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The Novel Use of Zwitterionic Bacterial Components and Polysaccharides in Immunotherapy of Cancer and Immunosuppressed Cancer Patients

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1. Introduction

The recognition of pathogenic antigens as foreign particles by adaptive immune cells induces T and B lymphocytes to start defensive humoral and cellular reaction. Latest research revealed that proteins and some lipids are the main molecules inducing protective T cell responses during microbial infections while polysaccharides which are important components of microbial pathogens and many vaccines were not regarded as important antigens. However, research regarding the role of the adaptive immune system by polysaccharides gained interest only recently. Traditionally, polysaccharides were considered to be T cell-independent antigens that did not directly activate T cells or induce protective immune responses, but chemically modified polysaccharides, namely zwitterionic polysaccharides (ZPSs) were recently found highly immunogenic. Therefore, in this chapter we will discuss the role of zwittrionized polysaccharides in immune reaction induction and their immunostimulatory effect in cancer patients. Several studies were conducted to use ZPSs to induce vigorous immune response and to establish immunostimulatory or immunomodulatroy effect which can be used in cancer immunotherapy. Bacterial ZPSs that are naturally zwitterionic or those that were artificially zwitterionized were recently identified as potent immune regulators. The immunomodulatory effect of ZPSs requires antigen processing and presentation by antigen presenting cells, the activation of CD4 T cells and subpopulations of CD8 T cells and the modulation of host cytokine responses.

In addition, a recent model research done by our team will be presented in this chapter. This research introduced, in a breakthrough approach, zwitterionic motifs experimentally into polysaccharides of pneumo-23 vaccine converting these polysaccharides into effective immunostimulatory agent in immunosuppressed cancer patients. This study intended to assess the in vitro immunostimulatory effect of zwitterionized penumoccocal vaccine

compared with the nonzwitterionized commercial pneumo-23 vaccine. The in vitro immunostimulatory potential of zwitterionized penumoccocal vaccine was clearly observed in cancer immunosuppressed patients as well as, to a lesser extent, healthy control subjects, stimulating the synthesis of core cytokines of T-helper 1, and primarily inducing CD4+ and CD8+T cells. These studies collectively open the door wide for a new field for effective cancer immunotherapy.

2. The role of ZPS in eliciting immune response

The majority of the natural polysaccharide molecules consist of anionic sugar molecules which fail to activate T cells, and do not induce B-cell antibody isotype switching (Tzianabos et al., 1992). However, it was identified that a small group of bacterial polysaccharides carries both positive and negative charges in the same repeating sugar molecules which were accordingly called zwitterionic polysaccharides (ZPSs) (Baumann et al., 1992; Tzianabos et al., 1992; Coyne et al., 2000; Kalka-Moll, et al., 2001). Unlike most of bacterial polysaccharides, zwitterionic PS possess both positive and negative ions, which in turn found to be able to trigger T cell-dependent immune response, initiate class switching and affinity maturation of immunoglobulins, and induce long-term memory immunity (Gallorini et al., 2007; Cobb & Kasper, 2005; Wack et al., 2008; Richard & Amyes, 2004). Polysaccharides with both positively and negatively charged sugar molecules, unlike negatively charged polysaccharides, are able to stimulate both CD4 and CD8 T cells as well as antigen presenting cells of the adaptive immune system. This feature shows that molecules other than proteinaceous antigens are capable of activating conventional αβT cells.

However, development of specific T-Cell receptor (TCR) transgenic mouse models are required to understand the selection and development of ZPS-specific T cells. These cells may be more enriched in the mucosae as ZPS-producing bacteria such as *Bacteroides fragilis* (*B. fragilis)* and *Streptococcus. Pneumoniae* (*S. pneomoniae*) are contained within the commensal flora of the gut and upper respiratory track, respectively. ZPSs display an extended right-handed helix structure in which two repeating sugar units per turn form grooves with positive charges exposed on the outer surface (Wang et al., 2000). Due to this unique structure, ZPSs possess immunomodulatory activities, unlike other polysaccharides. Experimental studies in rats and mice have shown that intraperitoneal challenges with ZPSs induced vigorous pathogenic conditions, such as intra-abdominal abscesses which never happen in response to traditional polysaccharides (Tzianabos et al., 2000; Mazmanian et al., 2005). When ZPSs are accidently introduced into a sterile area, such as the peritoneal cavity during intra-abdominal surgical procedures when abdominal contents can spill into the peritoneal cavity, or by intraperitoneal inoculation with ZPS and a sterile cecal adjutant, a rapid recruitment of ZPS-specific, proinflammatory CD4 T cells may be induced. This recruitment of CD4 T cells together with other innate cells results in the generation of intra-abdominal abscess, a defensive mechanism of the body to contain the infection. Recent studies have shown that ZPS-mediated intraabdominal abscess induction depends on ZPS processing and presentation on MHC class-II molecules by APCs for recognition and activation of CD4 T cells (Stephen et al., 2010).

ZPS-specific T cells are in constant contact with ZPS presented by antigen presenting cells (APCs) of the mucosal immune system. Accordingly, the steady-state presentation of this

unique immunomodulatory microbial antigen is beneficial to our immune system as it can play pivotal role in shaping a functionally competent, immune system which is a must element in any successful immunotherapy. Interestingly, upon subcutaneous inoculation, ZPSs induce, in a unique manner, both anti-inflammatory and immunosuppressive T cells at the same tine. The stimulated population of cells includes both regulatory CD4 and CD8 T cells, which secrete the immunosuppressive cytokine IL-10. Thus, the same immunogen can elicit both proinflammatory and anti-inflammatory responses dependent upon the route and mode of administration; this can be regarded as one of the reasons that ZPSs are able to play essential role in modulating/regulating immune system of humans.

3. Mechanisms of immune induction and regulation by ZPSs

ZPSs were found to possess immunostimulatory functions. The in vitro stimulation of human and murine CD4 T cells with ZPSs induces cellular proliferation and inhibits apoptosis of immune cells (Stephen et al., 2005; Groneck et al., 2009). And this activity of ZPSs was found to be conducted mainly via T cell receptor (TCR). In addition, co-stimulatory signals were also found necessary to induce remarkable T-cell responses to ZPSs; the activation of CD4 T cells by ZPS is found to be dependent on a several costimulatory factors. It was found that ZPSs can interact with lymphocytes and antigen presenting cells by particular mechanism(s). ZPSs possess specific immunostimulatory activity, leading to direct activation of antigen-presenting cells (APCs) through Toll-like receptor 2 (TLR2) and T-cell receptors (TCR) on the surface of T cells in co-culture systems. ZPS are therefore, considered TLR2 and TCR agonists, able to activate human and mouse APCs. Since T-regulatory cells and other T-cell subsets express TLR2, and TLR2 engagement modifies functionality and activation state of these cells, it is speculated that most effects induced by natural and chemically derived ZPS may be explained by their TLR2 and TCR agonist properties. Moreover, the presentation of fragmented ZPSs by MHC-class II molecules shows that ZPS-mediated T-cell activation is mediated by non-specific, generalized TCR recognition. Vβ chain repertoire analyses of ZPS-stimulated T-cell populations showed that a broad repertoire of subfamilies, including all of the Vβ subfamilies, was used more than specific Vβ genes (Stingele et al., 2004; Groneck et al., 2009). Therefore, the oligoclonal activation of T cells by ZPS seemed highly probable. Clonotype mapping of T cells that were stimulated in vitro with ZPSs showed oligoclonal T cell expansion. This nonrestricted Vβ usage indicates possible ZPS recognition by the CDR3 antigen-binding domain of the TCR. After TCR stimulation, CD69 is rapidly upregulated by the Ras-MAP kinase signaling pathway (D'Ambrosio et al., 1994); this implies to a notion that this pathway may be involved in ZPS-mediated T-cell activation (Stephen et al., 2010). However, no solid clues suggest the ability of ZPSs to stimulate gamma-delta T lymphocytes, NK or NKT cells.

4. The immunological role of bacterial ZPSs

Pathogenic strains of bacteria, such as *B. fragilis*, *S. pneumoniae*, and *S. aureus*, produce one of the most potent ZPSs in nature (Kolka-Moll et al., 2002); for this reason, bacterial ZPSs offer unique opportunity to exploit their extraordinary potential in stimulating or modulating immune system of humans. The capsular polysaccharide antigens PSA of B. fragilis (NCTC 9343 and 638R) (Kolka-Moll et al., 2001) and Sp1 of S. pneumoniae serotype 1 (Kolka-Moll et

al., 2002) are the most widely studied ZPSs. Intra-abdominal abscess formation, which commonly occurs during secondary peritonitis and abdominal surgeries, is actually a ZPS-based protective mechanism used by the body to limit the spread of microbial pathogens. Initially, bacterial ZPS-induced pathologies were shown to be T-cell dependent. In those experiments, T-cell-deficient mice were unable to form abscesses after inoculations with bacterial ZPSs (Shapiro et al., 1986). Further experiments with α/βTCR-knockout mice revealed that immune response towards bacterial ZPSs were dependent on α/β TCR+ T cells (Chung et al., 2003). The activation of CD4 T cells is required for ZPS-mediated intra-abdominal abscess induction because mice lacking CD4 T cells failed to develop abscesses (Chung et al., 2003). As abscess formation was inhibited by the transfer of T cells from animals that were immunized with ZPS, these results suggest that abscess inductions and protection in the presence of ZPS are likely mediated by T-cell components of the adaptive immune system. The immunomodulatory effects of ZPSs require both positive and negative charge motifs as neutralization of a single charge motif abrogates the biological activities of ZPSs in triggering elaborative immune response and in stimulating immune cells altogether (Stephen et al., 2010). In this regard, it was found that the conversion of a nonzwitterionic bacterial molecule into a zwitterion makes the molecule biologically active just like the natural zwitterionic molecules (Gallorini et al., 2009; Stephen et al., 2010). However, bacterial ZPSs exert both wanted and unwanted actions, namely immunostimulatory/ immunomodulatory action and vigorous antigen-based immune response, respectively. This provided evidence for the justification of creating novel approaches for converting bacterial non-ZPSs into ZPSs molecules using chemical in-laboratory methods in a way strengthening their immunostimulatory effect and at the same time lowering their antigen-based immune reaction induction.

5. Model study for the experimentally zwitterionized bacterial polysaccharides to induce immunostimulatory effect in immunosuppressed cancer patients

The unique effect of zwitterionized bacterial polysaccharides as a potent in vitro immunostimulatory agent for immunosuppressed cancer patients was investigated in a novel study. The introduction of zwitterionic motifs into some of bacterial capsular polysaccharides turn these modified polysaccharides into moderately immunogenic and highly immunostimulatory agents. This study was conducted to assess the in vitro immunogenic and immunostimulatory effect of novel zwitterionized polysaccharides of the commercial pneumo-23 vaccine. In vitro proliferation, ELISA-based in vitro cytokine synthesis (IL-2, IFN-γ, and IL-10), and immunofluorescence microscopy-based immune profiling (CD4+, CD8+, and CD21+ cells) assays were used to evaluate the immunostimulatory effect of 48h of zwitterionized pneumo-23 encounter with peripheral blood mononuclear cells (PBMC) of immunosuppressed cancer (CA) patients and healthy control subjects (HC) in comparison with PBMC exposed to Concanavalin A (Con A), the positive control, and to phosphate buffered saline (PBS), the negative control. Zwitterionized pneumo-23, induced remarkable proliferation of PBMC in both CA and HC groups, induced in vitro synthesis of IL-2 and IFN-γ but not IL-10 in CA and borderline increase in IFN-γ and IL-10 in HC group, and expanded CD4+, CD8+, and CD21+ lymphocytes in CA rather than HC group. On contrary, Con A induced proliferation in HC more than CA group, induced only IL-2 synthesis and expanded only CD4+ cells in HC

rather than CA group. Therefore, in this regard, it was concluded that a unique in vitro immunostimulatory potential of zwitterionized pneumo-23 vaccine was observed on PBMC of immunosuppressed more than immunocompetent subjects, stimulating the synthesis of core cytokines of T-helper 1, and inducing the CD8+ T cells, which are most probably CD8+ cytotoxic T-cells rather than suppressor cells. Accordingly, introducing zwitterionic motifs into polysaccharides of the commercial penumo-23 vaccine turned it into a unique and potent immunostimulator reverting suppressed immune cells back into normal levels.

Pneumo-23 is a polyvalent pneumococcal polysaccharide vaccine (Pasteur Merieux Connaught, 2004). This vaccine was designed mainly to confer immunity against severe pneumococcal infections in elderly patients and those who underwent splenectomy as part of a management of various hematological disorders, renders the patients unsusceptible to the development of overwhelming sepsis by Streptococcus pneumoniae (Pasteur Merieux Connaught, 2004; Rybachenko et al., 2009; vad der Harst, 2007; Kazancioglu et al., 2000). Pneumo-23 is a clear, colorless liquid prepared from purified pneumococcal capsular antigens. Each dose of 0.5 mL contains purified *S. pneumoniae* polysaccharides (PS), 25 µg of each of the included 23 serotypes (Rybachenko et al., 2009). Although pneumo-23 is composed of non- T cell-dependent polysaccharides, it was observed that pneumo-23 vaccine when is given to elderly people, or immunosuppressed patients, their immune response, in unexplained manner, was moderately enhanced non-specifically in addition to the immune protection achieved against pneumococcal infection(Rybachenko et al., 2009; vad der Harst, 2007; Kazancioglu et al., 2000). In addition, a recent study revealed another immune enhancing feature of pneumo-23 vaccine stating that pneumo-23 vaccine enhanced the opsonization and sustained normal compliment components of C3 and C4 in elderly cancer patients after splenectomy Uslu et al., 2006). This might be attributed to the fact that some of pneumo-23 PS is zwitterionic substances such as polysaccharides of type 1 pneumococci (Gallorini et al., 2007).

Although many studies discussed the specific immunogenicity of pmeumo-23 vaccine against pneumococcal bacterial serotypes in elderly or in HIV-infected patients (de Greef et al., 2007; Peetermans et al., 2005; Sumitani et al., 2008; Valenzuela et al., 2007), very few reports gave remarks on the unexplained potential of pneumo-23 to exert non-specific, generalized, immunostimulating effect and non-specific protection against microbes rather than pneumococci. A study revealed that the protection of pneumo-23 vaccine after splenectomy was perfect against the sepsis of many bacteria other than pneumococci which indicated a dubious role of the non-specific immunostimulatory effect of pneumo-23 vaccine in protecting subjects against bacterial infection in addition to its specific anti-pneumococcal infection (Uslu et al., 2005). Nevertheless, our team examined the possible in vitro immunostimulatory effect of the commercial pneumo-23 vaccine on human peripheral blood mononuclear cells (PBMC); the results showed some immunostimulatory potential of penumo-23 but it did not reach significant levels [data not shown]. This was predicted as the majority of pneumo-23 polysaccharides are T cell-independent substances except for small portion, serotype 1, which possess T cell-dependent zwitterionic motifs (Gallorini et al., 2007; Cobb & Kasper, 2005). Since the commercial pneumo-23 is a pure polysaccharides mixture and unlikely to possess TLR ligands, accordingly, it was believed that the observed slight immunostimulatory effect of the commercial pneumo-23 in the preliminary study of our team and in other studies might belong to the presence of low level of zwitterionic motifs in pneumo-23 vaccine which provide evidence that if polysaccharides of pneumo-23

vaccine were zwitterionized, then this might issue in a very potent and unique bacterial ZPSs that is able to modulate and stimulate immune system of immunosuppressed and debilitated human patients. Therefore, it was hypothesized that introducing zwitterionic motifs into polysaccharides of pneumo-23 vaccine might render it a potent and effective immunostimulatory agent. In the current research, polysaccharides of the commercial pneumo-23 vaccine were modified chemically by adding zwitterionic motifs. The ability of zwitterionized pneumo-23 vaccine to trigger T cell-dependent immunostimulatory effect on PBMC of immunosuppressed cancer patients in comparison with healthy control subjects was scrutinized.

5.1 Patients and methods

5.1.1 The research population

Since, cancer (CA) itself and the related chemotherapy are considered as a well-known condition of immunosuppression (Whiteside, 2006), three kinds of common CA were involved in this study, namely, lung, head and neck, and colorectal cancers. Sixty five immunosuppressed cancer patients of age ranged 42 to 62 years were involved in this study including 42 men with lung (22), head and neck (11), and colorectal (9) cancers and 23 women with lung (13), colorectal (7), and head and neck (3) cancers. They were selected out of 94 cancer patients after excluding the non-confirmed cases of immunosuppression. The selection criteria of the involved cancer patients were: confirmed diagnosis of advanced cancer (stages III and IV), pre-operative, immunosuppressed, engaged regularly in chemotherapy, 6 weeks interval from the last chemotherapy. The selection was done without any bias to certain type of the involved cancer types. This study was conducted in the period from November 2006 to February 2009. Cancer patients were retrieved from a number of central hospitals in Malaysia, Kuala Lumpur. The immunosuppression status of cancer patients was checked twice. First, the clinical presentation of patients which revealed patients' high susceptibility to frequent infections, patients' lymphopenia in complete blood count, and state of hypo-γ-globulinemia where serum IgG < 250 mg/dL, and serum IgA <5 mg/dL. Second, the immunosuppression status was confirmed by the conducted assays of the current study.

On the other hand, 100 age- and sex- matched healthy control (HC) were selected who attended hospitals for minor traumatic therapy. Medical examination was done for HC as well as their medical records were retrieved. They showed no current or previous major illness, no cancer, and normal blood and biochemical laboratory tests, namely erythrocyte sedimentation rate, complete blood count, liver function test, kidney function tests, and total serum proteins. Blood samples were taken from CA and HC groups after obtaining full written consent. Withdrawn blood was held in heparinized tubes for later isolation of PBMC. Permission was granted from the regional committee of ethics for biomedical research.

5.1.2 Zwitterionisation of pneumo-23 polysaccharides

Chemical modification of PSs of the commercial form of pneumo-23 (Sanofi Pasteur, France) was done by adding positive charge motifs in order to convert PS to zwitterionic PSs. The procedure of zwitterionisation was done according to a recent standardized method (Gallorini et al, 2007). Briefly, a chemical oxidation of the aliphatic chain from the terminal

NeuNAc residue using 0.01 M NaIO4, sodium metaperiodate, (Sigma, USA) for 90 min at room temperature was conducted leaving an aldehyde group. NaIO4 was used as limiting (30%) or stoechiometric (100%) reagent of the reaction. The periodate oxidation selectively cleaves the C8-C9 bond between vicinal hydroxyl groups (-CHOH-CH_2OH) of NeuNAc residues, leaving an aldehyde group (-CHO) at C8. This group was converted to a cationic – $NH3^+$ group by reductive amination using 300 mg/ml NH_4Ac, ammonium acetate, (Sigma, USA) and 49 mg/ml $NaBH_3CN$, sodium cyanoborohydride, (Sigma, USA) at pH 6.5 for 5 days at 37°C. PS obtained, R-CH_2NH_2, were treated with 37% formaldehyde (Merck, Germany) in the presence of sodium cyanoborohydride to convert the generated free amino group to a tertiary dimethylamine, R-$CH_2NH(CH_3)_2^+$ such that it retained a positive charge. This conversion was confirmed by the routine application of NMR spectroscopy Avance 600 MHz (Bruker, Germany) using 5-mm triple-resonance NMR probe as evidenced by the resonance at 2.9 ppm. The NMRShiftDB was used for processing data obtained (Fig. 1).

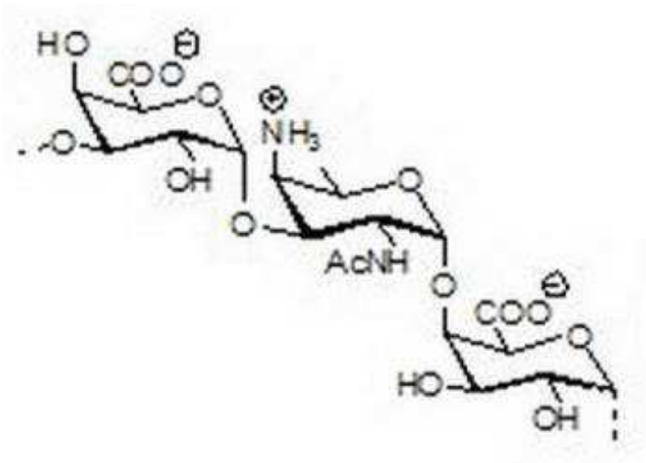

Fig. 1. The chemical structure of the zwitterionized polysaccharide (ZPS) of Streptococcus pneumoniae.

5.1.3 The strategy of conducting in vitro assays

The in vitro PBMC challenge with modified pneumo-23, the in vitro proliferation assay, the in vitro PBMC cytokine synthesis assay and the in vitro peripheral blood lymphocytes (PBL), part of PBMC, subsets markering assay were all conducted double blindly by two immunologists, the first from inside and the second from outside the research team. After finding that the results were minimally different between double blind runs of each assay, the final results were averaged. This strategy was necessary to keep the fidelity of the findings of the current study as this study is a pilot research on zwitterionized pneumo-23 vaccine and because some of the novel findings in this study need to be verified prudently in order to avoid causal or biased results.

5.1.4 Isolation of peripheral blood mononuclear cells

Isolation of PBMC from the heparinized whole blood of 65 CA and 100 HC subjects was conducted to prepare a population of cells containing T and B lymphocytes for the subsequent in vitro assays. PBMC separation was carried out in ultraviolet hood. The

procedure was based on the gradient density sedimentation technique by using Ficoll hypaque (Sigma) (Wahlstorm et al., 2005). Cell viability was done; >99% of cells were found viable. Final concentration of the isolated PBMC suspension was adjusted to $1x10^6$ cells/mL.

5.1.5 PBMC challenge with modified pneumo-23 and concanavalin A

One hundred µl of $1x10^6$ cells/mL of PBMC suspension in complete RPMI–1640 medium (Merck, Germany) with 200 U/mL penicillin G (Sigma, USA), 200 µg/mL streptomycin (Merck, Germany), and 10% human AB serum (BDH, UK) were added to 96 wells microtiter plate in duplicates. Initially, cells were cultured for 1 day in a humid sterile incubator; then, 10 µl of 40ug/mL Concanavalin A (Con A) mitogen were added as positive control, 10 µl of 5 µg/mL diluted in PBS of modified pneumo-23 vaccine were added as test sample, and 10 µl of PBS alone were added as negative control. The used concentration of modified pneumo-23 vaccine, 5 µg/mL, was adjusted repeatedly after many standardization trials. PBMC in complete RPMI-1640 medium were incubated with modified pneumo-23, Con A, and PBS for 48 hours at 37 °C. Afterwards, PBMC were subjected to three in vitro assays; MTT assay to measure the proliferative potential of PBL, ELISA for in vitro synthesis of PBMC cytokines, IL-2, IFN-γ, and IL10, and PBL subset profiling namely, CD4, CD8, and CD21.

5.1.6 In vitro prolifertative assay

After 48 hours of PBMC incubation with Con A, modified penumo-23, and PBS, 40 µl of 3-[4,5,dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide microculture, MTT, reagent (Sigma, USA) at 5 mg/mL were added to the microtiter plate and incubated for 4 hours at 37 °C. Finally, the supernatant was removed by Pasteur pipette and 100 µl of isopropanol was added. ELISA reader was used to measure the optical density (OD) at 540 nm Wigzell & Andersson, 1971). By using MTT assay, the proliferative activity of living PBMC exposed to mitogenic substances was measured (Shimoyama et al., 1989). The proliferative percentage of the challenged PBMC with Con A and zwitterionized penumo-23 was calculated in relation to the negative control, PBS, according to the below-mentioned equation (Kane e al., 1999):

Proliferative % of modified pneumo-23 = [absorbency of experimental wells "PBMC exposed to modified pneumo-23" / absorbency of negative control wells "PBMC exposed to PBS"] – 1 x 100

Proliferative % of the positive control (Con A) = [absorbency of positive control wells "PBMC exposed to Con A" / absorbency of negative control wells "PBMC exposed to PBS"] – 1 x 100.

5.1.7 ELISA for in vitro synthesis of PBMC cytokines

After 48 hours of PBMC incubation with modified penumo-23, Con A, and PBS, PBMC were centrifuged at 3000 g for 5 minutes and the supernatant was withdrawn separately. After a series of standardization steps, mouse monoclonal anti-human IL-2, IFN-γ, and IL-10 capturing antibodies (Dako, Denmark) were diluted 1:10 in carbonate-bicarbonate coating buffer (1.59g/L carbonate & 2.93 g/L bicarbonate) (Sigma, USA) at final concentration 0.1 mg/mL. Fifty µl/well of the diluted capturing antibodies in coating buffer were added and incubated overnight at 4 °C. Next day after washing step, 50 µl/well of the supernatant diluted 1:4 in blocking buffer, bovine serum albumin (Sigma, USA), were added in

duplicates and the microtiter plate was incubated for 1 hour at 37 °C. Horseradish-peroxidase-labeled rabbit monoclonal anti-human IL-2, IFN-γ, and IL-10 antibodies (ICN immunologicals, UK) were diluted 1:100 in the antibody dilution buffer (ICN immunologicals, UK) at final concentration, 0.01, 0.005, and 0.0.005 mg/mL respectively. After a washing step, 50 µl/well of the diluted horseradish-peroxidase-labeled rabbit monoclonal anti-human IL-2, IFN-γ, and IL-10 antibodies were added and incubated for 1 hour at 37°C. The conjugated antibodies recognize different epitopes in IL-2, IFN-γ, and IL-10 from these recognized by the capturing antibodies. After washing step, 50 µl/well of OPD-H2O2 as chromogen-substrate (Sanofi diagnostics, France) were added for 20 minutes at 37 °C. For each run, duplicate wells of the negative control of the run were used by adding antibody dilution buffer alone instead of monoclonal antibodies. ELISA readings in terms of OD were measured at wavelength 492 nm (Schuurs & Weeman, 1977). A series of standards for IL-2, IFN-γ, and IL-10 (Sigma, USA) were used to obtain the linear regression analysis of the resulted standard curve. Moreover, the median value of the standard curve was considered as the positive control of the ELISA run. The achieved correlation coefficient of the standard curve was 0.81 which indicated a high linear behavior of the customized ELISA. The minimal detection limits for IL-2, IFN-γ, and IL-10 were 0.1, 0.08, and 0.1 ng/mL, respectively.

5.1.8 Immune phenotyping via direct immune fluorescence microscopy

For the Fixation of PBMC cells, after 48 hours of PBMC incubation with modified penumo-23, Con A, and PBS, PBMC were centrifuged at 3000 g for 5 minutes and the pellet was resuspended in PBS. Fifteen µl of $1x10^6$ cells/mL of PBMC suspension were added onto circular depressions of the immunofluorescence slides. Slides were allowed to dry up for about 1 to 2 hours. Fifteen µl of fixative solution, which is buffered formal acetone (BFA) composed of 5.7% v/v PBS, 24% v/v 40 % formalin, and 43% v/v acetone from (Merck, Germany), were added per depression to cover all the dried lymphocytes and stored at –20 0C for later use Taylor et al., 1970).

For the Fluorescent microscopy, 15 µl of immunofluorescence-labeled monoclonal antibodies, namely anti-CD4, anti-CD8 and anti-CD21 (Dako, Denmark) diluted 1:5 in antibody dilution buffer (Dako, Denmark) at final concentrations 0.1, 0.1, and 0.05 mg/mL respectively. These antibodies were added onto slide depressions in duplicates for 1 hour at 37 °C with mild shaking. After dipping and stirring slides in PBS-filled jar for 10 minutes, 1-2 drops of mounting fluid were added. After adding cover slips, examination of slides was conducted under immunofluorescent microscope at 40X and 100X. At light microscopy phase, a suitable countable field was chosen to count the total number of lymphocytes. At UV light phase, the fluorescently stained cells were only counted. The average percentage of the fluorescently stained cells was calculated from the total PBL cells in 5 high power fields multiplied by 100 (Ben Trividi et al., 1990).

5.1.9 Statistical analysis

The statistical analysis was preformed using software SPSS version 10 and MS Excel 2000. Kolmogorov-Semirnov tests were used for confirming the normal distribution pattern of MTT, ELISA, and immunofluorescent CD marketing values. Therefore, mean ±SEM of the averaged values as well as parametric multivariate student t-tests were used to evaluate the significance of differences. P value <0.05 was considered significant.

5.2 Results

5.2.1 The confirmation of the immunosuppression status of cancer patients

As part of the research plan, the immunosuppression status of the involved CA patients, who were already shown as immunosuppressed at the clinical and laboratory hospital tests, was confirmed. All of the selected immunosuppressed CA patients showed significantly lower prolifertative rates of PBMC, lower IL-2 and IFN-γ concentrations, and lower CD4+, CD8+, and CD21+ cells percentages than in HC ($P<0.05$). These comparisons are clarified as dashed lines between the negative control group of HC to that of CA (Fig. 2- A&B, Fig. 4-A&B, and Fig. 5-A&B).

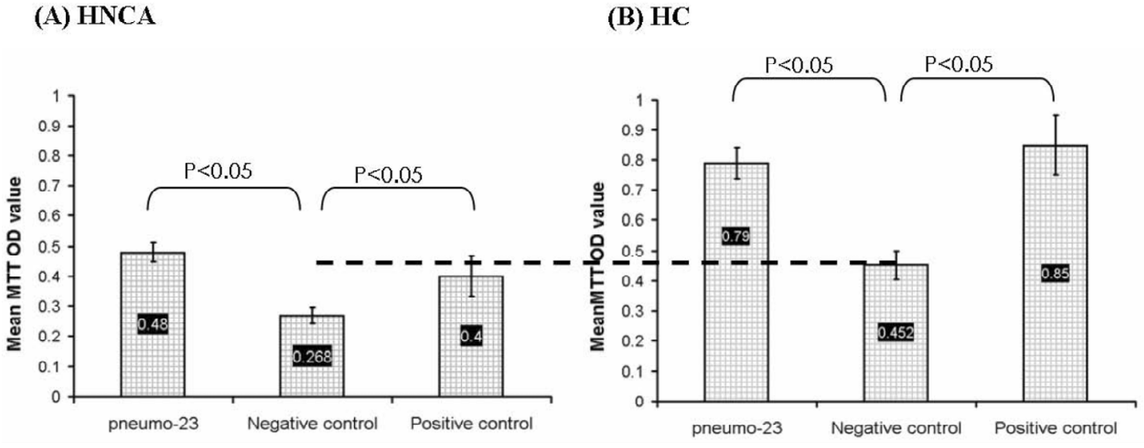

Fig. 2. Mean MTT OD values for negative control (PBS), zwitterionized pneumo-23 vaccine, and positive control (Con A) groups in both (A) CA patients and (B) healthy control subjects (HC). This histogram shows that pneumo-23 induces remarkable PBMC proliferation in immunosuppressed CA and immunocompetent HC subjects. The dashed line depicts the confirmed status of immune suppression represented by the significantly lower proliferation of un-stimulated PBMC, negative control, of CA patients than that of HC.

5.2.2 The in vitro proliferative assay

In this study, two groups were involved, CA and HC group. Each group was subdivided into three categories; test group where PBMC exposed to modified pneumo-23 vaccine, negative control group where PBMC cells exposed to PBS, and positive control group where PBMC exposed to Con A. It was found that the modified, zwitterionized, pneumo-23 vaccine exerted remarkable stimulatory effect on PBMC proliferation of immunosuppressed CA patients in that the mean MTT OD value of modified pneumo-23 group in CA, 0.48±0.033, and in HC, 0.79±0.053, were higher than in the negative control of CA, 0.268±0.027, and of HC, 0.452±0.048 respectively ($P<0.05$) (Fig. 2-A and 2-B). Moreover, the mean MTT readings for PBMC exposed to modified pneumo-23 vaccine were close to that of Con A in HC, 0.85±0.1, and borderline higher than Con A in CA, 0.4±0.068 ($P>0.05$) (Fig. 2-A and B). Nevertheless, by using the calculated proliferative percentage of MTT, a significant difference was clear and evident between the proliferative percentage of modified pneumo-23 and that of Con A in immunosuppressed CA patients. The mean

proliferative percentage of PBMC of CA patients exposed to modified pneumo-23, 81%,±7.6, was significantly higher than that exposed to Con A, 51%±8.9, ($P<0.05$) while in HC group, both con A, 89%±9.6, and penum-23, 75%±8.4, were close to each other ($P>0.05$) (Fig. 3). In other words, the zwitterionized pneumo-23, unlike the unmodified penumo-23 [data not shown], induced efficiently a remarkable proliferation in both immunosuppressed and immunocompetent groups while Con A induced proliferation efficiently only in immunocompetent group rather than the immunosuppressed group (Figure 2). Moreover, there was no differences in the modified pneumo-23 or Con A -driven proliferation in PBMC of CA patients in respect cancer type, age, or sex of patients ($P>0.05$).

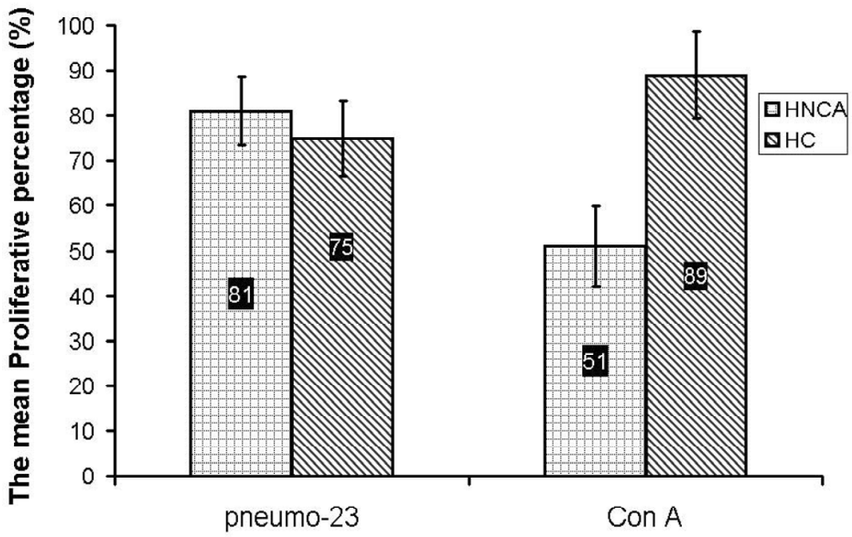

Fig. 3. The calculated proliferative percentage of PBMC in immunosuppressed CA and immunocompetent HC groups in response to zwitterionized pneumo-23 vaccine and Con A measured by MTT assay. The proliferative percentage of PBMC exposed to zwitterionized penumo-23 was closely high in both CA and HC groups ($P>0.05$) while Con A induced much higher PBMC proliferation in HC than in CA group ($P<0.05$).

5.2.3 The in vitro synthesis of cytokines

ELISA for in vitro synthesis of cytokines was conducted on PBMC of both CA and HC groups that each was subdivided into three groups; test group, negative control, and positive control where PBMC were exposed to modified pneumo-23, PBS, and Con A respectively, for 48 hours. In CA group, it was found that the mean synthesis of soluble IL-2, 0.61±0.063 ng/mL, and IFN-γ, 0.45±0.052 ng/mL, in PBMC exposed to modified pneumo-23 was much higher than that exposed to PBS, 0.41±0.03 and 0.29±0.051 ng/mL respectively and that exposed to Con A, 0.45±0.028 and 0.42±0.03 ng/mL respectively ($P<0.05$) (Fig. 4-A) while the mean synthesis of soluble IL-10, 0.36±0.042 ng/mL exposed to modified pneumo-23 was not different from that exposed to PBS and Con A, 0.4±0.041 and 0.42±0.027 ng/mL respectively ($P>0.05$) (Fig. 4-A).

On the other hand, in HC group, no similar upsurge was found in the mean synthesis of IL-2, 0.67±0.061 ng/mL in PBMC exposed to modified neumo-23 when compared to the

negative control, 0.62±0.034 ng/mL (P>0.05) and, on contrary to CA, it was lower than in PBMC exposed to Con A, 0.82±0.042 ng/mL (P<0.05) (Fig. 4-B). Regarding IFN-γ and IL-10, they were close to each other with borderline higher levels of IFN-γ and IL-10 in PBMC exposed to modified pneumo-23, 0.52±0.031 and 0.41±0.03 ng/mL respectively than in PBMC exposed to PBS, 0.45±0.034 and0.37±0.052 ng/mL respectively and PBMC exposed to Con A, 0.47±0.042 and 0.36±0.017 ng/mL respectively (P ranged 0.051 to 0.048). (Fig. 4-B). In addition, there was no significant differences in the pneomo-23 and Con A -driven synthesis of IL-2, IFN-γ, and IL-10 in PBMC of the CA patients in respect to cancer type, age, and sex (P>0.05). These results collectively indicated that zwitterionized pneumo-23 exerted a unique potent cytokine stimulatory effect, IL-2 and IFN-γ, on PBMC of immunosuppressed cancer patients while a powerful mitogenic substance, Con A, exerted a mild cytokine stimulatory effect, IL-2, on immunocompetent healthy group only.

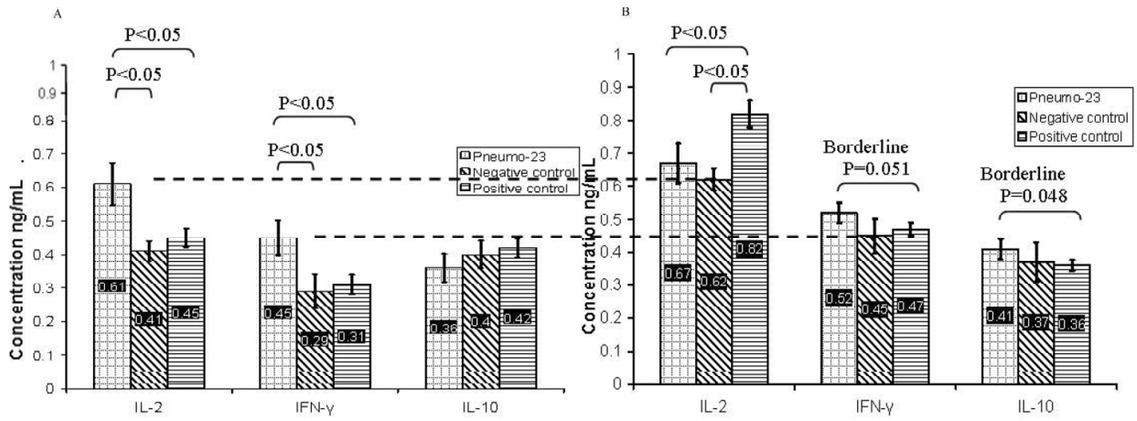

Fig. 4. (A) the concentrations of in vitro synthesis of IL-2 and IFN-γ but not IL-10 in CA PBMC exposed to zwitterionized pneumo-23 was higher than in PBMC of the negative control, PBS, and the positive control, Con A. (B) The concentration of IL-2 in HC PBMC exposed to Con A was higher than in PBMC exposed to zwitterionized penumo-23 whereas borderline higher IFN-γ and IL-10 synthesis is seen by HC PBMC exposed to zwitterionized penumo-23 than by PBMC exposed to Con A. The dashed line depicts the confirmed status of immune suppression represented by the significantly lower synthesis of IL-2 and IFN-γ by PBMC of the CA patients than that of negative control group of HC.

5.2.4 The immune phenotyping of lymphocytes subsets

The immune phenotyping of PBL exposed to modified pneumo-23, PBS, and Con A was investigated. Although the percentages of CD4+, CD8+, and CD21+ cells in CA group were lower than in HC group, these percentages were within the lowest tier of normal ranges, 30-60% for CD4+, 20-50% for CD8+ cells, and 4-14% for CD21 [14]. IN CA, the mean percentage of CD8+, CD4+, and CD21+ cells in modified pneumo-23 group, 39.3±4.7, 42.7±3.2, and 8.1±2.1% respectively, was higher than in PBS group, 25.6±3.1, 31.9±4.1, and 4.2±1.7% respectively, (P<0.05) and in Con A group, 27.2±2.8, 35.8±3.6, and 4.1±0.8 respectively (P<0.05) (Fig. 5-A) while no significant difference found between con A and PBS groups regarding the mean percentage of CD8+, CD4+, and CD21+ cells (P>0.05).

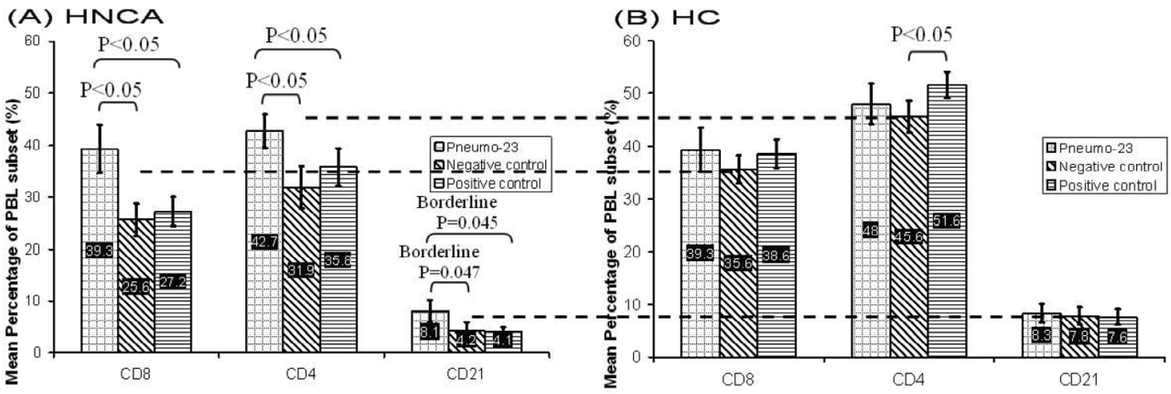

Fig. 5. (A) The mean percentage of CD8+, CD4+ and CD21+ cells was higher in CA PBMC exposed to zwitterionized penumo-23 than both these exposed to PBS or Con A. (B) CD4+ cells was higher in HC PBMC exposed to Con A than these exposed to PBS but not zwitterionized pneumo-23. The dashed lines depicts the confirmed status of immune suppression represented by the significantly higher percentages of CD8+, CD4+, and CD21+ cells in PBMC of the negative control group of HC than that of CA patients.

On the other hand, there was no significant difference in the percentage of CD8+, CD4+, and CD21+ cells in HC PBL exposed to modified pneumo-23, 39.3±4.2, 48±5.8 and 8.3±2.2% respectively, when compared to cells exposed to PBS, 35.6±2.6, 45.6±3.8, and 7.8±2.4% respectively ($P>0.05$) (Fig. 5-B). However, Con A was shown to increase the percentage of CD4+ cells, 51.6±2.4 more than PBS group ($P<0.05$) and pneumo-23 ($P>0.05$) (Fig. 5-B). In addition, there was no significant differences in the percentage of CD4+, CD8+, and CD21+ cells in PBMC exposed to modified pneumo-23 or Con A in respect to cancer type, age, or sex of CA patients ($P>0.05$).

5.3 Discussion

The immunostimulatory effect of the zwitterionized pneumo-23 was tested on PBMC of immunosuppressed CA patients in comparison with HC group regarding the in vitro proliferative potential, the in vitro cytokine synthesis, IL2, IFN-γ, and IL-10, and immune phenotyping, CD4+ as T helper cells, CD8+ as T cytotoxic/suppressor cells (Kalinski et al., 2006; Knustion & Disis, 2005), and CD21+ as B cells (Knustion & Disis, 2005) by using the accurate and simple microculture tetrazolium (MTT) assay for the in vitro prolifertative assay (Ben Trivdei et al., 1990; Iwatsuki et al., 2004), ELISA for in vitro cytokines synthesis, and direct immunofluoresecence microcopy for immune phenotyping assays.

Unlike the commercial pneumo-23 vaccine [data not shown], the chemically modified pneumo-23 vaccine, in a unique manner, augmented the proliferative potential of PBMC in both immunosuppressed, CA, and immunocompetent, HC, groups, inducing remarkable in vitro synthesis of PBMC soluble IL-2 and IFN-γ but not IL-10 in CA versus just borderline increase in IFN-γ and IL-10 in HC group, and augmented the proliferation of CD8+, CD4+, and CD21+ PBL selectively in CA rather than HC group. On contrary, the positive control, Con A, induced PBMC proliferation, increased in vitro synthesis of IL-2, but not IL-10 or

IFN-γ, and increased CD4+, but not CD8+ or CD21+ cells, in immunocompetent, HC, rather than immunosuppressed, CA group.

Unfortunately, no previous report was found utilizing zwitterionized pneumococcal PSs as enhanced immunogenic vaccine or as immunostimulatory agent to compare with. Nevertheless, the findings of the current research showed that the zwitterionized pneumo 23 exerted a highly remarkable in vitro T cell dependent and/or mitogenic triggering of a robust immune response in just 48 hours of exposure. Moreover, in unexplained manner, the immunosuppression status, interestingly, did not hinder the zwitterionized pneumo-23, unlike Con A, to stimulate immune cells proliferation, cytokines synthesis, and PBL subsets proliferation and expansion. Con A, a powerful mitogenic substance, failed in triggering an efficient immune stimulation in immunosuppressed PBMC like that seen in immunocompetent PBMC. Therefore, the immune triggering pathway of the zwitterionized pneumo-23 might be unique.

Collectively, the zwitterionized pneumo-23 vaccine was shown to act as a normalizing immunostimulatory agent for the immunosuppressed cells. In other words, it served to revert the suppressed 'lazy' immune cells back to normal ‘alert’ status while it did not stimulate much the already competent 'alert' immune cells. The findings of the current study might explain how the commercial pneumo-23 showed some signs of immune stimulation, which is most likely due to the presence of small fraction of natural zwitterionic type 1 PSs (Gallorini et al., 2007). Beside the report of the manufacturing company on the immunostimulatory effect of pneumo-23, there was a report supports the findings of the current research which showed that the protection of pneumo-23 vaccine after splenectomy was perfect against the sepsis of many bacteria other than pneumococci (Uslu et a., 2006). This suggests strongly the presence of non-specific immune stimulation, which was augmented and shown in the current study after the zwitterionisation of the commercial pneumo-23 vaccine

The modified pneumo-23 vaccine was shown to stimulate the cell-mediated immunity (CMI) more than the humeral arm of immune response as it induced CD8+ and CD4+ cells far higher than CD21+ cells. These findings might render zwitterionized pneumo-23 vaccine as a practicable choice for stimulating the suppressed CMI in attempts to augment immunity against diseases. The current study also showed that the immune stimulation of zwitterionized pneumo-23 vaccine in CA patients was attributed partially to the synthesis of IL-2 and IFN- γ, T helper 1 cytokines, rather than IL-10, T helper 2 cytokine, as well as stimulating vigorously CD8+ T cells. Collectively, this provided evidence that the zwitterionized pneumo-23 stimulate T-helper 1 profile of CMI. This might be supported indirectly by other studies which stated that the atopic bronchial asthma children, from a past case of outhospital pneumonia, which were given pneumo-23 vaccine showed tendency towards decreased level of serum IgE (Markelova et al., 2005; Ryzhov et al., 2005). This was either due to complete eradication of the chronic infection of pneumococcal bacteria and/or the deviation of the immune system towards T-helper one which counteracts the T-helper two atopic arm of immunity (Markelova et al., 2005; Ryzhov et al., 2005). Moreover, the nature of the remarkably increased CD8+ cells in response to the modified pneumo-23 was more likely cytotoxic rather than suppressive CD8+ cells because the enhanced prolifertative rate of PBMC, the in vitro high level of synthesis of IL-2 and IFN-γ, and the high rates of CD4+ and CD21+ cells in PBMC exposed to modified penumo-23 contradict strongly the

possibility of the suppressive nature of CD8+ cells. Accordingly, zwitterionized pneumo-23 more likely augments the cytotoxic arm of CMI which is essential against most of viral infections and tumor diseases.

A question might be presented, whether the zwitterionized penumo-23 is an immunostimulatory agent or it is just an antigen that induced immune reaction. The findings of the current study showed that pneumo-23 mostly acts as potent immunostimulator as well as an effective T cell-dependent antigen rather than just an antigen. The findings supporting the immunostimulatory action of pneumo-23 are: first, peumo-23 induced consistent upsurge in PBMC proliferation, IL-2 and IFN-γ synthesis, and CD 4+, CD8+, and CD21+ cells in immunosuppressed CA patients more than immune competent HC group which is exactly the contrary to what should happen in the antigenic stimulation. Second, the magnitude of increase in PBMC proliferation, cytokines synthesis and expansion percentage of PBL subsets in just 48 hours favors the immunostimulatory effect in addition to antigenic effect.

The mechanism of the observed immunostimulatory action of zwitterionized pneumo-23 vaccine has not yet been understood. It could be a combination of T cell-dependent antigen triggering pathway via T cell receptor (TCR), a non-specific mitogenic signal through bridging of multiple costimulatory receptors, or acting as a ligand on Toll-like receptors (TLR). A recent study revealed that TLR2 engagement in T-regulatory cells and other T-cell subsets with zwitterionic motifs of PS modifies functionality and activation state of these cells and the most effects induced by natural and chemically derived zwitterionized PS may be explained by their TLR2 agonist properties on T cells Wack & Gallorini, 2008). Regardless the previous study conclusions, the immunostimulatory effect of zwitterionized pneumo-23 vaccine needs further studies designed specifically for investigating the mechanism and pathways of its immunostimulatory effect in vivo using experimental animals to explore why it is more evident in immunosuppressed rather than immunocompetent subjects. And to explore its effects on natural killer cells, antigen presenting cells, TLR, other cytokines, and other CD markers. Moreover, a long-term prospective study might be needed to evaluate the in vivo immunostimulatory effect of pneumo-23 vaccine on laboratory animals and later, after proving its safety, on human beings.

Taken together, the zwitterionized, but not the commercial, pneumo-23 vaccine appeared to exert a remarkable in vitro immunostimulatory effect on the isolated PBMC of immunosuppressed cancer patients more than the immune competent individuals. In addition, it drives the immunity towards T-helper one profile and triggers largely CD8+ cells. Basing on the findings of the current study, it seems that the zwitterionized PSs exert a unique immunostimulatory pathway that tends to correct the immune suppression status of CA patients' immune cells to normal levels while such normalizing effect was not observed in the immunocompetent healthy subjects. Therefore, zwitterionized PSs, such as pneumo-23, can be developed, after securing its safety in humans, as a potent immunostimulatory drug for cancer immunosuppressed patients.

6. Conclusion

Zwitterionization can be one of the most recent approaches for immuno- stimulating /modulating the immune system of cancer patients. The process of zwitterionization

is a cheap, simple, and fast; it is a chemical procedure by which natural, non-immunostimulating and non-immunomodulating polysaccharides are converted to universal TCR-based immunostimulator and immunomodulators. ZPSs are found to be useful in correcting the immune suppression and immune deviation in incompetent immune systems in cancer patients. Patients with cancer might be immunosuppressed due to disease itself or due to cancer chemotherapy. In both cases, correction of the immune system of cancer patients is necessary taken into account that fatality in cancer patients due to infections is very high. In addition, immune suppression itself can be considered one of the serious causes for the development of some tumors because of the loss of immune surveillance exerted by immune system which daily scavenges any cancerous neoplastic cells. In addition, ZPSs can be used as adjuvant elements along with other modalities of cancer immunotherapy to overcome the long achieved failures in this field. It is well known that most cases of failures in cancer immunotherapy are attributed to the ability of cancerous cells to escape immune defense mechanisms as well as immunotherapy approaches used. In this instance, ZPSs can change the fate of cancer immunotherapy to a more optimistic modality of therapy. One of the essential conclusions incurred from the use of ZPSs in cancer patients is that ZPSs exert immunostimulatory action on cancerous cells much more remarkably than on non-cancerous cells. The mechanism underlying this is still unknown; however, this implies to a very important phenomenon which might be highly useful in combating immune evasive nature of cancerous cells without affecting other normal cells.

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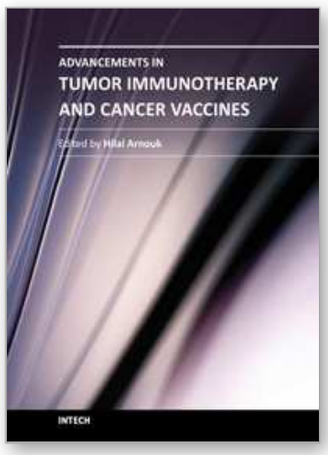

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