IgA Nephropathy. *Trends Mol Med. déc* 2015;**21**(12). 762-75.
9 Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, Feldman HI, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med. 5 mai* 2009;**150**(9). 604-12.
10 Moranne O, Froissart M, Rossert J, Gauci C, Boffa J-J, Haymann JP, et al. Timing of onset of CKD-related metabolic complications. *J Am Soc Nephrol JASN. janv* 2009;**20**(1). 164-71.
11 Chapal M, Le Borgne F, Legendre C, Kreis H, Mourad G, Garrigue V, et al. A useful scoring system for the prediction and management of delayed graft function following kidney transplantation from cadaveric donors. *Kidney Int. déc* 2014;**86**(6). 1130-9.
12 Komatsu N, Yamamoto M, Fujita H, Miwa A, Hatake K, Endo T, et al. Establishment and characterization of an erythropoietin-dependent subline, UT-7/Epo, derived from human leukemia cell line, UT-7. *Blood. 15 juill* 1993;**82**(2). 456-64.
13 Berthelot L, Papista C, Maciel TT, Biarnes-Pelicot M, Tissandie E, Wang PHM, et al. Transglutaminase is essential for IgA nephropathy development acting through IgA receptors. *J Exp Med. 9 avr* 2012;**209**(4). 793-806.
14 Oruc Z, Oblet C, Boumediene A, Druilhe A, Pascal V, Le Rumeur E, et al. IgA Structure Variations Associate with Immune Stimulations and IgA Mesangial Deposition. *J Am Soc Nephrol JASN* 2016. 29 janv.
15 Valentijn RM, Radl J, Haaijman JJ, Vermeer BJ, Weening JJ, Kauffmann RH, et al. Circulating and mesangial secretory component-binding IgA-1 in primary IgA nephropathy. *Kidney Int* 1984;**26**(5). nov760-6.
16 Martinez F, Kamar N, Pallet N, Lang P, Durrbach A, Lebranchu Y, et al. High dose epoetin beta in the first weeks following renal transplantation and delayed graft function: Results of the Neo-PDGF Study. *Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. juill* 2010;**10**(7). 1695-700.
17 Uffing A, Pérez-Saéz MJ, Jouve T, Bugnazet M, Malvezzi P, Muhsin SA, et al. Recurrence of IgA Nephropathy after Kidney Transplantation in Adults. *Clin J Am Soc Nephrol CJASN. août* 2021;**16**(8). 1247-55.
18 Tomana M, Novak J, Julian BA, Matousovic K, Konecny K, Mestecky J. Circulating immune complexes in IgA nephropathy consist of IgA1 with galactose-deficient hinge region and antiglycan antibodies. *J Clin Invest. juill* 1999;**104**(1). 73-81.
19 Mahesh E, Madhyastha PR, Kalashetty M, Gurudev KC, Bande S, John MM. Idiopathic erythrocytosis in IgA nephropathy. *Indian J Nephrol. févr* 2017;**27**(1). 72-3.
20 Woof JM, Kerr MA. The function of immunoglobulin A in immunity. *J Pathol. janv* 2006;**208**(2). 270-82.

21 Schena FP, Scivittaro V, Ranieri E, Sinico R, Benuzzi S, Di Cillo M, et al. Abnormalities of the IgA immune system in members of unrelated pedigrees from patients with IgA nephropathy. *Clin Exp Immunol*. *avr* 1993;**92**(1). 139-44.

22 Ancochea À, Álvarez-Larrán A, Morales-Indiano C, García-Pallarols F, Martínez-Avilés L, Angona A, et al. The role of serum erythropoietin level and jak2 v617f allele burden in the diagnosis of polycythaemia vera. *Br J Haematol* 2014;**167**(3). 411-7.

23 Mercadal L, Metzger M, Casadevall N, Haymann JP, Karras A, Boffa J-J, et al. Timing and Determinants of Erythropoietin Deficiency in Chronic Kidney Disease. *Clin J Am Soc Nephrol*. *1 janv* 2012;**7**(1). 35-42.

24 Berthoux F, Mohey H, Laurent B, Mariat C, Afiani A, Thibaudin L. Predicting the risk for dialysis or death in IgA nephropathy. *J Am Soc Nephrol JASN*. *avr* 2011;**22**(4). 752-61.

25 Rauen T, Eitner F, Fitzner C, Sommerer C, Zeier M, Otte B, et al. Intensive Supportive Care plus Immunosuppression in IgA Nephropathy. *N Engl J Med*. *3 déc* 2015;**373**(23). 2225-36.

26 Vlahakos DV, Marathias KP, Madias NE. The role of the renin-angiotensin system in the regulation of erythropoiesis. *Am J Kidney Dis Off J Natl Kidney Found*. *sept* 2010;**56**(3). 558-65.

27 Garg R, Yusuf S. Overview of randomized trials of angiotensin-converting enzyme inhibitors on mortality and morbidity in patients with heart failure. *Collaborative Group on ACE Inhibitor Trials. JAMA*. *10 mai* 1995;**273**(18). 1450-6.

28 Moranne O, Bakris G, Fafin C, Favre G, Pradier C, Esnault VLM. Determinants and changes associated with aldosterone breakthrough after angiotensin II receptor blockade in patients with type 2 diabetes with overt nephropathy. *Clin J Am Soc Nephrol CJASN* 2013;**8**(10). oct1694-701.

29 Wouters HJCM, Mulder R, van Zeventer IA, Schuringa JJ, van der Klauw MM, van der Harst P, et al. Erythrocytosis in the general population: clinical characteristics and association with clonal hematopoiesis. *Blood Adv* 2020. 22 déc4246353-63.