High Levels of Gut Homing IgA$^+$ B Lymphocytes Support the Pathogenic Role of Intestinal Mucosal Hyper-responsiveness in IgA Nephropathy Patients

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SUPPLEMENTARY MATERIALS

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 Board (protocol number 606). Written consent was obtained from all subjects. Three groups of Caucasian volunteers were included in the study: (i) 44 IgAN patients, (ii) 23 healthy subjects (HS) without known diseases, (iii) 22 controls with non-IgA glomerulonephritis (patients with membranous glomerulonephritis and minimal change disease), matched to cases by age and gender. We analyzed B cell subpopulations by FACS in 36 IgAN patients, 13 HS and 22 controls.

Exclusion criteria included the presence of type 1 or type 2 diabetes mellitus, neurological or gastro-intestinal diseases, acute myocardial infarction or stroke in the previous 6 months, severe uncontrolled hypertension (diastolic blood pressure ≥120 mmHg and/or systolic blood pressure ≥220 mmHg), evidence or suspicion of renovascular disease, severe liver diseases, malignancies, active peptic-ulcer disease, secondary IgAN, renal allograft, pregnancy, other immunological or autoimmune disorders, alcohol abuse, psychiatric disease and inability to assess the follow-up. Subjects included in the study were not treated with antibiotics, steroids, immunosuppressive drugs and/or functional foods (probiotics and/or prebiotics) for at least three months before enrollment. All volunteers confirmed that there were no remarkable changes in meals and medication for at least 1 month.

We collected clinical data (serum creatinine, 24h-proteinuria and estimated glomerular filtration rate) referring to the time of IgAN diagnosis, corresponding to the date of renal biopsy. Serum creatinine, 24h-proteinuria, estimated glomerular filtration rate (eGFR) and Body Mass Index (BMI) were evaluated at the time of biopsy.

Fecal microbiome analysis

The fecal microbiome and urinary and fecal metabolome of all subjects were previously characterized (11). Biochrom 30 series amino acid analyzer and gas-chromatography mass spectrometry/solid-phase microextraction (GC-MS/SPME) analysis were carried out for metabolomic analyses in our previous study (11). Canonical discriminant Analysis of Principal coordinates (CAP) analysis was also carried out for GC-MS/SPME data (12)and it allowed us to identify organic compounds upregulated in IgAN patients. The hypothesis of not significant differences in the multivariate location within groups was tested using the trace statistic based on 9999 permutations.

Determination of serum IgA

Total IgA content in serum from each participant was measured in duplicate using ELISA. Briefly, high-adsorption polystyrene 96-microwell plates (Corning Inc.) were coated overnight with 5 μg/ml of F(ab′)2 fragment of a goat anti-human IgA antibody (Jackson ImmunoResearch Laboratories) in PBS at 4°C. Plates were blocked with 1% BSA in PBS containing 0.05% Tween-20 for 90 minutes at room temperature. The captured IgA was then detected with biotin-labeled F(ab′)2 fragment of goat IgG anti-human IgA (Biosource). The binding was measured after addition of avidin–horseradish peroxidase conjugate (ExtrAvidin; Sigma-Aldrich), and the reaction was developed with the peroxidase chromogenic substrate o-phenylenediamine–H_2O_2 (Sigma-Aldrich). The color reaction was stopped with 2 NH_2SO_4, and the OD at 490 nm was determined in a microplate reader (GDV, Italy; model DV 990 B/V6) (13).

Detection of Gd-IgA1

Gd-IgA1 was detected by a Helix Aspersa Agglutinin (HAA) lectin binding assay (Sigma-Aldrich), as reported elsewhere (14–17). High-adsorption polystyrene 96-microwell plates (Corning Inc.)

were coated overnight with 3 μg/ml of $F(ab')_2$ fragment of goat anti-human IgA antibody (Jackson ImmunoResearch Laboratories) in PBS at 4°C. Plates were blocked for 3 hours at room temperature with 1% BSA/PBS containing 0.1% Tween-20. Two samples, working as standard inter-assay controls, were added for each plate. The captured IgA was subsequently desialylated by treatment for 3 hours at 37°C with 20 mU/ml neuraminidase from Vibrio cholerae (Sigma-Aldrich) in 10 mM sodium acetate buffer (pH 5). Samples were then incubated for 3 hours at 37°C with 2 μg/ml of GalNAc-specific biotinylated HAA lectin (Sigma-Aldrich) diluted in blocking buffer. The lectin binding was detected with avidin-horseradish peroxidase conjugate (ExtrAvidin) diluted in blocking buffer, and the reaction was developed with the peroxidase chromogenic substrate o-phenylenediamine-H2O2 (Sigma-Aldrich). The color reaction was stopped with 2 NH2SO4, and the optical density (OD) at 490 nm was determined in a microplate reader (GDV, Italy; model DV 990 B/V6).

Flow cytometry analysis

Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation with Ficoll-Hypaque. PBMC were then stained with the following monoclonal antibodies (mAbs) for flow cytometry analysis (FACS): Vio Blue-conjugated anti-CD19 (clone LT19), APC-conjugated anti-CD24 (clone REA832), PECy7-conjugated anti-CD38 (clone REA671), PerCP vio700-conjugated anti-IgA (clone REA1014), PE-conjugated anti-Integrin β7 (clone REA441) and FITC-conjugated anti-CCR9 (clone REA469) (or their corresponding isotype controls). PBMC were incubated for 20 min with the antibody mixes in the dark at 4°C, washed twice and resuspended in FACS buffer. All mAbs and their respective isotypes were purchased from Miltenyi Biotec (Bologna, Italy). The anti-CD19 antibody was used to identify total B cells ($CD19^+$ cells), then the anti-CD38 and anti-CD24 were used to distinguish B cells subsets: Naïve B cells ($CD24^{+dim}$ $CD38^{+\ dim}$ B cells), Memory B cells ($CD24^+$ $CD38^-$ B cells), plasmablasts ($CD24^-$ $CD38^+$ B cells) and regulatory B cells (Bregs; $CD24^{+\ high}$ $CD38^{+\ high}$ B cells). Within each of these subsets, we detected the presence of IgA and analyzed the expression of integrin β7 and CCR9 (two gut homing receptors). Stained cells were

then acquired on a Navios cytometer (Beckman coulter, Milan, Italy) and analyzed using the Flowjo software (Treestar Inc. USA). 500000 cells for each sample were analyzed (Supplemental Table 2).

Supplementary Table 1:

Metabolite	IgAN Patient mean value	HS mean value
1-butanol	0.113613422	0.066484238
1-trydecin-4-ol	0.058368396	0.093971787
2-butanone(CHETONE)	4.368852085	1.536423261
2,4-dimethyl-1-heptene	0.805443087	0.334016479
2,6-dimethyl-4-heptanone	0.123243592	0.029345184
2,6-octadien-1-ol, 3,7-dimethyl- (Z) ALCOL	2.31681671	0.306219288
4-heptanone	0.024139627	0.011377249
acetic acid	0.517115699	0.110686176
acetic acid, butyl ester	0.08900047	0.018695096
1,4-bis(1,1-dimethylethyl)- benzene,	2.451964023	6.553099852
benzoic acid, hexadecyl ester	0.053850809	0.065737351
butanoic acid (SCFA)	1.627721809	0.316222065
butanoic acid, ethyl ester ESTERE	3.275487005	0.717615463
ethyl acetate	0.724472503	0.110969141
ethyl alcohol ALCOL	1.557697438	0.451946628
Furanone A	1.864100089	1.51782679
indole	0.128874758	0.137613466
pentanoic acid	0.350491195	0.115220355
indole2	0.128874758	0.137613466
phenol, 4-(1,1,3,3-tetramethylbutyl)-ALCOL	0.290948745	0.310465876
phenol, p-tert-butyl-	0.063221793	0.062528059
phthalic acid, methyl neopentyl ester?	0.155014429	0.168858503
propanoic acid	0.312805969	0.058475403
propanoic acid, ethyl ester	1.660569897	0.278019133
methyl isobutyl ketone	0.063469766	0.062455313
tridecanal	0.217271856	0.003526314

Supplementary Table 2:

					Bregs				Memory B cells				Plasmablasts			
							IgA+ Bregs				IgA+ Memory B				IgA+ Plasmablasts	
	TOTAL CELL NUMBER	Lymphocytes | Freq. of Parent	B cells in lymphocytes	Naive B cells in B cells	Bregs in B cells	B7+ CCR9+ in Bregs	IgA+ Bregs in Bregs	B7+ CCR9+ in IgA+ Bregs	Memory B cells in B cells	B7+ CCR9+ in Memory B cells	IgA+ Memory B in Memory B cells	B7+ CCR9+ in IgA+ Memory B cells	Plasma blasts in B cells	B7+ CCR9+ in Plasma blasts	IgA+ Plasma blasts in Plasma blasts	B7+ CCR9+ in IgA+ Plasma blasts
Healthy subjects	500000															
MEAN HS		273923.08	6757.60	4695.29	206.51	18.73	7.14	0.59	1398.61	42.46	64.80	6.94	36.13	4.47	7.99	1.62
SEM		22308.43	757.85	559.46	40.52	7.78	3.39	0.33	203.71	13.30	10.67	2.78	6.10	1.28	1.96	0.50
IgAN	700000															
MEAN IgAN		412301.94	11766.45	7204.64	381.65	56.68	12.34	3.87	2944.75	248.10	180.31	39.26	351.35	89.60	144.63	64.19
SEM		28511.91	1155.00	768.49	50.10	12.90	3.05	0.83	402.24	69.91	31.69	10.30	120.47	30.19	52.84	26.28
CONTROLS	700000															
MEAN CTR		390285.00	10089.74	6040.76	425.75	51.14	8.28	3.50	1703.32	83.77	169.53	14.59	308.08	31.62	69.88	13.67
SEM		33678.34	1942.99	1210.04	75.79	14.71	3.15	1.56	621.85	28.82	88.16	4.81	148.78	5.87	32.08	3.92

Mean and standard error of the cells for each of the analyzed B-cell populations