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Characterization of a double-CRD-mutated Gal-8 recombinant protein that retains co-stimulatory activity on antigen-specific T-cell response

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Galectins (Gals) constitute a family of mammalian lectins with affinity for β-galactosides, characterized by the presence of conserved CRDs (carbohydrate-recognition domains). We have found previously that Gal-8, from the tandem-repeat group with two linked CRDs, exerts two separate actions on $CD4^+$ T-cells: antigen-independent proliferation and, at lower concentration, antigen-specific co-stimulation. Whereas proliferation can be ascribed to the pro-inflammatory role of Gal-8, the co-stimulatory activity of borderline T-cell-specific responses allows the proposal of Gal-8 as an adjuvant in vaccination. To study the relevance of glycan–lectin interaction on these T-cell activities, we generated a double-mutated protein (Gal-8mut) by replacing canonical arginine residues on each CRD, so to abolish sugar-binding capacity. As expected, Gal-8mut was unable to bind to lactosyl-Sepharose, confirming that lactose recognition was precluded; however, preservation of lectin activity was still evident since Gal-8mut displayed haemoagglutinatory effects and binding capacity to the T-cell surface. To search for glycan affinity, a glycan microarray analysis was conducted which revealed that Gal-8mut lost most low- and intermediate-, but retained high-, affinity interactions, mainly to polylactosamines and blood group antigens. These findings were supported further by molecular modelling. Regarding biological activity, Gal-8mut was unable to induce T-cell proliferation, but efficiently co-stimulated antigen-specific responses, both *in vitro* and *in vivo*. Therefore Gal-8mut represents a useful tool to dissect the specificities of lectin–glycan interactions underlying distinctive Gal-8 activities on T-cell biology. Moreover, given its distinguishing properties, Gal-8mut could be used to enhance borderline immune responses without the non-specific pro-inflammatory activity or other potential adverse effects.

Key words: galectin, glycan affinity, inflammation, T-cell proliferation, T-cell receptor co-stimulation.

INTRODUCTION

Galectins (Gals) constitute a family of secreted mammalian lectins with affinity for β-galactosides that bind glycoreceptors on target cells and induce multiple biological activities [1]. Gals are characterized by the presence of conserved CRDs (carbohydrate-recognition domains) and are classified as prototype (one CRD, such as Gal-1, -2 and -7), tandem-repeat (two linked CRDs, such as Gal-4, -8 and -9) and chimaera (one CRD fused to a non-lectin domain, Gal-3). A special feature of the galectin family is the involvement of many members, mainly Gal-1, -3, -8 and -9, in several aspects of the immune response such as inflammation, tumour escape, pathogen elimination and tolerance induction [2–4].

Gal-8, from the tandem-repeat group, is intrinsically a heterodimer because it has an N-terminal CRD (N-CRD) and a C-terminal CRD (C-CRD) joined by a linker peptide of variable length which defines the isoforms among species [5,6]. Gal-8 is widely expressed in many normal tissues and endothelium, as well as in pathological conditions such as inflamed synovia and tumours [7,8]. Given its particular heterodimeric structure, Gal-8 mediates many cellular processes such as cell–cell and cell–matrix adhesion [9]. Regarding the immune response, we have found that Gal-8 exerts two defined actions on naïve $CD4^+$ T-cells: it induces strong proliferation in the absence of antigen, whereas at lower concentrations, it co-stimulates T-cells in the presence of antigen-presenting cells and the corresponding antigen [10]. Besides T-cell activation, the pro-inflammatory role of exogenous Gal-8 was largely evident on different cellular contexts, such as neutrophils and platelets [11,12]; and more recently in the endothelium [13]. Regarding the innate immune response, it was reported that Gal-8 directly killed blood B-group-bearing bacteria [14], and, moreover, Thurston et al. [15] demonstrated the participation of endogenous Gal-8 in the clearance of damaged bacteria-containing vacuoles by autophagy.

Our group has studied the molecular requirements for both Gal-8 co-stimulatory and proliferative effects on mouse and human T-cells [16]. We have observed that the C-CRD is the principal domain involved in both effects and that the tandem structure is essential for the antigen-independent proliferative effect, but not for co-stimulation induction. In fact, both N- and C-CRD isolated domains were able to exert the co-stimulatory effect. In agreement, we have found that Gal-1, from the prototype group with a single CRD, shared with Gal-8 the co-stimulatory, but not the proliferative, activity [17]. However, when two Gal-1 CRDs were fused with Gal-8 linker peptide (Gal-1-8-1), the chimaeric

Abbreviations: C-CRD, C-terminal CRD; CRD, carbohydrate-recognition domain; Gal, galectin; Gal-Ara, 3-*O*-β-D-galactopyranosyl-D-arabinose; HRP, horseradish peroxidase; IL, interleukin; i.p., intraperitoneally; N-CRD, N-terminal CRD; OVA, ovalbumin; RFU, relative fluorescent unit(s); TDG, thiodigalactoside.

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protein readily induced proliferation. On the other hand, Gal-3 from the chimaera type with a CRD and a non-lectin domain not only was unable to induce antigen-specific co-stimulation, but also antagonized Gal-1 and Gal-8 co-stimulatory effects.

Gal-8 glycan preferences are well documented: whereas Gal-8 N-CRD displays affinity for sialylated and sulfated glycans, the C-CRD prefers blood antigens and poly-*N*-acetyl-lactosamine [18–20]. The presence of sugar residues such as lactose or its derivative TDG (thiodigalactoside) inhibits the Gal-8 proliferative effect almost completely, demonstrating the participation of lectin–glycan interaction in this activity. In contrast, co-stimulation is only partially inhibited in the presence of these sugars, but is readily prevented by other sugar compounds such as Gal-Ara (3-*O*-β-D-galactopyranosyl-D-arabinose) or Galα1–3[Fucα1–2]Galβ1 – 4GlcNAcβ (a blood group B antigen which is recognized by the C-CRD), suggesting the involvement of higher-affinity lectin–glycan interactions [16]. At present, we are particularly focused on understanding the antigen-specific T-cell response co-stimulation mechanism induced by Gal-8 *in vivo*, given the potential use of this lectin as an adjuvant to stimulate borderline immune responses. An interesting aspect to analyse is the actual involvement of lectin–glycan interaction in Gal-8-induced co-stimulation; however, sugar inhibitors should not be used *in vivo* as they could be metabolized or result in toxicity, and, besides, they are non-specific as they can also inhibit Gal-1 and Gal-3 effects. Therefore, owing to the physiological concerns in the use of sugar compounds as inhibitors *in vivo*, along with the fact that sugar addition could affect other endogenous galectins, the aim of the present study was to generate and characterize a Gal-8 protein with altered sugar-recognition ability as a tool to study the participation in the lectin–glycan interaction.

EXPERIMENTAL

Recombinant proteins and reagents

Gal-8mut was designed by replacing two arginine residues (Arg^{68}xHis and Arg^{241}xHis) from mouse Gal-8L isoform in a synthetic gene construction (Genscript). For protein expression, the same conditions described previously for Gal-8 were followed [6], except for purification, where two consecutive rounds of immobilized metal-affinity chromatography (GE Healthcare) were performed, owing to the lack of lactose-binding capacity of the mutated protein. Purity was checked by SDS/PAGE, and protein concentration was assessed using the Bradford method (Thermo Scientific). Imidazole (Sigma) was eliminated from the elution buffer by dialysis, and 10 % (v/v) glycerol (Sigma) was added in order to stabilize Gal-8mut protein. Recombinant mouse Gal-8L isoform (Gal-8) was obtained as described previously [6], and resuspended in the same final conditions as Gal-8mut. Lectin activity of these proteins was tested using haemoagglutination, as described in [6]. Lactose and Gal-Ara were from Sigma. 3′-Sialyl-lactose was from Carbosynth. Galα1–3[Fucα1–2]Galβ1–3GlcNAcβ (β tetra type 1), GalNAcα1–3[Fucα1–2]Galβ1–4GlcNAcβ (α tetra type 2) and Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ (Tri-LN) were obtained from the Consortium For Functional Glycomics (La Jolla, CA, U.S.A.).

Mice, cell lines and cell purification

C57BL/6J and C.Cg-Tg(DO11.10)10Dlo/J (DO11.10) breeding pairs were obtained from The Jackson Laboratory (Bar Harbor, ME, U.S.A.) and bred in our facilities. DO11.10 $CD4^+$ T-cell hybridoma was kindly provided by Dr P. Marrack (Howard Hughes Medical Center, Denver, CO, U.S.A.). Hybridoma cells were maintained at 37 °C in 5 % CO_2 in RPMI 1640 medium in the presence of 10 % FBS (Invitrogen), 2 mM glutamine and 5 μg/ml gentamicin. For mouse splenocyte purification, spleens from 4–8-weeks-old animals were removed and disrupted against a stainless steel mesh in RPMI 1640 medium. The cell suspension was washed and incubated with RBC lysis buffer (Sigma) and washed again with medium. MiniMacs columns and anti-CD4-coupled paramagnetic particles (Miltenyi Biotec) were used for $CD4^+$ T-cell purification, following the manufacturer's instructions. Cell purity (>90 %) was checked by flow cytometry. All procedures involving animals were approved by the Ethical Committee Board of the Universidad Nacional de San Martín.

Lactosyl-Sepharose pull-down assay

To determine the lactose-binding capacity of Gal-8mut, 100 μl of lactosyl-Sepharose (Sigma) was incubated with 25 μg of Gal-8 or Gal-8mut for 30 min at 4 °C, with shaking. Then, samples were centrifuged at 400 ***g*** for 5 min, and supernatants were kept (flowthrough). After washing three times with 1 ml of PBS, the resin was incubated with 100 μl of PBS plus 200 mM lactose for 15 min at room temperature and centrifuged. Supernatants were recovered (eluate), resolved by SDS/PAGE (10 % gel) and stained with Coomassie Blue (Sigma).

CD spectroscopy

CD spectra were recorded in the far-UV (200–260 nm) region using a Jasco J-815 spectropolarimeter equipped with a Peltier temperature-control system, using a 1-mm-pathlength quartz cell and the indicated protein concentrations. A scanning speed of 50 nm/min and a spectral bandwidth of 2 nm were set. For each sample, six spectra were recorded and the results are presented as the average spectra to reduce background noise. Data were collected at 25 °C in 10 mM Tris/HCl and 500 mM NaCl, pH 7.6. Thermal stability of the proteins was measured by heating the samples at a rate of 1 °C/min in a 1-mm-pathlength quartz cell and following the CD signal a 217 nm. CD signals were normalized and the melting temperature (T_m) was calculated by fitting the following equation to the normalized signal:

$$\mathrm{CD}(T) = (I_N + b_N.T) + (I_U + b_U.T) / \left[1 + \exp\left(-(T - T_m)/v\right)\right]$$

where I_N and I_U are the normalized CD signals of the native and unfolded states a 0 °C respectively, b_N and b_U is the thermal dependence of these signals, and v is a geometric factor that accounts for the co-operativity of the curves.

Gal-8 and Gal-8mut co-immunoprecipitation

DO11.10 hybridoma T-cells (25×10^6) were biotinylated with sulfo-NHS-LC-biotin [sulfosuccinimidyl-6-(biotinamido) hexanoate] (Thermo Scientific), following the manufacturer's protocol. Biotinylated cells were reacted with 0.1 μM Gal-8 or Gal-8mut for 30 min on ice and washed with PBS. Then, cells were resuspended in 1 ml of lysis buffer [20 mM Tris/HCl, pH 7.6, 1× protease inhibitor cocktail (Sigma), 1 mM PMSF (Sigma), 2 mM EDTA, 150 mM NaCl and 1 % Triton X-100].

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After 30 min of incubation on ice, cell lysates were centrifuged at 16000 ***g*** for 20 min at 4 °C and supernatants were kept at − 20 °C. For co-immunoprecipitation assay, 30 μl of Protein A–Sepharose (GE Healthcare) was incubated in the presence of 4 μg of rabbit affinity-purified anti-Gal-8 or normal IgG (control) in 1 ml of PBS at 4 °C with shaking. After washing, resin was incubated with 1 ml of biotinylated cellular extracts at 4 °C overnight with shaking and centrifuged, and a fraction of the supernatant was kept (input). After extensive washing, resin was incubated for 30 min with 100 μl of ice-cold PBS plus 200 mM lactose and centrifuged, and supernatant was recovered (lactose-elution). Then, 100 μl of 1× cracking buffer was added to the resin, boiled for 5 min at 100 °C and centrifuged, and supernatant was recovered (cracking-elution). Eluates were resolved by SDS/PAGE (7.5 %) and transferred on to a PVDF membrane (GE Healthcare). The blot was probed with a 1:5000 dilution of HRP (horseradish peroxidase)–avidin (Biolegend) and developed by chemiluminescence using SuperSignal West Pico substrate (Thermo Scientific).

Determination of anti-Gal-8 affinity

Maxisorp plates (Nunc) were seeded with 500 ng of Gal-8 or Gal-8mut per well in 100 μl of TBS (50 mM Tris/HCl and 150 mM NaCl, pH 7.6) at 4 °C overnight. Then, wells were washed four times with 200 μl of TBS and 0.05 % Tween 20 (Sigma), blocked with TBS plus 5 % dried non-fat skimmed milk powder for 1 h at 37 °C and washed. Dilutions (1:100, 1:500, 1:1000 and 1:5000) of homemade polyclonal rabbit affinity-purified IgG anti-Gal-8 were added and incubated for 1 h at room temperature. Wells were washed and incubated with 100 μl of 1:5000 dilution of HRP-conjugated anti-rabbit IgG (Sigma). After 1 h, wells were washed and revealed with 100 μl of TMB (3,3′,5,5′-tetramethylbenzidine) (Sigma) plus H_2O_2. Colour reaction was stopped with 100 μl of 0.2 M H_2SO_4, and absorbance was read at 450 nm in FilterMax F5 Microplate Reader (Molecular Devices).

Galectin-binding assays

CD4$^+$ T-cells (10^6) were resuspended in 100 μl of ice-cold PBS containing sodium azide and treated with 0.1 μM Gal-8 or Gal-8mut, washed with ice-cold PBS or PBS plus sugar compound and blocked with anti-Fc receptors (Becton Dickinson). After incubation with rabbit anti-Gal-8 followed by Alexa Fluor® 488-conjugated anti-rabbit IgG antibodies (Invitrogen), cells were fixed in 2 % (w/v) paraformaldehyde in PBS and analysed by flow cytometry. Cell samples that were processed without recombinant galectin were included as a negative control.

Glycan microarrays

Version 5.1 of microarray from the Consortium For Functional Glycomics containing 610 glycans printed in replicates of six were used. The highest and lowest point from each set of six replicates has been removed from the analysis to eliminate false hits that contain a single very high or low point. Also, points with high %CV (coefficient of variation) (>20) were not further considered. Glycochips were probed with 200 and 20 μg/ml Gal-8, and 200 and 50 μg/ml Gal-8mut, reacted with rabbit affinity-purified anti-Gal-8 IgG and revealed with a fluorescent-labelled secondary antibody. Anti-Gal-8 background signal [RFU (relative fluorescent units) < 100] was registered in a control array. Concentrations selected for comparison between galectins were made based on their similar maximum RFU signals on the arrays: RFU_{max} 12.651 for 20 μg/ml Gal-8 and RFU_{max} 13.158 for 50 μg/ml Gal-8mut (the scanner response is linear to a maximum RFU value of ~50000).

Gal-8 N- and C-terminal domain homology modelling

Wild-type full-length Gal-8 from mouse was homology-modelled using the model-building software Modeller (mod9v13) and the solved X-ray structure of the human Gal-8 as a 3D template (PDB code 4FQZ) [21]. The mutations Arg^{68}xHis and Arg^{241}xHis, located in the N- and C-terminal domains respectively, were introduced in the *.pir* alignment file of the corresponding wild-type protein. They correspond to Arg^{69} and Arg^{233} within human Gal-8 numbering. For each Gal-8, wild-type and mutant, 100 models were generated using Modeller, to satisfy the spatial restraints issued from the alignment with the target protein [22]. Models that combine lowest score function values (modeller function and DOPE score) and best stereochemistry, checked by Molprobity (http://molprobity.biochem.duke.edu/) were selected. Selected wild-type and variant models were superimposed on to the template and compared together, using the PyMOL visualization interface (The PyMOL Molecular Graphics System, version 1.2r3pre; http://www.pymol.org). The solved structures of holo human Gal-8 in complex with various ligands were also superimposed on the structural template to highlight the positioning of those ligands with respect to the crevice topology of the models. These superimpositions were carefully analysed using PyMOL, to obtain functional clues on the location of the mutation and the plausible consequences in terms of ligand binding to protein. Holo structures of human Gal-8 in complex with lactose, sialyl-lactosamine, lacto-*N*-fucopentaose III and blood group A tetrasaccharide ligands were retrieved from the pdb database, namely 4FQZ, 3VKO, 3AP9 and 1ULF respectively.

Cell proliferation and co-stimulation assays

Proliferation assays on mouse cells were performed as described previously [10]. Briefly, splenocytes (5×10^5 cells) from C57BL/6J mice were cultured at 37 °C under 5 % CO_2 for 48 h in flat-shaped 96-well plates in 0.2 ml of RPMI 1640 medium in the presence of 10 % (v/v) FBS, 2 mM glutamine and 5 μg/ml gentamicin (complete medium). For co-stimulation assays, splenocytes (3×10^5 cells) from DO11.10TCR_{OVA} mice were cultured for 48 h in 0.2 ml of complete medium in the presence of the cognate $OVA^{323–339}$ (ovalbumin) peptide at 1 μg/ml (Genscript). In all assays, 1 μCi of [^{3}H]methylthymidine (New England Nuclear) was added to each well 18 h before harvesting. Lactose was added 30 min before the addition of recombinant proteins. Concanavalin A (Sigma) was included at 5 μg/ml, as a positive control. Assays were performed in quadruplicate.

Determination of secreted IL-2

DO11.10 hybridoma T-cells (5×10^5) were seeded in a 24-well plate in 500 μl of complete medium in the presence of 2 μM Gal-8 or Gal-8mut or 1 μg/ml anti-CD3 (Becton Dickinson). After 72 h of culture, conditioned medium was recovered, centrifuged and kept at − 20 °C. Determination of IL (interleukin)-2 concentration in cell culture supernatants was assessed using a commercial ELISA (BioLegend) following the manufacturer's instructions.

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In vivo co-stimulation

In vivo co-stimulation was performed as described previously [17]. Briefly, 8-week-old female DO11.10 mice received i.p. (intraperitoneally) 0.5 mg of OVA (Sigma) in 0.2 ml of PBS, in combination with 25 μg of Gal-8 (OVA + Gal-8) or 25 μg of Gal-8mut (OVA + Gal-8mut); spleens were collected 5 days after immunization. *In vitro* re-stimulation of splenocytes was performed basically as for co-stimulation assays (see above), in the presence of 0.2 μg/ml cognate $OVA^{323–339}$ peptide.

Flow cytometry

A FlowMax cytometer PASIII (Partec) and WinMdi 2.9 software were used for flow cytometric analysis.

Statistical analysis

Student's *t* test was used for statistical analysis; P values <0.05 were considered significant.

RESULTS

Generation and characterization of a double-mutated Gal-8 recombinant protein (Gal-8mut)

A variant of murine Gal-8 recombinant protein was generating by replacing two canonical arginine residues from each carbohydrate-binding site (Arg^{68}xHis and Arg^{241}xHis), as it is well documented that the presence of these same mutations precludes lactose recognition [11,23]. The schematic representation of this double-mutated protein (Gal-8mut) is shown in Figure 1(A). First, we tested the binding capacity of Gal-8mut to lactosyl-Sepharose in order to confirm the absence of lactose recognition. As expected, Gal-8 bound efficiently to the resin and was completely displaced after washing with lactose, whereas Gal-8mut was only observable in the flowthrough fraction, indicating that the mutated protein no longer recognized lactose (Figure 1B). To rule out that the absence of lactose recognition was a result of a misfolded protein due to the inserted mutations, CD and unfolding thermic assays comparing native and mutated proteins were performed. As shown in Figure 1(C), Gal-8 and Gal-8mut far UV spectra were very similar with a minimum at 216 nm, which is typical of β-sheet conformation, indicating that both proteins share an almost identical secondary structure. In agreement, the unfolding thermic curves of both proteins displayed a co-operative transition, demonstrating further that Gal-8mut was folded properly. Taken together, these data confirm that the absence of lactose recognition of Gal-8mut is due to the loss of two key residues from the binding sites, rather than aberrant protein folding.

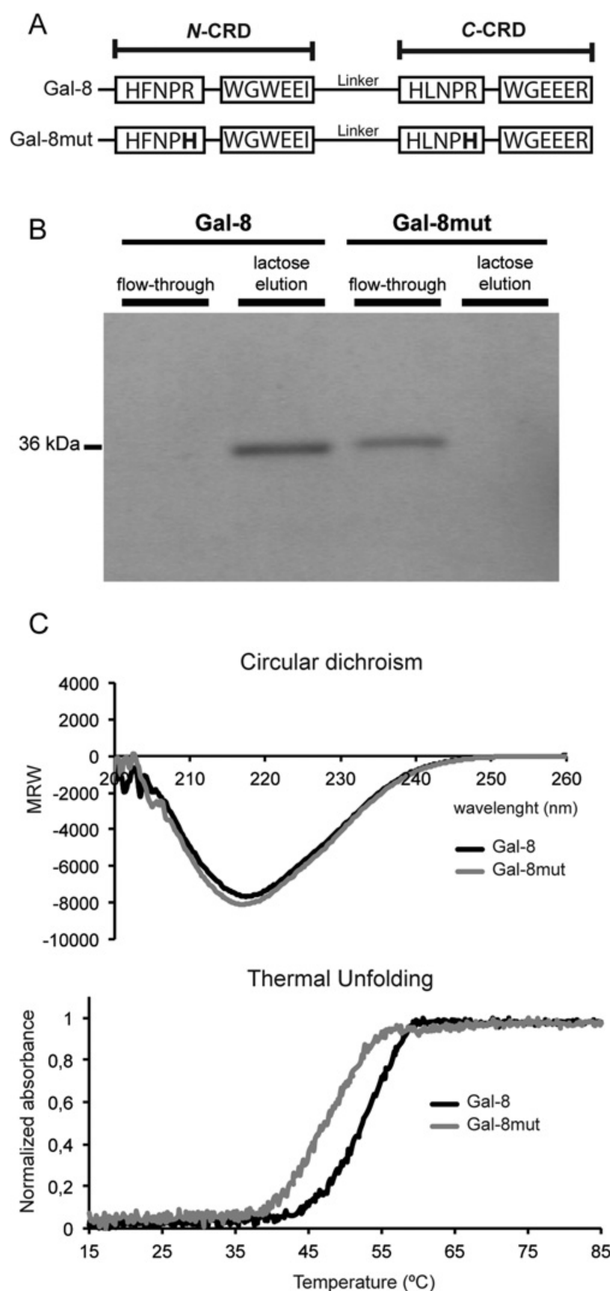

Figure 1 Molecular and functional characterization of Gal-8mut

(**A**) Scheme of Gal-8 and Gal-8mut CRDs showing the mutations at the N- and C-terminal carbohydrate-binding sites. Conserved arginine residues that are essential for lactose recognition were replaced by histidine. (**B**) Lactosyl-Sepharose pull-down assay. Gal-8 is absent from the flowthrough and it is effectively displaced from the resin after lactose washing. On the other hand, Gal-8mut can only be observed in the flowthrough, but not in the lactose elutate, indicating that Gal-8mut does not bind to lactosyl-Sepharose. (**C**) CD spectra and thermal unfolding curves for Gal-8 and Gal-8mut. The thermal unfolding was irreversible since aggregates were clearly visible when temperature was lowered; and the inserted mutations reduced the T_m (52.7 compared with 47.34 °C). These experiments were performed twice with two independent recombinant protein preparations. MRW, mean residue weight.

Gal-8mut recognizes binders at the cell surface in a lactose-independent manner

Despite its inability to recognize lactose, Gal-8mut still displayed lectin activity as assessed by haemoagglutination (Supplementary Figure S1). It was shown previously that Gal-8 binds many cell types, including thymocytes and splenocytes, by interacting with cell-surface glycans, since incubation with lactose reduced binding significantly [6,10]. Having demonstrated that Gal-8mut completely lacks lactose-binding capacity, but displayed lectin activity, we next studied whether this protein was still able to bind to the cell surface. For this purpose, cells from murine $CD4^+$ T-cell hybridoma (DO11.10 hybridoma) were incubated in the presence of Gal-8 or Gal-8mut. After washing in the presence or absence of lactose, cells were labelled with anti-Gal-8

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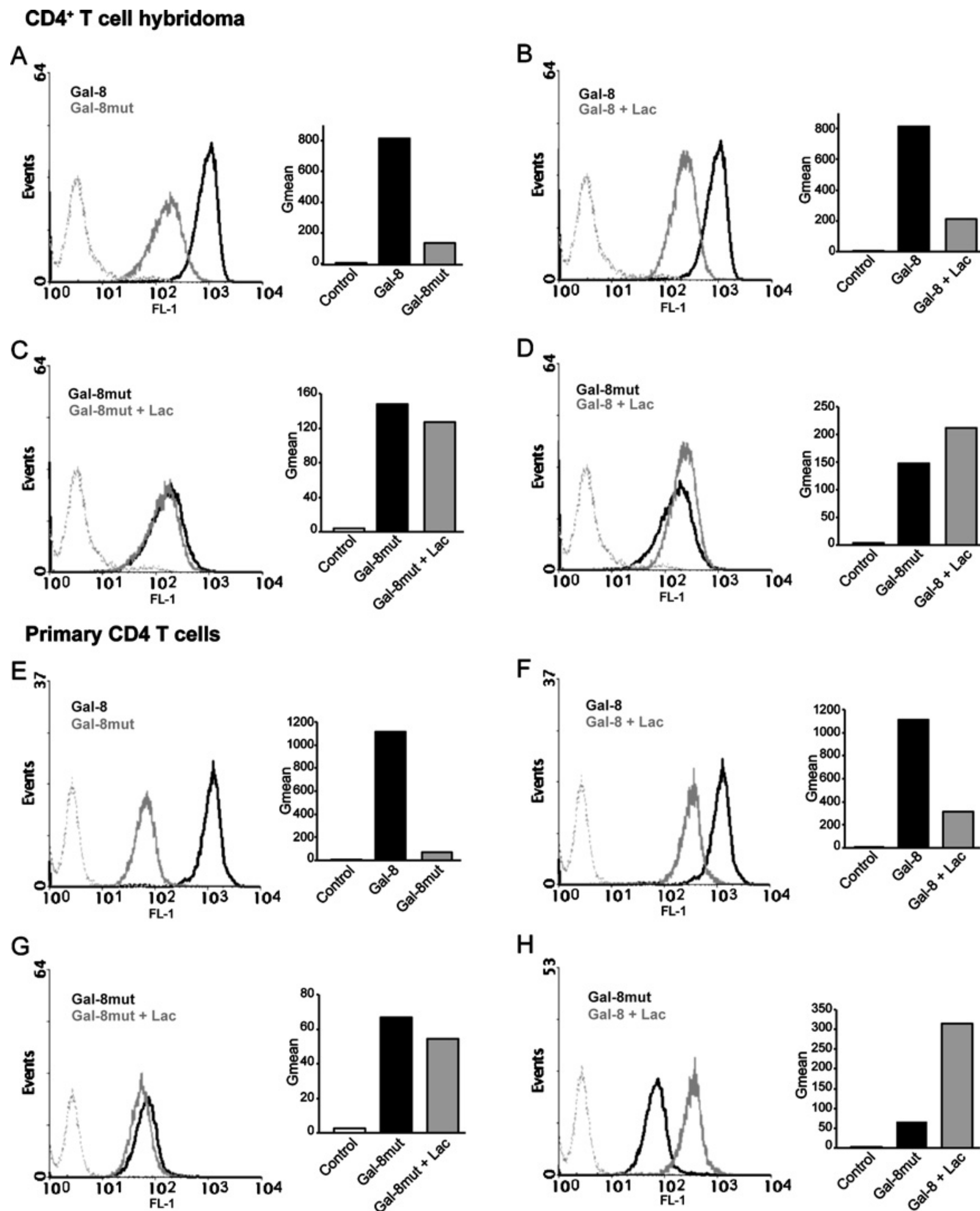

Figure 2 Both Gal-8 and Gal-8mut bind to T-cells, but only Gal-8 is displaced by lactose

Hybridoma (**A**–**D**) or primary CD4^{+} T-cells (**E**–**H**) were incubated in the presence of 0.1 μM Gal-8 or Gal-8mut, washed with PBS or PBS plus 100 mM lactose (Lac), then reacted with purified polyclonal antibodies anti-Gal-8 followed by Alexa Fluor® 488-conjugated anti-rabbit IgG, and analysed by flow cytometry. Both Gal-8 and Gal-8mut were able to bind to the T-cell surface, although Gal-8mut bound to a lesser extent (**A** and **E**). Lactose addition partially displaced Gal-8 from the cell surface (**B** and **F**), but did not affect Gal-8mut binding (**C** and **G**). Of note, cells treated with Gal-8mut displayed a similar fluorescence intensity (Gmean) to those treated with Gal-8 and washed with lactose in the hybridoma (**D**), but not in primary CD4^{+} T-cells (**H**). The broken line histograms correspond to control (no Gal added). Bars represent the Gmean values of duplicates. Results are representative of three independent experiments, each carried out in duplicate, with equal results.

antibodies and analysed by flow cytometry. As observed in Figure 2(A), Gal-8mut bound to T-cells, although into a lesser extent than Gal-8. This observation was not due to a differential recognition of Gal-8 and Gal-8mut by the polyclonal anti-Gal-8 antibodies, since they similarly recognized both proteins by ELISA, in a wide dilution range (Supplementary Figure S2). Unlike Gal-8, Gal-8mut was not displaced in the presence of lactose (Figures 2B and 2C), which is in agreement with the results depicted in Figure 1(B). Interestingly, fluorescence signals from the samples reacted with Gal-8mut and Gal-8 plus lactose coincided, suggesting that the Gal-8 fraction that is not displaced by lactose is actually binding to the same ligands as Gal-8mut

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(Figure 2D). Similar results were observed when primary CD4$^+$ T-cells purified from C57BL/6J mouse spleens were reacted with Gal-8 and Gal-8mut (Figure 2E), and after washing with lactose (Figures 2F and 2G). At difference with the hybridoma, primary CD4$^+$ T-cells seemed to display other ligands that, despite not competed in the presence of lactose, they were not recognized by Gal-8mut either (Figure 2H). To confirm further ligand recognition at the cell surface, biotinylated T-cells were reacted with Gal-8 or Gal-8mut, extensively washed and lysed. Then, cellular extracts were incubated with anti-Gal-8 antibodies immobilized to Protein A–Sepharose, and subjected to two consecutive elution steps, first with PBS/lactose, and then with cracking buffer at 100 °C. In strong agreement with our previous observations, lactose-eluted ligands were only evident when cells were incubated with Gal-8, but not with Gal-8mut (Figure 3). Notably, a similar band pattern was observed after cracking buffer elution from samples reacted with either Gal-8 or Gal-8mut. Specificity was confirmed since no signals were registered using purified normal rabbit IgG as control. These results not only demonstrate that Gal-8mut interacts with T-cell-surface ligands independently of the presence of lactose, but also support the idea that the mutated protein actually retains high-affinity recognition ability present in the wild-type molecule.

Given that sialylated sugars were shown to be specifically recognized by Gal-8 N-CRD [18], T-cells reacted with Gal-8 or Gal-8mut were incubated further in the presence of 3′-sialyl-lactose. Only Gal-8 was partially displaced in the presence of sialyl-lactose, indicating that other different sugar residues might be involved in Gal-8mut binding to glycoprotein receptors (Figures 4A and 4B). Then, blood group A and B as well as polylactosamine were tested, as they are specifically recognized by the C-CRD [20]. Gal-Ara, a galactose-containing sugar compound, was also included in these assays since it was shown previously to inhibit most of the effects of Gal-8 on T-cell response [16]. As expected, all of these compounds displaced Gal-8 binding from the cell surface (Figures 4C, 4E, 4G and 4I); however, Gal-8mut binding was only partially competed in the presence of blood group B (Figures 4D, 4F, 4H and 4J). *A priori*, these findings agree with the idea that Gal-8mut preserves high-affinity interactions with cell-surface glycoreceptors, partially resembling that of C-CRD.

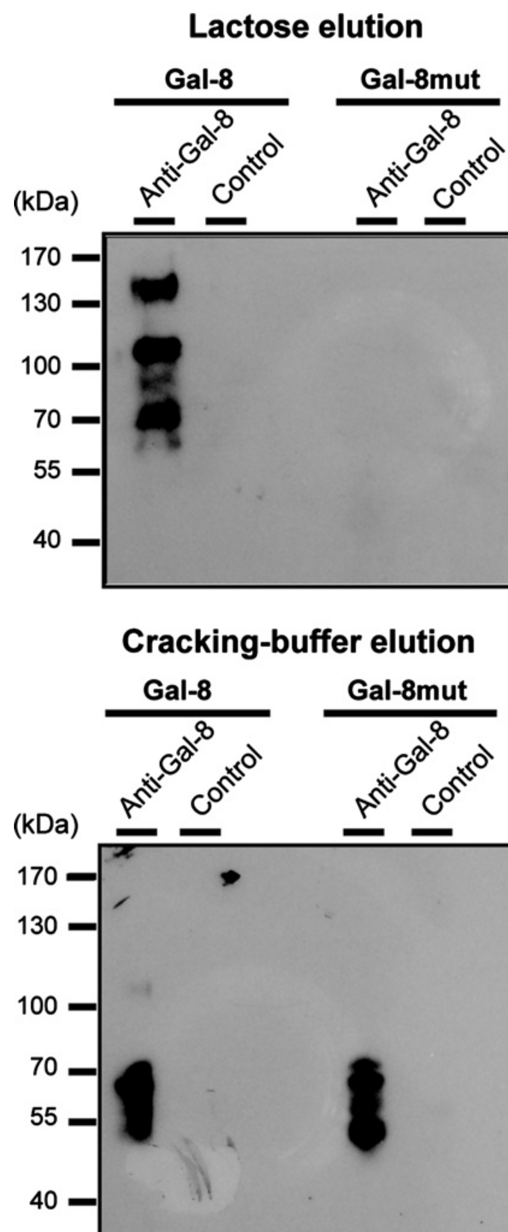

Figure 3 Gal-8mut binders are not competed with lactose

Murine hybridoma T-cell surfaces were biotinylated before incubation with 0.1 μM Gal-8 or Gal-8mut and cells were then washed and lysed. Cellular extracts were incubated with anti-Gal-8 antibodies immobilized to Protein A–Sepharose. After washing, resin was first eluted with PBS plus 100 mM lactose, followed by a second elution with cracking buffer at 100 °C. Samples were resolved by SDS/PAGE, transferred on to PVDF membranes and reacted with avidin–HRP. Consistent with previous results, Gal-8-binding proteins were displaced in the presence of lactose, whereas Gal-8mut-binding proteins were only observed after elution with cracking buffer. Control was purified normal rabbit IgG. Molecular masses are indicated in kDa.

Gal-8mut retains high-affinity glycan interactions

Results described in Figures 2–4 raised the hypothesis that recognition of high-affinity glycans is preserved in Gal-8mut, whereas recognition of low-affinity binders such as lactose are lost, rather than an overall reduction of glycan recognition ability. To identify those glycan structures that are specifically recognized by Gal-8mut, a glycan array profiling approach was performed. Glycan microarrays containing a total of 610 different glycan structures were probed with decreasing amounts of Gal-8 and Gal-8mut, and revealed with anti-Gal-8 antibodies followed by a fluorescent-labelled secondary antibody. Fluorescent signals (RFU) corresponding to bound glycans on the arrays, reacted with different concentration of each lectin, are shown in Figure 5(A). At high concentration, Gal-8 displayed recognition of a broad spectrum of glycans, including those with low affinity; however, at low concentrations, a more specific recognition emerged as motifs became apparent. Interestingly, Gal-8mut displayed only the last recognition pattern, even when tested at high concentration. In order to identify the ligand profile of each lectin, highly recognized glycans were selected further on the basis of its fluorescent signal on the array (RFU>1500) and ordered in groups on the basis of their common structures. As shown in Figure 5(B), Gal-8mut retained affinity to most polylactosamine-containing glycans, and to a few blood A and B group-containing glycans, whereas affinity to sialic acid-, sulfate- or lactose-containing glycans were no longer evident. The complete list of individual glycans depicted in Figure 5(B) is detailed in Supplementary Table S1. Of note, glycans recognized

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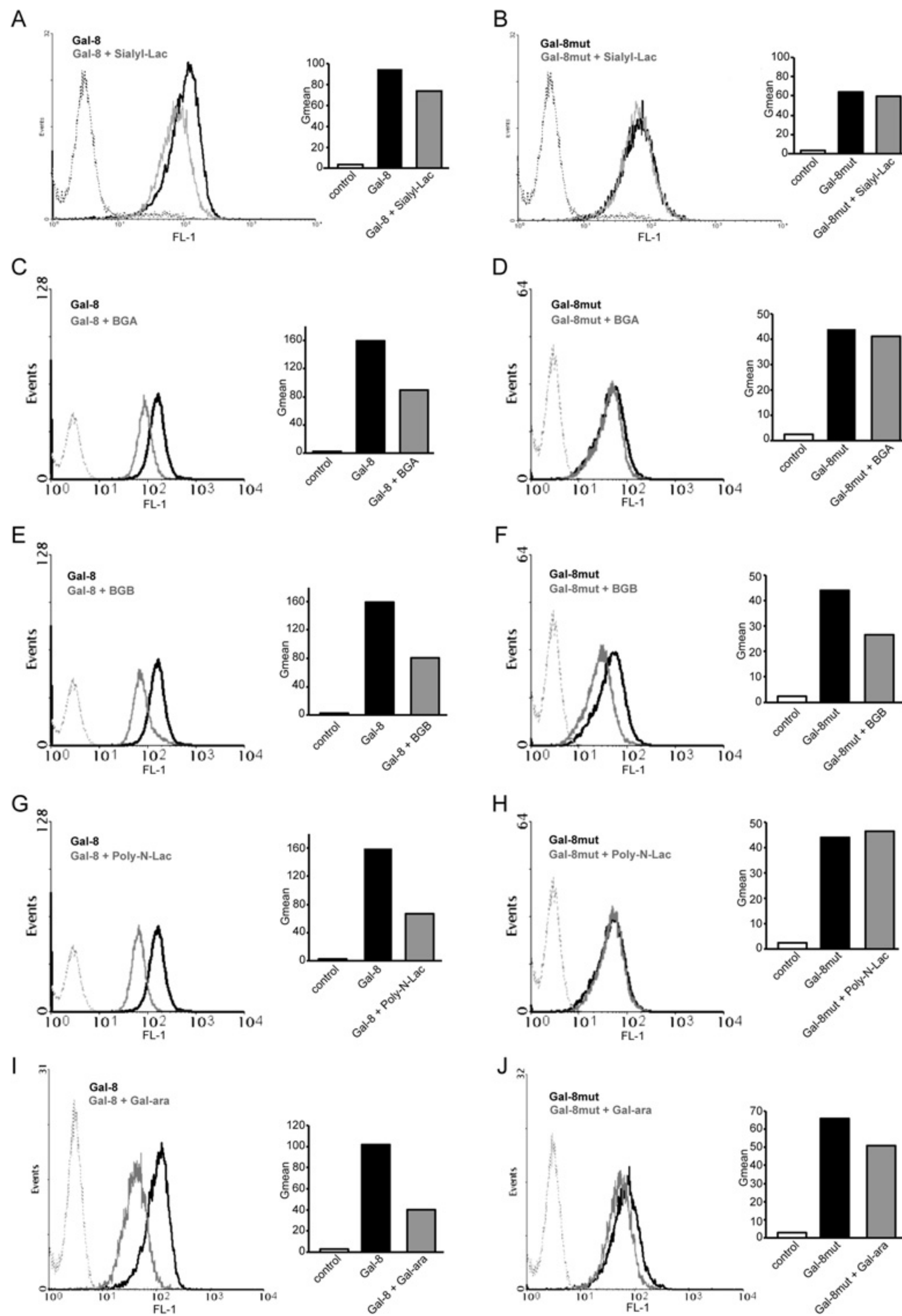

Figure 4 Gal-8mut affinity partially resembles that of C-CRD

Hybridoma CD4^{+} T-cells were incubated in the presence of 0.1 μM Gal-8 or Gal-8mut, washed with PBS or PBS plus 100 mM sialyl-lactose (Sialyl-Lac) (**A** and **B**), 10 mM blood group α tetrasaccharide type 2 (BGA) (**C** and **D**), 10 mM blood group β tetrasaccharide type 1 (BGB) (**E** and **F**), 10 mM tri-*N*-acetyl-lactosamine (poly-*N*-Lac) (**G** and **H**), 100 mM 3-*O*-β-D-galactopyranosyl-D-arabinose (Gal-Ara) (**I** and **J**), then reacted with polyclonal purified anti-Gal-8 followed by Alexa Fluor® 488-conjugated anti-rabbit IgG, and analysed by flow cytometry. Broken line histograms correspond to control (no Gal added). Bars represent the Gmean values of duplicates.

by Gal-8mut were also recognized by the wild-type protein, which indicates that Gal-8mut does not display novel interactions. These results are in agreement with previous studies that found Gal-8's affinity for polylactosamines and blood group sugars (C-CRD), as well as sialylated and sulfated sugars (N-CRD) [20]. Also in line with our previous results, lactose in the glycan array was no longer recognized by Gal-8mut. At a molecular level, these results were corroborated by homology modelling of Gal-8 and Gal-8mut. In the N-terminal domain of Gal-8, the mutation Arg[68]xHis impedes a salt bridge with Glu[88]. In the absence of this stabilizing

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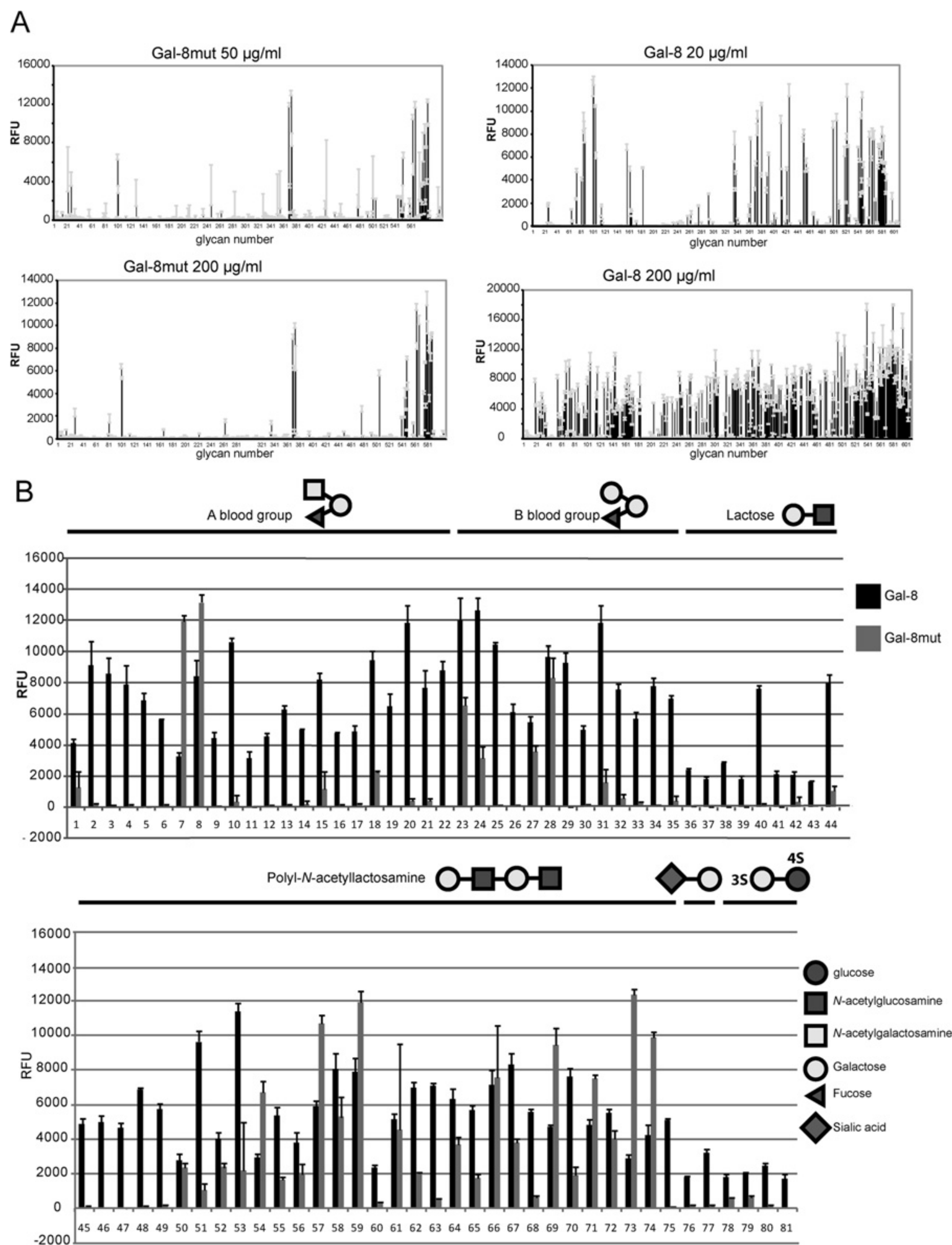

Figure 5 Gal-8mut conserves high-affinity interactions to poly-*N*-acetyl-lactosamines and blood group antigens

(**A**) Identification of glycan specificity of Gal-8mut by glycan microarrays from the Consortium for Functional Glycomics. Gal-8 was used at 200 and 20 μg/ml, and Gal-8mut at 200 and 50 μg/ml. Glycochips were reacted with affinity-purified anti-Gal-8 antibodies and revealed with a fluorescent-labelled secondary antibody. For details, refer to the Materials and methods section. Plots are glycan number against mean average RFU with S.E.M. (**B**) Glycans recognized by either Gal-8 (20 μg/ml) or Gal-8mut (50 μg/ml) with RFU $>$1500 were selected and grouped by common structures.

interaction, the histidine side chain flips backward so that its side-chain positioning is thus too far to accommodate a cognate lactose (Figure 6A). Conversely, in the C-terminal domain, the mutation Arg^{241}xHis does not generate a critical modification of ligand binding because the histidine side-chain positioning is kept with regard to the former arginine rotamer, mainly due to the interaction with a conserved salt bridge between Glu^{260} and Arg^{262}. This salt bridge also participates

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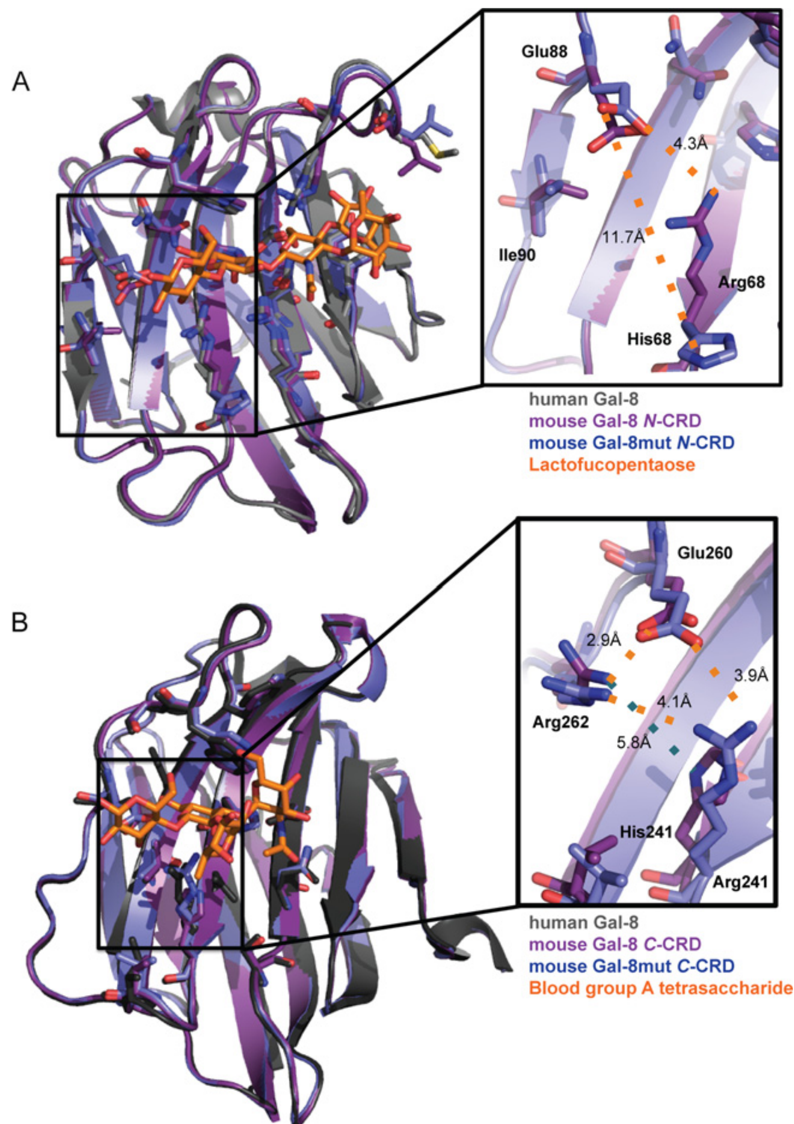

Figure 6 Homology models of N- and C-CRD of Gal-8 and Gal-8mut

(**A**) Homology models of the N-CRD of Gal-8 and Gal-8mut, coloured blue and purple respectively, are superimposed on the 3D template of human Gal-8 N-CRD coloured grey (PDB code 4FQZ). Also shown is a close-up view of the lactose-binding site highlighting the flipping backward of the His^{68} side chain, shown as a stick. (**B**) Homology models of the C-CRD of Gal-8 and Gal8mut are superimposed on the 3D template of human Gal-8 (PDB code 4FQZ), with the same colour code. Shown is a close-up view of the blood group A (BGA)-binding site highlighting the salt bridge between Glu^{260} and Arg^{262}, shown as sticks, of the motif GX**E**E**R** that stabilizes the side chain of His^{241}.

in ligand binding and closely interacts with ligands such as blood group A tetrasaccharide (Figure 6B). Taken together, these findings demonstrate that Gal-8mut lost most of the low- and intermediate-, but maintained some high-, affinity glycan interactions, especially at the C-CRD.

Gal-8mut lacks T-cell proliferation activity

We have demonstrated previously that Gal-8 is able to induce strong proliferation of mouse splenocytes, in the absence of antigen. This effect relies upon lectin activity, since it could be inhibited almost completely when cells were pre-incubated with 30–50 mM lactose or its TDG derivative [10]. In order to investigate whether Gal-8mut was able to induce the proliferative effect, splenocytes from C57BL/6J mice were incubated in the presence of 0.5 and 1 μM mutated protein. In contrast with native Gal-8, no proliferation induction was observed with Gal-8mut (Figure 7A). These results are consistent with the fact that Gal-8-induced proliferation is prevented by lactose and that Gal-8mut had lost lactose recognition, thus suggesting that Gal-8mut interacts with glycans different from that required to trigger cell proliferation. Since differences in glycan recognition between the native and mutated protein were evident using the DO11.10 hybridoma (Figures 2 –4), the absence of T-cell proliferation induction was also tested in this model. For this purpose, the presence of IL-2 was determined in the conditioned medium of Gal-8- or Gal-8mut-stimulated hybridoma cells. At difference

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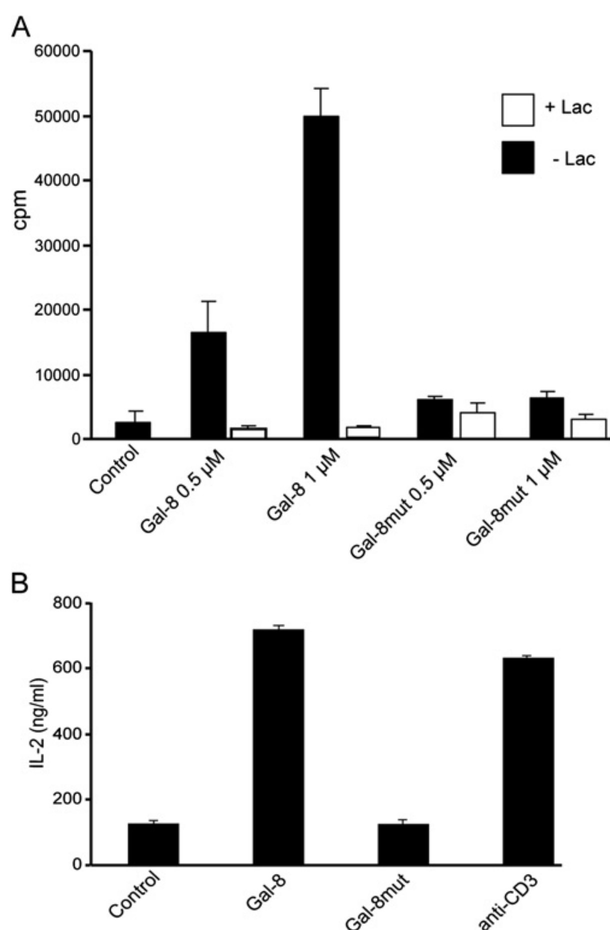

Figure 7 Gal-8mut is no longer able to induce antigen-independent T-cell proliferation

(**A**) Splenocytes from C57BL/6J mice were incubated for 48 h in the presence of the indicated amounts of Gal-8 or Gal-8mut. Lactose 50 mM (Lac) was added 30 min before Gal. Proliferation was assessed by [^{3}H]thymidine incorporation. The result is representative of, at least, four independent proliferation experiments. (**B**) Murine hybridoma T-cells were incubated in the presence of 2 μM Gal-8 or Gal-8mut for after 72 h, at which time supernatants were collected and quantification of secreted IL-2 was assessed by ELISA. The depicted experiment was carried out in triplicate and is representative of three independent assays. Results are means $\pm$ S.D.

with Gal-8, Gal-8mut was unable to induce secretion of IL-2, confirming further the absence of an antigen-independent proliferative effect (Figure 7B).

Gal-8mut is able to co-stimulate an antigen-specific response both *in vitro* and *in vivo*

When splenocytes from DO11.10TCR$_{OVA}$ transgenic mice are incubated in the presence of low concentrations of Gal-8 (0.1–0.2 μM), together with suboptimal doses of OVA antigen, a synergistic increase in T-cell-specific response is observed [10]. At difference with the antigen-independent proliferative effect, this co-stimulatory effect might involve higher-affinity interactions between Gal-8 and cell-surface glycoreceptors since it could be achieved with lower amounts of the lectin, and it is only partially inhibited in the presence of lactose or TDG. Another particular characteristic of the co-stimulatory effect is that it can be achieved with either isolated N- or C-CRD, then not requiring the tandem-repeat structure [16]. Accordingly, we have also observed that Gal-1, from the prototype group with only one CRD shared Gal-8 co-stimulatory effect; however, 100-fold more Gal-1 is needed to equal the effect of Gal-8 [17]. In order to analyse the co-stimulatory capacity of Gal-8mut, splenocytes from DO11.10TCR$_{OVA}$ mice were stimulated with the cognate peptide OVA$^{323–339}$ (dose of 1 μg/ml) in the presence of 0.2 μM Gal-8 or Gal-8mut. As shown in Figure 8(A), Gal-8mut displays a similar ability to enhance the antigen-specific response as the native protein. Having demonstrated the ability of Gal-8mut to induce co-stimulation *in vitro*, we next investigated whether Gal-8mut is also able to enhance the specific T-cell response *in vivo*. Notably, after 5 days post-administration of a single suboptimal dose of OVA antigen together with Gal-8mut, a significantly increased T-cell response was recalled upon re-stimulation with the cognate peptide OVA$^{323–339}$ (Figure 8B).

Taken together, these findings demonstrate that Gal-8mut retains co-stimulatory activity of the antigen-specific T-cell response, both *in vitro* and *in vivo*.

DISCUSSION

The glycan preferences of Gal have been largely studied by multiple and specialized approaches. In particular, differences in glycan affinity of each separate domain of tandem-repeat Gal-8 have been investigated and confirmed by means of frontal affinity chromatography, fluorescence cytometry, ELISA, surface plasmon resonance, glycan microarray, molecular dynamics simulations and X-ray crystallography [18,20,24–27]. In those studies, Gal-8 N- and C-CRD ligand affinities have been assessed by using divalent constructions containing a single amino acid substitution in one CRD (mainly Arg^{69}xHis and Arg^{233}xHis, which are the human counterparts of the mutations used throughout the present study, namely Arg^{68}xHis and Arg^{241}xHis) or N- and C-CRDs isolated monomers; however, glycan recognition of a divalent Gal-8 with the two CRDs mutated simultaneously had never been tested until now. Those same constructions were used to investigate the molecular requirements for Gal-8 biological effects [11,12,16,19,20,24,28]. Nevertheless, the relationship between Gal's biological effects on specific cellular functions and the implicated glycan ligands is, in most cases, undetermined. In the present study, we have distinguished further Gal-8 activities on the T-cell response, on the basis of differential glycan recognition, by using a double-CRD-mutated Gal-8 (Gal-8mut). Our results demonstrate that Gal-8mut maintained glycan affinity for polylactosamines and some blood antigens, together with the ability to induce the antigen-specific co-stimulatory activity, but not the antigen-independent proliferation. Gal-8mut seemed to retain the affinity for those glycans that are implicated in co-stimulation, but not in the proliferative induction, therefore it will represent a very useful tool to identify specific ligands required for each separate activity. In fact, the specific interaction of Gal-8mut with defined glycoproteins from the T-cell surface was demonstrated in the present study (Figure 3), allowing further the identification of those ligands involved in the Gal-8 co-stimulatory effect. In agreement with previous reports [11,23], the amino acid substitutions of Gal-8mut precluded lactose recognition. However, lactose is not the preferred ligand for intact Gal-8, as shown by many authors. For example, Vokhmyanina et al. [29] reported that Gal-8 binding to ligands with Galβ1–3GlcNAc or Galβ1–3GalNAc as basic motifs, was commonly better than that to canonical Galβ1–4GlcNAc; and Kumar et al. [27] reported that Gal-8 displayed higher affinity for blood group antigens than *N*-acetyl-lactosamine and lactose. In line with this

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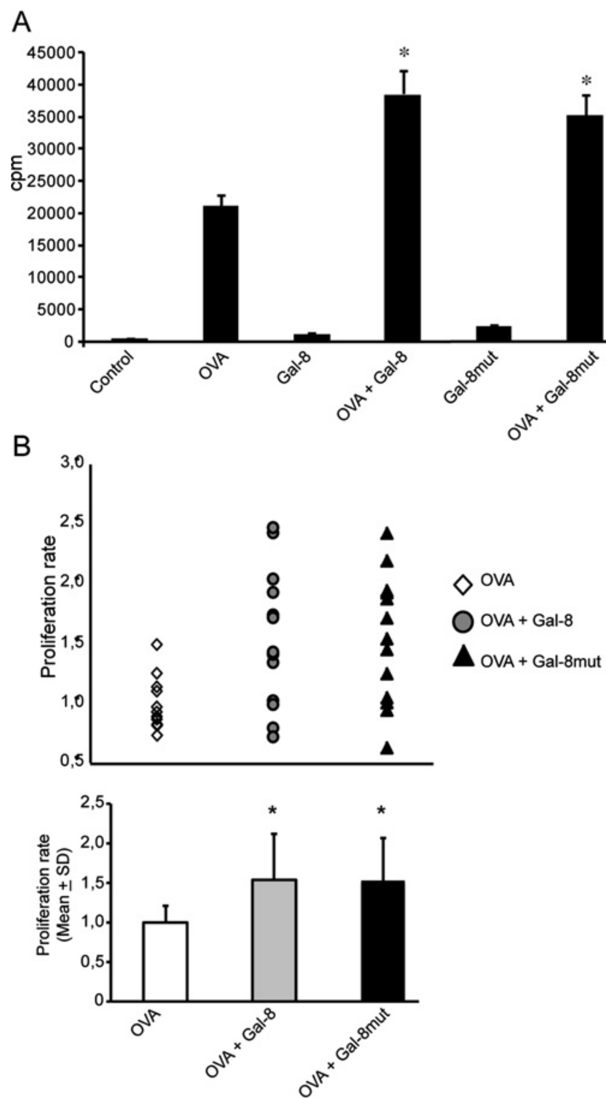

Figure 8 Gal-8mut co-stimulates the antigen-specific T-cell response

(**A**) Splenocytes from DO11.10 TCR_{OVA} mice were incubated in the presence of suboptimal dose of cognate peptide $OVA^{323-339}$ (OVA), together with 0.2 μM Gal-8 or Gal-8mut for 48 h. $*P < 0.01$ compared with OVA. (**B**) DO11.10TCR_{OVA} mice were immunized i.p. with 0.5 μg/ml OVA whole protein dissolved in PBS alone (PBS) or plus 25 μg of recombinant Gal-8 (Gal-8) or 25 μg of Gal-8mut (Gal-8mut). After 5 days, spleens were removed and splenocytes were re-stimulated *in vitro* with 0.2 μg/ml of cognate peptide $OVA^{323-339}$ for 48 h. OVA (n = 12), Gal-8 (n = 13) and Gal-8mut (n = 13). $*P < 0.01$ compared with OVA. Proliferation index (PI) was calculated as: c.p.m. (stimulated)/mean c.p.m. (unstimulated). To normalize data from independent experiments, a proliferation rate was calculated as PI(Gal-treated)/mean PI(OVA-treated). In all cases, proliferation was assessed by [^{3}H]thymidine incorporation. Results depicted in (**A**) are representative of, at least, four independent experiments, and results depicted in (**B**) are the sum of normalized PI values of individual animals from three independent *in vivo* assays. Results are means ± S.D.

evidence, we observed that intact Gal-8 bound to lactose with low affinity, as determined by glycan array analysis (Figure 5B). When Gal-8mut was probed, polylactosamines and blood group antigens were bound with high affinity, whereas lactose, sialylated and sulfated glycans were no longer recognized. A tight correlation between glycan array results and binding competition assays was evident, since the same blood group B type 1 tetrasaccharide that was recognized by Gal-8mut in the array (Supplementary Table S1) was also able to displace Gal-8mut binding at the cell surface (Figure 4F). Conversely, Gal-8mut could not be displaced in the presence of the glycans blood group α tetrasaccharide type 2 and tri-*N*-acetyl-lactosamine that were not specially recognized in the array (Figures 4D and 4H). It is worth pointing out that Gal-8mut did not recognize blood group antigens or polylactosamines to the same extension as the wild-type protein. This might be associated with differences in sugar chain length or complexity of side-chain residues. Further studies are required to analyse this possibility. In addition, Gal-8mut could not be displaced from the T-cell surface in the presence of sialyl-lactose (Figure 4B), confirming further the lack of sialylated sugar recognition. In agreement, it was reported previously that the same amino acid replacement in the N-CRD of Gal-8mut precludes sialic acid recognition [20]. Sialylated glycan recognition is a unique feature that enables Gal-8 to bind with high affinity to many cellular targets to exert distinct biological effects such as platelet activation and clearance of infected endosomes [12,15], although whether Gal-8mut is unable to exert such activities was not addressed in the present study. Nevertheless, despite lactose and other sugars such as sialic acid, no longer being recognized by Gal-8mut, cell-surface-binding activity was still evident, which suggested that Gal-8mut was actually functional. In fact, it seemed that Gal-8mut maintained much of the glycan affinity of the C-terminal domain, i.e. polylactosamines and blood group antigens. It is worth pointing out that C-CRD is the principal domain responsible for proliferation and co-stimulation induction on T-cells, as observed by *in vitro* assays using isolated domains and chimaeras [16]. Kumar et al. [27] suggested that conserved amino acid residues, other than Arg^{233} from the C-terminal domain of human Gal-8 were shown to favour interactions with high-affinity glycans. From the molecular analysis depicted in Figure 6, we strongly hypothesize that the salt bridge between Glu^{260} and Arg^{262} of the conserved motif GX**EER** located at the C-terminus is critical for recognition and high-affinity binding of these ligands. We propose that any substitution in these two residues would modify the topology and electrostatic profile of the crevice and would result in diminished binding. At difference with Gal-8mut which binding capacity to T-cells was readily evident in the present study (Figures 2 –4), the C-CRD monomer displayed almost no cell-surface-binding ability [16,25]. It seems that the N-CRD, even mutated, contributes to Gal-8mut cell-surface recognition. In this regard, Patnaik et al. [25] observed that whereas the N-CRD monomer lost its binding ability to undersialylated CHO (Chinese-hamster ovary) cells, the intact Gal-8 bound to the modified and parental cells with similar affinity. Considering that C-CRD did not bind to these cells at all, these results indicate that Gal-8 binding is not just a mere sum of the interactions of the their separate domains, but a concerted combination of both. In agreement, Carlsson et al. [19] observed that Gal-8 N-CRD binds to various non-sialylated or sulfated saccharides with a wide range of lower affinities, sufficient to act in concert with the C-CRD to bind cell surfaces and induce signals. This co-operation between the two CRDs may, in turn, explain why we could not infer the presence of glycan recognition in the double-mutated Gal-8 by only considering the glycan interaction of isolated CRDs. Overall, these observations reflect the broad specificity of intact Gal-8 for cell-surface binding and signal induction, although the presence of β-galactosides is always required.

In our laboratory, we have demonstrated that Gal-8 exerts multiple pro-inflammatory effects, such as strong T-cell proliferation induction, platelet spreading and degranulation, and endothelial cell activation [10,12,13]. All of these events were precluded when cells were pre-incubated with lactose, indicating that they rely upon lectin–glycan interactions, and, moreover,

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that they do not depend on further fine specificity. In the present study, we have demonstrated that Gal-8mut, which had lost lactose recognition, was unable to induce strong T-cell proliferation, whereas it retained the co-stimulatory effect on antigen-specific T-cell response, both *in vitro* and *in vivo*. We therefore hypothesize that Gal-8mut not only is devoid of T-cell-stimulatory activity, but also might lack the other lactose-dependent pro-inflammatory effects. This shortened activity will represent a very interesting feature for the feasible use of Gal-8mut as an enhancer of T-cell responses against weak antigens. In fact, the development of new-generation adjuvants is focused on the search for well-defined molecules for safety purposes [30]. Taken together, the use of Gal-8mut not only allows the characterization of distinctive T-cell responses, but also constitutes a suitable adjuvant alternative for Gal-8, as it might avoid a potential unspecific pro-inflammatory environment.

AUTHOR CONTRIBUTION

Matías Nicolás Schroeder, María Virginia Tribulatti, Julieta Carabelli and Valentina Cattaneo performed experimental work. Gwenaëlle André-Leroux performed protein–ligand modelling. Julio Javier Caramelo performed CD assays. María Virginia Tribulatti and Oscar Campetella conceived the project, designed experiments, supervised the work and wrote the paper.

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