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Original article

Elevated levels of IL-6 in IgA nephropathy patients are induced by an epigenetically driven mechanism modulated by viral and bacterial RNA

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ABSTRACT

Background: Immunoglobulin A nephropathy (IgAN) is the most frequent primary glomerulonephritis and the role of IL-6 in pathogenesis is becoming increasingly important. A recent whole genome DNA methylation screening in IgAN patients identified a hypermethylated region comprising the non-coding RNA Vault RNA 2–1 (VTRNA2–1) that could explain the high IL-6 levels.

Methods: The pathway leading to IL-6 secretion controlled by VTRNA2–1, PKR, and CREB was analyzed in peripheral blood mononuclear cells (PBMCs) isolated from healthy subjects (HS), IgAN patients, transplanted patients with or without IgAN. The role of double and single-strand RNA in controlling the pathway was investigated.

Results: VTRNA2–1 was downregulated in IgAN compared to HS and in transplanted IgAN patients (TP-IgAN) compared to non-IgAN transplanted (TP). The loss of the VTRNA2–1 natural restrain in IgAN patients caused PKR hyperphosphorylation, and consequently the activation of CREB by PKR, which, in turn, led to high IL-6 production, both in IgAN and in TP-IgAN patients. IL-6 levels could be decreased by the PKR inhibitor imoxin. In addition, PKR is normally activated by bacterial and viral RNA, and we found that both the RNA poly(I:C), and the COVID-19 RNA-vaccine stimulation significantly increased the IL-6 levels in PBMCs from HS but had an opposite effect in those from IgAN patients.

Conclusion: The discovery of the upregulated VTRNA2–1/PKR/CREB/IL-6 pathway in IgAN patients may provide a novel approach to treating the disease and may be useful for the development of precision nephrology and personalized therapy by checking the VTRNA2–1 methylation level in IgAN patients.

1. Introduction

Immunoglobulin A nephropathy (IgAN), also known as Berger's disease, is the most frequent primary glomerulonephritis characterized by the presence of IgA immune complexes in the glomeruli [1]. It generally appears in the second and third decade of life, has a higher incidence in males and is more common in whites than in blacks with a higher prevalence in Asians than Caucasians [1].

Recently, a multihit model has been proposed to describe the pathogenesis of IgAN. In this model, the first hit is given by the hypersecretion of deglycosylated IgA (Gd-IgA1) [2]. These Gd-IgA1 are recognized and attacked by autoantibodies (second hit) and this process leads to the formation of circulating immune complexes (third hit), some of which are deposited at the mesangial level in the glomeruli [2]. Kidney damage resulting from the deposition of the Gd-IgA1 is characterized by local inflammation, complement activation, cell proliferation, and finally fibrosis [3].

Moreover, in humans, the gut-associated lymphoid tissue (GALT) is the primary source of IgA, therefore the pathogenesis of IgAN is related to gut homeostasis. Indeed, the intestinal–renal axis is important in Berger's glomerulonephritis, where several factors (e.g. genetics [4,5], infections [6,7], and food antigens [8] may play a role in the disease

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complex pathogenesis and provide novel therapeutic targets to modify disease evolution.

These models explain the pathogenesis of IgAN caused by the production of aberrant IgA, but it is believed that further predisposing factors are present, including immunological, genetic, environmental, or nutritional factors that can influence the pathogenesis and that could be useful for the development of precision nephrology and personalized therapy.

We have recently showed that IgAN patients have a higher frequency of intestinal-activated B cells than healthy subjects (HS). IgAN patients showed greater BAFF cytokine blood levels, which were linked to higher levels of five microbiota metabolites, and high APRIL cytokine serum levels. In comparison to HS, IgAN individuals had a larger number of circulating gut-homing (CCR9 β7 integrin) regulatory B cells, memory B cells, and IgA memory B cells [9].

Moreover, several studies have shown the involvement of the IL-6 pathway in IgAN. Interleukin-6 is essential for glomerular IgA deposition and the development of renal pathology in Cd37-deficient mice [10]. In addition, the proliferation-inducing ligand (APRIL) and IL-6 are involved in the overproduction of aberrantly glycosylated IgA. In mice, the APRIL silencing blocked the overproduction of Gd-IgA1 induced by IL-6 and, conversely, neutralizing IL-6, the production of Gd-IgA1 was reduced [11]. Interestingly, the mycotoxins deoxynivalenol (DON) prolonged exposure causes the expansion of IgA secreting B cells by activating macrophages and T cells. In mice this stimulation results in the early stages of human IgA nephropathy [12,13] and the overproduction of inflammatory interleukins such as IL-6 [14], which in turn induces upregulation of IgA.

However, to date, the biological mechanisms leading to the elevate IL6 levels in IgAN patients are not clear. In a recent study, a whole-genome screening was performed for DNA methylation in $CD4^+$ T cells from IgAN patients, identifying three regions with altered methylation capable of influencing the gene expression of genes involved in cell response and proliferation T $CD4^+$ [15]. In particular, a hypermethylated region was identified comprising Vault RNA 2–1 (VTRNA2–1), a non-coding RNA also known as the precursor of miR-886 (Pre-mi-RNA).

Here we studied the VTRNA2–1/PKR/CREB/IL-6 pathway showing that it is upregulated in IgAN patients and that it is responsible for the elevated IL-6 levels characterizing the disease, thus providing for the first time an explanation of the abnormal levels of this cytokine. We found that this pathway is epigenetically controlled by VTRNA2–1 and modulated by bacterial and viral infections. It can explain both the high levels of IL-6 and the correlation to mucosal infections. Finally, we showed that the drug imoxin, targeting this pathway, can reduce IL-6 secretion by PBMCs of IgAN patients providing a new possible approach to treat the disease.

2. Materials and methods

2.1. Study design and patients

The study was carried out in accordance with the Helsinki Declaration and the European Guidelines for Good Clinical Practice and approved by our institutional ethics review. Five groups of Caucasian volunteers were included in the study after providing their written informed consent: 34 primary biopsy-proven IgAN patients, 22 IgAN subjects with a kidney transplant (T-IgAN), 14 patients transplanted for causes other than IgAN (TP), 11 HS without known diseases and 5 controls with non-IgA glomerulonephritis (minimal change disease), matched to cases by age and gender. The main clinical features of enrolled patients and HS included in the study are summarized in Table 1. All patients were enrolled before receiving drug administrations. No significant difference in age and sex distribution was observed among the groups.

Patients with biopsy-proven IgAN and with age ranging between 18 and 70 years old were included in the study based on the following criteria: eGFR (calculated using the Chronic Kidney Disease Epidemiology Collaboration [CKD-EPI] creatinine equation) >30 ml/min per 1.73 m^2; baseline proteinuria <1500 mg/d while on stable doses of angiotensin–converting enzyme inhibitor or angiotensin receptor blocker for at least 6 months. Baseline BP was controlled to <130/80 mmHg. Patients with secondary forms of IgAN, such as cirrhosis, were excluded. Patients were excluded if they had previously received rituximab, or were receiving other immunosuppressive therapy or other systemic corticosteroid therapy within 1 year of sample collection. The main clinical features of enrolled patients included in the study are summarized in Table 1. The main pathological features of IgAN patients, according to the Oxford classification MEST-C score, were reported in Table 2.

Table 1
Clinical features of studied IgAN patients.

Variable	IgAN Patients (n = 34)	Transplanted IgAN patients (n = 22)
Age (years), mean ± SD	46.88±14.55	59.23±11.85
Male,%	58.82%	68%
Serum creatinine (mg/dL), mean ± SD	1.13±0.41	1.506818182± 0.43
Proteinuria (g/day), mean ± SD	611.15±430.39	620.09±421.31
GFR (mL/min/1.73m^2), mean ± SD	78.65±27.52	51.27±19.1489943
Body mass index (kg/m^2), mean ± SD	25.95±3.51	25.5455±2.46591

Kidney transplant patients, ages 18–70 years old, with biopsy-proven IgAN in a native kidney biopsy and without signs of recurrence of IgAN in the renal graft were also included in the study on the basis of the same criteria. Patients were on stable doses of corticosteroids, calcineurin inhibitors, and mycophenolic acid. Baseline BP was controlled to <130/80 mmHg. The main clinical features of enrolled patients included in the study are summarized in Table 1.

PBMCs were isolated by gradient centrifugation with Ficoll-Hypaque (Euroclone, Italy) from heparinized venous blood from patients and HS. PBMCs were cultured in RPMI 1640 (Euroclone) supplemented with 10% Fetal Bovine Serum (Euroclone), 100 U/mL Penicillin/Streptomycin (Euroclone), 4mMGlutamine (Euroclone), 10 mM Hepes (Sigma), 0.1 mM non-essential amino acids (Euroclone), 1 mM sodium pyruvate and 50 U/mL, rhIL-2.

2.2. RNA extraction and real time-PCR expression of VTRNA2–1, CREB, and PKR

Total RNA was isolated from PBMCs of IgAN, T-IgAN, TP and HS using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction and quantified by NanoDrop One Spectrophotometer (ThermoFisher Scientific). Total RNA was retro-transcribed using the miScript II RT Kit with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions.

Real Time-PCR was performed on a StepOne Plus instrument (Applied Biosystems) by using VTRNA2–1, PKR and CREB primers (Integrated DNA Technologies, Coralville, IA, USA) GAPDH gene amplification was used as a reference standard to normalize the target signal. Real Time-PCR was performed in triplicate, and relative expression was calculated using the $2-\Delta C_t$ method.

2.3. pPKR/pCREB/IL-6 pathway ELISA quantification

Briefly, PBMCs of HS, IgAN, T-IgAN, and TP subjects were freshly isolated and seeded overnight with RPMI medium at 37 °C, 5% CO_2, in a 96-Well Cell Culture Clear-Bottom Microplate. The ELISA assay protocols were executed following manufacturer instructions.

Table 2
Frequency of pathologic features (%) according to the Oxford classification of IgAN patients (n = 34).

Mesangial Hypercellularity M0 M1	Endocapillary hypercellularity E0 E1	Segmental Glomerulosclerosis S0 S1	Tubular atrophy/interstitial fibrosis T0 T1 T2	Crescents C0 C1
32 68	53 47	18 82	65 32 13	85 15

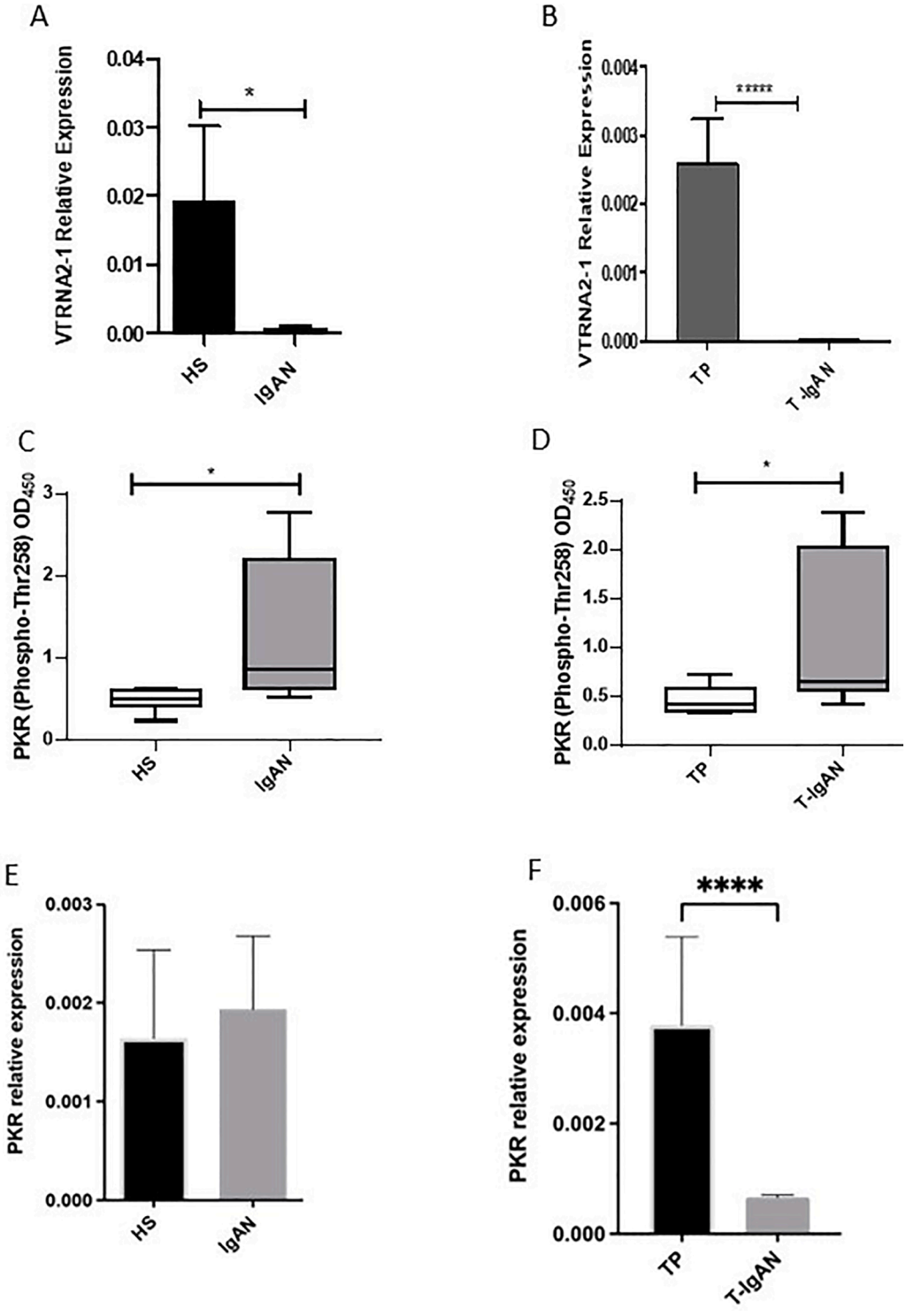

Fig. 1. IgAN patients showed extremely low VTRNA2–1 levels and high levels of phosphorylated PKR. (A) Real-time PCR of VTRNA2–1 transcript differentially expressed in PBMCs of IgAN (n = 22) and HS (n = 15). (B) Real-time PCR of VTRNA2–1 transcript differentially expressed in PBMCs of T-IgAN (n = 11) and TP (n = 7). (C) ELISA assay showed a significantly increased level of phosphorylated PKR in PBMCs from IgAN patients (n = 19) compared to that in PBMCs from healthy subjects (HS) (n = 15). (D) A strong increase in phospho-PKR levels was found in PBMC from T-IgAN (n = 10) patients compared to levels in TP group PBMCs (n = 7). (E) Gene expression of PKR in IgAN patients (n = 8) and HS (n = 8) PBMCs. (F) Gene expression of PKR in TP (n = 8) and T-IgAN (n = 8) patient PBMCs. Data are expressed as mean ± SEM, (* p-value < 0.05, ****p-value <0.001).

Colorimetric Cell-Based ELISA assays were used to detect the levels of CREB and phospho-CREB (pCREB (CREB (Phospho-Ser142) Aviva Systems Biology, USA), and PKR and phosphor-PKR (PKR (Phospho-Thr258) Aviva Systems Biology, USA). The absorbance at 450 nm (OD) values obtained for the phosphorylated target protein have been normalized using the OD values obtained for the non-phosphorylated target protein via the proportion, OD_{450} (Anti-CREB (Phospho-Ser142) Antibody)/OD_{450} (Anti- CREB Antibody) and OD_{450} (Anti- PKR (Phospho-Thr258) Antibody)/OD_{450} (Anti- PKR Antibody). Data were plotted as the normalized pPKR values.

To determine IL-6 levels, PBMCs of HS, IgAN, T-IgAN, and TP subjects, were freshly isolated and seededin a 96-Well Cell Culture Microplate supplemented with RPMI medium at 37 °C, 5% CO_2. After 48 h of culture, cells were collected and, centrifuged at 230 rpm for 15 min. PBMCs supernatants were recovered and analysed using Human IL-6 Quantikine ELISA Kit (R&D Systems, USA), following the manufacturer's protocol.

Optical densities were determined using a microplate reader (programmable MPT reader model DV 990BV6; GDV, Italy) set to 450 nm.

2.4. Stimulation experiments

PBMCs of HS and IgAN patients were seeded in 6-well plates at a density of 2×10^6 cells/cm^2 and were incubated with specific medium. Xfect Transfection Reagent (Clontech, Takara bio, France) was used to transfect 20 ug of Poly (I:C) (InvivoGen, Europe), a synthetic analogous of dsRNA, in PBMC cells, according to manufacturer's instructions. Imoxin (Chem Cruz) was added to PBMCs at a concentration of 1 μM. After two days of incubation with Poly (I:C) and/or Imoxin the secretion of IL-6 levels by PBMCs was measured in the cell culture supernatants. Data from experiments in which IL-6 or CREB values were detected following treatment of PBMCs from the same subject with a stimulus (Imoxin, poly(I:C), COVID RNA, and COVID vaccine) are plotted as the ratio of stimulated /unstimulated PBMC values.

2.5. Statistical analysis

Statistical analyses were performed using the Student's *t*-test, as appropriate. A p-value < 0.05 was considered significant. Data are expressed as means ± SEM.

3. Results

3.1. VTRNA2–1 is downregulated in IgAN patients

Recently, we showed that the non-coding RNA VTRNA2–1 was epigenetically downregulated in IgAN patients [15]. Since we hypothesize that it may be implicated in the pathway for the secretion of IL-6, we further confirm the involvement of VTRNA2–1 in IgAN studying its expression in PBMCs isolated from IgAN patients and healthy subjects.

VTRNA2–1 was strongly downregulated in IgAN patients compared to healthy subjects showing a decrease of 30 fold ($p < 0.05$, Fig. 1A). Moreover, recent clinical observations have shown that IL-6 production is implicated in renal allograft rejection [16,17] and that the cumulative risk of IgA nephropathy recurrence increased after transplant and was associated with a 3.7-fold greater risk of graft loss [17].

We therefore analyzed the expression of VTRNA2–1 in PBMCs isolated from IgAN patients with kidney transplant (T-IgAN) and in patients non-IgAN with a kidney transplant (TP). Also in this case, we found that the VTRNA2–1 was extremely downregulated in transplanted IgAN patients compared to their controls ($p<0.0001$, fold change=100; Fig. 1B), confirming that this modulation is a characteristic of IgAN disease.

3.2. Downregulation of VTRNA2–1 in IgAN patients lead to an increase in phosphorilated PKR

VTRNA2–1 can inhibit PKR, an interferon-inducible and double-stranded RNA (dsRNA) dependent kinase, by binding to this protein and preventing its auto-phosphorylation [18]. Thus, we studied whether in the IgAN patients with low levels of VTRNA2–1, the correspective upregulation of the PKR signaling was present. We measured the levels of the phosphorylated (pPKR) and of the total PKR in PBMCs and found that in IgAN patients the level of pPKR protein normalized on the total PKR was significantly increased compared to HS (FC 2.6; $p < 0.05$; Fig. 1C). At the same manner, also in T-IgAN we found the doubling of pPKR levels compared to TP (FC 2, $p < 0.05$; Fig. 1D).

To confirm that the incerase in pPKR levels was due to the auto-phosphorylation induced by the lack of VTRNA2–1 and not by a different mechanism regulating the gene expression, we checked the PKR mRNA levels in IgAN patients and in HS. We found no significant differences between the two groups (Fig. 1E). However, in T-IgAN compared to TP we found rather a decrease of the PKR transcripts (Fig. 1F, $P < 0.0001$), making data of the pPKR increase even more meaningful.

3.3. Phosphorylation of PKR corresponds to CREB activation in IgAN patients

In mice, pPKR induced the Cyclic adenosine monophosphate (AMP) response element-binding protein (CREB) activation by increasing its phosphorilation [12]. We studied whetherCREB phosphorilation levels were higher in PBMCs of IgAN patients compared to that in PBMCs from HS. A significant 3-fold increase of pCREB levels was found in IgAN patients compared to HS ($p < 0.01$; Fig. 2A). Also in T-IgAN pCREB levels were significantly increase compared to TP, even if to a lesser extent (1.67 fold, $p < 0.01$; Fig. 2B). However, no significant difference in CREB gene expression was present among IgAN patients and HS (Fig. 2C), nor in TX compared to T-IgAN (Fig. 2D), corroborating the data of CREB phosphorilation in IgAN patients.

3.4. CREB activation by phosphorylation leads to IL-6 increase in IgAN patients

Since several studies showed that CREB activation may lead to IL-6 expression [19–23], we analyzed the IL-6 levels secreted by PBMCs in our IgAN patient groups. IL-6 levels in PBMC supernatanats showed a significant increase in IgAN patients compared to HS ($p < 0.05$; Fig. 3A). In T-IgAN compared to TP a tendence in increased levels of IL-6 was found, even if it is not significant (Fig. 3B). Levels of pCREB in PBMC significantly correlated with IL-6 levels in PBMC supernatants of IgAN patients ($r = 0.89$, $p = 0.0064$, respectively), indicating that the high levels of IL-6 are due to the epigenetically regulated VTRNA2–1/pPKR/pCREB signaling (Fig. 3C). Both pCREB and IL-6 levels significanlty increased in IgAN patients and not in minimal change diseases patients (MCD) compared to healthy subjects, confirming that the PKR/CREB/IL-6 pathway is hyperactivated specifically in IgAN (Fig. 3D and E).

3.5. PKR inhibitor imoxin decreases IL-6 secretion by PBMCs from IgAN patients

To further validate the dependence of IL-6 secretion from PKR activation we investigated the effect of the PKR inhibitor imoxin, named also imidazolo-oxindole PKR inhibitor C16, as a blocking agent for the pCREB activation and IL-6 secretion. Results showed that both cellular CREB phosphorilation and IL6 secretion in supernatants of IgAN patient PBMCs were significantly reduced by 1 μM imoxin stimulation for 48 h (Fig. 4A and B, $p < 0.05$ and $p < 0.01$, respectively). However, imoxin stimulation in PBMC from MCD patients did not lead to CREB nor to IL-6

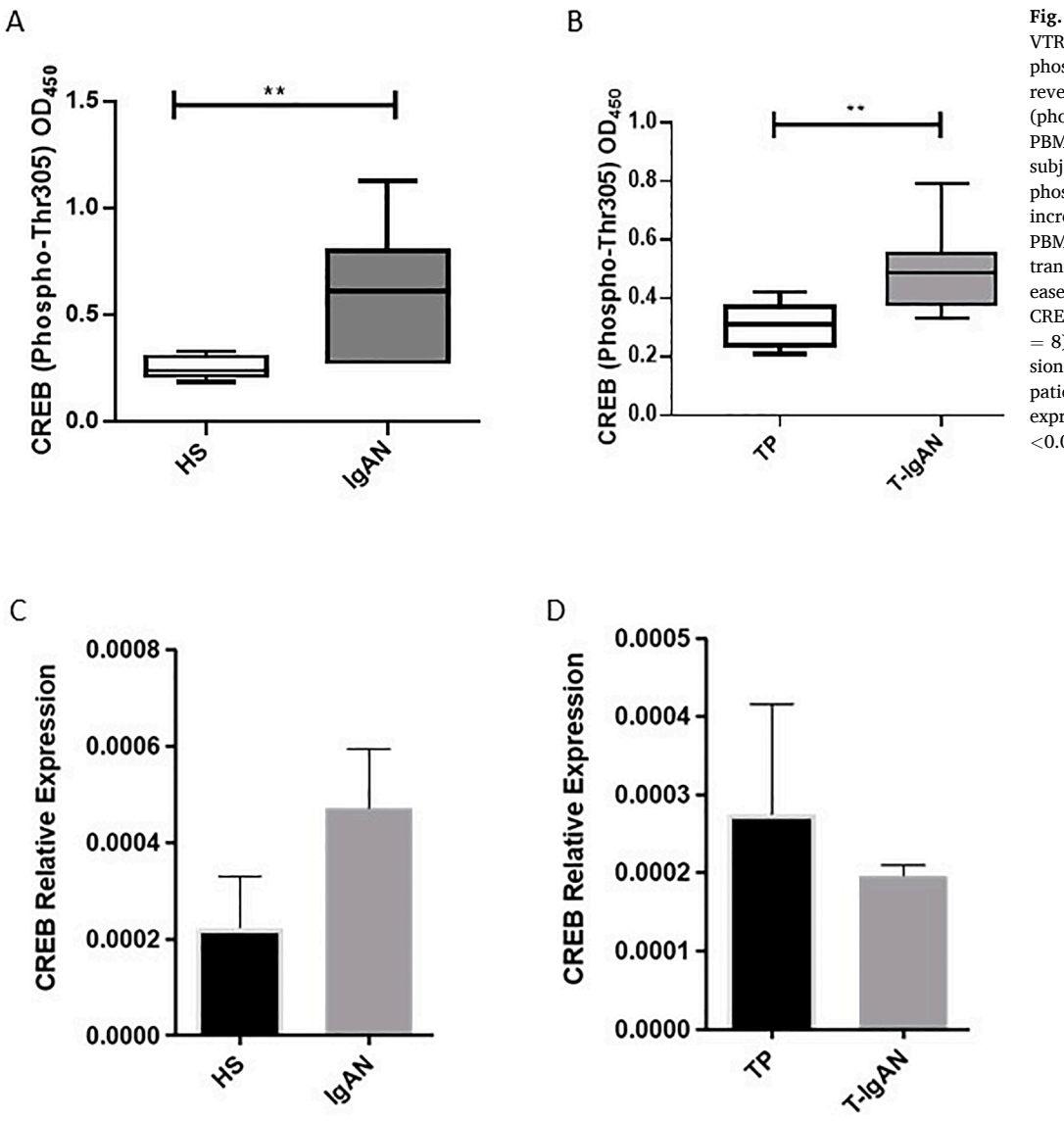

Fig. 2. IgAN patients with low VTRNA2–1 showed elevated levels of phosphorylated CREB. (A) The assay revealed systemic activation of CREB (phospho-Thr305-CREB) in IgAN group PBMCs (n = 19) compared to healthy subjects (HS) PBMCs (n = 15). (B) CREB phosphorylation was significantly increased in T-IgAN (n = 10) patient PBMCs compared to that in PBMCs from transplanted patients without IgAN disease (n = 7). (C) Gene expression of CREB in PBMCs from IgAN patients (n = 8) and HS (n = 8). (D) Gene expression of CREB in TP (n = 8) and T-IgAN patient (n = 8) PBMCs. Data are expressed as mean ± SEM (**p-value <0.01).

levels decrease(Fig. 4C and D). Since PKR is a selective inhibitor of PKR, these data confirmed that the PKR/CREB/IL-6 pathway is specifically activated in IgAN patients.

3.6. Double and single strand RNA or COVID vaccine stimulation increases IL-6 secretion in PBMCs from HS and has an opposite effect in PBMCs from IgAN patients

The biological effect of triggering the PKR/CREB/IL-6 pathway on PBMCs from IgAN patients and HS was studied. Since PKR can be activated by viral or bacterial RNA [24–27], we transfected lymphocytes with synthetic polyinosinic:polycytidylic acid (poly(I:C), which mimics dsRNA or with the RNA vaccine against SARS-COV2, the renowned virus causing COVID-19. PBMCs from HS treated for 48 h with Poly(I:C) showed a 3-fold increase in the IL-6 ratio as compared to the untreated condition, while a more modest increase in IL-6 secretion was detected after vaccine treatment. Nonetheless, both dsRNA and ssRNA trigger IL-6 production in healthy subjects (Fig. 5A and B, $p < 0.05$). Unexpectedly, when we extended our investigation to IgAN patients, we found that both the Poly(I:C) and the vaccine stimulation had an opposite effect compared to the HS stimulation, leading to a decreased IL-6 secretion in supernatants(Fig. 5C and D, $p < 0.05$). The RNA inhibitor effects in IgAN patients were confirmed also transfecting the PBMC directly with the SARS-CoV-2 RNA (Fig. 5E, $p < 0.001$). The restraint of the CREB phosphorilation following RNA stimulation in PBMC from IgAN patients confirmed the different PKR/CREB/IL-6 pathway control in IgAN patients compared to HS (Fig. 5F–H). Both the RNA Covid and the vaccine stimulation induced a significant decrease of CREB phosphorilation (Fig. 5F and E, $p < 0.05$ and $p<0.01$, respectively); however the Poly(I:C) did not change the CREB activation (Fig. 5H).

4. Discussion

The exact immunopathogenic mechanisms underlying IgAN are poorly understood. Very recently the role of Il-6 in IgAN pathogenesis is becoming increasingly important [10,28–31], even if the reason why levels of IL-6 are elevated in IgAN patients is not well understood.

It selectively increases the production of galactose-deficient IgA1 in IgA1-secreting cells from patients with IgA nephropathy [28,29] and IL6-/- mice displayed no glomerular IgA deposition and were protected from exacerbated renal failure following lipopolysaccharide treatment [10]. Interestingly, IL-6 signaling seems to be involved in this complex intestinal immune network, particularly in mediating the production of Gd-IgA [10,11].

One attainable hypothesis about high levels of IL-6 in IgAN comes

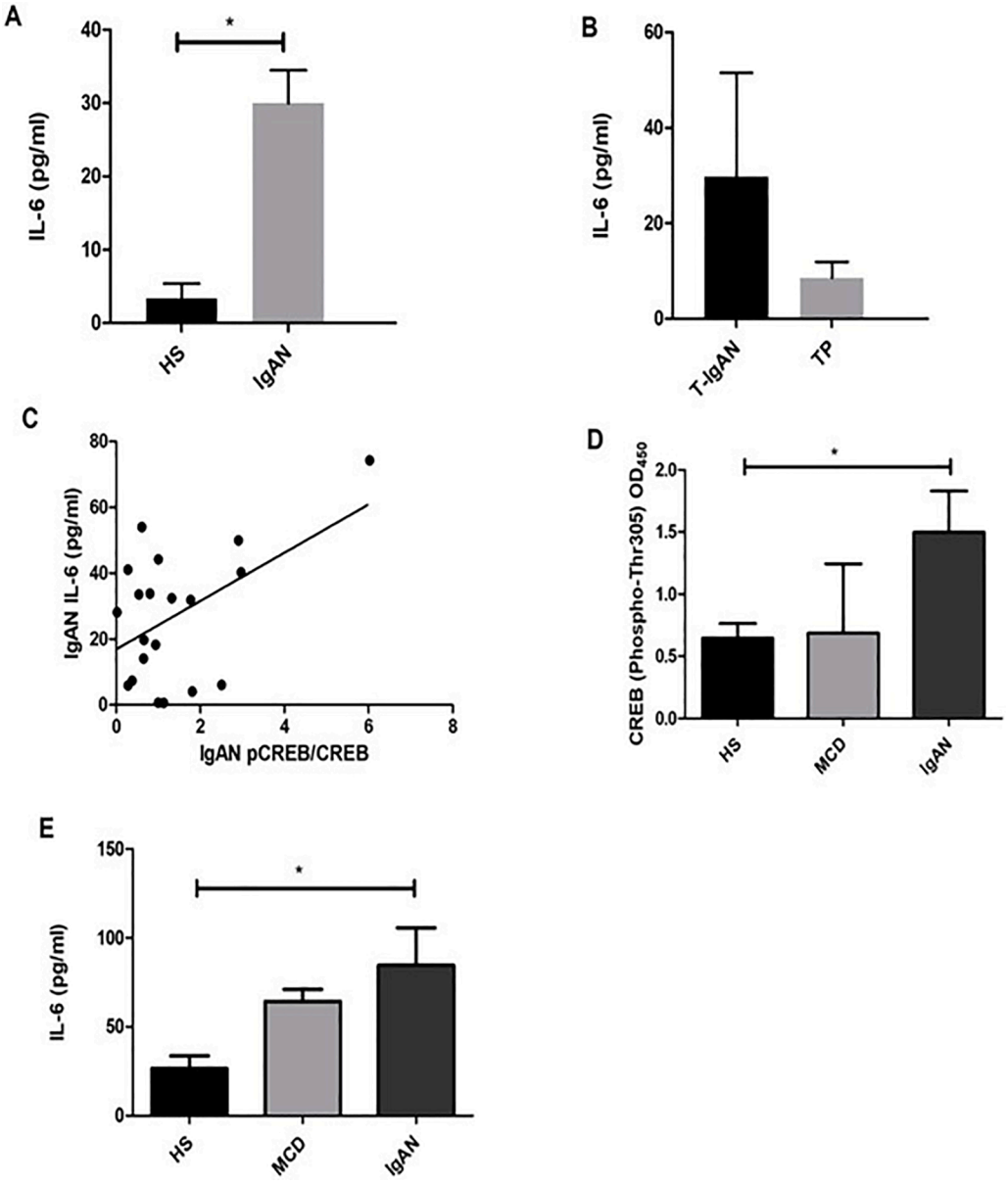

Fig. 3. VTRNA2–1/PKR/CREB activated pathway leads to elevated IL-6 secretion in IgAN patients (A) ELISA assay showed significantly increased levels of IL-6 in supernatants of PBMCs from IgAN patients with VTRNA2–1/PKR/CREB/IL-6 activated pathway respect to the healthy group. (B) An IL-6 increase was found in supernatants of PBMCs from T-IgAN patients with VTRNA2–1/PKR/CREB activated pathway compared to the TP group, even if not statistically significant. (C) The levels of pCREB significantly correlated with IL-6 levels in PBMC supernatants from IgAN patients (and $r = 0.5026$, $p = 0.0239$, respectively). Data are representative of 20 independent experiments. (D) CREB phosphorylation was significantly increased in PBMCs from IgAN patients ($n = 20$) but not in PBMCs from MCD patients ($n = 5$), compared to PBMCs from HS ($n = 15$). (E) IL-6 levels were significantly increased in supernatants of PBMC from IgAN patients ($n = 20$) but not in supernatants of PBMCs from MCD ($n = 5$) patients compared to supernatants of PBMCs from HS ($n = 15$). Data are expressed as means ±SEM; * p-value < 0.05.

out from our recent whole genome DNA methylation screening in IgAN patients, which identified, among others, three regions with altered DNA methylation capable of influencing the expression of genes involved in the response and proliferation of T and B cells [15]. We identified a hypermethylated region encompassing Vault RNA 2–1 (VTRNA2–1), a non-coding RNA also known as precursor of miR-886 (pre-mi-RNA). Consistently, the VTRNA2–1 expression was found down-regulated in IgAN patients [15].

Here we confirm that VTRNA2–1 is lower expressed in IgAN subjects compared to HS and we found that also in transplanted IgAN patients compared to non IgAN transplanted patients the VTRNA2–1 transcript was expressed at very low levels. However, the RNA product does not function as a vault or microRNA; rather, it acts as a direct inhibitor of protein kinase R (also known as eukaryotic translation initiation factor 2-alpha kinase 2, EIF2AK2), and thereby plays an important role in the regulation of cell growth. This gene is near a differentially methylated region (DMR), it is imprinted and may show allele-specific expression. VTRNA2–1 is a metastable epiallele with accumulating evidence that methylation at this region is heritable, modifiable, and associated with disease including risk and progression of cancer [32].

We found that, in IgAN patients with downregulated VTRNA2–1, PKR is overactivated, coherently with the role of VTRNA2–1 that binds to PKR and inhibits its phosphorylation [33]. The loss of this natural restrain causes the activation of CREB, a classical cAMP-inducible CRE-binding factor interacting with a region of the IL-6 promoter (that is known as CRE-like sequence), leading to IL-6 production [19–23].

The discovery of the upregulation of the PKR/CREB/IL-6 in IgAN patients is very suggestive. PKR is normally activated by double-strand bacterial [24,25] and single-strand viral [26,27] RNA and has long been recognized as a key mediator of the innate immunity response to viral infection. Expression of latent PKR is induced by interferon and it is activated upon binding to viral RNA containing duplex regions to undergo autophosphorylation.

We expected to find a further activation of PKR and of the downstream pathway following the PBMC stimulation by RNA, simulating a bacterial or viral infection, or also the COVID vaccination. Instead, this mechanism works in HS but not in IgAN patients. Our data cannot explain the reasons, but we can hypothesize that, in subjects with this pathway already hyperactivated, the further triggering led to an

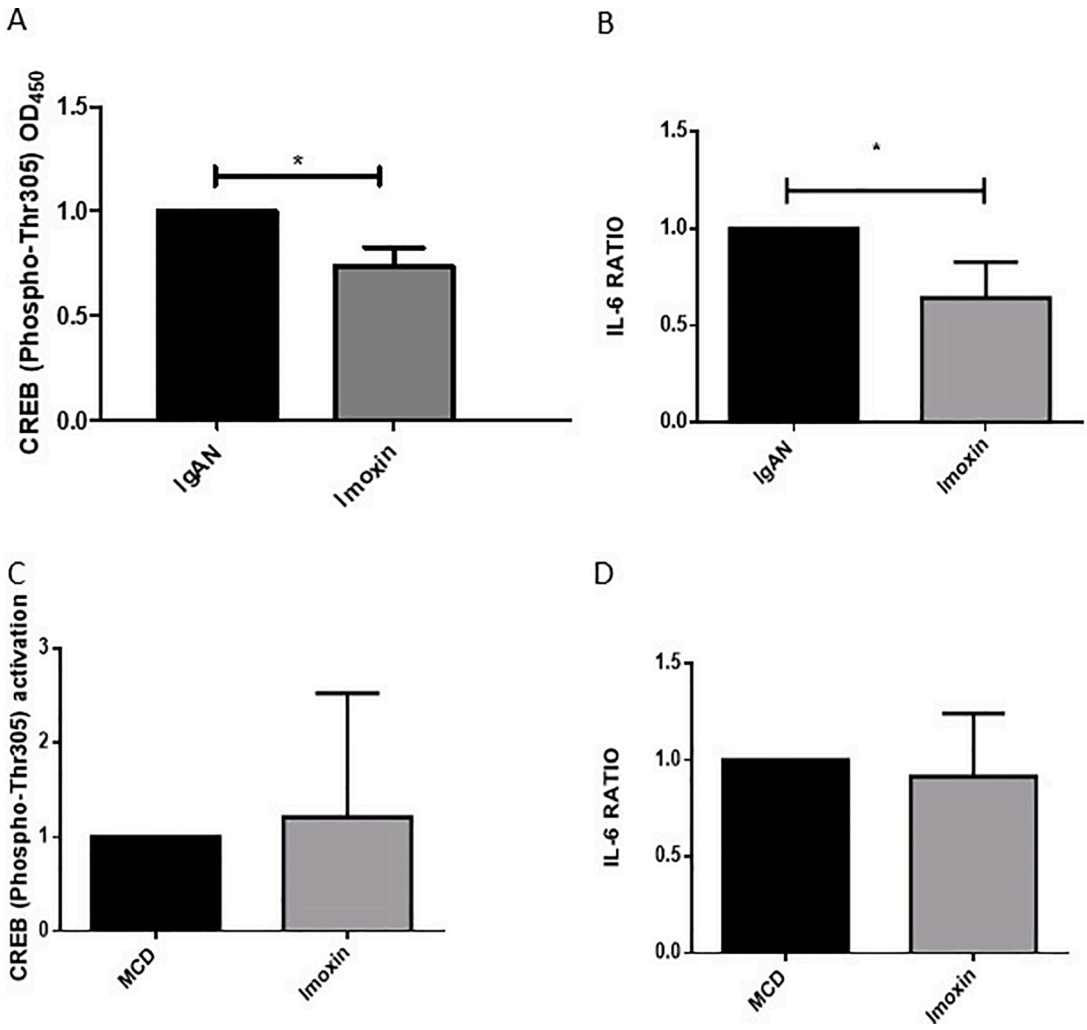

Fig. 4. PKR Inhibitor Imoxin decreases CREB activation and IL-6 secretion in PBMCs from IgAN patients. (A) ELISA assay showing that imoxin significantly decrease the CREB activation in PBMCS from IgAN patients ($n = 11$). (B) ELISA assay showing that imoxin significantly decreases the IL-6 secretion in PBMC supernatants PBMCS from IgAN patient ($n = 11$). (C) ELISA assay showing that imoxin did not decrease CREB activation in PBMCs from MCD patients ($n = 5$). (D) ELISA assay showing that imoxin did not decrease IL-6 secretion in PBMC supernatants of MCD patients ($n = 5$). Data are plotted as the ratio of stimulated /unstimulated PBMC values and are expressed as means ±SEM; * p-value < 0.05.

inhibition rather than an extra activation. This hypothesis is supported by studies showing that increasing nucleic acid concentration leads to PKR inhibition, likely because PKR monomers are diluted out on separate dsRNA molecules and have therefore decreased ability to dimerize [34–36]. However, the epigenetic hyperactivation of the PKR/CREB/IL-6 pathway may contribute to the IgAN onset together to the other determinants. The data showing that in IgAN patients the PKR signaling is already activated due to their epigenetic background could imply a sort of balance that may be perturbed by mucosal infections leading to clinical manifestation of hematuria following upper respiratory tract microbes challenge [37,38]. Interestingly, very recently it has been shown that PKR is regulated by adenovirus-associated noncoding RNA that functions by binding PKR but not inducing activation, thereby inhibiting the antiviral response [39]. This kind of mechanism, driven by the epigenetic silencing of VTRNA2–1, may therefore explain both the high levels of IL-6 and recent data showing microbiota involvement in IgAN [9,40,41].

The PKR/CREB/IL-6 pathway may be very important also in the setting of renal transplantation. We found that this pathway is upregulated also in IgAN transplanted patients. Recent studies showed that the cumulative risk of IgA nephropathy recurrence increases after transplant and is associated with a 3.7-fold greater risk of graft loss [42]. Besides, evidence suggests that IL-6 may play an important role in donor-specific antibodies generation and chronic active antibody-mediated rejection and that the treatment with an anti-IL-6 receptor monoclonal antibody may represent a novel approach for chronic antibody-mediated rejection and transplant glomerulopathy, stabilizing allograft function and extend patient lives [17].

Finally, we showed that the IL-6 secretion can be reduced by the PKR inhibitor imoxin (Fig. 6). This drug also known as C16 or imidazolo-oxindole PKR inhibitor C16, has shown beneficial effects on cell cultures and in vivo in animal models for numerous conditions, such as improvement in inflammation, oxidative stress, diabetes, suppression of tumor proliferation, and in the control of hypertension [43]. However, in light of our results, it may be considered as a possible therapeutic drug also in IgAN. Interestingly, in mice dietary exposure to the common foodborne mycotoxin deoxynivalenol (DON) upregulates serum immunoglobulin A (IgA) and IL-6 miming IgAN and activating PKR/CREB/IL-6 pathway. The dietary omega-3 fatty acids can invert these processes and ameliorate DON-induced IgA nephropathy [12,13].

Further studies will be needed to address this point and the weight of the involvement of the VTRNA2–1/PKR/CREB/IL-6 pathway in IgAN, also increasing the number of patients to study. Moreover, we analysed only Caucasian patients, but the results should be confirmed in other

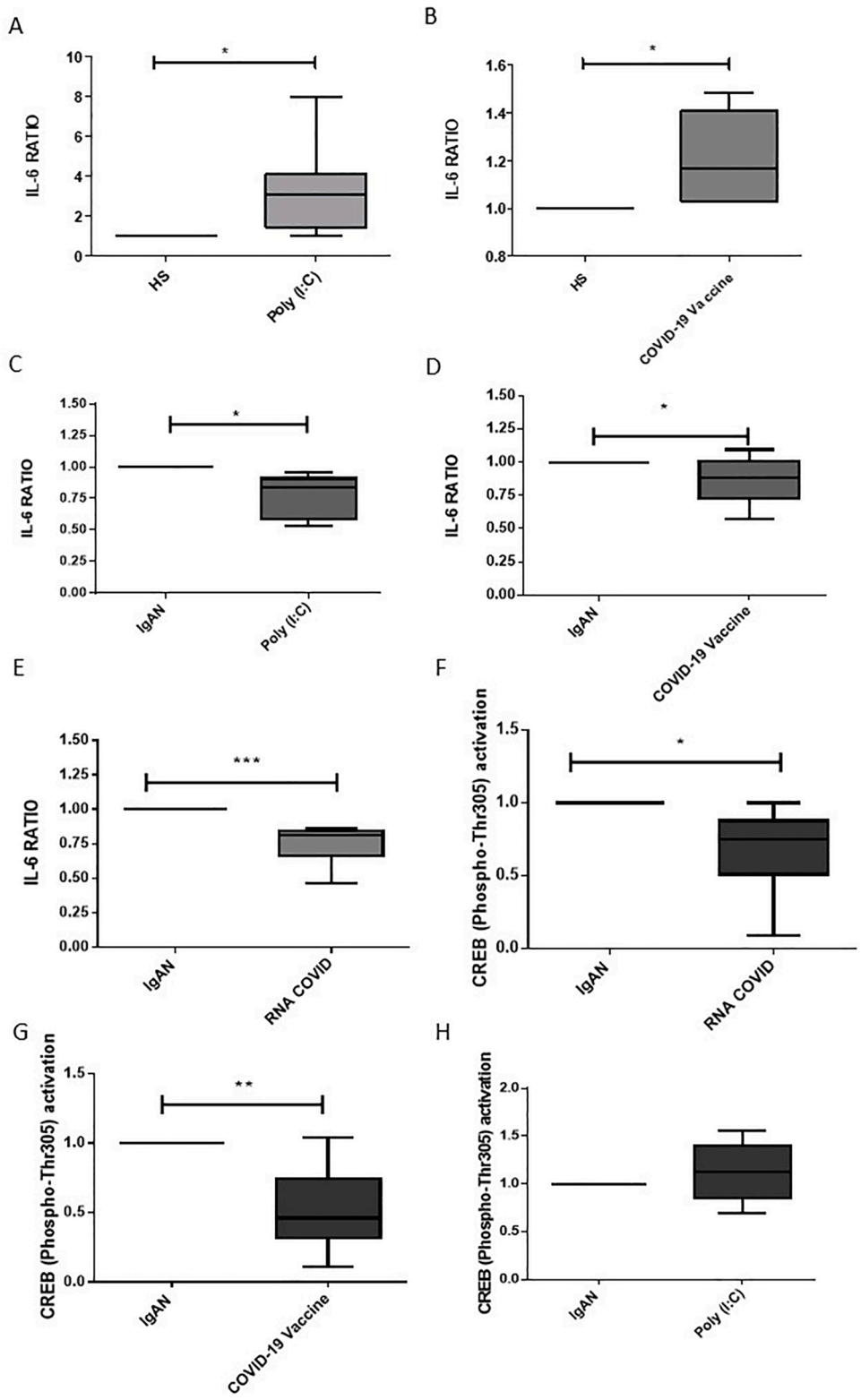

Fig. 5. Effect of poly(I:C), COVID vaccine, and SARS-CoV-2 RNA stimulation in IL-6 secretion in PBMCs from HS and IgAN patients. (A) ELISA assay showing that the poly(I:C) stimulation increased IL-6 secretion in PBMC supernatants of HS ($n = 8$). (B) ELISA assay showing that Covid-19 vaccine stimulation increased IL-6 secretion in PBMC supernatants of HS ($n = 8$). (C) ELISA assay showing that the poly(I:C) stimulation decreased IL-6 secretion in PBMC supernatants of IgAN patients ($n = 8$). (D) ELISA assay showing that Covid-19 vaccine stimulation decreased IL-6 secretion in PBMC supernatants of IgAN patients ($n = 10$). (E) ELISA assay showing that Covid RNA stimulation decreased IL-6 secretion in PBMC supernatants of IgAN patients ($n = 10$). (F) ELISA assay showing that Covid RNA stimulation decreased CREB activation in PBMCs from IgAN patients ($n = 10$). (G) ELISA assay showing that Covid-19 vaccine stimulation decreased CREB activation in PBMCs from IgAN patients ($n = 9$). (H) ELISA assay showing that the poly(I:C) stimulation did not change CREB activation in PBMCs from IgAN patients ($n = 7$). Data are plotted as the ratio of stimulated /unstimulated PBMC values and re expressed as means ±SEM; * p-value < 0.05; **p-value <0.01.

ethnicities.

In conclusion, the discovery of the upregulated VTRNA2–1/PKR/CREB/IL-6 pathway in IgAN patients opens new perspectives in the study of the disease onset and development and may provide novel approach to treat the disease, including the use of imoxin as a drug in IgAN patients showing the PKR, or the entire pathway, hyperactivated. Our results suggest that, in the future, screening for VTRNA2–1 methylation/expression may be useful for the development of precision nephrology and personalized therapy for the IgAN disease.

Data availability statement

The data underlying this article are available in the article and in its online supplementary material.

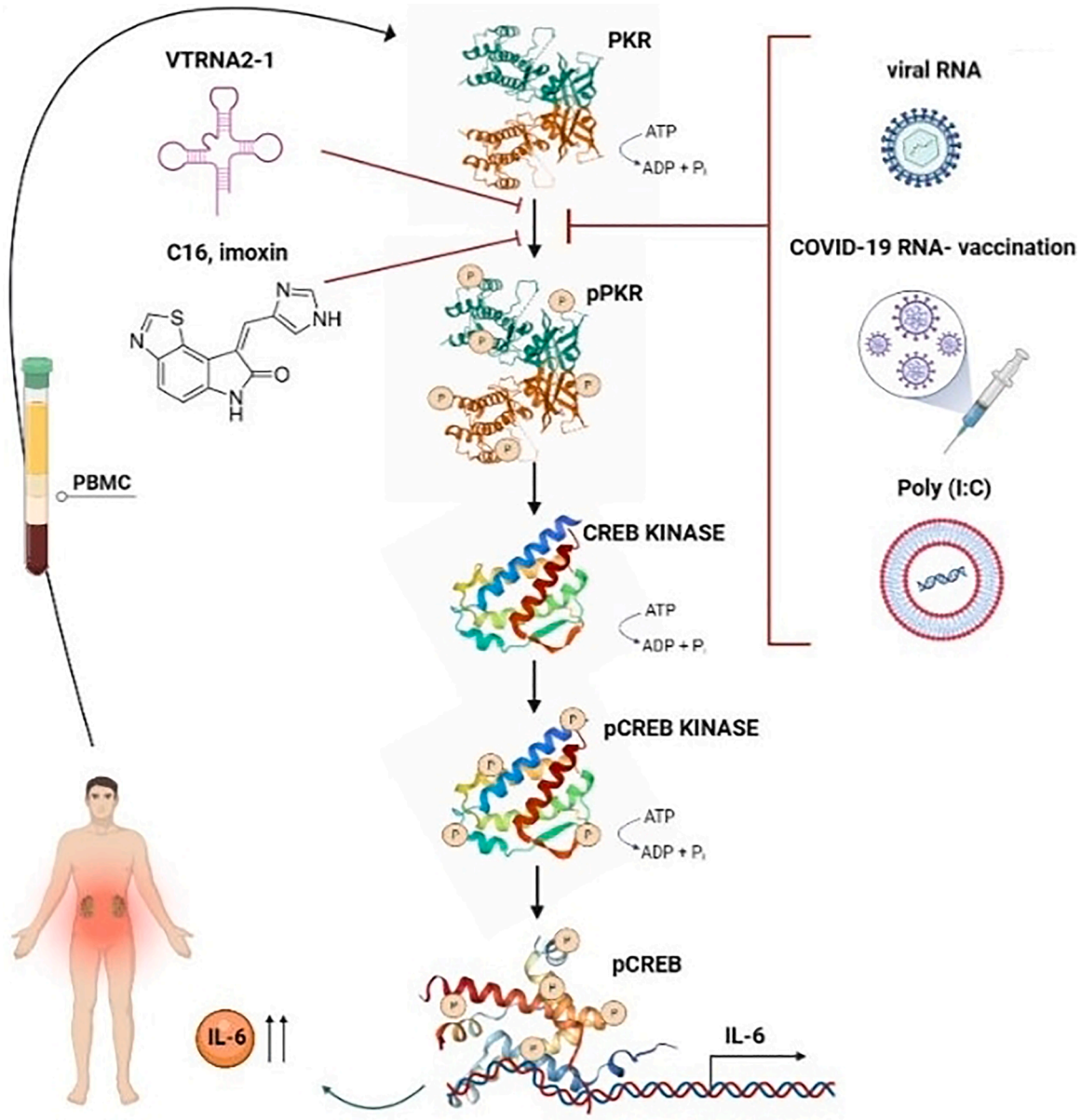

Fig. 6. The involvement of PKR/CREB/IL-6 pathway in IgAN patients and its modulation. In IgAN patients the PKR/CREB/IL-6 pathway is overexpressed and this overactivation leads to high levels of IL-6 secreted by patient PBMCs. The pathway is modulated by the non-coding RNA VTRNA2–1 that is downregulated (because it is hypermethylated) and therefore can not inhibit PKR phosphorylation. As a consequence, PKR is hyperphosphorylated and, in turn, activates phosphorylation of CREB kinase. Pospho CREB (pCREB) interacts with a region of the IL-6 promoter and leads to high levels of IL-6 production. PKR is also induced by bacterial and viral RNA, thus PBMC transfection with synthetic polyinosinic: polycytidylic acid (poly(I:C) and COVID-19 vaccine normally increase IL-6 levels in HS. Instead, in IgAN patients this stimulation had an opposite effect compared to the HS stimulation leading to a decreased IL-6 secretion. Moreover, the drug Imoxin is a PKR inhibitor, that prevents PKR phosphorylation and, consequently, inhibits the PKR/CREB/IL-6 pathway, significantly decreasing IL-6 secretion.

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CRediT authorship contribution statement

Fabio Sallustio: Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, Supervision, Funding acquisition, Writing – review & editing. **Angela Picerno:** Writing – original draft, Investigation, Data curation, Formal analysis, Writing – review & editing. **Maria Teresa Cimmarusti:** Data curation, Investigation, Formal analysis, Writing – review & editing. **Francesca Montenegro:** Writing – original draft, Investigation, Data curation, Formal analysis, Writing – review & editing. **Claudia Curci:** Investigation, Writing – review & editing. **Giuseppe De Palma:** Investigation, Writing – review & editing. **Carmen Sivo:** Resources, Data curation, Writing – review & editing. **Francesca Annese:** Resources, Data curation, Writing – review & editing. **Giulia Fontò:** Resources, Data curation, Writing – review & editing. **Alessandra Stasi:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing. **Francesco Pesce:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing. **Silvio Tafuri:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing. **Vincenzo Di Leo:** Writing – original draft, Resources, Data curation, Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Loreto Gesualdo:** Writing –

review & editing, Conceptualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

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