A Computational Study of the Oligosaccharide Binding Sites in the Lectin-Like Domain of Tumor Necrosis Factor and the TNF-derived TIP Peptide

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Abstract: The lectin-like domain of Tumor Necrosis Factor (TNF), mimicked by the TIP peptide, activates amiloride-sensitive sodium uptake in type II alveolar epithelial cells and as such increases alveolar liquid clearance in dysfunctional lungs. This protective effect is blunted upon mutation of residues T105, E107 and E110 in human TNF into alanine or upon pre-incubation of the cytokine with the disaccharide *N,N'*-diacetylchitobiose. In this study, we used molecular docking and molecular dynamics simulation to predict the binding sites for *N,N*'-diacetylchitobiose and trimannose-O-ethyl in the lectin-like domain of TNF and in the TIP peptide. Specific sites (K98, S99, P100, Q102 and E116) in the three loops of the lectin-like domain provide specific binding for both oligosaccharides, but none of the residues crucial for anti-edema activity are involved in hydrogen bonding with oligosaccharides or are subjected to steric hindrance by them. These results thus suggest that neither chitobiose nor trimannose affect crucial amino acids, while they occupy the cavity in the lectin-like domain. Consequently, both crucial amino acids and the emptiness of the cavity in the lectin-like domain may be critical for TNF's lectin-like activity. Analogously, the R4, E5, P7, Y16 amino acids of the TIP peptide are involved in forming hydrogen bonds with both oligosaccharides, whereas residues T6, E8 and E11 (corresponding to T105, E107 and E110 in hTNF) play an important role in stabilizing the peptide-oligosaccharide complex, supporting the hypothesis that amino acids in the polar region (TPEGAE) of the TIP peptide represent only a partial binding motif for sugars.

Keywords: lectin-like domain, tumor necrosis factor, TIP peptide, oligosaccharides, molecular docking, molecular dynamics simulation, alveolar liquid clearance.

INTRODUCTION

Tumor Necrosis Factor (TNF) is a widely studied pleiotropic cytokine that exerts a broad range of effects in a variety of mammalian cells [1]. In addition to its binding to mammalian 55 kDa (TNF-R1) and 75 kDa (TNF-R2) receptors, the 51 kDa trimeric cytokine also has a lectin-like activity, which associates with specific oligosaccharides, such as *N,N'*-diacetylchitobiose and branched trimannoses [2, 3]. These sugars can be present in specific glycoproteins, such as uromodulin, a glycoform of Tamm-Horsfall protein expressed in the loops of Henle in pregnant women [2, 3], shown to bind pro-inflammatory cytokines, such as TNF, IL-1β and IL-2, which could represent a mechanism to clear excessive levels of these mediators from the circulation during pregnancy [2, 3]. As uromodulin-bound TNF is still able to exert cytotoxic effects in L929 fibrosarcoma cells, it was proposed that the lectin-like domain of the TNF should be spatially distinct from its receptor binding sites [2]. Specific oligosaccharides, such as *N,N*'-diacetylchitobiose, known to bind to the lectin-like domain of the TNF, inhibit the lytic activity of TNF in bloodstream forms of African trypanosomes, which occurs upon binding to the variant surface glycoprotein (VSG) of the parasites, but they do not interfere with cytokine's cytotoxic activity in L929 cells [4, 5]. Moreover, lectins with a similar oligosaccharide specificity as TNF, such as *Urtica Dioica* Agglutinin, but not those with a different specificity, block the trypanolytic effect of the cytokine [4]. TNF's trypanolytic activity is not inhibited by soluble TNF receptor 1, which neutralizes the cytokine's cytotoxic effect in L929 cells. Thus, the lectin-like domain of TNF differs both spatially and functionally from the receptor binding sites. Molecular graphics comparisons of tertiary structures of TNF (trypanolytic) and the highly homologous lymphotoxin-α (non-trypanolytic), led us to select a dissimilar structure mediating the lectin-like activity [4]. This structure, present at the tip of the TNF molecule and spatially distinct from the TNF receptor binding sites, is mimicked by a circular 17 amino acid peptide, called the TIP peptide. Antibodies to this peptide inhibit TNF's trypanolytic activity and moreover the TIP peptide exerts trypanolytic activity [4]. Since alanine substitution of residues T105, E107 and E110 in hTNF completely blunts the cytokine's trypanolytic activity, these amino acids were proposed to be crucial for TNF's lectin-like activity.

An increase in alveolar liquid content, which severely hampers efficient gas exchange, can occur when the rate of fluid movement into the alveoli exceeds alveolar liquid clearance capacity (ALC), the latter of which is regulated by alveolar sodium transport [6]. Pharmacological treatment with β2-adrenergic agonists, which leads to cAMP-dependent activation of both the epithelial sodium channel (ENaC), situated at the apical membrane of type II alveolar epithelial cells, and the Na^+-K^+-ATPase, located at the basolateral side, induces a sustained stimulation of alveolar fluid reabsorption, thus facilitating recovery from experimental pulmonary edema [7]. However, these agents readily induce tolerance [8, 9] and can have side effects, such as tachycardia [10], thus hampering their use in patients with heart disease, which often suffer from cardiogenic edema. Moreover, in conditions associated with increased generation of reactive oxygen species (ROS), these agents no longer work [11], making the search for alternative candidates augmenting transepithelial sodium transport in injured lungs of high therapeutic importance.

The lectin-like domain of TNF mediates an increase of amiloride-sensitive sodium transport in pulmonary microvascular endothelial and alveolar epithelial cells [12, 13]. Moreover, the TIP peptide, which mimics the lectin-like domain of the cytokine [4], efficiently activates ALC in blood-perfused isolated flooded rat and

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rabbit lung models *ex vivo* [14, 15], as well as in flooded rat lungs *in vivo*, when applied intratracheally [16]. Recent studies moreover indicate that the TIP peptide can also blunt hyperpermeability mediated by the G^+ bacterial toxins listeriolysin (LLO) and pneumolysin (PLY) *in vitro* and *in vivo*, upon inhibiting protein kinase C-α activation and reactive oxygen species (ROS) generation [17, 18]. The peptide moreover activates ENaC in resting or toxin-treated alveolar epithelial cells [19, 20] and improves rat lung function upon lung transplantation in rats [19]. These results indicate that the lectin-like domain of TNF has a potential therapeutic value in preventing the formation and activating the reabsorption of pulmonary edema [21].

To date, the mechanism by which the TIP peptide activates ENaC-mediated sodium uptake remains elusive. We currently put forward the hypothesis that glycosylated interaction partners or ion channels on the apical side of alveolar epithelial cells get activated upon a protein-carbohydrate interaction mediated by the lectin-like domain of the TNF [4]. This assumption is strengthened by the observation that the TIP peptide mediates edema resolution only when applied to the apical side of the lung epithelium [19], thus most likely through ENaC. This activation can be, however, abolished by preincubation of the TIP peptide with the *N,N'*-diacetylchitobiose (chitobiose), but not cellobiose [19], or by introducing critical mutations, T105A, E107A, E110A or T6A, E8A, E11A in the lectin-like domain or in the TIP peptide, respectively [4, 13, 19]. Trimannose-*O*-ethyl (trimannose), as well as chitobiose, were shown to inhibit receptor-independent TNF binding to the glycosylated protein uromodulin, whereas other mono- and oligosaccharides had no inhibitory effect [3]. Taken together, these data emphasize the importance for ENaC activation of specific protein-carbohydrate interactions between the lectin-like domain of the TNF (or the TIP peptide) and its putative interaction partners, mediated by mannose- and glucosamine-rich carbohydrate moieties.

In this study, we wanted to better characterize the nature of the protein-carbohydrate interactions between the lectin-like domain of TNF and its interaction partners *N,N'*-chitobiose or branched trimannose. To that end, we have investigated whether the previously detected Thr and Glu residues are directly or rather indirectly involved in the oligosaccharide binding activity of TNF or the TIP peptide. Since no experimentally determined 3D structure of the TIP peptide is available, we have made use of a model structure of the TIP peptide, based on the crystal structure of TNF [22]. Molecular docking and molecular dynamics (MD) simulations, which allow the identification of specific binding sites and key elements involved in the interactions, revealed that cellobiose is theoretically unable to form stable complexes with TNF and the TIP peptide, as was indeed confirmed by experimental results [3, 4, 19, 23]. In general, the results of this study can thus improve our understanding of the lectin-like interactions between a cytokine domain or its peptide mimic and specific oligosaccharides. In particular, this investigation can stimulate the development of novel peptide-based therapeutics tackling pulmonary edema.

METHODS

Molecular Structures

Cellobiose, *N,N'*-diacetylchitobiose, and trimannose-*O*-ethyl were built in YASARA Structure version 11.2.18 (YASARA Biosciences GmbH, Vienna, Austria). 3D coordinates of human TNF (PDB: 1TNF) [22] were used as a template to build the TIP peptide model, using YASARA. The structural alignment of the three TNF monomers showed differences in side-chains of the amino acids 100-116 of the lectin-like domain (PCQRETPEGAEAKPWYE), yielding three different models of the TIP peptide: TIP_A, TIP_B, and TIP_C. The mutations rendering the sequence of the lectin-like domain of TNF identical to the one used for the TIP peptide in experimental studies (CGQRETPEGAEAKPWYC) [4, 16, 19] were introduced and the disulfide bond between terminal cysteines was added to retain the circular structure. The models were placed in the 5×5×5 nm simulation cells and were subjected to energy minimization and MD equilibration with YASARA, as described below. A 10 ns equilibration run (backbone atoms fixed) was followed by a 90 ns production run (all atoms free). Three-dimensional alignment of the equilibrated TIP peptides with the crystal structure of the TNF using MUSTANG [25] showed that the TIP_A peptide has the closest conformational resemblance to the lectin-like domain of the TNF, with the distances between the T6-T105, E8-E107 and E11-E110 C-α atoms below 0.2 nm.

Docking of TNF and the TIP Peptide with Oligosaccharides

Molecular docking was performed in AutoDock Vina, version 1.1.2 which shows higher performance and better accuracy of the binding mode predictions, as compared to AutoDock 4.0 [24]. Hydrogen atoms and charges were added using AutoDockTools version 1.5.4 [26], using Babel for hydrogens and the Gasteiger PEOE method for charges [27]. All rotatable bonds in the oligosaccharides were set as such (Fig. **S1** supplementary materials) and side chains of residues T6, E8 and E11 of the TIP peptide were also set flexible. Global docking was performed to get a "blind" docking result of the *N,N'*-diacetylchitobiose, trimannose, and cellobiose to TNF and the minimized TIP_A model. For the TNF docking, the search space was subsequently narrowed to a 5×5×5 nm box, in which the lower part of the TNF molecule was placed to increase docking accuracy. The exhaustiveness of the search was set to 200. Additionally, Glide version 5.7 (Schrödinger, LLC, New York, NY) was used in order to verify the gained data independently by means of a different technique. Blind docking with Glide flexible docking and standard precision was followed by extra precision docking with subsequent post-docking minimization. Receptor and ligands were pre-processed by assigning bond orders only, using hydrogens added by YASARA. Here, Glide assigns its own partial charges to be used for the Glide flexible docking. The potentials for non-polar parts of the ligands were softened by introducing a scaling factor of 0.8 to the van der Waals radii of ligand atoms with partial atomic charge less than 0.15.

Molecular Dynamics (MD) Simulations of TNF and the TIP Peptide with Oligosaccharides

After docking, the TNF- and the TIP peptide-oligosaccharide poses with the highest binding affinities were subjected to energy minimization and MD equilibration with YASARA. The receptor binding sites of TNF, which are far from the sugar binding domain, were removed, leaving the extended lectin-like domain (LLD) with the adjacent loops (S60-T79, L94-L120 residues from each monomer). The simulation cell with dimensions of 7×7×7 nm was filled in each case with explicit solvent (TIP3P water). Periodic boundary conditions were applied and the YAMBER3 force field [28] was employed. The cut-off for the Lennard-Jones potential and the short range electrostatics was 1 nm. Long-range electrostatics were calculated using the Particle Mesh Ewald (PME) method [29] with a grid spacing <0.1 nm, 4^{th} order PME-spline, and PME tolerance of 10^{-5} for the direct space sum. Force field parameters for the oligosaccharides were generated using the AutoSMILES approach [30], which employs a semi-empirical AM1 geometry optimization and an assignment of charges, followed by an assignment of AM1BCC atom and bond types with refinement using RESP charges, and finally the assignments of general AMBER force field atom types. YASARA's pKa utility was used to assign pKa values at pH 7.3. The simulation cell was neutralized with NaCl (0.9% final concentration; % by mass) by iteratively placing sodium and chlorine ions at the coordinates with the lowest electrostatic potential. The entire systems were then energy-minimized using steepest descent minimization, in order to remove conformational stress, followed by a simulated annealing minimization until convergence (<0.05 kJ/mol/200 steps). Three independent simulations were run for each pose (five

in the LLD-cellobiose) including controls at 298 K, with integration time steps for intra-molecular and inter-molecular forces of 1 fs and 2 fs, respectively. After 10 ns of equilibration, the restraints on the backbone atoms of the LLDs were removed in all models (except for the terminal C-α in the LLDs) and simulations were further continued for another 50 ns and 90ns for the LLD and TIP peptide respectively. Evaluation of the LLD- and the TIP peptide-oligosaccharide complexes over time was done on the basis of root mean square deviation (RMSD) of C-α atoms, hydrogen bond occurrence and binding energies between the receptors and the corresponding oligosaccharides. Binding energies were calculated with YASARA using the following formula:

$$E_{binding} = \left(E^{protein}_{internal} + E^{sugar}_{internal} + E^{protein}_{solvation} + E^{sugar}_{solvation}\right) - \left(E^{complex}_{internal} + E^{complex}_{solvation} + A^{accessible}_{surface} \times 0.15\right)$$

where E – energy, A – area in Å^2, and 0.15 is a guesstimate of the entropic cost of exposing one Å^2 to the solvent (kJ/mol× Å^2) [YASARA's documentation and 31]. The energy was calculated using the YAMBER3 force field parameters.

Constant acceleration steered molecular dynamics (SMD) simulations were performed in YASARA. After the 90 ns production run, constant acceleration to the center of masses to both the TIP peptide and the appropriate oligosaccharide (triplets of each) was applied in opposite directions to each other. The following constant accelerations were used: 3500, 3000, 2500, 2000, 1500, and 1000 pm/ps^2. All other parameters were the same as described above for a regular MD.

RESULTS AND DISCUSSION

In silico modeling of the full process of diffusion and ligand binding without any approximations is still beyond present computational capabilities. Docking programs are widely used to discover novel ligands efficiently and to predict protein-ligand complex structures with reasonable accuracy and speed. However, there is an emerging demand for better performance of the scoring functions. A combination of molecular docking with classical MD simulations represents a fitting compromise that encompasses the advantages of both. Upon performing the rigid docking procedure, we could localize potential "hot spots" on the TNF and the TIP peptide surfaces, representing suitable binding sites for the oligosaccharides. The dynamics of these TNF- and TIP peptide-oligosaccharide complexes were further studied by means of MD simulations that take into account solvation and thermal motion, allowing the physical movement of all atoms and molecules in the protein and the peptide. In the MD simulation the predicted binding poses were allowed to evolve over time, thus allowing the identification of persistent interactions.

Docking and Molecular Dynamics of TNF with Oligosaccharides

Molecular docking allowed us to identify unique binding sites for *N,N'*-diacetylchitobiose and trimannose in the lectin-like domain of TNF. The cavity formed by the three lectin-like domain loops of trimeric TNF (Fig. **1A**) is solvent-accessible, ~1.2 nm in width and ~1.9 nm deep, with charged residues (R103, E104) lining the cavity entrance. Partial unfolding of TNF at acidic pH, which promotes surface exposure of hydrophobic residues (like L157, W28, and W114) [32], could also lead to an increase in cavity volume, making it more accessible to the ligands. Due to the size of the TNF trimer and our primary interest in interactions mediated by the lectin-like domain of TNF, we have truncated the cytokine molecule for the subsequent MD equilibration of the complex. The remaining extended lectin-like domain (LLD) contained only one-third of the atoms and amino acids, as compared to native TNF, including the elongated anti-parallel β-pleated sheet sandwich, in order to preserve the "jelly-roll" topology of the molecule. This modification significantly lowered the contact area between the monomers, which could eventually lead to the dissociation of the trimer. To prevent the latter, we have fixed C-α of the terminal residues in the LLD (see materials and methods for details), which appears to be a good compromise between stabilizing the LLD trimeric structure on the one hand and restraining protein-oligosaccharide complexes on the other hand. After 15 ns of MD simulations, the root mean square deviation of the LLD C-α atoms reaches a stable plateau at 0.15-0.26 nm from the initial conformation, representing a value typical for systems of this size [33]. Additionally, the hydrogen bonding network between the protein and the corresponding oligosaccharides was stabilized and showed persistent interactions.

As such, we can state that after 15 ns we have a fully equilibrated system that can be used to run production MD. After 35 ns of production, the LLD-chitobiose and LLD-trimannose complexes showed positive binding energies of 63–310 kJ/mol and 31–113 kJ/mol, respectively (Fig. **S2**, supplementary materials). By contrast, the LLD-cellobiose complexes showed negative binding energy values of −167 to −17 kJ/mol, indicating much weaker or non-binding. Moreover, in two out of five models the LLD-cellobiose interactions lead to a distortion in the structure, resulting in a large (>0.27 nm) change in RMSD of Cα atoms (Fig. **S3,** supplementary materials). This conformational change was irreversible, as the RMSD value did not decrease after removing cellobiose from the binding pocket (data not shown). Such large values correspond to a distortion in the LLD conformation, which is impossible to be induced by the small cellobiose structure *in vivo*, and therefore must be considered an artifact. As such, the binding energy values for these two cases could not be taken into account and consequently were neglected. In comparison, RMSD values of the control models of the LLD alone have not exceeded 0.23 nm. The RMSD of the LLD-chitobiose and LLD-trimannose models were even smaller than in control proteins and were generally below 0.2 nm (Fig. **S3,** supplemental materials). Interestingly, the average binding energy between chitobiose and LLD was ~2.8 higher than between trimannose and LLD; while it was reported that a ~3.7 higher molar concentration of trimannose, as compared to chitobiose, is required to obtain the same extent of inhibition of the interaction between recombinant TNF and uromodulin [3].

Approximately six hydrogen bonds were constantly present between chitobiose and LLD and eleven between trimannose and LLD. The amino acids K98, S99, P100, Q102 and E116 were highly preferred for hydrogen bond formation between the LLD and both chitobiose and trimannose, whereas Y115 was more specific for chitobiose and E104 was more specific for trimannose (Figs. **1B**, **1C**). Interestingly, none of the polar amino acids of the potential carbohydrate binding motif (T105-E110) [4, 19] was involved in hydrogen binding. Moreover, amino acids of the lectin-like domain (T105, E107 and E110) that are crucial for its trypanolytic and anti-edema activity were >0.7 nm away from bound chitobiose and >0.5 nm from bound trimannose. All in all, both chitobiose and trimannose occupy the same binding site in the TNF molecule, however with different theoretical binding affinities. None of the amino acids crucial for anti-edema activity of the lectin-like domain of the TNF were involved in hydrogen bonding with oligosaccharides or were subjected to their imposed steric hindrance (Figs. **1B**, **1C**). This observation is of crucial importance since both pre-incubation of TNF with chitobiose, as well as inducing specific mutations T105A, E107A, and E110A, lead to inactivation of its lectin-like activity.

Our results suggest that neither chitobiose nor trimannose affect crucial amino acids, while occupying the cavity in the lectin-like domain, indicating that both crucial amino acids and the emptiness of the cavity in the lectin-like domain are critical for the lectin-like activity of TNF. We propose that TNF is capable to interact with glycosylated binding partners, the sugar moieties of which can enter its lectin-like domain cavity and as such stabilize such a complex. It is plausible that the crucial amino acids are subsequently involved

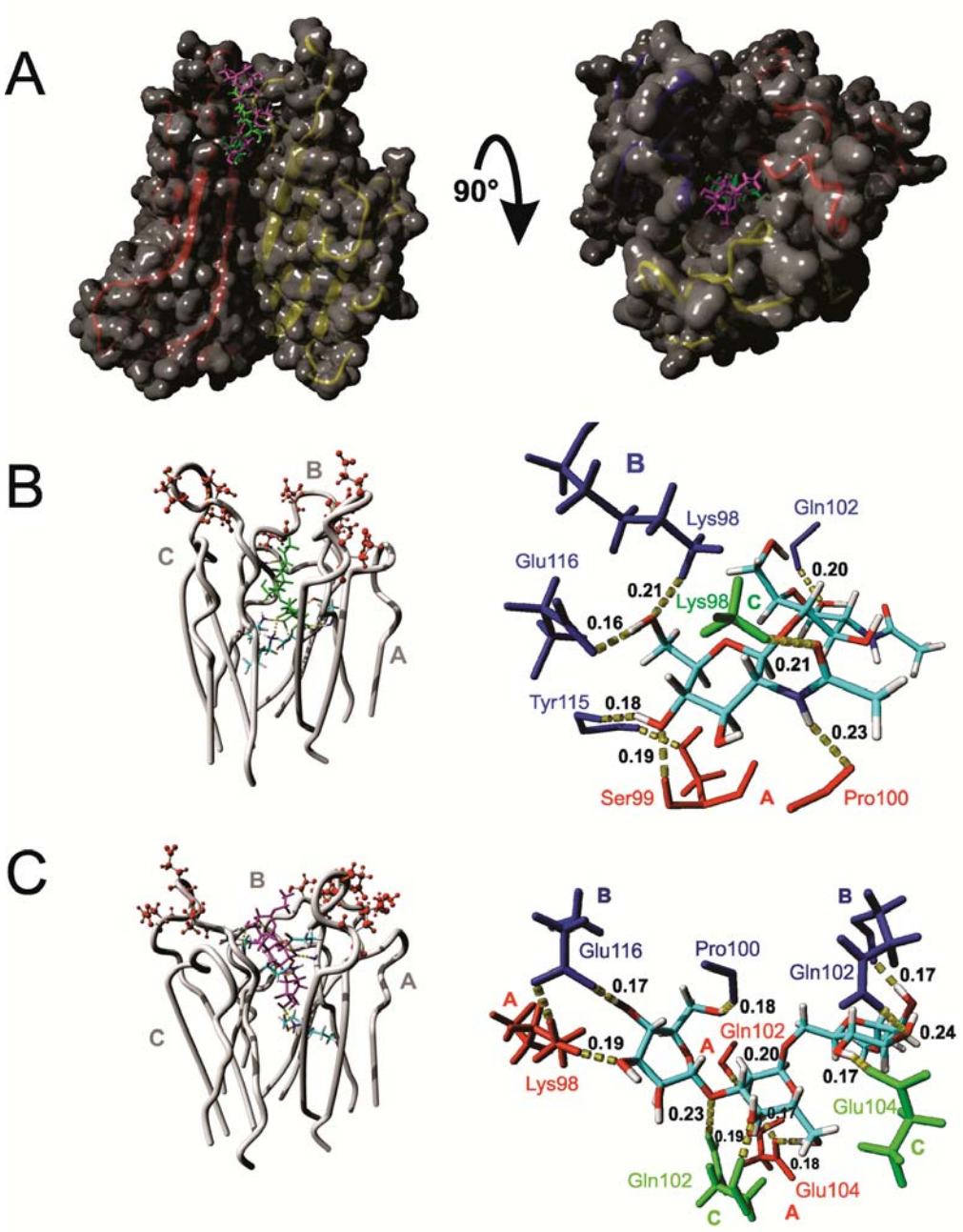

Fig. (1). Docking of *N*,*N*'-diacetylchitobiose and trimannose-*O*-ethyl to TNF. **A.** Van der Waals surface representation of TNF are shown with docked *N*,*N*'-diacetylchitobiose (green) and trimannose-*O*-ethyl (magenta) located in the middle of the lectin-like domain and shown with sticks (monomer B removed for better visibility). It is visible that both molecules are deepened to the same extent. **B.** Localization of *N*,*N*'-diacetylchitobiose (green) in the LLD (grey tube) after docking and 50 ns of MD. **Left,** crucial amino acids (T105, E107, and E110, balls and sticks) are colored in red. **Right,** schemes of hydrogen bonds formed between the LLD and *N*,*N*'-diacetylchitobiose (colored elements), with letters A, B, and C corresponding to the LLD subunits and hydrogen bond distances (black) are showed in nm. **C.** Localization of trimannose-*O*-ethyl (magenta) in the LLD (grey tube) after docking and 50 ns of MD. **Left,** crucial amino acids (T105, E107, and E110, balls and sticks) are colored in red. **Right,** schemes of hydrogen bonds formed between the LLD and trimannose-*O*-ethyl (colored elements), with letters A, B, and C corresponding to the LLD subunits and hydrogen bond distances (black) are showed in nm. Graphics created with YASARA (www.yasara.org) and POVRay (www.povray.org).

in the protein-protein interactions, which might be crucial for triggering the signaling activity of the LLD of TNF.

Docking and Molecular Dynamics of the TIP Peptide with Oligosaccharides

The three-dimensional alignment of the TNF monomers revealed differences in side chains between the TNF monomers A, B, and C. Based on that fact, we have built three different models of the TIP peptide (TIP_A, TIP_B and TIP_C), derived from the corresponding monomers of the TNF molecule. After energy minimization and MD equilibration with YASARA, the folding of the TIP_A peptide resembled most the crystal structure of native TNF, demonstrating its structural stability and was therefore selected as the only model for the further study. Even though TNF represents

the trimer of the lectin-like domains (or TIP peptides), no trimer formation of TIP peptides was reported [23], leading to the conclusion that single TIP peptide molecules are able to mediate the lectin-like activity of TNF [4, 19]. Our docking results obtained with the TNF trimer have identified a unique binding site for chitobiose and trimannose ligands. In this binding site, the oligosaccharide molecules interact with the lectin-like domain only from one side of its loops structure, which we denote here as the *inner* side, since it faces the interior of the cavity (Fig. **S4**, supplementary materials). The other sides of the lectin-like domain loops (the *outer* sides) were not accessible to oligosaccharide binding, also due to steric hindrance by the adjacent loops (S60-T79). This is however not the case for the TIP peptide, where both sides are accessible. The equilibrated model of the TIP_A peptide was globally docked with the oligosaccharides, capable to approach the peptide from both sides. Nevertheless, in the best binding poses generated by AutoDock Vina and Glide, the oligosaccharides were placed on the *inner* side of the TIP peptide, thereby highlighting this side as the most probable place for oligosaccharide binding. Interestingly, the inner side of the TIP peptide appears to be more electronegative, due to the presence of polar amino acids (Figs. **2A**, **2B**). Subsequent MD simulations of the best poses have shown that chitobiose, trimannose and also cellobiose have positive binding energies to the TIP peptide (Fig. **S5**, supplementary materials).

A reported mass spectroscopic study of the interaction between the TIP peptide and oligosaccharides has shown that cellobiose binds with a 20-fold lower affinity to the TIP peptide than does chitobiose [23]. This disagreement between the experimental and our computational methods could be due to the fact that 100 ns of MD simulation was not long enough to observe dissociation of cellobiose. Since the dissociation constant (K_d) for most lectin-oligosaccharide interactions lies within the micro- to millimolar range [34-36], the time required for the complexes to dissociate is expected to be between 0.1 and 100 milliseconds, which is three to

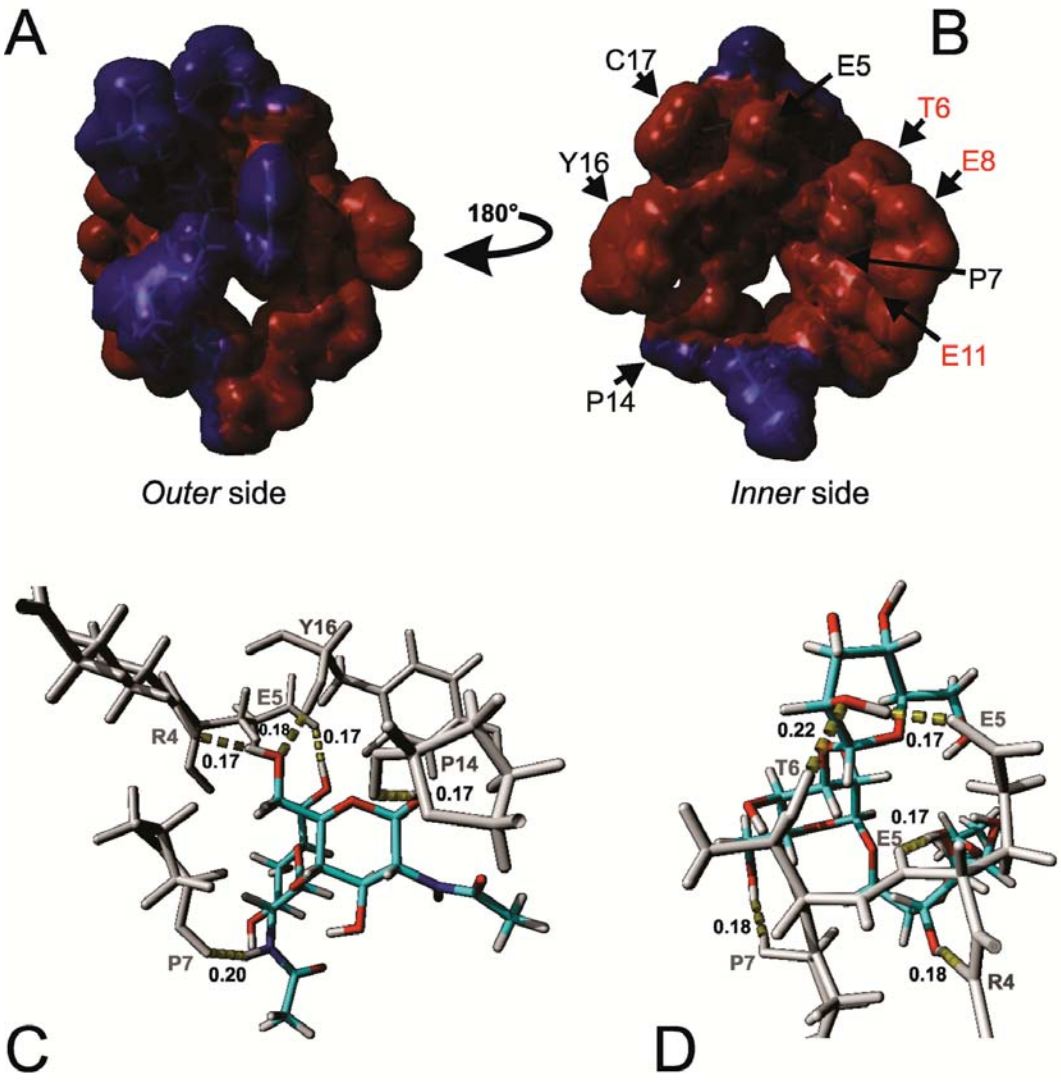

Fig. (2). A-B. Localization of the *inner* and *outer* binding sides on the TIP peptide. Van der Waals surface representation of the TIP peptide colored according to the surface charge (red – negative, blue – positive). Amino acids involved in hydrogen bonding with oligosaccharides (black) and the crucial amino acids (red) are highlighted. **C.** Localization of *N,N'*-diacetylchitobiose (colored elements) on the *inner* side of the TIP peptide (grey) after docking with AutoDock Vina and 100 ns of MD. Hydrogen bond distances (black) are showed in nm. **D.** Localization of trimannose-*O*-ethyl (colored elements) on the *inner* side of the TIP peptide (grey) after docking with AutoDock Vina and 100 ns of MD. Hydrogen bond distances (black) are showed in nm. Graphics created with YASARA (www.yasara.org) and POVRay (www.povray.org).

six orders of magnitude longer than the length of our experiment. Performing such a long MD simulation is still not common practice, due to the high computational expense.

One way to "speed-up" the dissociation process is to apply external stimulation to the binding complex by means of heat or external force. Gradual increase of the temperature during the MD simulations of the TIP peptide with the oligosaccharides from 298K to 353K have not lead to the complex dissociation, nor to the significant changes in the binding energies (data not shown). However, perturbation by constant acceleration applied to the center of masses to the both TIP peptide and the corresponding oligosaccharides in the opposite directions to each other led to discrimination between the binding kinetics between the oligosaccharides. Constant acceleration rather than constant force was used, due to the more uniform pulling, which is not dependent on the atomic masses and therefore causes lower internal stress. During such steered MD (SMD) simulations we observed the temporal evolution of the distances between the centers of masses of the two interacting molecules under applied accelerations. Complexes with peptide-oligosaccharide distances of more than 1 nm can be considered as dissociated. The SMD simulations were repeated with various accelerations of 3000, 2500, 2000, and 1500 pm/ps^2. In case of the acceleration being above 3000 pm/ps^2, we observed "immediate" dissociation of the complexes, regardless of the oligosaccharide used. If the acceleration was below 1500 pm/ps^2, the three independent repetitions led to three different results with large differences in the time of the complex dissociation (T_D), demonstrating that the external acceleration applied is similar to the strength of the interactions between ligand and receptor and therefore competing with various outcomes. However, within the range of accelerations of 3000–1500 pm/ps^2, we observed a straight dependence of T_D on acceleration for a given TIP peptide-oligosaccharide pair (Fig. **3**). Though all oligosaccharides have shown equal binding energies to the TIP peptide during the MD simulations, oligosaccharides dissociated differently, regardless of the acceleration used when the constant acceleration was applied. Cellobiose always dissociated first, trimannose always second, and chitobiose was always dissociating the last with the longest time required to break the peptide-oligosaccharide complex at a given acceleration.

The results obtained during SMD for the TIP peptide are in a good agreement with the ones we obtained for the whole TNF trimer, where chitobiose showed the highest binding affinity to TNF, with trimannose displaying a more moderate and cellobiose only a low binding. Moreover, these data correspond well with the experimental data obtained by mass spectrometry [23]. The following amino acids of the TIP peptide were involved in forming hydrogen bonds with oligosaccharides: chitobiose: R4, E5, P7, P14, Y16 (Fig. **2C**); trimannose: R4, E5, T6, P7, Y16 (Fig. **2D**). Interestingly, the hydrogen bonding between the crucial amino acids T6 and E8 and oligosaccharides becomes significant after some time of the SMD simulation when the oligosaccharides were moved out of the initial binding site at about 0.2 nm, and forming sort of a transition binding state. Such a state was detected by a little hump present on a plot displaying the simulation time versus the distance between the binding partners. Amino acids T6 and E8 were playing the major role in stabilizing such a transition state and were therefore important in the formation of the TIP peptide-chitobiose and TIP peptide-trimannose complexes. The crucial amino acid E11 was not involved in hydrogen bond formation, mainly due to its location on the opposite (*outer*) side in our model of the TIP peptide. Nevertheless, together with the other two crucial amino acids, E11 does increase the overall electronegativity of the TIP peptide, therefore making it more favorable for oligosaccharide binding (Fig. **2B**).

Even though initially all peptide-oligosaccharide complexes were stabilized by a network of hydrogen bonds, the SMD simulations led to different T_D values unique for the particular oligosaccharide used at a given acceleration. Our study suggests that the amino acids R4, E5, T6, P7, P14, and Y16 are playing the major role in hydrogen bond formation and therefore in stabilization of the TIP peptide-chitobiose and the TIP peptide-trimannose complexes. Crucial amino acids T6 and E8, but not E11, were also involved in hydrogen bond formation, particularly in stabilizing the transition binding state, as well as in increasing the overall electronegativity of the TIP peptide. Though the importance of the crucial amino acids of the TIP peptide in binding chitobiose was reported, it was also shown that they are not a prerequisite for the TIP peptide-chitobiose interaction [23]. Recent results have demonstrated the importance of the bulky hydrophobic region introduced by hydrophobic side chains of P14, W15, and Y16 residues, as an essential feature of the TIP peptide for ENaC activation [20]. Since our computationally obtained results suggest that amino acids P14 and Y16 are important in the TIP peptide-oligosaccharide binding by introducing the hydrogen bonding, this not only validates the importance of our study for the mechanisms involved in the inhibition of the TIP peptide lectin-like activity, but also for the mechanisms responsible for the alveolar liquid clearance activating capacity of the peptide.

The SMD simulation has provided further insights into the kinetics of the dissociation of the peptide-oligosaccharide complexes. The time required for the complexes to dissociate at applied zero acceleration (T_D^0), extrapolated from the linear fits of the plots T_D vs. the acceleration yielded values, is not compatible with our results from the MD simulations. This could be because all T_D^0 were shorter than the length of the MD productive runs, during which we did not detect any signs of the complexes' dissociation (Fig **3**). Moreover, the dissociation time of such complexes is expected to be around milliseconds as the K_d values for most lectin-saccharide interactions are found to be in the millimolar range [34-36]. A plausible explanation for this discrepancy is that the dissociation of the TIP peptide-oligosaccharide complexes follows a different path under lower accelerations. Measurements of the SMD simulations at lower accelerations are extremely inaccurate, as the external force applied gets more similar to the interaction energy between the ligand and the receptor, which then start to compete with various outcomes. Nevertheless, the extrapolated T_D^0 values for different oligosaccharides displayed a remarkable agreement with the experimentally reported affinities of the various oligosaccharides. For example, our results have shown that a 44-fold longer time is required for chitobiose, as compared to cellobiose, to dissociate from the TIP peptide, while a 20 times higher binding affinity to the peptide of chitobiose over cellobiose was reported [23]. Even though no comparative binding study between trimannose and the TIP peptide was performed, our findings have shown that *N,N'*-diacetylchitobiose is a 4 times stronger binder to the TIP peptide than trimannose, which corresponds well with the reported ~3.7 higher molar concentration of trimannose, as compared to chitobiose, required to inhibit to the same extent the lectin-like interaction between recombinant TNF interaction and uromodulin [3].

CONCLUSION

Alveolar edema represents a life-threatening pathological state associated with e.g. acute lung injury, severe pneumonia and the acute respiratory distress syndrome [37], for which no standard treatment exists to date. The lectin-like domain of the TNF, as well the TIP peptide, mimicking its structure, are able to stimulate ALC and therefore represent a potential therapeutic option in the treatment of pulmonary edema. Our study on the molecular interactions between the TNF trimer or its derived TIP peptide and specific oligosaccharides that abrogate the ALC increasing activity provides new insights into the nature of the lectin-like interactions involved. Our computationally obtained results structurally characterize the unique binding sites on the lectin-like domain of the TNF for both chitobiose and trimannose, as formed by the three lectin-like domain loops at the center axes of the TNF trimer and as such identify key interactions involved in ligand binding. The average binding

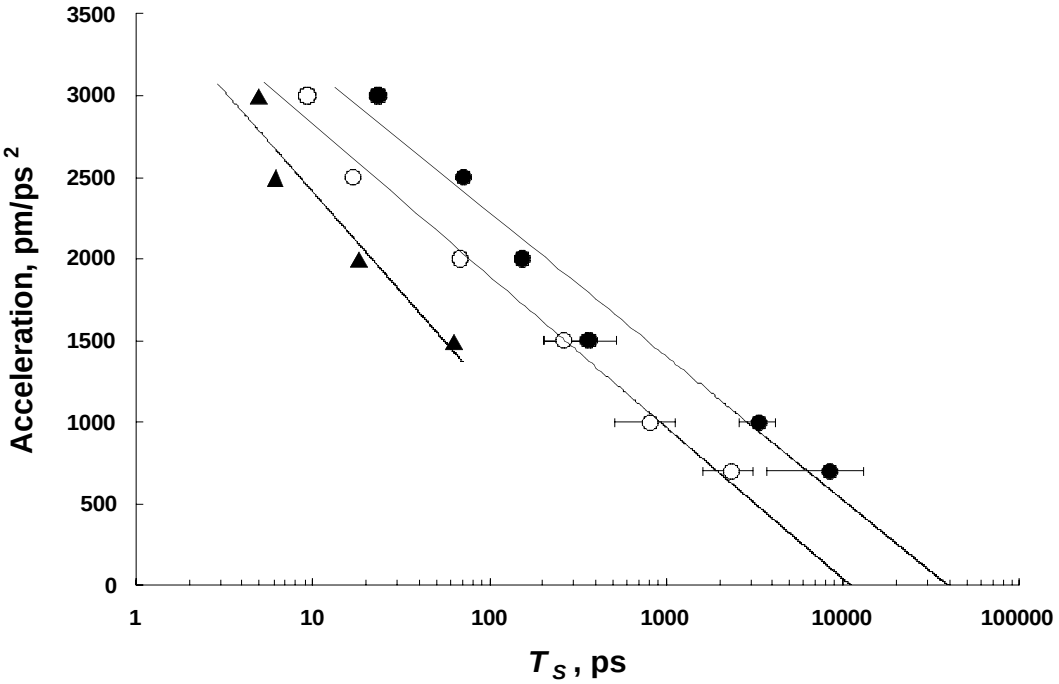

Fig. (3). Logarithmic dependence of the time required to separate the peptide-oligosaccharide complexes (T_D) on acceleration (filled circles – *N,N'*-diacetylchitobiose-TIP peptide complex; empty circles – trimannose-*O*-ethyl-TIP peptide complex; filled triangles – cellobiose-TIP peptide complex). During the SMD simulations, various accelerations were applied to the center of masses to both the TIP peptide and the appropriate oligosaccharides (triplets of each) in opposite directions to each other. Complex separation means that the distance between the TIP peptide and oligosaccharide is >1 nm.

energy of chitobiose was 2.8 times higher than for trimannose, whereas cellobiose was shown to be unable to bind, in agreement with previous results obtained experimentally. Amino acids K98, S99, P100, Q102, and E116 where specific for the binding of TNF to both chitobiose and trimannose, with Y115 being specifically important for chitobiose and E104 being specifically involved in trimannose binding. The amino acids reported to be important for ALC activation and trypanolytic activity (T105, E107, and E110) were unaffected by the bound oligosaccharides, highlighting the importance of the empty cavity in the lectin-like domain interaction with its putative receptor. The generated model of the TIP peptide showed a good structural agreement with the crystal structure of the lectin-like domain, particularly with regard to the localization of the C-αs of the crucial amino acids. Our results obtained using our model structure of the TIP peptide highlighted the preference of the *inner* (more electronegative) side of its loop-like structure for oligosaccharide binding. As such, the major peptide-oligosaccharide stabilizing amino acids were R4, E5, P7, P14, and Y16. The crucial amino acids T6 and E8, but not E11 were involved in stabilizing perturbed complexes, therefore highlighting their importance in the TIP peptide-oligosaccharide binding, as was experimentally reported. The specificity for the bound oligosaccharides was assessed using steered MD simulations, identifying cellobiose as the weakest binder for the TIP peptide. These results provide significant insight in our understanding of the mechanisms involved in the lectin-like activities of TNF and the TIP peptide, which can give rise to the development of peptides with improved bioactivity.

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CONFLICT OF INTEREST

None declared.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

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