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*J Immunol* 2007; 179:8208-8215; doi: 10.4049/jimmunol.179.12.8208
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Introduction of Zwitterionic Motifs into Bacterial Polysaccharides Generates TLR2 Agonists Able to Activate APCs

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It was shown previously that bacterial polysaccharides (PS), which naturally contain both positive and negative charges, are able to activate T cells and APCs. However, the vast majority of bacterial PS are anionic and do not have these properties. In this study, we show that chemical introduction of positive charges into naturally anionic bacterial PS confers to the resulting zwitterionic PS (ZPS) the ability to activate pure human monocytes, monocyte-derived dendritic cells, and mouse bone marrow-derived dendritic cells, as do natural bacterial ZPS. Cells are induced to up-regulate MHC class II and costimulatory molecules and to produce cytokines. In mixed monocyte-T cell cocultures, ZPS induce MHC II-dependent T cell proliferation and up-regulation of activation markers. These stimulatory qualities of ZPS disappear when the positive charge is chemically removed from the molecules and thus the zwitterionic motif is destroyed. The ability of natural and chemically derived ZPS to activate APCs can be blocked by anti-TLR2 mAbs, and TLR2 transfectants show reporter gene transcription upon incubation with ZPS. In conclusion, the generation of a zwitterionic motif in bacterial PS confers the ability to activate both APCs and T cells. This finding has important implications for the design of novel polysaccharide vaccines. The Journal of Immunology, 2007, 179: 8208–8215.

Pathogenic bacteria pose a major threat to the organism, and a multitude of immune mechanisms has evolved to recognize and target bacterial surface structures. For instance, many components of the bacterial envelope, such as peptidoglycans and lipoteichoic acid in the bacterial cell wall and LPS and porins embedded in the outer membrane, are recognized by the innate immune system and are key to the initiation of an immune response. In addition, most pathogenic bacteria are coated by a capsule composed of repetitive units of polysaccharides (PS), and induction of Abs specific to the capsular PS confers protection against infection. This is the reason why a number of vaccines against bacterial infections aim exclusively at the induction of Abs against capsular PS. Because PS are considered T cell-independent Ags, i.e., they are not recognized by the TCR and therefore do not induce the T cell help necessary for the generation of high-affinity, class-switched Abs, in most vaccines PS are conjugated to carrier proteins, eliciting the desired cognate T cell help.

A number of publications have shown in the past that capsular PS from Bacteroides fragilis, Staphylococcus aureus, and type 1 Streptococcus pneumoniae, which naturally contain both positive and negative charges, i.e., zwitterionic polysaccharides (ZPS), are able to activate T cells and APCs (1–7). The initial findings demonstrated that abscess formation in a rat model was induced by PS containing zwitterionic charge motifs (8) and that abscess formation was T cell dependent and transferable with ZPS-activated T cells (9, 10). The integrity of the zwitterionic motif was essential for this biological activity, because removal of one of the two charges also removed the ability to induce abscesses (8). In vitro experiments with unfraccionated splenocytes or with mixed populations showed that natural ZPS were able to induce T cell activation in these conditions, whereas the coculture of fixed APCs with T cells was not sufficient to induce proliferation (2). The alternative, but not mutually exclusive hypotheses to explain these findings are that ZPS require processing to activate T cells directly through TCR recognition of MHC class II-ZPS complexes (11) or that ZPS activate APCs to up-regulate MHC class II, costimulatory molecules and cytokines, and thus generate conditions that favor the activation of T cells (2). In fact, a very recent publication from the same group shows that the natural ZPS, B. fragilis capsular PS A (PSA), is also a TLR2 agonist and able to activate a number of different APCs (12). MHC class II-blocking Abs inhibit T cell activation, and up-regulation on APCs of a number of molecules involved in T cell activation has been demonstrated (4).

Because the vast majority of naturally occurring bacterial capsular PS are neutral or anionic and are not able to activate T cells or APCs, we decided to test whether the introduction, by chemical modification, of a zwitterionic charge motif into a naturally anionic PS would yield a molecule with the described stimulatory qualities. We chose the PS from type Ia, Ib, and III of group B streptococcus (GBS), an emerging pathogen against which a glycoconjugate vaccine is currently being developed (13). These PS are naturally anionic, because the otherwise neutral sugar backbone carries anionic groups such as carboxyls, which are present as
carboxylate ions at physiological pH. Positive charges were chemically introduced, and the ability of these chemically derived ZPS to activate APCs and T cells was tested on a variety of human and mouse cell types.

Materials and Methods

Purification of GBS capsular PS

Capsular PS were prepared from Streptococcus agalactiae bacteria using a modified version of the procedure previously published (14). Isolated PS were found to contain low concentrations of nucleic acids (<10 μg/mg), proteins (<10 μg/mg), group B saccharides (<10 μg/mg), and LPS (<0.001 U/ml).

Chemical modifications of GBS PS

Cationic protonated forms of amines can be introduced into PS at the following: 1) free N-acetyl groups, which are part of N-acetylneuraminic acid (NeuNAc) and N-acetylgalactosamine residues, and 2) the terminal aliphatic chain of NeuNAc (see Fig. 1A). De-N-acetylation was achieved by basic hydrolysis (1 M NaOH for 60 min at 80°C) to form free amino groups available for further reaction (scheme a in Fig. 1A). Alternatively, chemical oxidation of the aliphatic chain from the terminal NeuNAc residue (scheme b in Fig. 1A) with sodium metaperiodate (Sigma-Aldrich; 0.01 M NaIO4 for 90 min at room temperature) leaves an aldehyde group at C8. This group was converted to a cationic –NH3+ group by reductive amination using 300 mg/ml ammonium acetate (NH4Ac; Sigma-Aldrich) and 49 mg/ml sodium cyanoborohydride (NaBH3CN; Sigma-Aldrich) at pH 6.5 and T = 37°C for 5 days (16).

PS obtained by the reaction schemes a and b (Fig. 1) were treated with 37% formaldehyde (H2CO; Carlo Erba) in the presence of sodium cyanoborohydride to convert the generated free amino group to a tertiary dimethylammonium such that it retained a positive charge (6).

To remove the anionic charge on ZPS2 (Fig. 1A, scheme c), the carboxyl group was reduced to an aliphatic group by treatment with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma-Aldrich) and sodium borohydride (NaBH3CN; Sigma-Aldrich) at pH 6.5 and T = 37°C for 5 days (16).

As for the native PS, contamination with proteins, nucleic acids, and group B saccharide was also determined in all ZPS preparations and found to be below 10 µg/mg. Because reagents used for the modifications contained detectable levels of these contaminants, the purity of ZPS products should be higher than the native PS due to additional purification steps performed after the chemical treatments.

Nuclear magnetic resonance (NMR) spectroscopy was used to assess the structural identity of the purified PS and the chemically derived ZPS molecules. NMR spectra were recorded at 25°C on Bruker DRX 600 MHz spectrometer and using 5-mm triple- resonance NMR probe (Bruker). For data acquisition and processing, XWINNMR software package (Bruker) was used. The 1-D proton NMR spectra were collected using a standard one-pulse experiment with 32 scans. 1H NMR spectra were obtained in quantitative manner using a total recycle time to ensure a full recovery of each signal (5 × longitudinal relaxation time T1).

NMR samples were prepared by dissolving lyophilized product in 0.75 ml of deuterium oxide (D2O; Sigma-Aldrich) to a uniform concentration and transferred to 5-mm NMR tubes (Wilmad).

Cell preparation and culture

PBMCs were collected from buffy coats of healthy donors who had given written informed consent by Ficoll-Hypaque (Amersham Biosciences) density gradient centrifugation. Highly purified (>98%) monocytes were obtained from PBMCs by positive selection of CD14+ cells using anti-CD14-coated magnetic microbeads and MACS technology (Miltenyi Biotec). Monocytes (2 × 10⁷ per well) were cultured for 24 h in RPMI 1640 (Invitrogen Technologies) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM solution (Invitrogen Life Technologies) (RPMI-PSG), and 5% human serum (Sigma-Aldrich) using U-bottom 96-well plates. T cells (>98%) were prepared from PBMCs by MACS by negative selection using the Pan T Cell Isolation kit (Miltenyi Biotec). T cells (2 × 10⁶ per well) were cocultured with monocytes (1 × 10⁷ per well) for 6 or 8 days in the same conditions as described above for monocytes.

Immature monocyte-derived dendritic cells (Mo-DCs) were obtained from culturing monocytes for 6 days in RPMI-PSG supplemented with 10% FCS (HyClone) (complete medium) with IL-4 (10% of supernatant from IL-4-secreting cell line; provided by A. Lanzavecchia, Institute for Research in Biomedicine, Bellinzona, Switzerland) and 50 ng/ml GM-CSF (Gentaur). Immature dendritic cells were washed and cultured for the experiments in complete medium, using 96-well flat-bottom cell culture plates.

Mouse bone marrow-derived dendritic cells (BM-DCs) were generated, culturing femoral bone marrow with murine rGM-CSF (PeproTech), as described (18). At day 6, BM-DC were washed and cultured in complete medium with 50 μM 2-ME (Sigma) and 100 U/ml mGM-CSF, using porcine U-bottom 96-well plates (BD Biosciences).

Where indicated, cells were treated with LPS, macrophage-activating lipopeptide-2, or N-palmitoyl-S-[2,3-bis(palmitinloyloxy)-(2R5)-propyl]-cysteiny1]-S-erythrose-4-phosphate) (PS-Pam3CSK4), which are part of native GBS serotype III capsular PS. A, De-N-acetylation by basic treatment leading to ZPS1 (scheme a), and periodate oxidation and reductive amination leading to ZPS2 (scheme b), of one repeating unit of GBS serotype III PS. The additional reaction with formaldehyde to obtain tertiary amines is also shown. Scheme c, Reduction of the carboxyl group leading to ZPS2 reduced. B, NMR spectra of the native form (bottom line) and the ZPS2 modification (top line) of serotype Ib PS from GBS. The peaks generated by the newly formed methyl group (CH3)2-NH– and by other groups are annotated.

Flow cytometry

The following mAbs were used for flow cytometry: for human cells, FITC-conjugated anti-CD14 or anti-CD83, PE-conjugated anti-CD80, allophycocyanin-conjugated anti-CD86, and PerCP-conjugated anti-MHC class II, and PerCP-conjugated streptavidin (BD Pharmingen). Rabbit serum was used as a blocking agent. After incubation for 20 min on ice, cells were washed and analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).
ELISA

TNF-α production in culture supernatants was quantified by specific standard sandwich ELISA, using capture B154.9 and biotinylated B154.7 mouse mAbs provided by G. Trinchieri (Center for Cancer Research, National Cancer Institute, National Institutes of Health, Frederick, MD).

Plasmid production

A DNA fragment coding for human TLR2 with no signal peptide was obtained by RT-PCR from human PBMC cDNA using TLR2-specific primers and then cloned in the pFLAG-CMV-1 vector (Sigma-Aldrich) to attach an N-terminal FLAG epitope to TLR2; the FLAG-TLR2 sequence was then subcloned in pcDNA3.1 (Invitrogen Life Technologies).

Plasmid production

The NF-kB-regulated promoter of the Igκ-luc plasmid (a gift from A. Leonardi, University of Naples, Naples, Italy) was subcloned in pEGFP-1 vector (BD Clontech) upstream to the EGFP coding sequence to obtain pNFκB-d2EGFP plasmid; the hygro gene with an upstream SV40 promoter, excised from the pTK-Hygro vector (Invitrogen Life Technologies), was then inserted in an uninfluential region to obtain pNFκB-d2EGFP-Hygro.

Enzymatic treatments

Treatments were adapted, as described previously (19). Before addition to cells, ZPS and controls were incubated in PBS with 200 µg/ml lipoprotein lipase (LPL) from bovine milk or from pseudomonas sp. (Sigma-Aldrich) at 37°C for 7 h. Results shown were obtained using bovine milk-derived LPL. Alternatively, immobilized trypsin (Pierce) or proteinase K-acrylic beads (Sigma-Aldrich) were incubated with samples at a 1:20 ratio at 37°C for 1 h. Proteases were removed from the reaction by centrifugation. The enzymatic treatments were performed on ZPS and controls that were concentrated 10^3 compared with their final concentration in the in vitro experiments.

HEK-293 stable transfectants

HEK-293 cells were grown in DMEM supplemented with glucose (4500 g/L) glutamine (2 mM), 10% FCS, penicillin, and streptomycin. Cells were transfected using lipofectamine 2000 (Invitrogen Life Technologies) with the pcDNA-FLAG-TLR2 construct, and a stable clone was derived by Geneticin (Invitrogen Life Technologies) selection. FLAG-TLR2 expression was verified by surface staining with FLAG-M2 mAb (Sigma-Aldrich) and FACS analysis. This clone was then transfected with pNFκB-d2EGFP-Hygro, and a stable clone was derived by selection with hygromycin B (Invitrogen Life Technologies). For experiments, cells were cultured for 24 h with PS and controls, washed, and analyzed by flow cytometry. The HEK-293 triple transfectants TLR4/MD-2/CD14 were obtained from InvivoGen, and surface expression of CD14 was confirmed by FACS. In experiments comparing directly TLR2 and TLR4 agonist activity, the respective transfectant cell lines were incubated with PS for 20 h, supernatant was obtained, and IL-8 content was determined using the FlexSet kit (BD Biosciences), according to manufacturer’s instructions. IL-8 produced by the transfectants was normalized to the amount of IL-8 present in the supernatant of the respective unstimulated transfectant line.

Proliferation assays

T cell proliferation was assessed by [3H]thymidine incorporation using 2 x 10^5 T cells and 1 x 10^5 γ-irradiated monocytes (3000 rad) per well in round-bottom 96-well plates. After 6 days of culture, cells were pulsed with 0.5 µCi/well [3H]thymidine, incubated for 18 h, and harvested onto filter plates (Packard Instrument), and counts were analyzed using a Top Count NXT beta counter (Packard Instrument).

Statistical analysis

Statistical significance was determined using two-tailed Student’s t test analysis. Significance was reconfirmed using nonparametric statistical analysis.

FIGURE 2. ZPS activate human and mouse APCs. Human CD14^+ monocytes (A–C), Mo-DCs (D), or mouse BM-DCs (E and F) were incubated with the indicated compounds for 24 h (A, B, D, E, and F) or as indicated (C), and up-regulation of the indicated surface markers was measured by flow cytometry (A, B, E, and F) or TNF-α concentration in the culture supernatants was determined by ELISA (C and D). LPS, 1 µg/ml; Pam3CSK4, 100 ng/ml; all PS in A and C–F, 6 µg/ml. Error bars indicate SD of triplicate samples; results are representative of at least three experiments. *, p < 0.05; **, p > 0.05 compared with medium.
Results

Chemical modifications of the native GBS serotype III PS

Fig. 1A shows, as an example, the modifications introduced into the repeating unit of the capsular PS from GBS serotype III (GBS III). The same type of modifications was also introduced into GBS 1a and 1b. According to the annotations shown in Fig. 1III), the same type of modifications was also introduced into GBS 1a and 1b. According to the annotations shown in Fig. 1A, ZPS1 is the zwitterionic PS obtained from the chemical modification (scheme a) that converts the N-acetyl groups to free amino groups. Chemical modification (scheme b) generates the ZPS2 resulting from a periodate oxidation to generate an aldehyde group, which is also converted to an amino group by a reductive amination reaction. Furthermore, all amino groups are converted to tertiary amines that retain their positive charges. Thus, in each repeating unit, ZPS1 molecules from all three serotypes contain two positive and one negative charge, whereas ZPS2 contain a balanced motif of one charge each. To remove the anionic charge from ZPS2, the carboxyl group of ZPS2 is reduced by a carbodiimide-mediated reaction with NaBH4 (modification scheme c). All structural analyses and chemical modifications thereof were confirmed by NMR spectroscopy (Fig. 1B). In particular, the zwitterionic structure is confirmed by detecting the methyl group (CH3)-N+H–, which has been generated by the chemical modification scheme b (Fig. 1A). All labeled signals have been assigned using bidimensional homonuclear (1H-1H) and heteronuclear (1H-13C) NMR experiments (data not shown).

Additional saccharide contaminations (i.e., group B polysaccharide) were also excluded by analyzing the NMR profiles. Only a little residual amount of ethanol used in the purification procedure has been detected.

Chemically derived ZPS are able to activate APCs

To test the ability of ZPS to activate APCs, purified human monocytes and Mo-DCs were incubated with either the natural ZPS purified from B. fragilis, the PSA, or the ZPS derived from GBS PS by the chemical modification 1, as indicated in Fig. 1A, leading to ZPS1, or by the modification 2 (ZPS2). The dot plots in Fig. 2A show that both PSA and the chemically derived ZPS2 are able to induce up-regulation of MHC class II and CD80 on human monocytes, whereas the native anionic GBS PS (Fig. 2A) or the ZPS1 derived by chemical modification 1 (data not shown) do not activate monocytes. The dose-response relation of this activation is shown in Fig. 2B. Similarly, ZPS2 of all three GBS serotypes induce TNF-α production by purified human monocytes, whereas the native forms are inactive (Fig. 2C). The amount of TNF-α induced in monocytes by PSA is comparable to that induced by serotype 1b ZPS2 (data not shown). The production of TNF-α is induced in human Mo-DC by the ZPS2 form, but not the native or ZPS1 form of the GBS capsular PS, again indicating that the zwitterionic motif introduced is essential for the biological activities observed (Fig. 2D). Similar results were found for up-regulation of the maturation marker CD83 on human Mo-DCs (Fig. 3A). Finally, mouse BM-DCs from BALB/c mice also are activated by ZPS2, but not the native PS or ZPS1 (Fig. 2E). The dot plots and gates used for the determination of marker up-regulation are shown in Fig. 2F. As previously reported (20), BALB/c mice are more responsive to TLR2 agonists than C57BL/6 mice, and this is reflected in the stronger response of BALB/c BM-DCs to the positive control Pam3CSK4, as compared with control Pam3CSK4. In conclusion, these results indicate that the chemical introduction of a zwitterionic charge motif into anionic PS confers the ability to activate a variety of human and mouse APCs.

The stimulatory activity of ZPS depends on the integrity of the zwitterionic motif and is not extracted by phenol

The above results indicate that the chemical generation of ZPS leads to molecules with new biological activity. To confirm that the integrity of the zwitterionic motif is essential for the observed stimulatory abilities, we removed the negative charge from ZPS2 (see Fig. 1A) and tested whether the resulting cationic molecule can stimulate APCs. As shown in Fig. 3A, the ability to stimulate Mo-DCs disappears when the positive charge is removed from the molecules, and thus the zwitterionic motif is destroyed. This result demonstrates that the zwitterionic motif is required for the ability of PS to stimulate APCs. A recent publication shows that the ability of Pneumococcus PS to induce strong immune responses in mice depends on associated TLR2 agonists that can be separated from the PS by phenol extraction (17). To ensure that the APC stimulatory abilities are not due to a lipophilic contamination, we...
subjected ZPS2 to phenol extraction and tested the residual biological activity. Fig. 3B shows that the ability of ZPS2 to activate monocytes is not affected by phenol extraction. Similar results were obtained for the ability to stimulate Mo-DCs (data not shown). In addition, *limulus* amebocyte lysate tests performed on the native PS and on ZPS2 before and after phenol extraction resulted in extremely low endotoxin values, and no correlation was found between endotoxin content and the biological activity observed in this study (data not shown). All subsequent experiments were done with ZPS2 that had undergone phenol extraction. Because the native PS did not show any ability to activate APCs, it can be excluded that APC stimulation was due directly to bacterial contaminants that remained after PS purification. To exclude also the possibility that contaminants were introduced during chemical modification, we subjected native PS to the whole chemical modification with the exception of the first step, thus not generating the ZPS molecule, but allowing for all factors that may contribute to the introduction of contaminants ("no first step"). Fig. 3C shows that this preparation has no biological activity.

**APC activation by ZPS is mediated by TLR2**

The results shown in Fig. 2E suggest an involvement of TLR2 in the APC-activating properties of ZPS. In addition, it was recently reported that the natural ZPS, PSA, is able to activate APCs through TLR2 (12). To analyze in detail TLR2 involvement, we tested whether a TLR2-blocking Ab can inhibit the observed effects. Fig. 4A shows that anti-TLR2 mAbs block the induction on human monocytes of MHC II and costimulatory molecules by both the natural and the chemically derived ZPS. The canonical TLR2 agonist Pam3CSK4 induces the same effects on monocytes that can...
also be blocked by the anti-TLR2 mAb. This mAb did not block APC activation induced by TLR4 (Fig. 4A) or TLR7/8 agonists (data not shown), thus confirming the specificity of the reagent. ZPS2- and PSA-induced TNF-α production by monocytes was also blocked by these mAbs (data not shown). An isotype-matched control Ab was not able to block the effects described in this study (data not shown). To reconfirm the specific interaction with TLR2, we used stable TLR2 transfectants and observed reporter gene transcription upon incubation with both natural and chemically derived ZPS, but not with the native or the ZPS1 form of the GBS PS (Fig. 4B). GFP expression induced by Pam3CSK4 and the natural and chemically derived ZPS was blocked by the anti-TLR2 mAb, confirming the specificity of this induction. As a further reconfirmation of absence of LPS contamination or TLR4 agonist activity of the ZPS2, we performed in parallel experiments using TLR2 and TLR4/MD-2/CD14 transfectants and assayed for the same readout, namely IL-8 production (Fig. 4, C and D). Although all natural and chemically derived ZPS induce significant IL-8 production in TLR2 transfectants at the two different doses tested, none of these molecules did so in the triple transfectants at any of the doses tested. Hence, this very sensitive assay confirms that TLR4-mediated activation does not play a significant role in the phenomena described in this work. TLR2 transfectants were also used to exclude a number of other contaminants (Fig. 4, E and F). To exclude lipopeptide contaminants in the preparations, ZPS were treated with LPL. As shown in Fig. 4F, pretreatment with LPL does not reduce the TLR2 agonist activity of ZPS, whereas Pam3CSK4 activity is greatly reduced. Similarly, pretreatment with proteases does not alter the biological activity of ZPS, whereas Pam3CSK4 activity is reduced to different degrees by these two proteases (Fig. 4E). In conclusion, this set of experiments indicates that the biological activity of ZPS is mediated by TLR2, and that LPS, lipopeptide, or protein contaminations do not play a role in this activity.

**ZPS induce T cell activation in APC-T cell coculture**

A series of previous publications show that natural ZPS can induce T cell proliferation in vitro coculture experiments (2, 3, 5–7). When cocultures of purified T cells and purified, γ-irradiated syngeneic monocytes were incubated with PSA or ZPS2, both proliferation and up-regulation of activation markers on T cells were observed, whereas single populations of purified cells did not proliferate in the presence of PSA or ZPS2 (Fig. 5A and data not shown). Fig. 5B shows that only ZPS2, but not ZPS1, are able to induce T cell proliferation. To test the relative contribution of MHC II and TLR2 to the T cell activation observed, blocking experiments with mAbs for both molecules were performed. Fig. 5C shows that ZPS-induced T cell proliferation was consistently blocked by MHC II-blocking mAbs, whereas anti-TLR2 mAb significantly blocked proliferation induced by serotype Ib ZPS, but not by serotype III ZPS or PSA. However, it is interesting to note that all TLR agonists used as controls stimulate T cell proliferation that can be blocked by anti-MHC II mAbs. As expected, LPS-induced T cell proliferation is not blocked by the TLR2 mAbs, whereas TLR2 agonist-induced T cell proliferation is. In conclusion, these results confirm that the chemically derived ZPS show the same range of activities described for natural ZPS, and that APC activation is in some cases, but not always, an essential component of the T cell stimulation induced by ZPS. It is also clear from these experiments that MHC II dependency of stimulation may not be sufficient to establish whether a molecule is a MHC II-dependent T cell Ag or whether MHC II up-regulation is part of the APC activation that eventually leads to T cell proliferation in this experimental set-up.

In conclusion, we demonstrate that the generation of a zwitterionic motif in naturally anionic bacterial PS generates TLR2 agonists that are able to activate both APCs and T cells.

**Discussion**

In this study, we show that introduction by chemical means of a zwitterionic charge motif into anionic PS can generate molecules with the same biological activity as described for natural ZPS. In addition, we demonstrate that ZPS-induced activation of a variety of APCs is mediated by TLR2, and therefore identify ZPS as a new class of agonists for these receptors. From our study, a clear structure-activity relationship emerges: the same PS repetitive unit...
shows biological activity in the presence of a zwitterionic motif of alternating charges, but not in the presence of either negative or positive charges only. It remains to be clarified why one of the two chemical modifications leading to ZPS did not yield biologically active molecules. Two possible explanations can be envisaged, as follows: either a specific spatial relationship between the positive and negative charges is required that was met with one, but not the other modification, or the fact that ZPS1 contain two positive vs one negative charge per repetitive unit and thus represent cationic molecules prevents their biological activity. Among the natural ZPS, some have a negative net charge and some are neutral, but none of them is cationic.

It was shown previously that conversion of a primary amine to a tertiary amine on PSA was associated with a loss of T cell stimulatory activity (6). The chemically derived ZPS2 forms shown in this study all contain tertiary amines and are able both to activate APCs and to induce T cell proliferation. This indicates that the presence of primary amines is not mandatory for the ability to induce T cell proliferation.

Our findings have important implications with regard to PS-based vaccines. The conjugation of a vaccine Ag to a TLR2 agonist was shown to increase both Ab and T cell responses, and thus, TLR2 agonists can clearly function as adjuvants (21). In addition, vaccines against yellow fever and against *Pneumococcus* have been shown to be efficient partly due to associated TLR2 agonists (17, 22). In line with these results, our observations on human and mouse APCs strongly suggest an adjuvant function for ZPS, and we are currently investigating this in in vivo experiments.

Our studies using TLR2 transfectants clearly show that ZPS can act through this receptor. However, we have also noted that the canonical TLR2 agonist Pam3CSK4 is rather inefficient at activating Mo-DCs, whereas ZPS appear to be equally potent activators in both systems. A number of explanations for this discrepancy can be envisaged: first, on Mo-DCs, ZPS may use cooperatively a second receptor that enhances a weak TLR2-mediated signal; second, the multivalent nature of the ZPS may lead to cross-linking of a higher number of TLR2 molecules per cell than is the case for Pam3CSK4; thus overcoming inefficient activation. It remains to be determined whether the multiplicity of binding sites on a TLR agonist influences its efficiency in activating target cells.

The possibility to introduce T cell-activating properties into a PS by chemical modification may open an avenue for developing an alternative to glycoconjugate vaccines, but it remains to be determined by which mechanism T cells are activated. It was shown recently that PSA, the natural ZPS from *B. fragilis*, is a TLR2 agonist (12). TLR2 expression on T cells has been observed (23), and both direct and indirect T cell costimulatory properties have been ascribed to TLR2 agonists (24, 25). In addition, it has been shown in vitro that TLR2 agonists alone are able to activate T cells (26). Similar observations were reported for the TLR4 agonist LPS (27, 28). We confirm these findings in a coculture system of human monocytes and T cells and show that, in all cases, activation is blocked by MHC II-blocking mAbs, indicating that MHC II is essential for T cell activation in this set-up, even when no T cell Ag is involved. This suggests also that ZPS-mediated T cell activation may be a consequence of APC activation, leading to strongly enhanced T cell costimulation, possibly in combination with direct effects on TLR2-expressing T cells.

The alternative hypothesis is that ZPS represent both TLR2 agonists and true MHC class II-dependent T cell Ags. This second hypothesis implies that a separable set of structural requirements may apply for each of the two different biological activities, and that the two functions can be introduced separately by different chemical modifications. A hint in this direction is that another natural ZPS, namely Sp1, was reported to be a T cell Ag, but not a TLR2 agonist (12). In line with these observations, we find that among all of our chemically derived ZPS2 forms, those most effective at activating T cells were not those inducing the strongest TLR2-dependent APC activation. In this context, it is of note that the best APC activator, namely the serotype Iib ZPS, is the one whose T cell-activating ability is completely abolished by the TLR2-blocking mAb. Importantly, whereas T cell activation induced by Pam3CSK4, macrophage-activating lipopeptide-2, and serotype Iib ZPS2 is abolished by TLR2 block, the same is not true for PSA and serotype III ZPS2, indeed suggesting that these latter molecules have an activity beyond the TLR2 agonist activity shown in this study. Additional experiments are underway to dissect these effects of different ZPS molecules.

In conclusion, we describe in this work that both natural and chemically derived ZPS are TLR2 agonists and determine for a vaccine-relevant PS a structure-activity relation in which molecules with the zwitterionic charge motif, but not those with positive or negative charges only, are able to activate human and mouse APCs via TLR2.

Acknowledgments

We are grateful to Dennis Kasper for reading the manuscript, helpful discussions, and the gift of *B. fragilis* PSA; to Isabella Pesce and Simona Tavarini for experimental help; and to Giorgio Corsi for art work.

Disclosures

The authors have no financial conflict of interest.

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