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Perspective/Review

Biological chemistry of immunomodulation by zwitterionic polysaccharides

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Abstract

Capsular polysaccharides isolated from pathogenic bacteria are comprised typically of many repeating units from one to eight or more monosaccharides in length. These polysaccharides stimulate the murine humoral immune system to elicit primarily IgM antibody responses. Studies conducted primarily in the mouse have characterized these polymers as T cell-independent antigens. These mouse studies and the relatively poor immunogenicity of polysaccharides in human hosts have led to the design of vaccines by coupling these polysaccharides to protein carriers to stimulate a T cell-dependent response. However, a newly described class of bacterial polysaccharides has been characterized that have the ability to modulate the cellular immune system. They are structurally diverse, but all share a zwitterionic charge motif that allows them to directly interact with T cells and antigen-presenting cells to initiate an immunomodulatory T cell response. These polymers, termed zwitterionic polysaccharides (ZPSs), elicit T cell-derived chemokines and cytokines that influence the immune response governing at least one classic host response to bacterial infection: abscess formation. This review will describe the biological and structural aspects of ZPSs that convey these activities.

Keywords: Polysaccharides; T Cells; Structure/function

Contents

1. ZPS structure

Several immunomodulatory zwitterionic polysaccharides (ZPSs) have been identified from different bacterial species, including PS A1 and PS B from *Bacteroides fragilis* strain 9343, PS A2 from *B. fragilis* 638, and capsule polysaccharide from type 1 *Streptococcus pneumoniae* (Sp1) (Fig. 1). Although they share similar biological properties, these ZPSs have very different chemical structures (Fig. 1). PS A1 is composed of a branched tetrasaccharide repeating unit with three monosaccharides in the polymer backbone and one residue at the side chain. The repeating unit of PS A1 has the following sequence: [→3)-β-D-Gal*p*-(1→3)-α-

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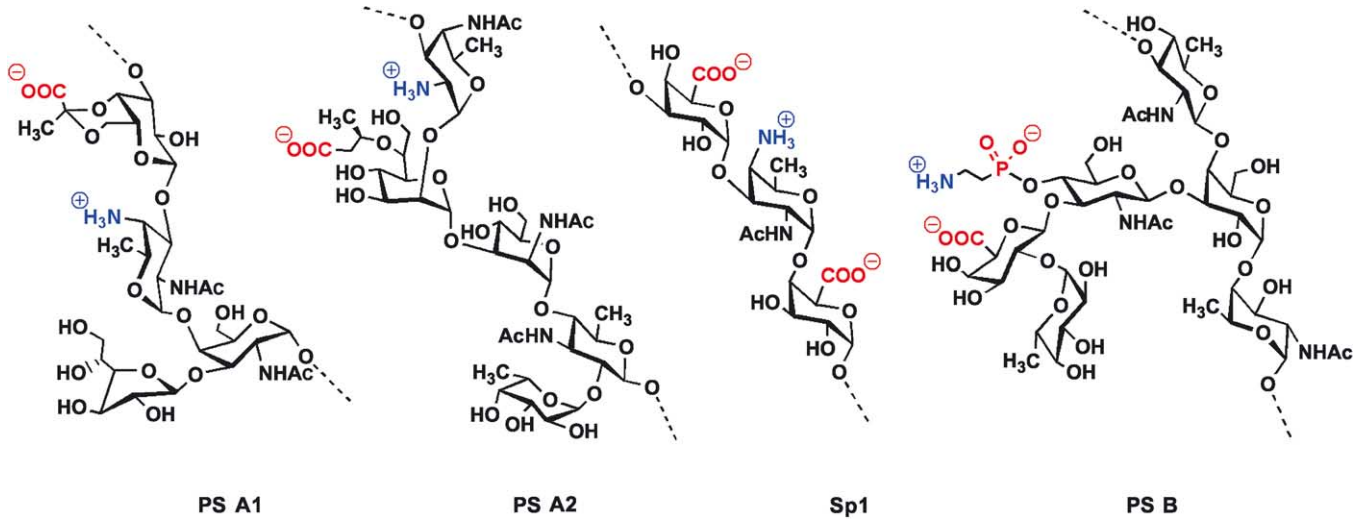

Fig. 1. Chemical structures of the repeating units of various zwitterionic polysaccharides. Positive and negative charges are located on adjacent residues and form multiple zwitterionic disaccharides in each ZPS. PS A1, PS A2, and PS B are capsules from *B. fragilis.* Sp 1 is the capsule from *S. pneumoniae* type 1.

D-Sug*p*-(1→4)[β-D-Gal*f*-(1→3)]-α-D-Gal*p*NAc-(1→], where Sug is 2-acetamido-4-amino-2,4,6-trideoxygalactose. A pyruvate substituent with *R* configuration spans O-4 and O-6 of the β-D-galactopyranosyl residue.[1] PS B contains a hexasaccharide repeating unit with three residues on the polymer backbone and three sugars forming the side chain. The sequence of its repeating unit is as follows: [→3)-β-D-Qui*p*NAc-(1→4)[α-L-Fuc*p*-(1→2)-β-D-Gal*p*A-(1→3)-β-D-Glc*p*NAc-(1→3)]-α-D-Gal*p*-(1→4)-α-L-Qui*p*NAc-(1→].[1] The *N*-acetyl-β-D-glucopyranosyl residue is substituted at O-4 with a 2-aminoethylphosphonate. PS A2 contains branched pentasaccharide repeating units with the following sequence: [→3)-α-D-AAT*p*-(1→2)-α-D-Hep*p*NAc-(1→3)-α-D-Man*p*NAc-(1→4)[α-L-Fuc*p*-(1→2)]-β-D-ADG*p*A-(1→].[2] AAT is 2-amino-4-acetamido-2,4,6-trideoxygalactose and ADG denotes 4-acetamido-4,6-dideoxyglucose. HepNAc is *N*-acetyl-*manno*-hepatose, which bears a 3-hydroxybutanoic acid substituent at O-6. Sp1 is a simpler polymer with a linear trisaccharide repeating unit with the sequence of [→3)-α-D-Gal*p*A-(1→3)-α-D-Sug*p*-(1→4)-α-D-Gal*p*A-(1→], where Sug denotes the same 2-acetamido-4-amino-2,4,6-trideoxygalactose as that in PS A1.[3]

This is not a complete list of the ZPSs that exist in nature as several other bacterial species synthesize polymers that have a zwitterionic charge motif. However, there are relatively few bacterial polysaccharides that possess a zwitterionic charge motif. Understanding of the structural basis for the unique immunological properties of these ZPSs required a detailed examination of their structures. Despite the difference in their chemical compositions and monosaccharide sequences, PS A1, PS A2, Sp1, and PS B share a striking structural feature, i.e., they are zwitterionic polymers that display a high density of positive and negative charges. PS A1, PS A2, and Sp1 carry positively charged amino and negatively charged carboxylate groups, whereas PS B contains positively charged amines and negatively charged carboxylate and phosphonate groups. As zwitterionic charge motif is shared by all these ZPSs but is generally rare among natural PSs, it is logical to reason that charges play essential roles in the immunological function of these ZPSs. This hypothesis is supported by several studies[4–6] in which chemical conversion of charged groups to neutral components (e.g., conversion of amines to *N*-acetyl and carboxyl to hydroxymethyl groups) eliminates the activity of these ZPSs. These findings reveal the vital importance of molecular charges of ZPSs to their immunological function.

2. Role of ZPS in clinical disease

The understanding of ZPSs as important biologic molecules emanated from the study of the role of anaerobic bacteria, particularly *B. fragilis*, in the pathogenesis of intraabdominal sepsis. Clinical studies demonstrated that *B. fragilis* is the major isolate from abscess formation associated with intraabdominal sepsis in humans.[7] Abscess formation is a classic host response to bacterial infection and is a cause of severe morbidity and can be fatal in some patients. The high frequency of clinical abscess formation associated with *B. fragilis* is reflected by this organism's singular ability to induce abscess formation in a rat model of intraabdominal sepsis.[8] Studies of the virulence factors responsible for abscess formation by this organism showed that the capsular polysaccharide of *B. fragilis* potentiates the development of this host response in rodent models of

intraabdominal sepsis. In these models, intraperitoneal challenge with the capsule along with a sterile cecal contents adjuvant induced abscess formation in animals.

Immunochemical characterization of the capsule from the reference strain NCTC 9343 revealed that it is comprised of multiple polysaccharides. For this reason, the capsule was termed a capsular polysaccharide complex or CPC. Initially, two polymers were purified from the complex, PS A1 and PS B.[1,9] Subsequent studies by Comstock and colleagues have used a molecular and immunochemical approach to determine that this organism produces at least eight distinct capsular polysaccharides. This is a number far greater than previously reported for any encapsulated bacterium. *B. fragilis* is able to exhibit a wide array of distinct surface polysaccharide combinations by regulating the expression of these different capsules in an on–off manner by the reversible inversion of DNA segments containing the promoters for their expression.[10] It is not known at this time if these additional capsules all possess a zwitterionic charge motif.

PS A1 was chosen as the prototype for the ZPS and used to investigate the role of zwitterionic structure on experimental abscess formation. Conversion of the free amino groups into N-acetyl groups abrogated the ability for this polymer to induce abscess formation, while conversion of negatively charged carboxyl groups into hydroxymethyl groups via carbodiimide reduction had a similar effect.[5] Chemical neutralization of the charged groups of other ZPS also disrupted abscess formation by these polysaccharides. Moreover, chemical conversion of a polysaccharide comprised of a single monosaccharide repeating unit that possessed a negatively charged carboxyl group into a zwitterionic polymer conferred the ability to induce abscess formation in the animal model.[5] These data showed that the zwitterionic charge motif is the critical structural aspect associated with these polymers that potentiates their ability to induce abscess formation in experimental models.

3. Cellular host response to ZPS: role of T cells in abscess formation

Although bacterial polysaccharides are considered to be classic T-independent antigens, a series of studies on the host response to ZPS has demonstrated that CD4+ T cells are critical in the pathogenesis of intraabdominal abscess formation by a variety of abscess-inducing bacteria.[6,11] Depletion of T cells or administration to animals of CTLA4Ig, a molecule that inhibits T-cell activation via blockade of the CD28-B7 costimulatory pathway, results in the abrogation of intraabdominal abscess formation following challenge with different bacterial pathogens such as *S. aureus*, *B. fragilis*, or a combination of *Enterococcus faecium* and *B. distasonis*.[11] A common effector mechanism requiring T cell activation seems to be essential for abscess induction by a broad range of pathogenic bacteria.

Other data suggested that ZPSs interact directly with T cells to promote abscess formation. Several ZPS were shown to activate CD4+ T cells both in vitro and in vivo.[4] This result was unanticipated given that bacterial polysaccharides have not been described previously with the ability to directly activate T cells. A direct connection between T cell activation by ZPS and abscess formation by these polymers was made in a subsequent series of studies. The most compelling data from these experiments showed that CD4+ T cells activated in vitro by ZPS induce abscesses when adoptively transferred to the peritoneal cavity of naïve rats along with sterile cecal contents adjuvant.[6,11] The adjuvant, by itself, did not induce abscesses. Addition of CTLA4Ig to block ZPS-mediated T cell activation in vitro prevented abscess formation in these experiments. The role of CD4+ T cells in abscess induction is shown in Fig. 2.

4. T Cell activation by ZPS

The observation that ZPSs activated T cells prompted our investigation into the ZPS/T cell interaction. Rigorous structural and immunochemical studies first determined that this activity was specific for ZPS structure and not contaminating proteins or peptides.[4] The ZPSs specifically activated CD4+ and not CD8+ T cells in these assays. It was also shown that chemical alteration of the zwitterionic charge motif to be either positive or negative, but not zwitterionic, abrogated the ability of ZPS to activate T cells.

An ozonolytic method for depolymerizing polysaccharides was employed to examine the influence of the molecular size of PS A on T cell activation by ZPS.[12] PS A with average molecular sizes of 129 (native), 78, 47, and 17 kDa each stimulated CD4+-cell proliferation in vitro to the same degree, whereas the 5.0-kDa saccharide was much less stimulatory than the control 129.0-

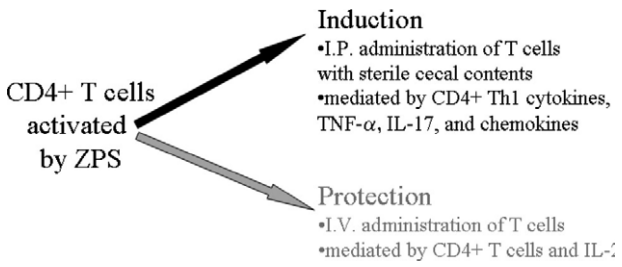

Fig. 2. Paradoxical role of CD4+ T cells activated by ZPS in the modulation of abscess formation. Abscess induction or prevention depends on where T cells are administered, the presence of sterile cecal contents adjuvant, and the type of cytokines produced.

kDa PS A. These results demonstrated that a ZPS as small as ~22 repeating units (~88 monosaccharides) can elicit a strong T cell-dependent immune response.

The activation of T cells by ZPS requires APCs.[13] A broad range of APCs can facilitate this activity including B cells, monocytes, and dendritic cells. APCs lacking major histocompatibility complex (MHC) class II molecules do not support this activity and blockade of the human MHC restriction element, HLA-DR, with monoclonal antibodies inhibits ZPS-mediated T cell activation. Similar experiments targeting class I MHC had no effect. Further study of the role of MHC class II showed that ZPS colocalize with HLA-DR on the eukaryotic cell surface and in intracellular compartments of the endocytic pathway. These results suggest that binding of ZPS to HLA-DR may be required for T cell activation.

Further study of requirements for T cell activation by ZPS focused on the role of TCR-MHC class II (Signal 1) interactions as well as the interaction of the CD28-B7 T cell costimulatory pathway (Signal 2). Both signals are required for T cell activation by conventional protein antigens and are shown in Fig. 3. ZPS-mediated T cell activation requires the involvement of the αβ T cell receptor (TCR) since the addition of antibodies specific for this ligand inhibits T cell activation by ZPS.[11] Further analysis of the role of the CD28-B7 costimulatory pathway showed the interaction of CD28 present on T cells with the B7 ligand CD86 (B7-2) on APCs mediates T cell activation by ZPS.[11]

Currently, our studies are focused on understanding the antigenic nature of the ZPS. Superantigens do not require internalization and antigen processing in order to stimulate T cells, since they bind directly to MHC class II on the surface of APCs. Thus far the data suggest that ZPS do not behave as classical superantigens since fixation of APCs prior to the addition of ZPS disrupts T cell activation.[14] Analysis of the T cell Vβ repertoire used by the ZPSs suggests a pattern that is distinct from superantigens and conventional protein antigens (unpublished data). Further studies are now underway to better understand the exact nature by which APCs handle ZPS and activate T cells.

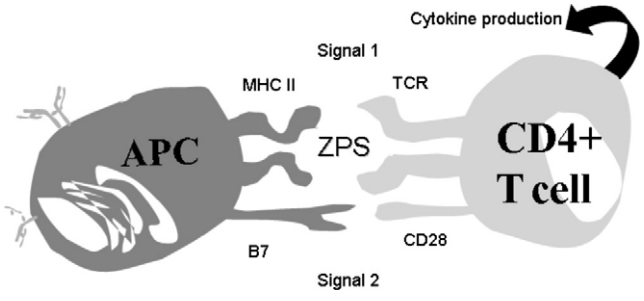

Fig. 3. T Cell–APC interactions. T cell activation generally depends on two signals. Signal 1 is characterized by the interaction of the TCR on T cells with MHC class II on APCs. In order for T cells to become fully activated, Signal 2 is required. This consists of ligation of CD28 on T cells with B7 on APCs. ZPS-Mediated T cell activation requires both signals and this results in the production of cytokines by CD4+ T cells. These T cell-derived cytokines play a role in abscess induction and prevention by the ZPS.

5. T Cell interactions with other host cell types

Investigation of the mechanism of abscess induction by ZPS led to analysis of the interaction of CPC from *B. fragilis* with host cells that comprise or reside in the peritoneal cavity. We have shown that the CPC stimulates the production of chemokines such as IL-8 from T cells and peritoneal macrophages,[15,16] which in turn leads to the recruitment of PMNs to peritoneal cavity. In addition, the CPC stimulates the pro-inflammatory cytokines TNF-α and IL-1 from peritoneal macrophages, which interact with mesothelial cells lining the peritoneal cavity to induce the production of the cell adhesion molecule ICAM-1. ICAM-1 expressed by mesothelial cells tethers infiltrating PMNs (recruited by the production of IL-8) to inflamed tissue and leads to further PMN recruitment and sequestration within the peritoneal cavity. These events are necessary for the development of experimental intraabdominal abscess formation. A proposed model for the induction of intraabdominal abscesses by ZPS is shown in Fig. 4.

6. Prevention of abscess formation by ZPS

Ongoing parallel studies have also focused on the ability of ZPSs to prevent intraabdominal abscess formation. Shortly after the discovery of the distinct biologic properties of ZPS, it was found that subcutaneous "treatment" with the different ZPSs shortly before and following intraperitoneal challenge with *B. fragilis* prevented subsequent abscess formation.[17] Interestingly, this protective activity could prevent abscesses following challenge with not only *B. fragilis*, but also with an array of organisms capable of causing intraabdominal abscesses, including *S. aureus*.[4,17] The ability to protect animals against abscess formation by antigenically distinct, heterologous bacterial species suggests that the ZPSs do not behave as "classic" immunogens. Rather, they appear to modulate the immune system to suppress a generalized host response that leads to abscess formation.

The structure/function relationship that governs abscess induction by ZPSs also accounts for their ability to protect against abscess formation. Naturally occurring ZPSs or polysaccharides chemically modified to possess both positively and negatively charged groups are

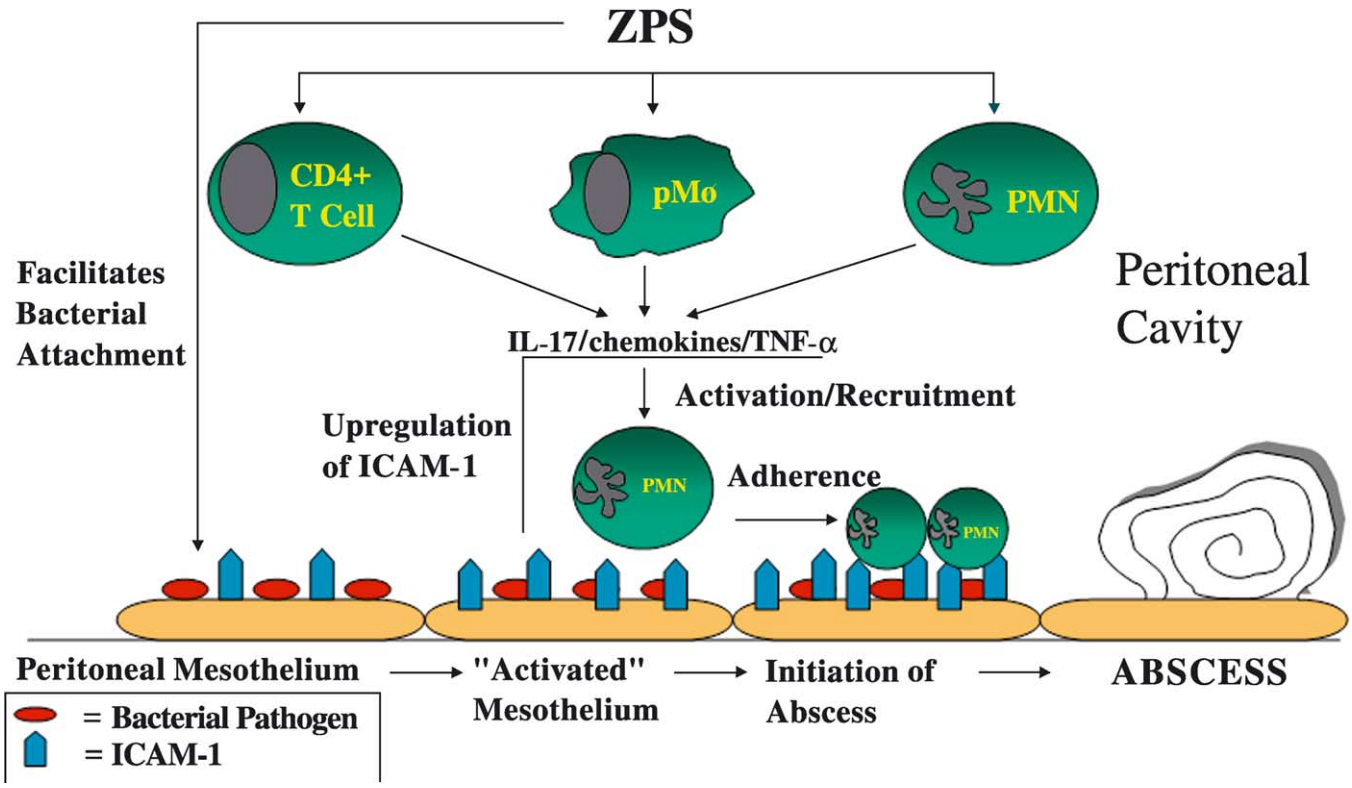

Fig. 4. Proposed model of intraabdominal abscess formation. We have used *B. fragilis* as the model organism to study the mechanism by which bacteria induce abscess formation and the role of ZPS in this process. The ZPS from this organism allows for localization of the organism within the abdominal cavity and the stimulation of pro-inflammatory cytokines and chemokines, with consequent expression of ICAM on host cells and recruitment of PMNs to the abdominal cavity. The recruitment of PMNs into the peritoneal cavity and the subsequent adherence of these cells to "activated" mesothelial tissue most likely represent the first stages of intraabdominal abscess formation in the infected host.

capable of protecting animals against abscess formation.[4,17]

7. Role of T cells in protection against abscess formation

The role of T cells in mediating protection against abscess formation by *B. fragilis* was originally demonstrated in rodent models of intraabdominal sepsis in the 1980s.[18] Recent work in this area has further elucidated the mechanism for this activity. These studies now show that CD4+ T cells activated by ZPSs can confer protection against a broad range of pathogens[4] when transferred via the intracardiac route 24 h prior to intraperitoneal challenge with abscess-inducing bacteria. The fact that ZPS-activated CD4+ T cells have the paradoxical ability to transfer the ability to both induce and protect against abscess formation depends on the route and timing of cell transfer as well as the intraperitoneal administration of the cecal contents adjuvant (in the case of peritoneal abscess induction).[6] The role of CD4+ T cells in the prevention of abscesses is shown in Fig. 2. At present, it is not known whether the particular CD4+ T cell responsible for abscess induction is the same as that required for protection against abscess formation. However, it is clear that in certain disease states such as inflammatory bowel disease, phenotypically different CD4+ T cells can have both a pathogenic and protective role.[19] Currently, we are investigating the ZPS-specific CD4+ T cell type(s) responsible for modulating abscess formation.

Protection by ZPS-specific CD4+ T cells can also be conferred by the transfer of a soluble factor(s) extracted from these cells. Experiments designed to determine the nature of these factors showed that the cytokine IL-2 has a prominent role in the protective response.[20] Addition of an IL-2-specific monoclonal antibody to these lysates abrogated the protective activity in recipient animals. Further, treatment of rodents with purified, recombinant IL-2 prior to bacterial challenge conferred complete protection against abscess formation. We hypothesize that ZPSs induce the production of a series of cytokines from T cells and/or APCs that are responsible for the protective activity associated with these polymers. IL-2 appears to be a central component of this cytokine cascade. Our recent work in this area is now directed towards the elucidation of the network of cytokines that have a role in the protective process.

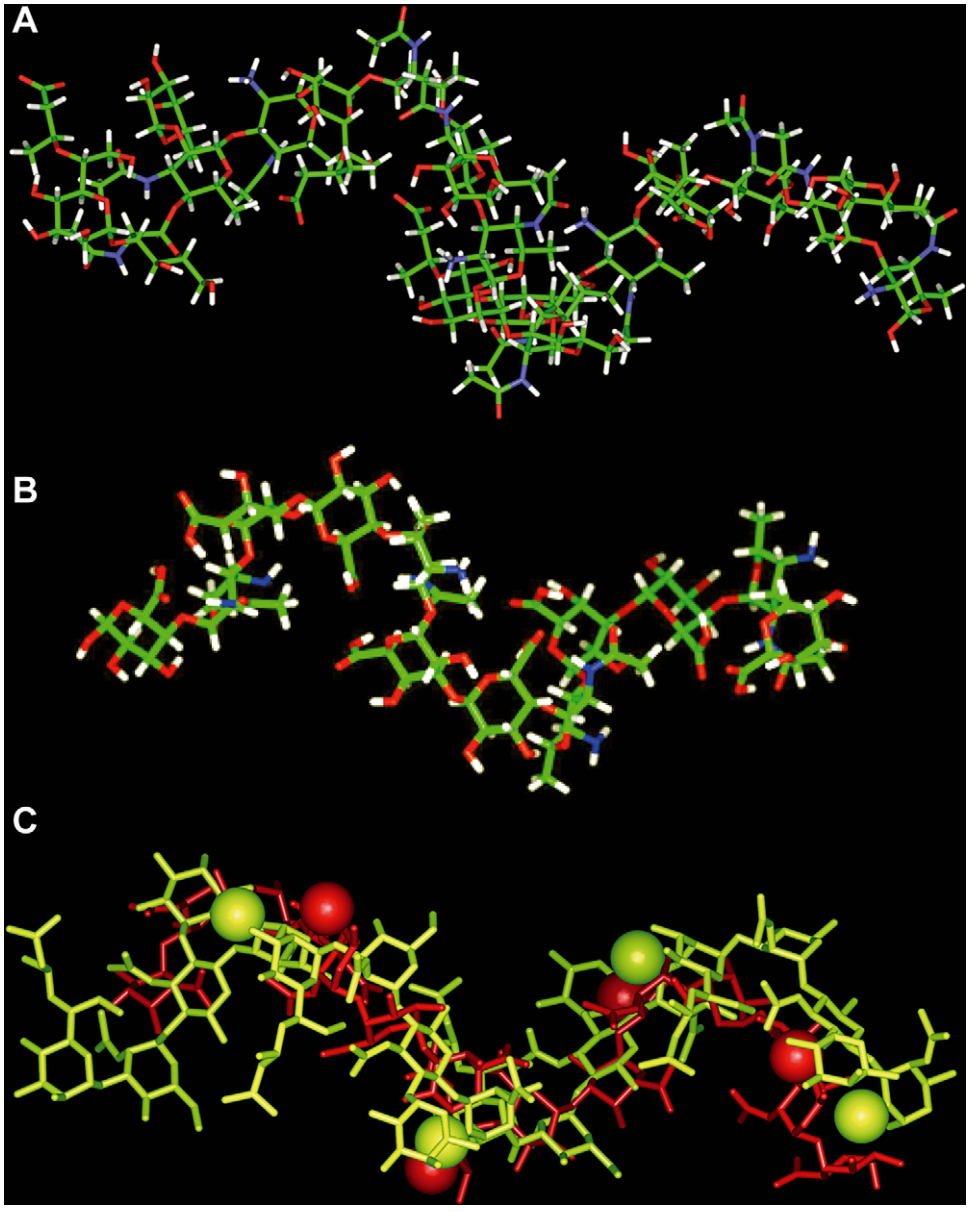

Fig. 5. Stick models of the 3D structures of PS A2 (**A**) and Sp1 (**B**). Carbons are colored green, oxygens red, nitrogens blue, and hydrogens white. (**C**) Conformations of Sp1 (red) and PS A2 (yellow) superimposed on the basis of their amino groups. Red and yellow dots represent positive charges from Sp1 and PS A2, respectively. Sp1 and PS A2 display a similar zigzag positive charge pattern with roughly equidistant charge separation of 15 Å.

8. Conformation of ZPS structure: model for interaction with the cellular immune system

Although zwitterionic charge motifs are present in all these ZPSs, PS A1 and PS A2 are overall neutral molecules, whereas Sp1 and PS B are polyanionic. Therefore, it is unlikely that nonspecific electrostatic interactions between ZPSs and receptors in the immune system account for the specific immunological activity shared by various ZPSs.

To explain why ZPSs comprising different chemical structures elicit similar immunological responses, one hypothesis is that the structural basis for the immunomodulating activity of ZPSs lies in their three-dimensional conformation and overall spatial charge organization. Different ZPSs may assume similar 3D structures that define a common scaffold for the presentation of charges. To test this hypothesis, the 3D structures of PS A2 and Sp1 were determined by NMR spectroscopy and molecular mechanics and

dynamics calculations.[2,21] PS A2 forms an extended right-handed helix with two repeating units per turn and a pitch of 20 Å (Fig. 5). All charges are exposed on the outer surface of the polymer in a regularly spaced pattern, which renders them easily accessible to other molecules. Every helical turn defines a large groove whose four edges are occupied by two pairs of zwitterionic charges. Strikingly, Sp1 also forms a similar helical conformation with spatial arrangement of positive charges nearly identical to that of PS A2 (Fig. 5).[21] This finding supports the hypothesis that a common 3D structural or charge pattern shared by compositionally dissimilar ZPSs accounts for their specific immunological activity.

The conformational models of PS A2 and Sp1 allow us to envision plausible mechanisms for the interaction of ZPSs with receptors of the immune systems. In one scenario, PS A2 or Sp1 could bind to receptors primarily along its longitudinal sides, which display a high density of alternating opposite charges. High binding affinities could be achieved via abundant electrostatic interactions supplemented by the potential for numerous hydrogen bonds to hydrophilic hydroxyls and, to a lesser extent, van der Waals interactions. In another scenario, the zwitterionic grooves of PS A2 or Sp1 serve as the primary binding domains. For example, the geometry of each groove can accommodate the insertion of an α helix from a protein.[2] The charges at the edges of a groove could strengthen the binding of receptor sites via multiple electrostatic interactions. Given their easy accessibility in ZPSs, charged groups can contribute directly and significantly to binding of receptors in many binding scenarios, which would explain why charges are essential determinants of the biological activity of ZPSs.

A better understanding of the structural basis for the immunomodulating activity of ZPSs entails a still more detailed examination of the 3D structures and charge patterns of these and other immunomodulating polysaccharides. A few questions remain to be clarified. First, are positive and negative charges equally important? PS A2 and Sp1 share a nearly identical positive pattern, but no similar negative charge pattern is easily identifiable. This finding underlines a more important role for positive amines than for negatively charged residues, although an earlier study revealed that both positive and negative charges are required for the abscess-regulating activities of ZPSs.[5] Second, are repetitive charges required? A recent study showed that partially *N*-deacetylated CP8 from certain strains of *Staphylococcus aureus* are remarkably active in stimulating T-cell proliferation and regulating abscess formation.[6] CP8 is one of the two most common *S. aureus* serotypes isolated from human infections. CP8 consists of a linear trisaccharide repeating unit, containing *N*-acetylmannosaminuronic acid and two *N*-acetyl fucosamines with a sequence of [→4)-3-O-Ac-β-D-Man*p*NAcA-(1→3)-α-L-Fuc*p*NAc-(1→3)-β-L-Fuc*p*-NAc-].[6] CP8 is naturally polyanionic, but N-deacetylation can generate positively charged amines, thereby converting CP 8 to a ZPS. Unlike other ZPSs that have regular repeating units, partially N-deacetylated CP8 carries positive charges at random sites. It is therefore intriguing to know whether repetitive charge patterns are required for the immunological activity of ZPSs. If not, perhaps it is because only a very small fragment of ZPS is recognized by the immune receptors. For example, it may be that only zwitterionic disaccharides are critical (Fig. 1). The clarification of these questions will provide further insight into the immunological recognition of carbohydrates.

9. Conclusion

A new class of carbohydrates has been identified that are recognized by T cells and can modulate the cellular immune system. These polysaccharides are displayed primarily on organisms associated with a classic host response to bacterial infection: abscess formation. The zwitterionic charge motif that characterizes these polymers is rare among bacterial polysaccharides and confers the ability to activate CD4+ T cells in a manner that governs the host's ability to develop abscesses in response to bacterial infection. The regulation of abscess formation by ZPS-specific CD4+ T cells represents a novel paradigm by which bacterial pathogens interact with the host immune system to cause disease.

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