Total synthesis of alginate and zwitterionic SP1 oligosaccharides

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Synthetic Zwitterionic Polysaccharides

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2.1 Zwitterionic polysaccharides: structure and activity

Bacteria are generally covered in polysaccharides featuring rare monosaccharide constituents and structural elements that differ from the mammalian glycan repertoire.[1] As such they represent excellent targets for the (human) innate and adaptive immune system to respond to.[2] However, bacterial polysaccharides behave poorly as stand-alone vaccine entities. Carbohydrates in general are poor immunogens and only trigger B-cell mediated IgM response, without switching to IgG production and memory development. Thus, in designing carbohydrate-based vaccines, bacterial oligo/polysaccharides are conjugated to a carrier protein to induce T-cell response to peptide epitopes embedded in

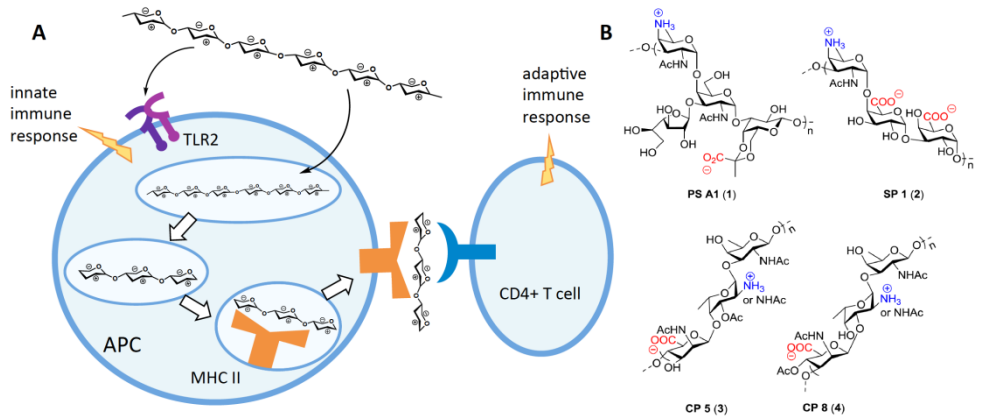

Figure 2.1 Zwitterionic polysaccharides. A) ZPS can stimulate the innate and adaptive arm of the immune system through interaction with TLR2 and by binding to MHC II, respectively. B) Examples of naturally occurring ZPS from *Bacteroides fragilis* (PS A1, **1**), *Streptococcus pneumonia* (SP1, **2**), *Staphylococcus aureus* (type 5, **3** and type 8, **4**).

the carrier protein.[3,4,5] This holds not true however for a unique class of carbohydrates that are characterized by the presence of both positively and negatively charged functionalities: the zwitterionic polysaccharides (ZPSs). These bacterial polysaccharides feature amino groups, protonated at physiological pH, and carboxylates or phosphates that are negatively charged at neutral pH. It is now well established that these unique structural features endow these saccharides with exceptional immunological properties.[6] Zwitterionic polysaccharides are T-cell dependent antigens as they can be processed by antigen presenting cells, loaded onto MHC II molecules and presented to T-cells, and thus are able to elicit an immune response.[7] As such, ZPSs behave like foreign proteins and this surprising activity has been show to arise from their unique structural elements.[8,9] Besides their activity in the adaptive part of the immune system, ZPS also interact with the innate arm of our immune system: various ZPS have been implied to interact with Toll like

receptor (TLR) 2.[10,11] Figure 2.1 represents the structures of the most prominent ZPSs: PS A1 from *Bacteroides fragilis* (**1**),[7] the *Streptococcus pneumonia* Sp1 saccharide (**2**)[7] and the capsular polysaccharides of *Staphyococcus aureus* type 5 (**3**) and type 8 (**4**).[12] Most mechanistic work on these ZPS has been conducted with ZPS **1** and **2**, isolated from the parent bacteria. Chemical modification of the isolated material (acetylation of the amines to remove the positive charge, reduction of the carboxylates) has shown the prerequisite of the zwitterionic motif for activity.[6] NMR studies combined with molecular dynamic calculations and supported by circular dichroism (CD) measurements revealed that ZPS **1** and **2** take up a helical structure, positioning their positive and negative charges at approximately equal distance.[13,14,15]

Because of the appealing biological activities and their unique structures, the ZPSs have been the subject of several synthetic endeavors.[16-27] Given the structural features of the target molecules (*cis*-glycosidic linkages, the presence of multiple functional groups, *i.e.* amines, acetamides, carboxylates and the rare monosaccharide constituents), these total synthesis campaigns have not been without a challenge. This chaper present an overview of the accomplished syntheses to date and the - limited - biological data that has been gathered with the resulting zwitterionic oligosaccharides.

2.2 Zwitterionic polysaccharides: synthesis

Several synthetic routes towards fragments of the ZPS depicted in Figure 2.1B have been disclosed. Because the synthesis strategies towards capsular polysaccharides of *S. aureus* are not compatible with the incorporation of free, positively charged amine functionalities (next to the acetamides) in the generated fragments[23-27] these syntheses will not be reviewed here. The overview of the successful syntheses of Sp1 and PS A1 fragments clearly illustrate the challenges associated with the complex structures of these molecules. One of the bottlenecks in the assembly of these structures is represented by the requirement of sufficient amounts of a suitably functionalized trideoxydiaminogalactose (TDDAG) building block. A recent review details the variant approaches taken to generate such building blocks.[30]

2.2.1 Synthesis of Sp1 polysaccharide fragments

The Sp1 polysaccharide is built up from trimer repeats composed of α-D-2-*N*-acetamido-4-amino-2,4,6-trideoxy-D-galactopyranose and α-D-galacturonic acid residues (See Figure 2.1B). The presence of the rare TDDAG and galacturonic acid (GalA) residues and the fact that they are all interconnected through *cis*-glycosidic linkages present a huge synthetic challenge. Bundle and co-workers were the first to complete the assembly of a fragment of this ZPS.[16] They reported the synthesis of a monomer and dimer of the repeating trisaccharide as depicted in Figure 2.2. The TDDAG motif was generated from a rather advanced disaccharide synthon (**7** to **8**). After coupling with galactose donor **10**, the trisaccharide **12** was obtained. Of this intermediate the primary alcohol functions were unmasked to set the stage for the double oxidation step. The two carboxylates were installed using a TEMPO/NaOCl oxidation procedure, after which global

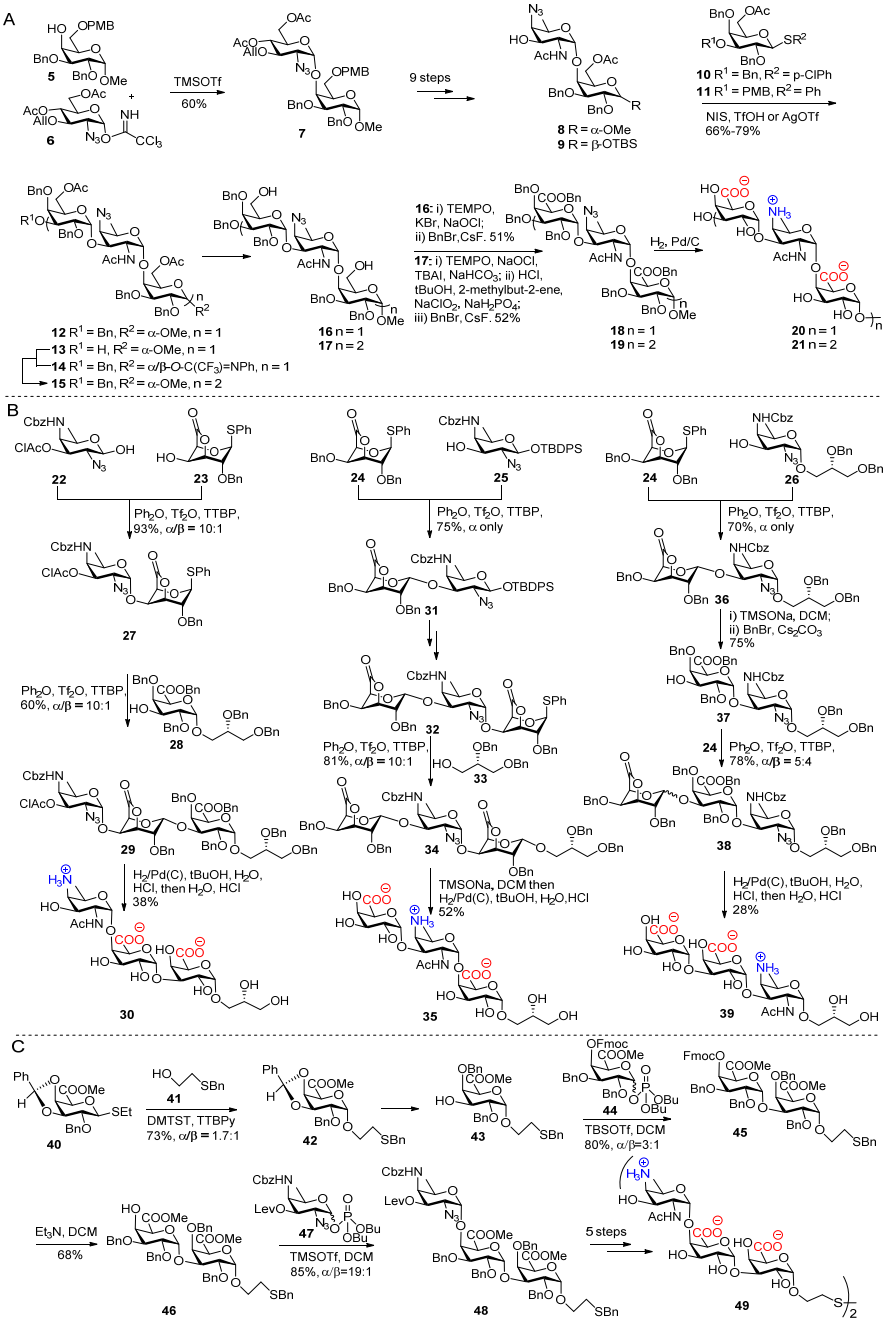

Figure 2.2 Syntheses towards fragments of ZPS Sp1. A) Approach by Bundle and co-workers generating a trisaccharide and a hexasaccharide. B) GalA-[3,6]-lactones in the synthesis of all three repeating units of Sp1. C) Assembly of Sp1 ready for conjugation.

deprotection of the trisaccharide was accomplished by a catalytic hydrogenation event. The assembly of the hexasaccharide, encompassing two repeating units, required the generation of a new trisaccharide. Again, a disaccharide synthon (**9**) was generated, this time bearing an anomeric *tert*-butyldimethyl (TBS) group as a temporary protecting, to allow for the generation of a trisaccharide donor (**14**). The crucial condensation of the trisaccharide donor **14** and acceptor **13** required careful tuning of the reaction conditions and hexasaccharide **15** could be obtained in 85% yield. Deacetylation of **15** then provided the tetraol, ready for the crucial oxidation step. Complete oxidation of the tetraol **17** proved more difficult that the corresponding oxidation of diol **16**. It is commonly observed in the assembly of uronic acid containing oligosaccharides that it is significantly more challenging to perform multiple oxidations on a large substrate than to oxidize smaller fragments. Using Huang's oxidation procedure that entails a biphasic TEMPO oxidation, followed by a Pinnick oxidation of the formed aldehydes, the tetra uronic acid was obtained. After benzylation of the esters the hexasaccharide **19** could be purified and it was obtained in 52% yield starting from tetra-acetate **15**. A single hydrogenation event then delivered the target hexasaccharide **21**. The authors report that the tri- and hexasaccharide **20** and **21**, respectively, were evaluated for their T-cell activating capacity but that no activity was found. However, no details for these experiments have been disclosed. It has been postulated that ZPSs take up helical shapes and that this 3-dimensinal structure may be relevant for the unique MHC-II binding capacity of the ZPSs. The NMR spectra of the tri- and hexasaccharide **20** and **21** were compared to the NMR of the native polysaccharide. Although there were clear similarities between the spectra, also significant differences were observed, indicating that the structures of the synthesized

fragments were quite distinct from their polymeric counterpart. Given the synthetic challenges that were encountered, Bundle and co-workers reached the conclusion that the solution to the question what the minimal structure is for MHC-II binding will not be delivered through an organic synthesis approach.

After this first synthetic effort, Christina *et al.* described the assembly of the three unique trimer repeats of the Sp1 ZPS (see Figure 2.2B).[17] To circumvent the challenges associated with late stage introduction of multiple carboxylates, to tackle the generally poor reactivity of galacturonic acid acceptors and to install the desired α-GalA linkages in a stereoselective manner, galacturonic acid lactone synthons **23** and **24** were produced. GalA-[3,6]-lactones are reactive GalA donors that normally provide condensation products in excellent *cis*-selectivity. The lactone bridge in GalA **23** places the C4-OH in a reactive and accessible equatorial position, which explains productive couplings with this building block. As depicted in figure 2.2B, the three trimer repeats **30**, **35** and **39** were assembled through a modular approach employing building blocks **22-26** and spacer **33**. All glycosylations towards the three trimer repeats proceeded in good yield and stereoselectivity, except for the condensation of donor **24** and acceptor **37**. The reason for the poor selectivity in the latter glycosylation is not clear. This result does indicate that the use of a GalA-TDDAG-GalA trisaccharide building block with a GalA-[3,6]-lactone donor moiety is not attractive to assemble larger Sp1 oligosaccharides. Nonetheless, the GalA-[3,6]-lactones have proven their merits in these syntheses. No biological evaluation of the trisaccharides has been reported yet.

Schumann *et al.* reported on the synthesis of a Sp1 trisaccharide, functionalized with a thiol handle for conjugation purposes. As depicted in Figure 2.2C, they built the

trisaccharide from the monomeric building blocks **40**, **44** and **47**. The TDDAG building block was assembled using a *de novo* approach.[18] The trisaccharide chain was built up by first condensing mercaptoethanol spacer **41** and GalA donor **40**. This glycosylation proceeded with poor selectivity to give the spacer-GalA building block **42** in 73% (α/β = 1.7 : 1). After regioselective benzylidene ring opening the resulting GalA was coupled with donor **44** again with moderate stereoselectivity. After Fmoc-removal the TDDAG building block was attached to give the fully protected trisaccharide. Unmasking of the trimer required the exchange of the C3"-levulinoyl (Lev) ester for a benzyloxymethyl (BOM) ether, to prevent base mediated carbamate formation on the TDDAG moiety, a side reaction also observed by Christina *et al*.[17] After installation of the acetamides, Birch reduction provided the desired trimer as a disulfide. This trisaccharide was used to interrogate rabbit sera raised against the Sp1 polysaccharide in a microarray binding study. To this end disulfide **49** was reduced *in situ* and coupled to a maleimide functionalized micro array glass slide. Binding was observed between the immobilized synthetic trisaccharide and the serum indicating that structural elements of the polysaccharide that are recognized by antibodies in the serum are also present in the trisaccharide. The structure was not recognized by serum raised against PS A1 (*vide infra*).

2.2.2 Synthesis of PS A1 polysaccharide fragments

To date only two synthetic approaches have been reported towards the assembly of the ZPS of *Bacteroides fragilis*, PS A1. Just like Sp1 saccharide, the PS A1 ZPS is built up from galactose configured monosaccharides and contains the TDDAG residue as well. As shown in Figure 2.1B, next to the TDDAG, it contains a GalNAc, a galactofuranose (Gal*f*) and a pyruvate functionalized galactopyranose residue. Van den Bos *et al.* were the first to

describe the synthesis of a protected tetrasaccharide (Figure 2.3A).[20] The trisaccharide **55** was assembled using an efficient one-pot sequential glycosylation strategy in which first

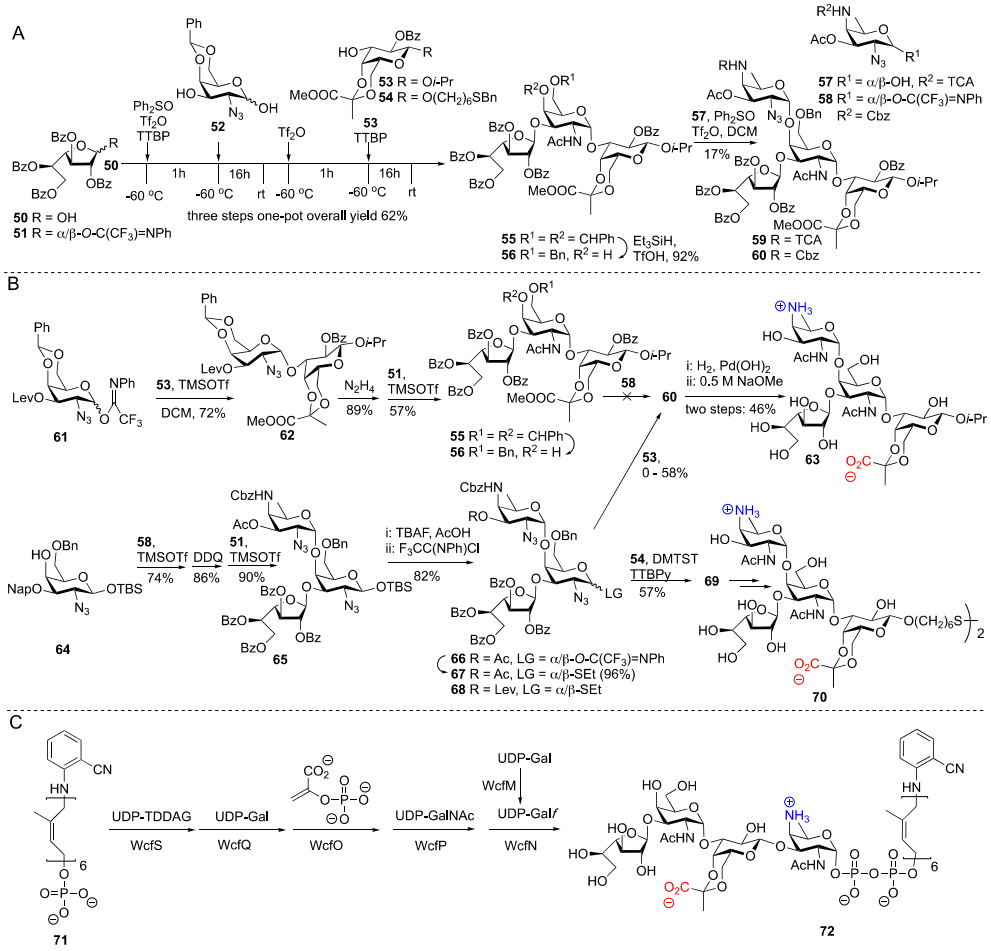

Figure 2.3. Syntheses toward the repeating unit of the ZPS PS A1. A) First assembly of a protected tetrasaccharide **59**. B) First completed synthesis of the PS A1 tetrasaccharide. C) Reconstitution of the biosynthesis of PS A1.

the Gal*f* hemiacetal **50** was coupled to the diol **52** in a chemoselective dehydrative glycosylation following Gin's protocol. Next, triflic anhydride (Tf_2O) was added to activate

the thus-generated disaccharide lactol. Ensuing addition of pyruvate galactose **53** led to the desired trisaccharide in 62% yield. After benzylidene opening the final condensation was attempted with the TDDAG donor **57**. Unfortunately, the desired tetrasaccharide **59** was formed in disappointing yield and not enough of the material was procured to deprotect the tetramer.

Pragani and Seeberger described a similar approach to generate the PS A1 tetrasaccharide and they also found that the condensation of a TDDAG donor (**58**) and the trisaccharide acceptor (**56**) was unproductive (Figure 2.3B).[21] According to molecular mechanics (MM2) energy minimized models of nucleophile **56**, the α-pyruvylated galactose preferentially occupies the space below the galactosamine residue near the C6 benzyl ether. This steric crowding likely forces the C6 benzyl ether to the top face of the galactosamine residue, thereby shielding the already poorly nucleophilic C4-OH. Therefore, the synthesis route was adapted, changing the order of the glycosidic bond formations. The TDDAG (**58**) and GalN3 (**64**) building blocks could be coupled in 71% yield. Next the Nap ether was removed and the Gal*f* attached (90%), eventually leading to the trisaccharide donor **66**. It proved challenging to couple this trimer in a stereoselective fashion to the pyruvate galactose acceptor. After screening several condensation conditions, it was found that the use of a thioglycoside donor (**67**) in combination with a mild activator (DMTST) performed best and tetramer **60** could be obtained in 58% yield. Deprotection of the tetrasaccharide **60** had to be done under carefully controlled conditions to prevent *O*→*N* acyl migration. By transformation of the azides into acetamides, reduction of the benzyl ether and carbamate and ensuing saponification the final tetrasaccharide **63** was obtained in 46%. The NMR of this tetrasaccharide proved to

be remarkably similar to the NMR of the native polysaccharide, leading Pragani and Seeberger to conclude that the crowded nature of the tetrasaccharide repeating unit determines -to a large extent- the overall structure of the saccharide. This synthetic strategy also allowed for the assembly of spacer containing tetrasaccharide **69**. Deprotection of this molecule again required the Lev to BOM exchange as described above for the Sp1 saccharide. Ensuing azide to acetamide conversion and final dissolving metal reduction gave tetrasaccharide **70** as a disulfide. This molecule was reduced *in situ* and immobilized on a micro array slide and probed with rabbit sera raised against *Bacteroides fragilis*. Minimal interaction was detected using this experimental set-up (in contrast to the Sp1 micro array results described above).

Very recently, Troutman and co-workers reconstituted the biosynthesis pathway of the PS A1 repeating unit using heterologously expressed glycosyl and pyruvate transferases (Figure 2.3C).[22] They showed that in a one-pot operation the tetrasaccharide repeat could be built up on a fluorescently labelled bactoprenyl phosphate (BP). Through the consecutive action of the TDDAG-transferase WcfS (obtained in a membrane franction) using UDP-TDDAG and the galactosyl transferase WcfQ (also in a membrane fraction) employing UDP-Gal as the donor substrate the BP linked TDDAG-Gal disaccharide was formed. This was shown to be the substrate for the pyruvate transferase, WcfO, which uses phosphoenol pyruvate (PEP) for pyruvate incorporation. Of note WcfO was incapable of transferring the pyruvate to simpler galactose substrates, such as UDP-Gal or nitrophenyl-Gal. The pyruvylated disaccharide serves as a substrate for the next transferase, WcfP, that introduces the GalNAc moiety. The non-pyruvylated BP-TDDAG-Gal is not elongated by this enzyme. Finally, WcfN transfers Gal*f* from UDP-Gal*f*, obtained from UDP-Gal through the

action of the galactopyranose mutase WcfM, to the BP-trisaccharide to complete the assembly of the PS A1 tetrasaccharide. It is expected that the polyprenylpyrophosphate tetrasaccharide can be used as a substrate to study the down-stream biosynthesis enzymes, including the polymerase that generates the polymeric PS A1 and the flippase that transposes the polysaccharide over the membrane to the outside of the bacterial cell wall. Generating larger oligomers or larger quantities of the fragments will represent a significant challenge as it will require harnessing the flippase and polymerase enzymes and demands the availability of sufficient donor glycosides or co-expression of the complete biomachinery to assemble these (the rare UDP-TDDAG donor is not readily available). Finally, controlling the length of the growing polymer will represent a significant challenge.

2.3 Conclusion

Synthetic organic chemistry is now at the level that short fragments of ZPSs are accessible, and the repeating units of the "archetype" ZPS, PS A1, and the Sp1 have been assembled by different laboratories. The longest ZPS to date, the Sp1 hexasaccharide encompassing two repeating units, has been synthesized by Bundle and co-workers. This molecule was evaluated for its T-cell stimulating activity, but shown to be inactive. The synthetic challenges encountered during the synthesis led Bundle and co-workers to conclude that "the resolution of the unresolved issue of the minimal size oligosaccharide for biological activity is unlikely to be addressed by oligosaccharide synthesis". Although no syntheses have appeared yet to challenge this statement, there have been important advancements in synthetic oligosaccharide synthesis that set the stage for the construction of longer fragments. Effective routes of synthesis have been laid out to assemble the rare TDDAG building blocks[28-30] and our knowledge of reactive intermediates and their

stereoselectivity in glycosylation reactions has significantly deepened. New oxidation protocols have been introduced and ever more effective functional and protecting group chemistry is introduced. Finally, automated synthesis now allows for the generation of very large oligosaccharides in an automated manner.[31-33] It is therefore not unlikely that these complex and intriguing molecules will be man-made in the future. The availability of synthetic long fragments will allow the study of the interaction of these with MHC molecules in atomic detail to explain their mode of action in a definitive manner. Likewise, they can be used to unravel how these saccharides interact with players of the innate immune system and how this activity may be used to elicit a tailor made immune response.

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