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Yeast expressed ArtinM shares structure, carbohydrate recognition, and biological effects with native ArtinM

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ABSTRACT

Recent advances in glycobiology have revealed the essential role of lectins in deciphering the glycocodes at the cell surface to generate important biological signaling responses. ArtinM, a D-mannose-binding lectin isolated from the seeds of jackfruit (*Artocarpus heterophyllus*), is composed of 16 kDa subunits that are associated to form a homotetramer. Native ArtinM (n-ArtinM) exerts immunomodulatory and regenerative effects, but the potential pharmaceutical applicability of the lectin is highly limited by the fact that its production is expensive, laborious, and impossible to be scaled up. This led us to characterize a recombinant form of the lectin obtained by expression in *Saccharomyces cerevisiae* (y-ArtinM). In the present study, we demonstrated that y-ArtinM is similar to n-ArtinM in subunit arrangement, oligomerization and carbohydrate binding specificity. We showed that y-ArtinM can exert n-ArtinM biological activities such as erythrocyte agglutination, stimulation of neutrophil migration and degranulation, mast cell degranulation, and induction of interleukin-12 and interleukin-10 production by macrophages. In summary, the expression of ArtinM in yeast resulted in successful production of an active, recombinant form of ArtinM that is potentially useful for pharmaceutical application.

1. Introduction

Lectins are a heterogeneous group of proteins containing at least one domain that selectively and reversibly binds to glycans, on proteins or lipids [1]. Lectins are ubiquitous in nature, and they are found in a variety of organisms ranging from viruses to animals. The binding to cell surface glycans can promote cell signaling and can trigger diverse phenomena such as proliferation, migration, and cell death [2]. Due to their important biological activities, lectins are increasingly being studied, and the growing understanding of lectin–carbohydrate interactions at the molecular level provides new and important insights that can be applied in biomedical research.

ArtinM (also known as KM+ or Artocarpin) is a D-mannose-binding lectin isolated from the seeds of the *Artocarpus heterophyllus* (jackfruit). It consists of a 64 kDa homotetramer formed by the association of 16 kDa non-glycosylated subunits [3,4]. This association, although non-covalent, is resistant to detergents such as sodium dodecyl sulfate (SDS), and thermal denaturation is required to obtain electrophoretically detectable monomers from these homotetramers. ArtinM has a high specificity for Manα1–3(Manα1–6)Man, the trimannoside core of *N*-glycans [5]. By interacting with *N*-glycans of cell surface receptors, ArtinM triggers cellular responses such as neutrophil migration and activation by binding to the CXCR2 receptor [6,7], mast cell degranulation by binding to immunoglobulin (Ig) E or its receptor FcεR [8,9],

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and interleukin12 (IL-12) production by inducing activation of macrophages and dendritic cells triggered by interaction with the Toll-like receptor 2 (TLR2) ectodomain [10,11].

Because of its pleiotropic action, ArtinM is an interesting immunomodulatory agent. Systemic administration of ArtinM to mice induces Th1 immunity that confers protection against various intracellular pathogens [10,12–15]. Topical administration of ArtinM promotes regeneration of injured corneal epithelium and oral mucosa in rabbits and rats, respectively [16,17]. Because of its potential pharmaceutical applications, there is considerable interest in large-scale production of ArtinM, and obtaining active, recombinant forms of the protein is a first step toward attaining this goal.

By cloning and expressing the ArtinM gene in *Saccharomyces cerevisiae* and *Escherichia coli*, we have produced the recombinant forms y-ArtinM and b-ArtinM, respectively [18]. In this study, we analyzed the carbohydrate binding specificity of y-ArtinM and examined its ability to mimic the activities exerted by the native ArtinM (n-ArtinM). The results showed that y-ArtinM has biological properties that are similar to those previously described for n-ArtinM, indicating that expressing the protein in yeast cells is a promising method for large-scale production of ArtinM for the development of new immunomodulatory drugs.

2. Materials and methods

2.1. Ethics statement

The animal studies were approved by the Committee on Ethics in Animal Research (CETEA) of the College of Medicine of Ribeirão Preto of the University of São Paulo, and were conducted in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) Protocol no. 025/2010. Human erythrocytes and neutrophils were isolated from blood samples obtained from healthy donors who provided written informed consent, and with approval of the Ethics Committee and the Institutional Review Board of the Clinical Hospital of Ribeirão Preto, University of São Paulo, Brazil (approval number 10012/2009).

2.2. Lectins

n-ArtinM was purified, as previously described [3], from a saline extract of *A. heterophyllus* (jackfruit) seeds *via* affinity chromatography on sugar columns. y-ArtinM was expressed in *S. cerevisiae* and purified as previously reported [18]. Briefly, the pYES-DEST52 (Invitrogen, Carlsbad, CA, EUA) expression vector containing the ArtinM coding sequence was used to transform *S. cerevisiae* strain BJ3501 (MATalpha pep4: HIS3 prb1-delta1.6R his3-delta200 ura3-52 gal2 can1). Transformants were selected on uracil-devoid minimal induction medium (SC-U) agar plates. Selected transformants were cultured in SC-U in which glucose was replaced by 2% galactose and 1% raffinose and collected by centrifugation. The cell pellets were suspended in sodium phosphate buffer supplemented with protease inhibitors and the cells were lysed by passage through a French pressure cell press (SLM-AMINCO, Urbana, IL, USA). y-ArtinM was purified by affinity chromatography on D-mannose columns (Sigma–Aldrich, St Louis, MO, EUA). Before use for biological assays, n-ArtinM and y-ArtinM preparations were incubated with a polymyxin solution (Sigma–Aldrich) for 1 h. The purity of the preparations was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration in the samples was estimated by densitometric scanning of the SDS gels.

2.3. Analytical ultracentrifugation

Sedimentation velocity measurements were performed using a Beckman XL-A analytical centrifuge equipped with both absorbance and interference optics. All data were acquired at a rotor-speed of 50,000 rpm at 20 °C using a Beckman An60Ti rotor. For each sample, 100 scans were acquired at 120 s intervals. The data were analyzed with the program SEDFIT [19] using a c(S) distribution. Buffer density and viscosity as well as the partial specific volume of the protein were calculated using SEDNTERP (Alliance Protein Laboratories, Thousand Oaks, CA, USA).

2.4. Glycan array analysis

The native and recombinant ArtinM forms were biotinylated as previously described [20] and quantified by determining their absorbance at 280 nm (OD_{280}). Microarrays were composed of lipid-linked oligosaccharide probes robotically printed in duplicate on nitrocellulose-coated glass slides at 2 and 7 fmol per spot (in-house designation sets 18–21bis) using a non-contact instrument, as previously described [21]. The microarray binding assays of biotinylated ArtinM proteins were performed at 19–20 °C, as previously described [22]. In brief, the arrayed slides were blocked with 1% w/v bovine serum albumin (BSA; Sigma–Aldrich A8577) in casein blocker solution (Pierce Chemical Co, USA) for 1 h. The biotinylated ArtinM preparations were overlaid at 50 μg/mL, and binding was detected with Alexa Fluor 647-labeled streptavidin (Molecular Probes-Life Technologies, CA, USA) at 1 μg/mL in blocker solution. Glycan array data analysis was performed with dedicated software [23]. The binding signals were probe-dose dependent.

2.5. Animals

Male BALB/c mice were acquired from the animal house of the Campus of Ribeirão Preto, University of São Paulo and housed in the Animal Facility of the Department of Cell and Molecular Biology of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, under optimized hygienic conditions. All experiments were conducted in accordance with the ethical guidelines of the Committee on Ethics in Animal Research. The mice were used for experiments at 6–8 weeks of age.

2.6. Human neutrophil purification

Neutrophil purification was performed as previously described [7]. The cell preparation had a purity of 98% as determined by flow cytometry, and over 95% of the neutrophils were viable as determined by trypan blue exclusion.

2.7. Hemagglutination assay

Human group O blood was collected in a heparinized tube, the erythrocytes were separated, and the hemagglutination assay was performed, as previously described [24]. Both ArtinM forms (800 μg/mL) were serially diluted (2-fold) in PBS and added to suspensions of human erythrocytes (3% in PBS) in each well. The lectin titer is represented as the reciprocal of the highest dilution of ArtinM protein able to cause detectable agglutination of erythrocytes. One hemagglutination unit (HU) is defined as the minimum lectin concentration required for complete agglutination. Specific hemagglutination activity corresponds to HU per microgram lectin (HU/μg).

2.8. Neutrophil migration assays

The induction of neutrophil migration by native and recombinant ArtinM forms *in vivo* was tested in 5 BALB/c mice per group. Two hundred microliters of n-ArtinM or y-ArtinM (5 μg/mouse) were injected into the peritoneal cavity. Carrageenan kappa (60 μg/animal; Sigma–Aldrich) and PBS were used as positive and negative controls, respectively. Six hours later, the mice were sacrificed by cervical dislocation. The neutrophils were immediately harvested in 5 mL of PBS. Total counts of harvested cells were determined by flow cytometry. The results are reported as the number of neutrophils per milliliter of cavity wash.

In vitro haptotaxis was assayed using the Transwell Matrigel invasion assay (Corning Incorporated Costar, NY, USA). Inserts containing polycarbonate 5-μm-pore size filters coated with Matrigel containing approximately 80% laminin were placed in the wells of 24-well plates containing n-ArtinM or y-ArtinM (5 μg/mL) dissolved in RPMI 1640 medium; Sigma–Aldrich). fMLP (10^{-4} M) and RPMI were used as positive and negative controls, respectively. The inserts were seeded with 1×10^5 human neutrophils and the plates were incubated at 37 °C in a humidified incubator with 5% CO_2 in air for 90 min. The migrated cells were fixed in 70% methanol and stained with a panoptic coloring agent (Laborclin, Paraná, Brazil). Five fields were counted for each assay and each sample was assayed in triplicate. The results are reported as the number of neutrophils per field.

In vitro chemotaxis was assayed in a modified 48-well Boyden chamber (NeuroProbe, Cabin John, MD, USA). y-ArtinM or n-ArtinM (5 μg/mL), fMLP (10^{-4} M), or serum-free RPMI, was added to the lower wells of the chamber, separated from the upper wells by a polycarbonate membrane filter (25 mm × 80 mm; Poretics, Livermore, CA, USA). Human neutrophils were seeded in the upper wells (5×10^4 cells/well) and the chambers were incubated at 37 °C in a humidified incubator with 5% CO_2 in air for 45 min. Cells that had migrated through the entire filter were counted. Five fields were counted for each assay and each sample was assayed in triplicate. The results are reported as the number of neutrophils per field.

2.9. Myeloperoxidase (MPO) assay

Human neutrophils were seeded in 96-well plates at 1×10^5 cells/well and stimulated by the n-ArtinM or y-ArtinM (5 μg/mL) at 37 °C for 30 min. The supernatants were collected and mixed with 0.02 M o-dianisidine (Sigma–Aldrich). Subsequently, the mixture was homogenized and incubated at 37 °C. After the incubation, the absorbance at 460 nm was measured using a microwell plate reader (PowerWave, Bio-Tek Instruments, Winooski, VT, USA). fMLP (10^{-4} M) was used as the positive control and Hank's medium, as the negative control.

2.10. β-Hexosaminidase assay

Rat mast cells line RBL-2H3 were incubated at 37 °C for 60 min with n-ArtinM or y-ArtinM (5, 10, or 15 μg/mL), DNP_{48}-HSA (positive control; 50 ng/mL; Sigma–Aldrich), or RPMI alone (negative control). Following incubation, 25 μL of supernatant or 25 μL of mast cells that had been solubilized in Tyrode's buffer (pH 7.3) containing 1% Triton-X 100 was mixed with 25 μL of the synthetic substrate p-nitrophenol-N-acetyl-β-glucosaminidase (Sigma–Aldrich) dissolved in 0.1 M citrate-acetate buffer (pH 4.5). The samples were incubated at 37 °C for 30 min and the reaction was stopped by adding 50 μL of a solution containing 0.2 M NaCl, 0.2 M NaOH, and 0.2 M glycine. The absorbance at 405 nm was measured using a microwell plate reader (PowerWave).

2.11. Cytokine production

Thioglycollate-elicited macrophages harvested from C57BL/6 (WT) or TLR2 knockout ($TLR2^{-/-}$) mice were used to determine the level of IL-12 p40 and IL-10 in response to stimulation with ArtinM. Macrophages (2×10^6/well) were seeded into a 24-well microplate and cultured at 37 °C in a humidified atmosphere with 5% CO_2 for 16 h. After washing with PBS, the adherent cells were incubated with ArtinM (2.5 μg/mL) or Pam3CSK4 (1 μg/mL) for 48 h. The IL-12 p40 and IL-10 concentrations in the supernatants were assayed by ELISA using specific antibodies (BD Biosciences, San Jose, CA, USA), according to the manufacturers protocol. Murine recombinant cytokines were used to create standard curves, which were used to determine the cytokine concentrations. The absorbance at 450 nm was measured using a microwell plate reader (PowerWave).

2.12. Statistical analysis

Results are presented as the mean ± SD, and all data were analyzed using Prism (GraphPad Software, San Diego, CA, USA). Differences between group means were determined by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. A p-value <0.05 was considered significant.

3. Results

3.1. Homogeneity and primary structure identity of n-ArtinM and y-ArtinM

We used SDS-PAGE to compare the homogeneity of the n-ArtinM and y-ArtinM preparations. After thermal dissociation, y-ArtinM and n-ArtinM exhibited identical electrophoretic profiles corresponding to a single 16 kDa band (Fig. 1A, lane 1 and lane 2, respectively), which is in accordance with the previously established criteria for ArtinM purity [3]. The analytical ultracentrifugation analysis confirmed that, in solution, n-ArtinM is a tetramer as shown by the X-ray structure [25]. Similarly, y-ArtinM had a tetrameric configuration as it exhibited a molecular weight of approximately 66 kDa (Table 1). The molecular weights obtained from the sedimentation velocities were confirmed by sedimentation equilibrium, which makes no assumption regarding the hydrodynamic shape of the protein. It is noteworthy that n-ArtinM can form higher order oligomeric species (Fig. 1B; minor peaks at *S*-values of approximately 6, 7, 9, 10). A similar feature was observed in y-ArtinM (Fig. 1C). These results indicated that *S. cerevisiae* transformed with the ArtinM construct expresses a tetrameric form of ArtinM.

3.2. y-ArtinM and n-ArtinM share carbohydrate-binding specificities

To assess the carbohydrate-binding properties of n-ArtinM and y-ArtinM, we performed glycan microarray analyses using a panel of 255 lipid-linked oligosaccharide probes representing diverse mammalian glycan sequences and their analogs. n-ArtinM and

Table 1
Sedimentation velocity of n-ArtinM and y-ArtinM. The sedimentation coefficient values (*S*) and calculated molecular weights (MW) are indicated.

Sample	*S*	MW
n-ArtinM	4.24	64 kDa
y-ArtinM	4.09	66 kDa

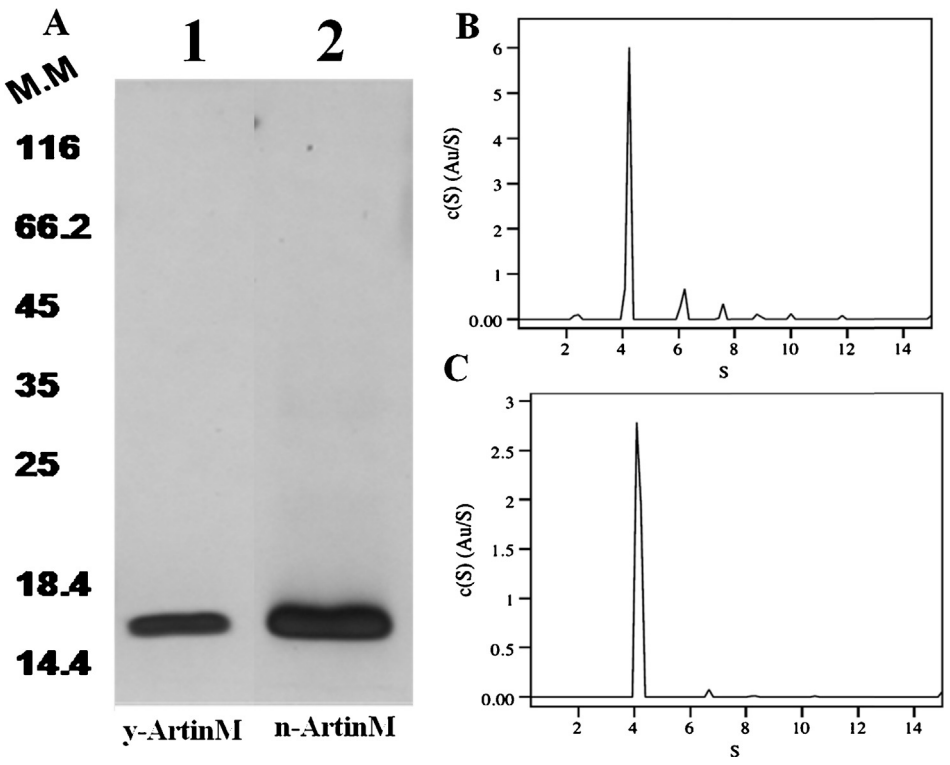

Fig. 1. Purification and structural characterization of n-ArtinM and y-ArtinM. (A) n-ArtinM and y-ArtinM were analyzed by SDS-PAGE. For each protein form, 5 μl of the preparation was heated at 100 °C for 10 min prior to loading onto a 12% SDS-PAGE gel. After gel electrophoresis, the protein bands were visualized by silver staining. Lane 1: y-ArtinM. Lane 2: n-ArtinM. Sedimentation velocity profiles of n-ArtinM (B) and y-ArtinM (C) at 20 °C. Fit and residuals after fitting to a c(S) model in SEDFIT, as well as the obtained distribution of sedimentation coefficients are shown. The sedimentation coefficient of tetrameric ArtinM form is 4 S.

y-ArtinM bound exclusively to the *N*-glycan probes present on the array (Fig. 2 and [26]), which is consistent with the previously reported specificity of the native form [5]. There was no significant difference in the carbohydrate-binding specificities of the two lectins, suggesting that the three-dimensional structure of the carbohydrate recognition domain (CRD) which is responsible for carbohydrate binding by the protein expressed in *S. cerevisiae* was similar to that of the natural protein. In the matrix presentation in Fig. 2, the relative binding intensities of both ArtinM forms toward 15 selected *N*-glycan probes are shown. Among the good ligands for the two lectins were the pauci-mannose structure Man3GN2 (position 129), its core-fucosylated analog Man3FGN2 (position 131), and the α1–6 mannose branch-elongated Man4aGN2 (position 133). Additionally, strong binding was observed for mono- or bi-antennary structures, in particular, those with core fucose residues (e.g. positions 147, 152, 159). Substantially weaker or no binding was observed to high-mannose *N*-glycans, the multi-antennary and bisected structures. These observations are in overall good agreement with previous findings from inhibition assays [5] and frontal affinity chromatography studies [27]. Unexpectedly, we detected very little binding to the Man3 structures with xylose (positions 132 and 134), although the horseradish peroxidase *N*-glycan Manα1–6(Manα1–3) (Xylβ1–2)Manβ1-4GlcNAcβ1–4(Fucα1–6)GlcNAc has previously been reported as the best ligand for n-ArtinM [5]. One possible explanation is that the α1–3-linked core fucose is an essential element for the interaction, but was not well presented in the ring-opened neoglycolipid probe prepared by reductive amination [28]. Thus the n- and y-ArtinM have the same carbohydrate-binding specificity. However, the y-ArtinM seems to have a higher avidity to these glycans [26]. Additional studies are required to elucidate the molecular basis of these differences.

Table 2
Hemagglutination activity of n-ArtinM and y-ArtinM. Hemagglutination unit (HU) is defined as the minimal concentration necessary to induce cell agglutination. The specific agglutination activity is given as HU per microgram of lectin (HU/μg).

Sample	Minimal agglutination concentration (HU/μg)	Minimal agglutination concentration (μg/mL)
n-ArtinM	1.6	6.24 μg/mL
y-ArtinM	0.1	117 μg/mL

3.3. *y-ArtinM and n-ArtinM induce hemagglutination*

The hemagglutination assay is the most commonly used tool to evaluate the carbohydrate-binding ability of a lectin. Hemagglutination results from the concurrent linking of oligomeric lectins to the surfaces of the target cells. The ability of both forms of ArtinM to agglutinate human erythrocytes was evaluated by incubating human group O red blood cells with serially diluted ArtinM (31.2 to 800 μg/mL) in PBS. n-ArtinM and y-ArtinM induced detectable cell agglutination at concentrations as low as 6 μg/mL and 117 μg/mL, respectively, with specific activities of 1.6 and 0.1 HU/μg, respectively (Table 2). These results are consistent with the oligomeric nature of y-ArtinM and n-ArtinM.

3.4. *y-ArtinM and n-ArtinM induce neutrophil migration and degranulation*

n-ArtinM has been reported to induce haptotactic neutrophil migration by establishing concomitant interactions with CXCR2 on the cell surface and laminin in the extracellular matrix [6,29].

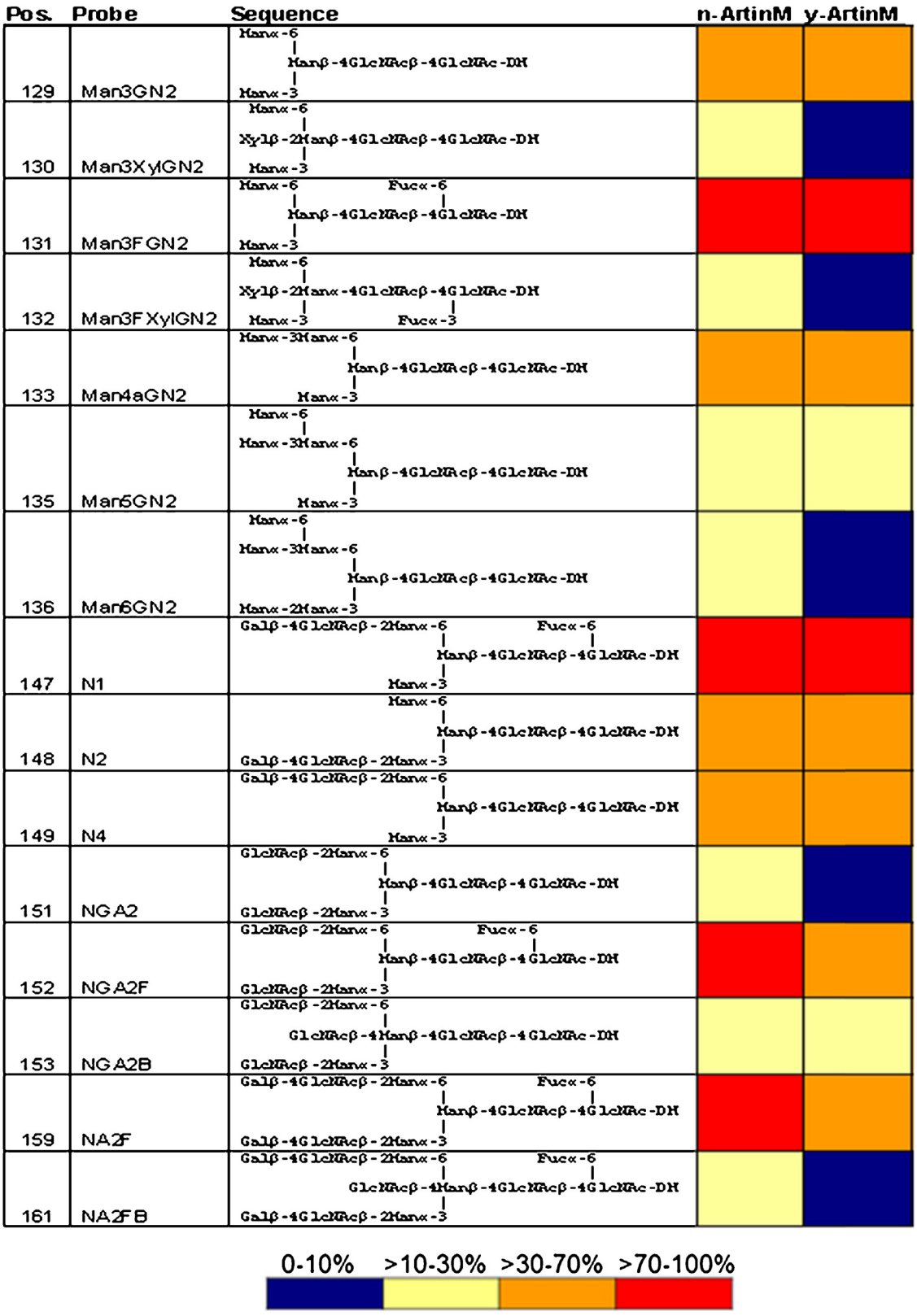

Fig. 2. Relative binding intensities of n-ArtinM and y-ArtinM to 15 *N*-glycan microarray probes. Relative binding intensities (7 fmol per spot) are shown as the percentage of the fluorescence signal intensity relative to that given by Man3FGN2, the probe most strongly bound by both proteins. Pos.: probe position in the microarray in [26].

Therefore, we investigated the ability of y-ArtinM to induce haptotaxis. *In vivo* neutrophil migration was assayed by injecting ArtinM into the peritoneal cavity of BALB/c mice. As shown in Fig. 3A, n-ArtinM and y-ArtinM induced neutrophil migration similarly to or more strongly than the positive control carrageenan. Fig. 3B shows that the rates of *in vitro* neutrophil haptotaxis induced by n-ArtinM (100 neutrophils/field) or y-ArtinM (380 neutrophils/field) were similar or superior to that induced by the positive control

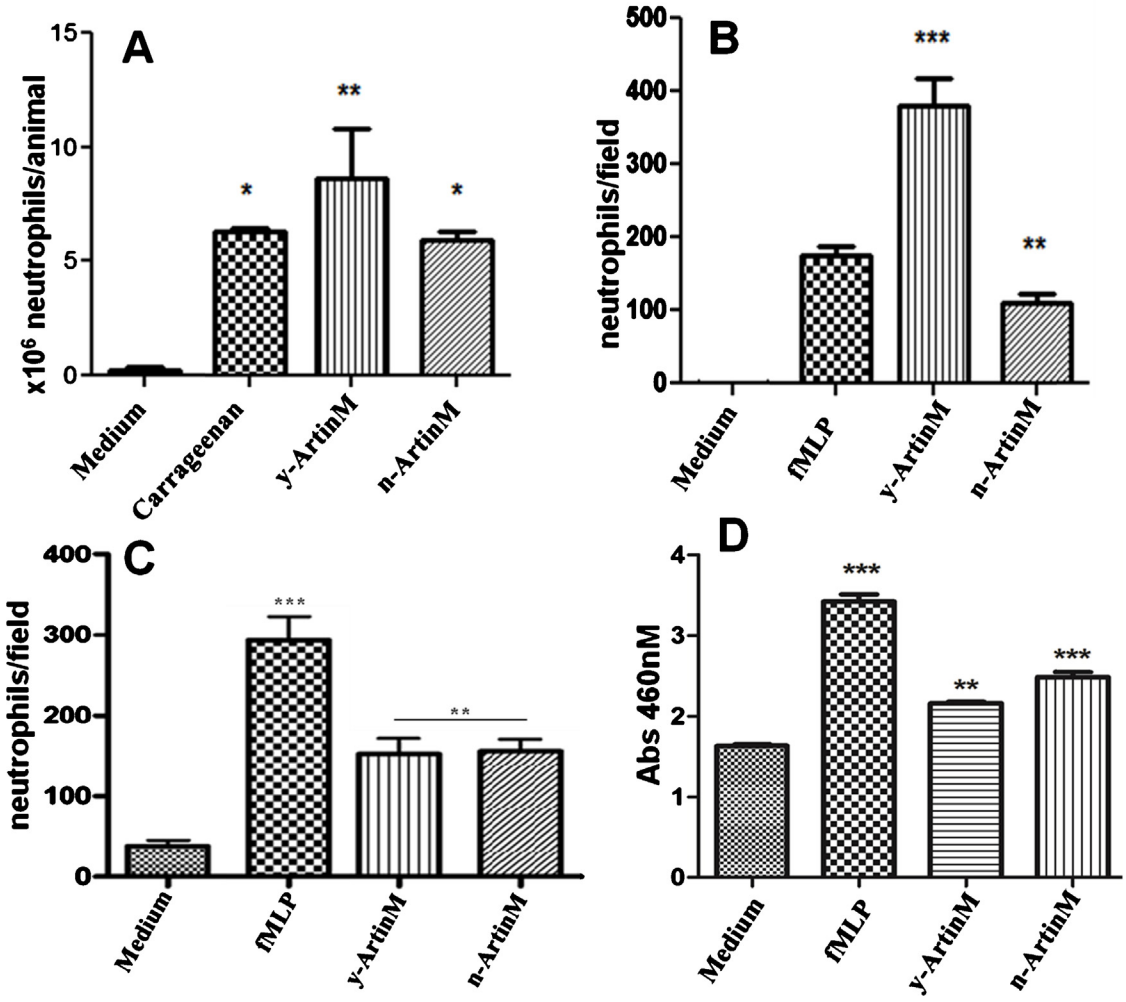

Fig. 3. y-ArtinM and n-ArtinM induce neutrophil migration and degranulation *in vivo* and *in vitro*. (A) *In vivo* neutrophil migration: BALB/c mice (5 animals/group) were injected intraperitoneally with 5 μg n-ArtinM or y-ArtinM. Saline and carrageenan (60 μg/animal) were used as the negative and positive control, respectively. The peritoneal fluid was collected after 6 h and migrant neutrophils were counted by flow cytometry; (B) *In vitro* neutrophil haptotaxis: human neutrophils were subjected to a Transwell Matrigel haptotaxis assay using 5 μg/mL of y-ArtinM and n-ArtinM; (C) *In vitro* neutrophil chemotaxis: human neutrophils were subjected to a Boyden chamber chemotaxis assay using 5 μg/mL of y-ArtinM and n-ArtinM. In both migration assays, RPMI and fMLP (10^{-4} M) were used as negative and positive controls, respectively. For each assay, the cells were counted in five fields and each sample was assayed in triplicate. The results are expressed as the number of neutrophils per field; (D) Myeloperoxidase activity assay: human neutrophils were stimulated with both forms of ArtinM (5 μg/mL). The supernatant was collected and added to a solution of 0.02 M o-dianisidine, and the OD_{460} of the mixture was determined. fMLP (10^{-4} M) and Hank's medium were used as the positive and negative controls, respectively. Each sample was assayed in duplicate. Results are expressed as the mean ± SD; $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.0001$ compared to the negative control (one-way ANOVA followed by Bonferroni's multiple comparison test).

fMLP (180 neutrophils/field), and significantly higher than that induced by the negative control. Fig. 3C shows that the rates of *in vitro* neutrophil chemotaxis induced by n-ArtinM and y-ArtinM (140 and 150 neutrophils/field, respectively) were similar to that in the positive, and significantly higher than that in the negative control. Notably, in the *in vitro* haptotaxis and the *in vivo* neutrophil migration assay, but not in the *in vitro* chemotaxis assay, y-ArtinM more efficiently promoted migration than n-ArtinM did. The reason for this is not known. In order to evaluate whether y-ArtinM also stimulates neutrophil degranulation as with n-ArtinM [7], we performed MPO release assay. We found that y-ArtinM as with n-ArtinM was able to stimulate MPO release by neutrophils at levels close to those found for neutrophils induced by fMLP (Fig. 3D). Taken together, these results indicated that, like n-ArtinM, y-ArtinM is able to stimulate neutrophil migration and degranulation.

3.5. *y-ArtinM and n-ArtinM induce mast cell degranulation*

Previously, we have demonstrated that in addition to stimulating neutrophil migration and degranulation, n-ArtinM induces mast cell activation, as shown by the massive degranulation of rat peritoneal mast and RBL-2H3 cells in response to *in vivo* and *in vitro* stimulation with the lectin, respectively [8,9]. Activated cells release mast cell granule components and the allergic mediator β-hexosaminidase [30]. We tested the ability of the y-ArtinM to induce mast cell degranulation by measuring the β-hexosaminidase activity in the cell supernatant of y-ArtinM- and n-ArtinM-stimulated RBL-2H3 cells. Compared to the negative control, stimulation with n-ArtinM and y-ArtinM induced a 1.86-fold and 2.67-fold increase in the amount of β-hexosaminidase released, respectively, at the optimal concentration of 10 μg/mL (Fig. 4). These results supported the hypothesis that y-ArtinM is

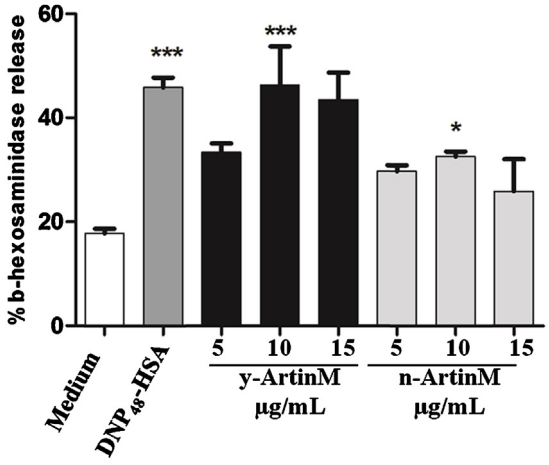

Fig. 4. y-ArtinM, like n-ArtinM, induces RBL-2H3 degranulation at an optimal concentration of 10 μg/mL. RBL-2H3 cells were stimulated with n-ArtinM or y-ArtinM at various concentrations (5, 10, or 15 μg/mL) and incubated for 30 min at 37 °C. The β-hexosaminidase release was calculated from the absorbance at 405 nm obtained using an ELISA Power Wave X microplate reader. The total amount of β-hexosaminidase in each well was determined by adding the value measured in the supernatant to the value obtained for the cells solubilized in 1% Triton-X 100. This value was then used to calculate the percentage of β-hexosaminidase released in the supernatant. The results are representative of 2 independent experiments. Negative control: culture medium in the absence of stimulus. Positive control: DNP_{48}-HAS (50 ng/mL). Results are expressed as the mean ± SD; $^{*}p < 0.05$ and $^{***}p < 0.0001$ compared to the negative control (one-way ANOVA followed by Bonferroni's multiple comparison test).

able to cross-link with receptors on mast cell surfaces that induce degranulation.

3.6. y-ArtinM and n-ArtinM induce IL-12 p40 and IL-10 production by murine macrophages in a TLR2-dependent manner

The activation of cellular process by binding of n-ArtinM to the cell surface receptors leads to IL-12 production and establishment of Th1 immunity, balanced by IL-10 production [10,11]. Previously, we demonstrated that n-ArtinM induces the release of IL-12 and IL-10 by macrophages in a TLR2-dependent manner [11]. In this study, we investigated the ability of the y-ArtinM to induce IL-12 and IL-10 production in murine macrophages and evaluated whether the activity was TLR2-dependent. Peritoneal macrophages obtained from WT and $TLR2^{-/-}$ mice were cultured with n-ArtinM or y-ArtinM for 48 h and the cytokine concentrations in the supernatants were assayed by ELISA.

We found that y-ArtinM, like n-ArtinM, stimulated the production of high levels of IL-12 p40 in wild-type macrophages but not in $TLR2^{-/-}$ macrophages, which released IL-12 p40 at levels that were 82% and 87% lower, respectively, than wild-type macrophages and were closer to those detected in the negative control (Fig. 5). In addition, y-ArtinM and n-ArtinM stimulated IL-10 production in wild-type macrophages, while $TLR2^{-/-}$ macrophages released IL-10 at levels 47% and 61% lower than in wild-type macrophages after y-ArtinM and n-ArtinM stimulation, respectively. These findings indicated that TLR2 is crucial for activation of macrophages by y-ArtinM.

4. Discussion

In the present study, we characterized the biological activities of a recombinant form of the immunomodulatory lectin, ArtinM, expressed in *S. cerevisiae* (y-ArtinM), compared with the well-established properties of native lectin (n-ArtinM) obtained from *A. integrifolia* seeds. Additionally we focused on the relationship between the detected biological properties and the quaternary structure of the protein, as a former recombinant ArtinM obtained by expression in *E. coli* differed from n-ArtinM by virtue of being monomeric and requiring 30-fold higher concentration than n-ArtinM to trigger proliferation in murine splenic cells [31]. Here, we demonstrated that y-ArtinM qualitatively and quantitatively reproduced the biological effects of n-ArtinM on innate immune cells, consistent with the proteins having the same carbohydrate-binding specificity and quaternary structure.

The specificity of carbohydrate binding, as assessed by glycan microarray analysis, was indeed similar for the two ArtinM forms. The results obtained are in agreement with a previous report demonstrating that ArtinM CRD has primary and secondary subsites for interaction with complex glycans [32]. The primary subsite interacts with Manα1–3(Manα1–6) Man *via* numerous hydrogen bonds, whereas the secondary subsite interacts with other sugar

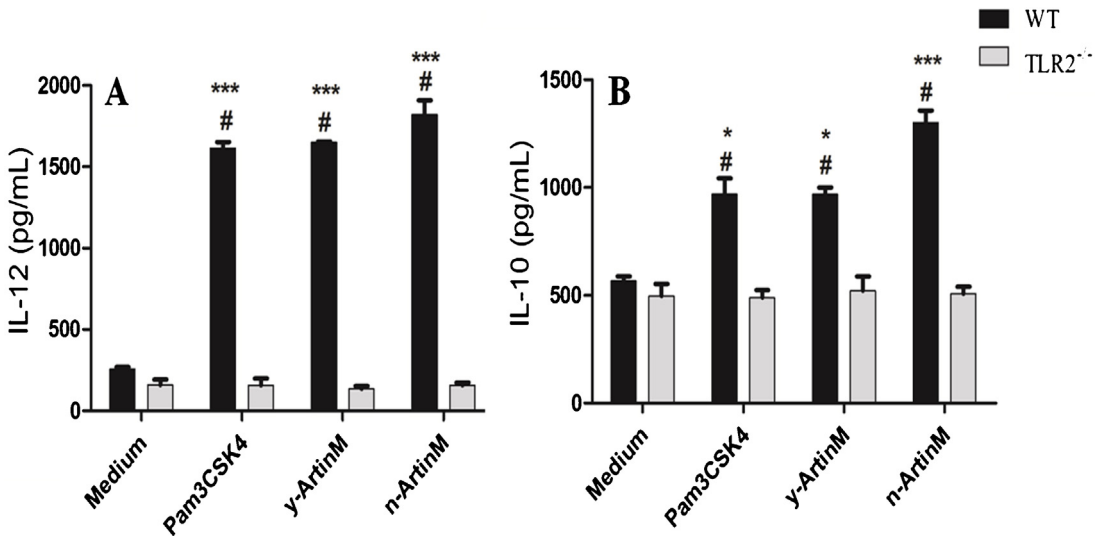

Fig. 5. y-ArtinM, like n-ArtinM, induces IL-12 and IL-10 production in a TLR2-dependent manner. Peritoneal macrophages from C57BL/6 (WT) or $TLR2^{-/-}$ mice were cultured for 48 h in the presence of 5 μg/mL n-ArtinM or 5 μg/mL y-ArtinM, 1 μg/mL PamCSK3 (positive control), or RPMI medium (negative control). The amount of IL-12 p40 in the supernatant was determined by capture ELISA. Assays were performed in triplicate, and the results represent the mean ± SD of at least 3 independent experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$stimulated *versus* negative control group, $^{\#}p < 0.001$ WT *versus* $TLR2^{-/-}$ group (ANOVA followed by Bonferroni's multiple comparison test).

residues, such as GlcNAc and Fuc, located close to the trimannoside core. The results of the glycan microarray were also consistent with the carbohydrate-binding specificity of ArtinM determined by frontal affinity chromatography [26]. The y-ArtinM has a binding profile similar to that of n-ArtinM; further studies are required to compare the fine specificities of the two lectin forms, using precisely matched samples at different concentrations that interact with a larger repertoire of *N*-glycan sequences.

The electrophoretic profiles of thermally denatured samples of n-ArtinM and y-ArtinM were similar; both gave a single 16 kDa band. Analytical ultracentrifugation showed similar molecular masses for n-ArtinM (64 kDa) and y-ArtinM (66 kDa), which is compatible with the tetrameric organization of the lectin molecule [4]. It is well known that oligomerization enhances the avidity of lectins for multiple targets on the cell surface contributing to the triggering of signaling and biological responses [33]. Herein, we demonstrated that both y-ArtinM and n-ArtinM promoted events dependent on lectin multivalency. Previous studies have shown that n-ArtinM induces neutrophil haptotaxis by simultaneous interactions of its CRDs with glycoligands in the extracellular matrix (laminin) and receptors in neutrophils, such as CXCR2 [6]. The induction of neutrophil haptotaxis is in agreement with the occurrence of an oligomeric structure of y-ArtinM.

Lectin binding to cell surface glycoproteins may influence the molecular structure and dynamics of the membrane. This was clearly demonstrated for galectin-1, the binding of which to T cells results in the redistribution of specific surface glycoproteins into discrete membrane microdomains and triggers signaling for cell apoptosis [34]. Similar events were demonstrated to occur with other members of the galectin family [reviewed in 35]. Clustering of selected glycopoteins in microdomains is an attractive hypothesis to explain how ArtinM can discriminate among several potential glycosylated receptors. Our previous studies have demonstrated that ArtinM can discriminate among surface *N*-linked glycoproteins, since it binds to CXCR2 on neutrophils [6], to TLR2 and CD14 on macrophages [10,11, and unpublished data], and to IgE and FcεRI on mast cells [8,9]. We are currently investigating the occurrence of glycoprotein segregation on the leukocyte membrane following stimulation with ArtinM.

Although it is established that lectins induce neutrophil migration *via* direct interaction with appropriate surface glycotargets, an additional mechanism may be involved in the concomitant interaction of the lectin with resident cells. It was reported for lectins from *Pisum arvense*, *Arum maculatum*, and *Dioclea rostrata*, as well as for concanavalin A, wheat germ agglutinin, and ArtinM [reviewed in 36]. In the case of y-ArtinM, we found that, in a manner similar to that of n-ArtinM, it promoted mast cell degranulation and macrophage activation. The mast cell degranulation triggered by ArtinM was previously demonstrated to cause the release of IL-8, providing an amplification loop for the induction of neutrophil migration [8,9].

Regarding the ArtinM-induced activation of macrophages, it is largely due to the recognition of TLR2 *N*-glycans [10,11], leading to the increased production of IL-12. This property, which was also demonstrated by y-ArtinM, induces Th1 immunity. The administration of ArtinM to mice consistently conferred protection against infections by intracellular pathogens. The Th1-skewed response induced by ArtinM is balanced by the induction of IL-10 production, which prevents exacerbated inflammation [12–15]. As shown in the present study, the IL-10 inducing property was also demonstrated by y-ArtinM.

The absence of effective therapies against most intracellular pathogens necessitates the development of new immunostimulatory agents, such as agonists of innate immunity receptors, especially Toll-like receptors (TLRs) [37]. Our observations demonstrate that the recombinant counterpart of a well-known immunomodulatory lectin reproduces the structural and biological features of the native protein. Because these features account for the capacity of lectins to confer protection against infection by several intracellular pathogens, the study may represent an important step toward the design of new immunostimulatory agents whose action is based on carbohydrate recognition.

Conflict of interest

The authors confirm that there are no conflicts of interest pertaining to the material in this manuscript.

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