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B-cell *O*-galactosyltransferase activity, and expression of *O*-glycosylation genes in bone marrow in IgA nephropathy

KS Buck[2,4], AC Smith[1,4], K Molyneux[1], H El-Barbary[1], J Feehally[1] and J Barratt[1,3]

[1]*John Walls Renal Unit, Leicester General Hospital, Leicester, UK;* [2]*Renal Unit, Queen Margaret Hospital, Dunfermline, UK and* [3]*Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, UK*

In IgA nephropathy (IgAN), pathogenic IgA1 is likely derived from bone marrow (BM) cells and exhibits reduced *O*-galactosylation. Defective *O*-galactosylation may arise from the compromised expression or function of the enzyme β-galactosyltransferase and/or its molecular chaperone (Cosmc). We measured B-cell *O*-galactosylation activity and the relative gene expression of β-galactosyltransferase and Cosmc in peripheral blood and BM taken from patients with IgAN and controls. *O*-galactosylation activity was measured in peripheral and BM B cells by the incorporation of radiolabeled galactose into an asialo-mucin acceptor. Gene expression of β-galactosyltransferase and Cosmc was measured by real-time PCR and related to that of the enzyme GalNAc-T2 (UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase-2), which synthesizes the core *O*-glycan. Neither the B-cell *O*-galactosylation activity nor the gene expression of the enzyme or chaperone was different between patients and controls. However, the relationships between the *O*-glycosylation of serum IgA1, galactosylation activity, and β-galactosyltransferase gene expression showed different patterns in IgAN and controls. In IgAN, *O*-galactosylation activity correlated with β-galactosyltransferase gene expression, but not with IgA1 *O*-glycosylation, suggesting that factors other than the availability of β-galactosyltransferase or Cosmc are responsible for altered IgA1 *O*-glycosylation.

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Correspondence: *AC Smith, John Walls Renal Unit, Leicester General Hospital, Leicester LE4 5PW, UK. E-mail: aa50@le.ac.uk*

[4]These authors contributed equally to this work

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IgA nephropathy (IgAN) is characterized by the deposition of IgA in the glomerular mesangium, and is among the most common forms of glomerulonephritis in developed countries.[1] Although the mechanism of mesangial IgA deposition in IgAN is not well understood, it is likely that physicochemical properties of the molecule are involved. Of the two human IgA subclasses IgA1 and IgA2, IgA1 has been most implicated in the pathogenesis of IgAN. IgA1 has a heavily *O*-glycosylated hinge region between the CH1 and CH2 domains, which is absent from IgA2 and all other serum immunoglobulins except IgD.[2] Indeed, only a few serum proteins carry *O*-linked glycans and IgA1 is the most abundant of these.

The IgA1 *O*-glycans are of the Core-1 type, short and simple sugar chains based on *N*-acetyl galactosamine (GalNAc) *O*-linked to serine or threonine residues. This can occur alone, but is usually extended with the addition of galactose (Gal) and sialic acid (*N*-acetylneuraminic acid, NeuNAc), giving rise to different *O*-glycan chain variants (I–V, Figure 1a). The *O*-glycan chains are formed in the Golgi apparatus during the synthesis of IgA1 by the sequential action of glycosyltransferases (Figure 1b). Initial linking of GalNAc to the protein backbone is catalyzed by one of the UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase family, known as GalNAc-T2.[3] Galactosylation is then effected by a core-1 β1-3 galactosyltransferase, C1Gal-T1.[4] C1Gal-T1 activity requires the co-expression of the chaperone protein Core-1-β3-Gal-T-specific molecular chaperone (Cosmc),[5] the availability of which may be a limiting factor in galactosyltransferase function.

Much research interest has been devoted to the *O*-glycosylation of IgA1 in IgAN, and it is now well established that abnormalities exist,[6–9] which are of potential pathogenic significance,[10–16] and may have diagnostic value.[17] The exact structural details of the IgA1 *O*-glycosylation abnormality found in IgAN has proved difficult to characterize, although most studies conclude that reduced galactosylation of some of the moieties is most likely.[9,10,17,18] This leads to the hypothesis that reduced activity of the galactosylating enzyme C1Gal-T1 may be responsible, due to

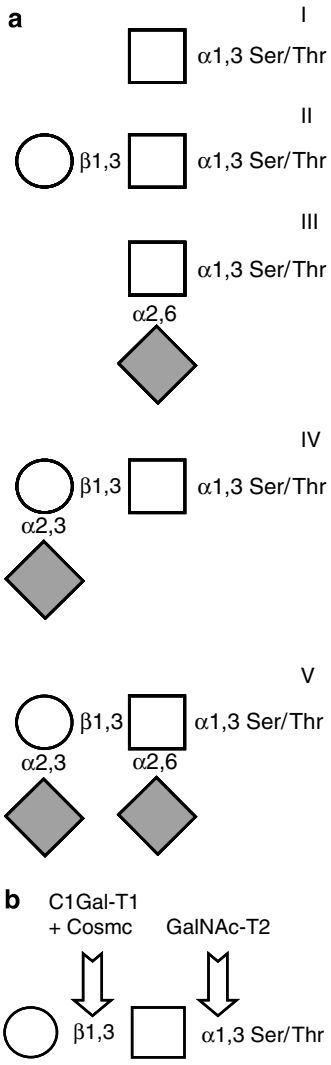

Figure 1 | *O*-glycosylation of human IgA1. (**a**) The hinge region of human IgA1 carries a series of approximately five Core-1 type carbohydrates per heavy chain. The diagram illustrates the various possible structures of these carbohydrate moeities. All the variations are based on *N*-acetyl galactosamine (GalNAc, open squares) *O*-linked to serine or threonine residues (I). GalNAc can be extended with the addition of galactose (Gal, open circles) in β1,3 linkage to form the basic Core-1 structure (II), which may be sialyated with the addition of one (III and IV) or two (V) *N*-acetylneuraminic acid units (NeuNAc, shaded diamonds). As the hinge regions of different IgA1 molecules can potentially carry a varied number and mixture of the different chain configurations, a wide array of *O*-glycoforms is theoretically possible. (**b**) *O*-glycosylation of IgA1 is catalyzed by the sequential action of highly specific glycosyltransferases. GalNAc is first added to serine or threonine by UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase-2 (GalNAc-T2). The moiety is galactosylated in the β1,3 configuration by Core-1 β1-3 galactosyltransferase-1 (C1Gal-T1), which requires the presence of the Core-1-β3-Gal-T-specific molecular chaperone (Cosmc).

either some defect in the enzyme or its expression, or secondary to the reduced availability of the chaperone protein Cosmc. In the hematological disorder Tn syndrome and in some cancers, a total lack of C1Gal-T1 activity in the affected clones results in undergalactosylation of the core-1 *O*-glycans of membrane proteins resulting in the abnormal surface expression of the Tn antigen (GalNAc),[19] an analogous situation to IgA1 in IgAN. In a Tn-expressing T-cell line (Jurkat), the lack of C1Gal-T1 activity is due to a mutation in the Cosmc gene rather than in C1Gal-T1 itself.[5] Some support exists for both these possibilities in peripheral blood (PB) B cells in IgAN: we previously published a study showing indirect evidence of reduced *O*-galactosylation activity,[20] and a more recent report demonstrated reduced Cosmc gene expression.[21] Any defect is unlikely to affect all *O*-glycosylated proteins in IgAN, as we have shown that C1 inhibitor, another serum protein carrying *O*-glycans, is normal in IgAN,[7] as is the *O*-glycosylation of IgD.[22] Therefore, changes in C1Gal-T1 or Cosmc activity are likely to be restricted to IgA1-producing B cells only.

Circulating B lymphocytes are mostly naive or memory cells, and are not in the process of producing the secreted IgA1 molecules found in the serum and deposited in the kidney. Although the majority of IgA production normally occurs at mucosal sites, this does not appear to be the source of abnormal IgA1 production in IgAN. Mesangial IgA1 in IgAN is polymeric: this type of IgA1 production is reduced in mucosal tissue in the patients,[23,24] but increased in the bone marrow (BM)[25,26] and tonsil,[27] and these latter sites are therefore the most likely origin of pathogenic IgA1 molecules. Therefore, the aim of this study was to look for defects of *O*-galactosylation factors that may account for abnormal IgA1 *O*-glycosylation in IgAN, focusing on the BM. We directly measured B-cell *O*-galactosylation activity effected by the galactosylating enzyme C1Gal-T1 with its molecular chaperone Cosmc. We also measured BM C1Gal-T1 and Cosmc mRNA expression in relation to that of GalNAc-T2.

RESULTS

Lectin binding of plasma IgA1

As is generally found in IgAN, serum IgA1 from patients had significantly higher *Helix aspersa* (HA) lectin binding than did control IgA1 (mean A_{492}HA/A_{492}IgA1 ± s.e.m.: IgAN 1.838 ± 0.0397, control 1.562 ± 0.0473, $P = 0.014$).

Purity and yield of B-cell preparations

Representative PB and BM preparations were 89 and 70% positive for the B-cell surface marker CD22, and negative for T cell and monocyte markers (CD3, CD14). The lower percentage of B cells in BM is probably due to the earlier expression of CD19 (used for selection) than CD22 (used for detection) by B cell precursors: CD22-negative cells had the morphological appearance of immature lymphocytes. PB and BM B-cell lysates with total protein concentrations of more than 100 μg were obtained from all subjects.

C1Gal-T1/Cosmc *O*-galactosylation activity in PB and BM B cells

The *O*-galactosylation activity of PB and BM B cells in each subject is shown in Table 1. We found no significant difference between patients with IgAN and controls in the

Table 1 | B-cell *O*-galactosylation activity (AU μg^{-1} total cell protein)

Subject no.	IgAN		Control	
	PB	BM	PB	BM
1	1.18	1.33	1.57	1.46
2	0.80	0.88	1.35	0.82
3	1.25	1.28	1.30	1.50
4	1.11	1.50	3.78	2.34
5	1.22	1.02	1.26	0.73
6	1.15	0.50	1.40	1.62
7	1.16	0.77	1.18	0.87
8	1.44	1.27	1.65	1.60
9	0.92	1.04	1.15	0.58
10	1.38	2.70	1.10	0.85
11	1.45	2.90	0.80	0.73
12	1.24	2.19	0.74	4.40
13			1.40	1.70
Mean (s.e.m.)	1.19 (0.06)	1.45 (0.22)	1.44 (0.21)	1.48 (0.29)
P-value paired *t*-test; PB vs BM	0.205		0.905	
P-value unpaired *t*-test; IgAN vs control	0.275	0.937		

AU, arbitrary units; BM, bone marrow; IgAN, IgA nephropathy; PB, peripheral blood.

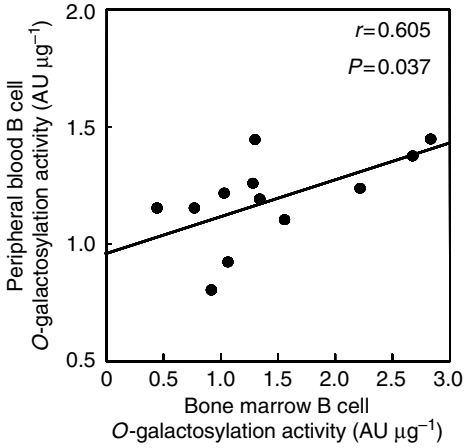

Figure 2 | *O*-galactosylation activity in B cells from peripheral blood and bone marrow in patients with IgAN. The C1Gal-T1/Cosmc activity of lysates of purified B cells was measured in an assay based on incorporation of radiolabeled UDP-Gal into an ovine submaxillary mucin acceptor. The results were expressed as arbitrary units per microgram total lysate protein (AU μg^{-1}). In IgAN (closed circles), there was a significant positive correlation between the C1Gal-T1/Cosmc activity of B cells from peripheral blood and bone marrow. No such relationship was seen in controls.

C1Gal-T1/Cosmc activity of either PB or BM B cells. Nor was there any significant difference in activity between PB and BM B cells in either the IgAN or the control groups. However, in IgAN, there was a significant positive correlation between the *O*-galactosylation activity of PB and BM B cells (Figure 2); this relationship was not seen in the controls ($r = 0.139$, $P = 0.650$, data not shown).

Correlation of B-cell enzyme activity and lectin binding of IgA1

In IgAN, HA lectin binding of serum IgA1 showed a significant positive correlation with *O*-galactosylation activity in BM B cells, but not with PB B cells (Figure 3a and b). By contrast, in controls, there was a significant inverse correlation between lectin binding of serum IgA1 and *O*-galactosylation activity of both PB and BM B cells (Figure 3c and d).

Real-time PCR

Table 2 shows the gene expression ratios in patients with IgAN and controls. There was no difference between the subject groups in the ratios of C1Gal-T1:GalNAc-T2 or C1Gal-T1:Cosmc in PB or BM lysates.

Correlation of enzyme gene expression with lectin binding of plasma IgA1 and C1Gal-T1 activity

There was no correlation between the lectin binding of plasma IgA1 and the PB or BM gene expression ratios of C1Gal-T1:GalNAc-T2 or C1Gal-T1:Cosmc in either the IgAN or control groups (data not shown).

In the IgAN group only, B-cell C1Gal-T1/Cosmc activity showed negative correlations with both C1Gal-T1:Cosmc C_t (threshold cycle) and C1Gal-T1:GalNAc-T2 ratios in PB and BM samples (Figure 4), although most of these did not reach statistical significance. As C_t is inversely proportional to gene expression, these correlations indicate a positive relationship between expression of C1Gal-T1 and *O*-galactosylation in these samples. No such correlation was found in the ratio of Cosmc:GalNAcT-2 (PB $r = 0.336$, $P = 0.342$; BM $r = -0.364$, $P = 0.24$), suggesting that the C1Gal-T1 gene expression is responsible for this relationship. There was no indication of any correlation of gene expression ratios and *O*-galactosylation activity in the controls (data not shown).

DISCUSSION

The *O*-glycosylation of IgA1 has received much attention in IgAN since the original reports of altered lectin binding to serum IgA1. It is apparent that there is over-representation of IgA1 molecules with certain *O*-glycan patterns in the serum of patients with IgAN and that these *O*-glycoforms predominate in the mesangial deposits.[14,28] The detailed structure of these abnormal IgA1 *O*-glycoforms has yet to be fully elucidated, but most of the available evidence suggests under-galactosylation, leading to increased exposure of the inner core GalNac moieties and the underlying protein backbone, and to reduced sialylation (Figure 1). It has been suggested that reduced *O*-galactosylation may result from reduced activity of C1Gal-T1, the intracellular enzyme that catalyzes the addition of galactose to *O*-linked GalNAc moieties.[20,29,30] We previously reported evidence that the activity of this enzyme may indeed be reduced in PB B cells in IgAN.[20] However, the assay used in that study measured enzyme activity only indirectly by monitoring changes in lectin binding of an acceptor prepared by degalactosylation of

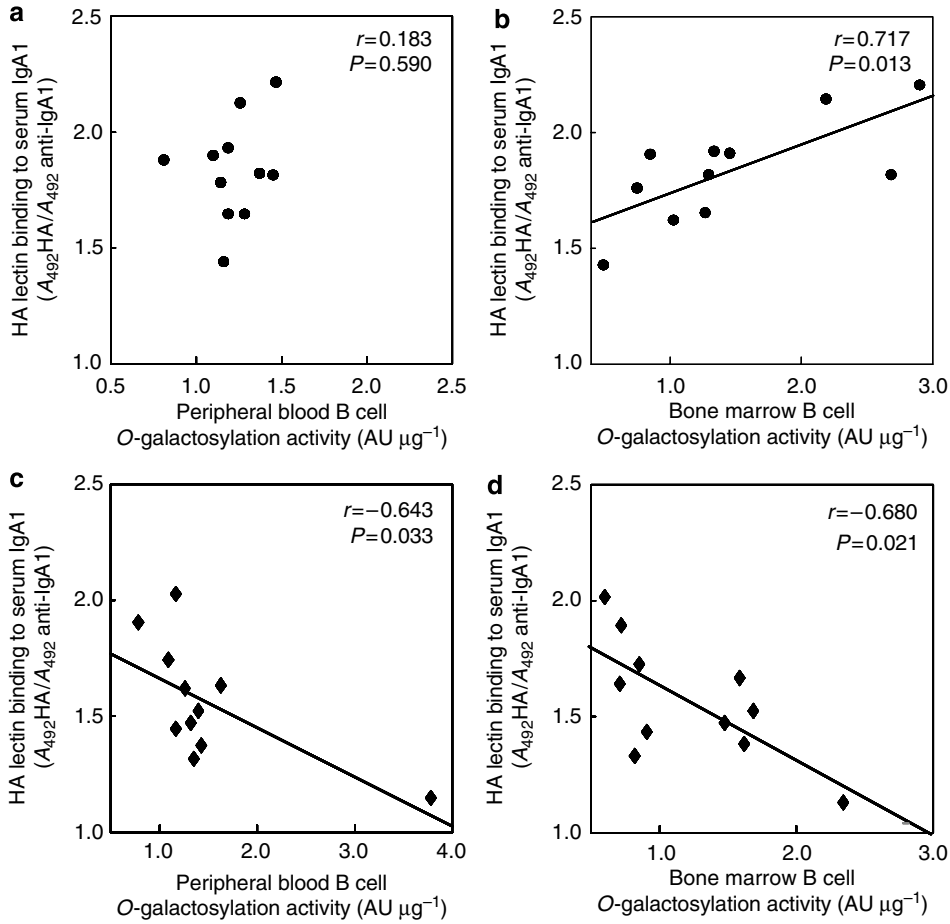

Figure 3 | Serum IgA1 *O*-glycosylation and B-cell *O*-galactosylation activity in IgAN and controls. The *O*-glycosylation pattern of serum IgA1 was shown by its binding of the lectin from *Helix aspersa* (HA), which recognizes ungalactosylated *O*-linked GalNAc, in an ELISA-type system. The higher the HA binding, the lower the overall galactosylation of the IgA1 sample. In IgAN (closed circles, **a** and **b**), the HA lectin binding of serum IgA1 showed no relationship with the C1Gal-T1/Cosmc *O*-galactosylation activity of peripheral blood (PB) B cells (**a**), but a significant positive correlation with that of bone marrow (BM) B cells (**b**). By contrast, in controls (closed diamonds, **c** and **d**), HA lectin binding of serum IgA1 had a significant inverse correlation with the C1Gal-T1/Cosmc activity of both PB (**c**) and BM (**d**) B cells.

Table 2 | Gene expression ratios

	IgAN		Control	
	PB	BM	PB	BM
	C1Gal-T1:GalNAc-T2 ratio			
Mean (s.e.m.)	1.17 (0.07)	0.94 (0.03)	1.12 (0.02)	0.95 (0.03)
P-value IgAN vs control	0.504	0.756		
	C1Gal-T1:Cosmc			
Mean (s.e.m.)	1.19 (0.07)	0.93 (0.01)	1.24 (0.03)	0.95 (0.03)
P-value IgAN vs control	0.600	0.426		

BM, bone marrow; C1Gal-T1, Core-1 β1-3 galactosyltransferase-1; Cosmc, Core-1-β3-Gal-T-specific molecular chaperone; IgAN, IgA nephropathy; PB, peripheral blood.

pooled IgA1 after incubation with cell lysates. The theoretical strength of this earlier assay lies in its use of IgA1 as the acceptor, which is clearly more relevant for our purpose than ovine submaxillary mucin (OSM). However, the changes in lectin binding could have been due to factors other than C1Gal-T1-mediated regalactosylation of the *O*-glycan moieties, and the results are therefore less specific and reliable than the direct measurement of galactose incorporation into the mucin acceptor preparation used here, which has previously been validated for the measurement of C1Gal-T1.[31] As the functions of C1Gal-T1 and Cosmc are intimately related, any assay of C1Gal-T1 in cell lysates, including that used here, will also include the activity of Cosmc. Differences in *O*-galactosylation activity between different lysates may reflect alterations in the function of either or both of these proteins. It is neither possible nor physiologically relevant to separate them.

In this study, in addition to PB B cells, we studied *O*-galactosylation activity in B cells from BM, the likely site of abnormal IgA1 production in IgAN. We also used real-time PCR to investigate gene expression of GalNAc-T2 and C1Gal-T1, the two major enzymes involved in synthesis of these *O*-glycan chains, and Cosmc, the essential chaperone protein of C1Gal-T1.

Compared to the controls, the serum IgA1 of the cohort of IgAN patients studied displayed abnormally high HA lectin binding. HA lectin recognizes GalNAc and its binding is high

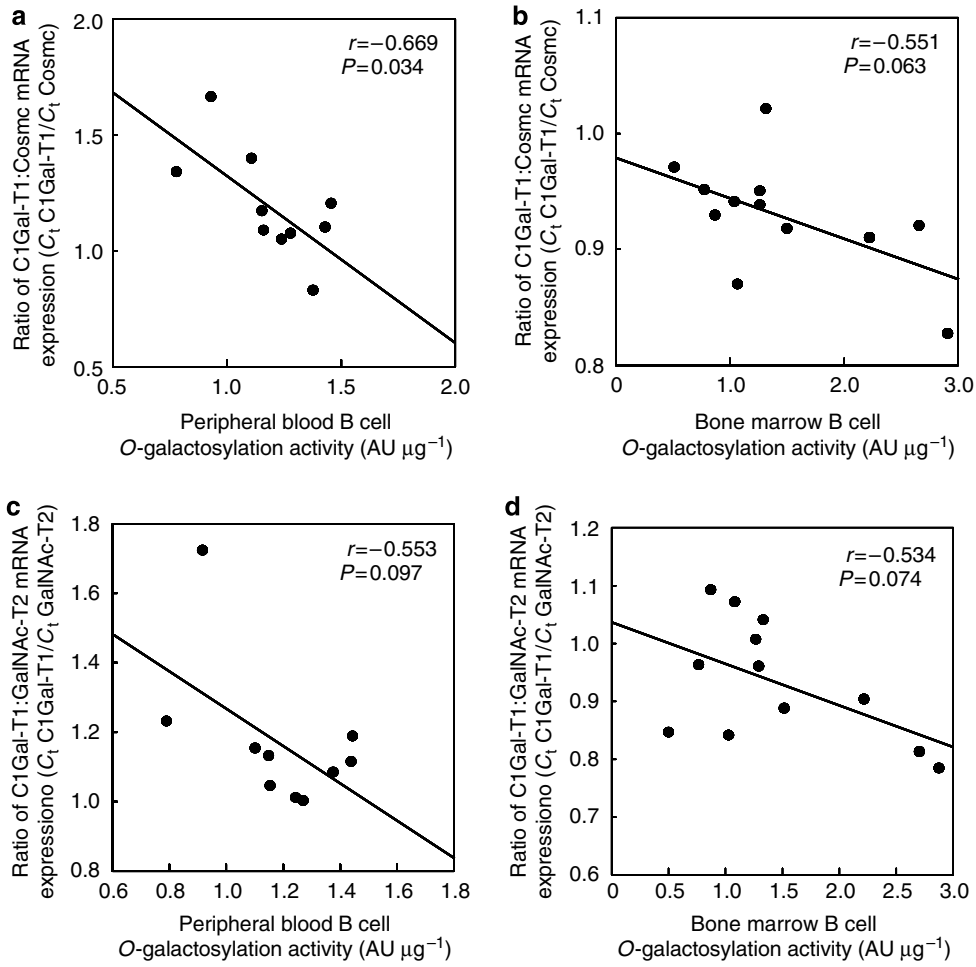

Figure 4 | B-cell *O*-galactosylation activity and galactosyltransferase gene expression ratios in IgAN. The mRNA expression of GalNAc-T2, C1Gal-T1, and Cosmc in peripheral blood (PB) and bone marrow (BM) lysates was measured by real-time PCR. The results were expressed as ratios of the threshold cycle (C_t) values obtained for each gene, as the two enzymes and Cosmc are functionally related and balanced availability of each is required for the assembly of fully galactosylated Core-1 *O*-glycans. In IgAN (closed circles), a negative relationship was observed between the ratios of C1Gal-T1:Cosmc (**a** and **b**) and C1Gal-T1:GalNAc (**c** and **d**) and B-cell *O*-galactosylation activity in both PB and BM. As C_t is inversely proportional to gene expression, this indicates a positive relationship between C1Gal-T1 gene expression and *O*-galactosylation activity. Although most of these correlations did not reach statistical significance, their consistent pattern implies a biologically significant association. No such relationship between gene expression ratios and *O*-galactosylation activity was observed in the controls.

when IgA1 is undergalactosylated,[32] leading to increased exposure of terminal GalNAc moieties. This observation confirmed that these patients displayed the usual *O*-glycosylation defect seen in IgAN and were a representative group of subjects for this work. The relationship between IgA1 lectin binding and C1Gal-T1/Cosmc *O*-galactosylation activity differed in patients and controls, as is logical if abnormal IgA1 *O*-galactosylation in IgAN arises from alterations in intracellular processing. In the controls, lectin binding showed a significant inverse correlation with enzyme activity in both PB and BM B cells. This is as expected, as the higher the *O*-galactosylation activity, the more galactosylated the *O*-glycans of IgA1 will be, and therefore the fewer ungalactosylated moieties will be available for HA binding. This correlation supports the validity of the assays used in this study for both the enzyme activity and lectin binding. However, this inverse relationship was not seen in IgAN. There was no correlation between HA lectin binding to serum IgA1 and PB B-cell *O*-galactosylation activity in the patients, but there was a positive correlation with BM B-cell activity. This suggests that in the BM, some factors other than the availability or activity of C1Gal-T1 and/or Cosmc underlie reduced *O*-galactosylation of IgA1.

In the controls, there was no correlation between the *O*-galactosylation activity of PB and BM B cells, suggesting that the two populations are distinct from one another in this respect. However, in IgAN, there was a significant positive correlation between B cells from these two sources, indicating a link, such as a common origin or involvement in the same type of immune activity. This is interesting in light of other studies from our group, which suggest that the 'abnormal' *O*-glycosylation pattern of IgA1 in IgAN actually represents a normal *O*-glycoform that is usually found in mucosal but not in systemic IgA1.[33] In controls, the majority of circulating

IgA1 B cells are trafficking to the mucosa, whereas those resident in the BM are systemic: these are different populations. In IgAN, the BM contains increased numbers of plasma cells producing IgA1 that contains J chain and is therefore polymeric,[23] another mucosal feature. The relationship between circulating and BM B cells observed here may be a further indicator of the abnormal presence of mucosal-type B cells in the BM in IgAN.

We found no difference between IgAN and controls in the *O*-galactosylation activity of either PB or BM B cells in this study. In agreement with the above discussion, this suggests that some factors other than absolute levels of C1Gal-T1/Cosmc activity are responsible for abnormally low *O*-galactosylation of IgA1 in patients with IgAN. This finding conflicts with our previously published results,[20] which did suggest that *O*-galactosylation activity was reduced in PB B cells in IgAN. This reason for this discrepancy is not clear, but may be a consequence of the more accurate assay system used here.

To assess mRNA expression, we used ratios of the different genes to one another. Absolute gene expression levels are irrelevant as they vary with cell number, type, and activity in each sample, and there is no suitable housekeeping gene for such experiments. However, the three genes examined here are functionally related to one another, as they are involved in the sequential construction of the Core-1 *O*-glycan chains. For a given degree of galactosylation, the ratios C1Gal-T1:GalNAc-T2 and C1Gal-T1:Cosmc should remain constant. Low expression of C1Gal-T1 in relation to that of GalNAc-T2 would be expected to result in reduced galactosylation of the *O*-glycans. The same effect would occur with relative underexpression of Cosmc as compared to C1Gal-T1, as this chaperone protein is essential for the activity of C1Gal-T1. Therefore, if reduced availability of either C1Gal-T1 or Cosmc is responsible for the under-galactosylation of IgA1 in IgAN, we would expect to find this reflected in the gene expression ratios. However, we found no difference between IgAN and controls in the relative expression of C1Gal-T1 and either GalNAc-T2 or Cosmc in PB or BM, arguing against a fundamental imbalance in the expression of the genes encoding these *O*-galactosylating proteins in the patients.

In IgAN PB cells, there was a significant negative correlation between the ratio C1Gal-T1:Cosmc and B-cell *O*-galactosylation activity (Figure 4a). As C_t is inversely proportional to gene expression, this indicates that low enzyme activity may be associated with low C1Gal-T1 gene expression in relation to Cosmc; that is, either C1Gal-T1 is low or Cosmc is high. The same relationship was seen in the BM in IgAN, although this correlation did not reach statistical significance (Figure 4b). In both PB and BM, the ratio C1Gal-T1:GalNAc-T2 also showed a non-significant trend toward a negative correlation with C1Gal-T1 activity (Figure 4c and d), suggesting that C1Gal-T1 expression is low in relation to GalNAc-T2. Although these correlations were not statistically significant, their consistent pattern suggests that they may indicate a biologically significant imbalance in C1Gal-T1 expression compared to the other two genes. As the ratio of Cosmc:GalNAcT-2 did not correlate with the C1Gal-T1 expression at all, relatively low C1Gal-T1 expression rather than raised Cosmc or GalNAc-T2 expression is most likely to underlie the above correlations.

The results of this study show no absolute deficiency in B-cell C1Gal-T1/Cosmc-catalyzed *O*-galactosylation activity or the expression of *O*-galactosylating enzymes in IgAN that might account for abnormal IgA1 *O*-galactosylation. However, we did find a number of subtle imbalances in the pattern of C1Gal-T1/Cosmc activity and expression in the patients. The *O*-galactosylation activity in control B cells showed a positive correlation with the degree of *O*-galactosylation (inverse to HA lectin binding), as would be expected. However, the opposite was found in BM B cells in IgAN, suggesting that some factors other than C1Gal-T1 activity dictate the degree of IgA1 *O*-galactosylation in IgAN. Although the availability of Cosmc is a possible limiting factor, this did not appear to be the case in our study: the *O*-galactosylation activity correlated with the C1Gal-T1 gene expression rather than with that of Cosmc. Overall, our results suggest that abnormal *O*-glycosylation in IgAN is not due to an *O*-galactosyltransferase or Cosmc deficiency or defect, although we cannot exclude the possibility that a minor subpopulation of B cells, not detected in these experiments, is abnormal in this regard. This hypothesis is consistent with our previous reports in which we found that in both IgAN and controls, the pattern of *O*-glycosylation varies at different stages of B-cell maturation,[22] and also in different immune responses,[33] indicating that the *O*-glycosylation profile of IgA1 is influenced by control mechanisms related to the immunological situation in which it is produced. Patients are able to produce IgA1 with a range of galactosylation profiles, and it seems likely that the presence of hypogalactosylated IgA1 in the serum in IgAN is a reflection of aberrant immune activity rather than a fundamental defect in *O*-glycosylation capacity. Future work should focus on the factors controlling the profile of IgA1 *O*-glycosylation in different immunological situations.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all consumables were from Sigma Chemical Co. (Poole, UK).

Subjects and samples

Twelve patients with IgAN (10 males, median age 39 years, range 29–69 years; details shown in Table 3) and 13 matched controls undergoing elective orthopedic surgery (7 males, median age 37 years, range 17–75 years) were recruited. All patients had biopsy-proven IgAN with microscopic hematuria and/or proteinuria less than 1.4 g per 24 h. At the time of the study, no subject had an intercurrent illness, and none of the IgAN patients had macroscopic hematuria. The median serum creatinine in the IgAN patients was 105 $\mu mol\,l^{-1}$ (range 58–162), and the median time from renal biopsy was 3 years (range 0.5–8). No subject was receiving

Table 3 | Patient details

Patient no.	Sex	Age	Serum creatinine	BP (mm Hg)	BP Rx: no. of drugs	ACEI or ARB	Proteinuria	Hematuria
				Patients				
1	M	50	87	No other information—notes lost				
2	F	32	78	110/70	0	No	0	3+
3	M	69	153	140/75	2	No	0	0
4	M	51	96	146/100	1	No	1+	0
5	M	63	161	170/95	4	Yes	2+	3+
6	M	52	96	125/80	0	No	0	0
7	M	29	103	130/100	1	No	2+	3+
8	F	39	69	138/84	0	No	1+	3+
9	M	39	103	140/90	0	No	2+	0
10	M	36	93	128/78	0	No	2+	3+
11	M	40	468	156/100	1	No	2+	3+
12	M	34	101	120/80	1	Yes	3+	3+

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BP, blood pressure; F, female; M, male.

immunosuppressive treatments at the time of the study or in the previous 24 months. BM aspirates from the posterior iliac crest and 60 ml venous blood were collected at the same time. The study was approved by the LNR Research Ethics Committee, and all subjects gave written informed consent.

IgA1 lectin binding

The binding of the *O*-glycan-specific lectin *Helix aspersa* (HA) to plasma IgA1 was measured by modified enzyme-linked immunosorbent assay, exactly as described previously.[22] All samples were run in a single assay to allow direct comparison.

Preparation of B-cell lysates for C1Gal-T1 assay

Plasma was separated from the blood samples and stored at −80 °C until required, and the remaining PB cells resuspended in Hank's balanced salt solution (HBSS). BM aspirates were diluted with an equal volume of HBSS and vortexed to break up cell clumps. Mononuclear cells were isolated from PB and BM by density gradient centrifugation over Ficoll 400 (density 1.077), washed in HBSS, and resuspended in culture medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU ml^{-1} penicillin, and 100 µg ml^{-1} streptomycin). The cells were then kept overnight at 37 °C in a sterile 5% CO_2 humid atmosphere.

B lymphocytes were separated from the BM and PB mononuclear cell samples by positive selection on CD19 magnetic beads (Dynabeads; Invitrogen, Paisley, UK) following the manufacturer's instructions. Purity of the recovered cell populations was assessed by morphological examination and immunohistochemical staining for surface markers. Finally, the B cells were lysed in 2% Triton X-100 in Tris-buffered saline.

Two different human monocyte cell lines, THP-1 (ECACC no: 88081201) and U937 (ATCC CRL 1593), were used as a standard and an internal control in the C1Gal-T1 assay. These were maintained in standard culture conditions in culture medium as above, feeding and passaging as necessary for consistent, healthy cell growth. When required for the assay, the cells were washed in protein-free HBSS and lysed in 2% Triton X-100/Tris-buffered saline.

The total protein concentration of the B-cell lysates was measured using a commercial kit (DC Protein Assay; Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's instructions.

Preparation of asialo-OSM acceptor for C1Gal-T1/Cosmc assay

Sialyl-OSM (sOSM) (derived from homogenized sheep submaxillary salivary glands) was kindly donated by Dr Tony Corfield (University of Bristol, UK). Desialylation was achieved by three sequential mild acid hydrolyses in 0.1 M HCl at 80 °C, followed by neutralization with 1 M NaOH. After dialyzing against ultrapure water, the acceptor was lyophilized and stored at 4 °C. This acceptor preparation was evaluated against an equivalent prepared, characterized, and routinely used by Dr Corfield, and was shown to perform satisfactorily in preliminary assays, the two acceptors incorporating equivalent amounts of (^{14}C)Gal when incubated with the same lysate samples.

C1Gal-T1/Cosmc assay

The *O*-galactosylation activity measured in this assay relies on the function of both C1Gal-T1 and its chaperone protein Cosmc. The assay was carried out in 75 µl volumes. A volume of 50 µl of substrate solution (50 mM Tris (pH 7.0) containing 2 mg ml^{-1} asialyl OSM (aOSM), 20 mM $MnCl_2$, 1% Triton X-100, 0.25 mM UDP-Gal, and 10 µl ml^{-1} UDP(^{14}C)Gal) was added to 25 µl of cell lysate in 1.5 ml polypropylene tubes and vortexed. After 1 h of incubation at 37 °C, the reaction was quenched by the addition of 500 µl of phosphotungstic acid/trichloroacetic acid (5%/15% (v/v)). After cooling at 4 °C for 30 min, the mixture was centrifuged at 14 000 *g* and the supernatant discarded. The product was washed twice in 1 ml 95% ethanol, drained, dissolved in 500 µl of 0.2 M KOH, and transferred to scintillation vials containing 2 ml Ecoscint A scintillation fluid (National Diagnostics, Atlanta, GA, USA). After the chemiluminescence had faded, scintillation counting was performed with ^{226}Ra external standardization and quench correction. The results were expressed in disintegrations per minute (DPM).

Each test lysate sample was run in duplicate. A standard curve of eight serial dilutions of U937 cell lysate, and internal controls of three dilutions of THP-1 lysate and one blank were also set up each time the assay was performed.

The U937 lysate protein was arbitrarily assigned an enzyme activity of 1 arbitrary unit/µg total protein (1 AU $µg^{-1}$), and standard curves constructed from which the *O*-galactosylation activity of both the test and the internal control lysates were read and expressed as AUµg total lysate protein.

Table 4 | PCR primers

GalNAc-T2	Forward	ATCCAGCCAGTTGGTGCTAC
	Reverse	TAACCGTCTCCCAGGTTCAC
C1Gal-T1	Forward	TCCAGAGATACCATTGGAAAA
	Reverse	CAAGATCAGAGCAGCAACCA
Cosmc	Forward	TTCCTCCTTCCATACCCACA
	Reverse	GCCCCACTACGTTTGCTATC

C1Gal-T1, Core-1 β1-3 galactosyltransferase-1; Cosmc, Core-1-β3-Gal-T-specific molecular chaperone; GalNAc-T2, UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase-2.

O-glycosyltransferase gene expression

RNA extraction and cDNA synthesis. Total cellular RNA was extracted from PB mononuclear cells and BM aspirates using RNAqueous-4 PCR (Ambion, Austin, TX, USA). The optional DNase 1 and DNase inactivation steps were included to remove any residual genomic DNA. cDNA was synthesized from the RNA using Reverse Transcriptase System (Promega, Southampton, UK), using random primers.

Real-time PCR. cDNA sequences were amplified in an MX4000 thermocycler (Stratagene, Amsterdam, The Netherlands) using primers detailed in Table 4. A volume of 50 µl amplification reaction mixes containing 45 mM Tris-HCl (pH 8.8), 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 20 µM each of dATP, dCTP, dTTP, dGTP, 0.11 mg ml^{-1} bovine serum albumin, 6.7 mM β-mercaptoethanol, 0.44 µM Na_2EDTA, 75 pM passive reference dye (Stratagene), SYBR Green I (Invitrogen) diluted 1:30 000, 1 U Jumpstart Taq polymerase and 10 pmol forward and reverse primers were used. The reactions were run in for 1 cycle of 10 min at 95 °C and then for 40 cycles of 30 s at 95 °C, 1 min at 62 °C (1,3 C1Gal-T1) or 65 °C (GalNAc-T2 and Cosmc), and 30 s at 72 °C Each reaction was run in duplicate. The results were obtained as C_t at which a standard fluorescence level (set to 0.1) was reached in each reaction. The higher the expression of each gene in the original sample, the lower the cycle number at which this threshold is reached, or in other words, a high gene expression gives a low C_t value and vice versa. Results were expressed as ratios of the C_t values of the genes to one another. As GalNAc is the core sugar to which the Gal is subsequently added, we expressed C1Gal-T1 in terms of GalNAc-T2, that is, C1Gal-T1:GalNAc-T2. The activity of C1Gal-T1 requires the chaperone protein Cosmc, and therefore we calculated the ratio C1Gal-T1:Cosmc.

Statistical analysis. Lectin binding results of serum IgA1, and *O*-galactosylation activity and gene expression ratios of cell lysates from IgAN and controls were compared by unpaired Student's *t*-tests. The *O*-galactosylation activity of PB and BM B cells from the same subjects was compared by paired *t*-tests. Linear regression analysis was used to investigate the relationships between B-cell *O*-galactosylation activity, gene expression, and IgA1 lectin binding. Results quoted in the text are mean values of groups, ± s.e.m.

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REFERENCES

1. Barratt J, Feehally J, Smith AC. Pathogenesis of IgA nephropathy. *Semin Nephrol* 2004; **24**: 197–217.
2. Kerr MA. The structure and function of human IgA. *Biochem J* 1990; **271**: 285–296.
3. Iwasaki H, Zhang Y, Tachibana K *et al.* Initiation of *O*-glycan synthesis in IgA1 hinge region is determined by a single enzyme, UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 2. *J Biol Chem* 2003; **278**: 5613–5621.
4. Ju T, Brewer K, D'Souza A *et al.* Cloning and expression of human core 1 beta1,3-galactosyltransferase. *J Biol Chem* 2002; **277**: 178–186.
5. Ju T, Cummings RD. A unique molecular chaperone Cosmc required for activity of the mammalian core 1 beta 3-galactosyltransferase. *Proc Natl Acad Sci USA* 2002; **99**: 16613–16618.
6. Andre PM, Le Pogamp P, Chevet D. Impairment of jacalin binding to serum IgA in IgA nephropathy. *J Clin Lab Anal* 1990; **4**: 115–119.
7. Allen AC, Harper SJ, Feehally J. Galactosylation of *N*- and *O*-linked carbohydrate moieties of IgA1 and IgG in IgA nephropathy. *Clin Exp Immunol* 1995; **100**: 470–474.
8. Hiki Y, Iwase H, Saitoh M *et al.* Reactivity of glomerular and serum IgA1 to jacalin in IgA nephropathy. *Nephron* 1996; **72**: 429–435.
9. Odani H, Hiki Y, Takahashi M *et al.* Direct evidence for decreased sialylation and galactosylation of human serum IgA1 Fc *O*-glycosylated hinge peptides in IgA nephropathy by mass spectrometry. *Biochem Biophys Res Commun* 2000; **271**: 268–274.
10. Tomana M, Matousovic K, Julian BA *et al.* Galactose-deficient IgA1 in sera of IgA nephropathy patients is present in complexes with IgG. *Kidney Int* 1997; **52**: 509–516.
11. Kokubo T, Hiki Y, Iwase H *et al.* Protective role of IgA1 glycans against IgA1 self-aggregation and adhesion to extracellular matrix proteins. *J Am Soc Nephrol* 1998; **9**: 2048–2054.
12. Hiki Y, Kokubo T, Iwase H *et al.* Underglycosylation of IgA1 hinge plays a certain role for its glomerular deposition in IgA nephropathy. *J Am Soc Nephrol* 1999; **10**: 760–769.
13. Amore A, Cirina P, Conti G *et al.* Glycosylation of circulating IgA in patients with IgA nephropathy modulates proliferation and apoptosis of mesangial cells. *J Am Soc Nephrol* 2001; **12**: 1862–1871.
14. Allen AC, Bailey EM, Brenchley PE *et al.* Mesangial IgA1 in IgA nephropathy exhibits aberrant *O*-glycosylation: observations in three patients. *Kidney Int* 2001; **60**: 969–973.
15. Xu LX, Zhao MH. Aberrantly glycosylated serum IgA1 are closely associated with pathologic phenotypes of IgA nephropathy. *Kidney Int* 2005; **68**: 167–172.
16. Novak J, Moldoveanu Z, Renfrow MB *et al.* IgA nephropathy and Henoch-Schoenlein purpura nephritis: aberrant glycosylation of IgA1, formation of IgA1-containing immune complexes, and activation of mesangial cells. *Contrib Nephrol* 2007; **157**: 134–138.
17. Moldoveanu Z, Wyatt RJ, Lee JY *et al.* Patients with IgA nephropathy have increased serum galactose-deficient IgA1 levels. *Kidney Int* 2007; **71**: 1148–1154.
18. Allen AC, Bailey EM, Barratt J *et al.* Analysis of IgA1 *O*-glycans in IgA nephropathy by fluorophore-assisted carbohydrate electrophoresis. *J Am Soc Nephrol* 1999; **10**: 1763–1771.
19. Berger EG. Tn syndrome. *Biochim Biophys Acta* 1999; **1455**: 255–268.
20. Allen AC, Topham PS, Harper SJ *et al.* Leucocyte beta 1,3 galactosyltransferase activity in IgA nephropathy. *Nephrol Dial Transplant* 1997; **12**: 701–706.
21. Qin W, Zhou Q, Yang LC *et al.* Peripheral B lymphocyte beta1,3-galactosyltransferase and chaperone expression in immunoglobulin A nephropathy. *J Intern Med* 2005; **258**: 467–477.
22. Smith AC, de Wolff JF, Molyneux K *et al.* *O*-glycosylation of serum IgD in IgA nephropathy. *J Am Soc Nephrol* 2006; **17**: 1192–1199.
23. Harper SJ, Pringle JH, Wicks AC *et al.* Expression of J chain mRNA in duodenal IgA plasma cells in IgA nephropathy. *Kidney Int* 1994; **45**: 836–844.
24. de Fijter JW, Eijgenraam JW, Braam CA *et al.* Deficient IgA1 immune response to nasal cholera toxin subunit B in primary IgA nephropathy. *Kidney Int* 1996; **50**: 952–961.

25. van den Wall Bake AW, Daha MR, Haaijman JJ *et al.* Elevated production of polymeric and monomeric IgA1 by the bone marrow in IgA nephropathy. *Kidney Int* 1989; **35**: 1400–1404.
26. Harper SJ, Allen AC, Pringle JH *et al.* Increased dimeric IgA producing B cells in the bone marrow in IgA nephropathy determined by *in situ* hybridisation for J chain mRNA. *J Clin Pathol* 1996; **49**: 38–42.
27. Harper SJ, Allen AC, Bene MC *et al.* Increased dimeric IgA-producing B cells in tonsils in IgA nephropathy determined by *in situ* hybridization for J chain mRNA. *Clin Exp Immunol* 1995; **101**: 442–448.
28. Hiki Y, Odani H, Takahashi M *et al.* Mass spectrometry proves under-*O*-glycosylation of glomerular IgA1 in IgA nephropathy. *Kidney Int* 2001; **59**: 1077–1085.
29. Mestecky J, Tomana M, Crowley-Nowick PA *et al.* Defective galactosylation and clearance of IgA1 molecules as a possible etiopathogenic factor in IgA nephropathy. *Contrib Nephrol* 1993; **104**: 172–182.
30. Inoue T, Sugiyama H, Kikumoto Y *et al.* Downregulation of the beta1,3-galactosyltransferase gene in tonsillar B lymphocytes and aberrant lectin binding to tonsillar IgA as a pathogenesis of IgA nephropathy. *Contrib Nephrol* 2007; **157**: 120–124.
31. King MJ, Chan A, Roe R *et al.* Two different glycosyltransferase defects that result in GalNAcα-*O*-peptide (Tn) expression. *Glycobiology* 1994; **4**: 267–279.
32. Moore JS, Kulhavy R, Tomana M *et al.* Reactivities of *N*-acetylgalactosamine-specific lectins with human IgA1 proteins. *Mol Immunol* 2007; **44**: 2598–2604.
33. Smith AC, Molyneux K, Feehally J *et al.* *O*-glycosylation of serum IgA1 antibodies against mucosal and systemic antigens in IgA nephropathy. *J Am Soc Nephrol* 2006; **17**: 3520–3528.