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B Cell and Antibody Responses in Mice Induced by a Putative Cell Surface Peptidase of *Pneumocystis murina* Protect against Experimental Infection

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Abstract

Rationale—*Pneumocystis* pneumonia is a major cause of morbidity and mortality in HIV-infected subjects, cancer patients undergoing chemotherapy and solid organ transplant recipients. No vaccine is currently available. By chemical labeling coupled with proteomic approach, we have identified a putative surface protein (SPD1, Broad Institute gene accession number PNEG_01848) derived from single suspended *P. murina* cysts. SPD1 was expressed in an insect cell line and tested for vaccine development.

Methods—Mice were immunized with SPD1 plus adjuvant MF-59 by subcutaneous injection. Three weeks after the last immunization, CD4⁺ cells were depleted with anti-CD4 antibody GK1.5. The mice were then challenged with 2×10^5 *Pneumocystis* organisms. Mice were sacrificed at 4 and 6 weeks after PC challenge. Spleen/lung cells and serum were harvested. B cells and memory B cells were assessed via flow cytometry. Specific *Pneumocystis* IgG antibody was measured by ELISA before and after challenge. Infection burden was measured as real-time PCR for *P. murina* rRNA.

Results—Normal mice infected with *Pneumocystis* mounted a serum IgG antibody response to SPD1. Serum from rhesus macaques exposed to *Pneumocystis* showed a similar serum IgG response to purified SPD1. SPD1 immunization increased B cell and memory B cell absolute cell

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CONFLICT OF INTEREST STATEMENT

Conflicts of interest: none

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counts in CD4-depleted Balb/c mice post *Pneumocystis* challenge in spleen and lung. Immunization with SPD1 significantly increased specific *Pneumocystis* IgG antibody production before and after challenge. Mice immunized with SPD1 showed significantly decreased *P. murina* copy number compared with mice that did not receive SPD1 at 6 weeks after challenge.

Conclusion—Immunization with SPD1 provides protective efficacy against *P. murina* infection. SPD1 protection against *Pneumocystis* challenge is associated with enhanced memory B cell production and higher anti-*Pneumocystis* IgG antibody production. SPD1 is a potential vaccine candidate to prevent or treat pulmonary infection with *Pneumocystis*.

Keywords

Pneumocystis vaccine; *Pneumocystis* pneumonia; CD4 T-cell deficiency

INTRODUCTION

Pneumocystis species constitute a group of fungi belonging to the Taphrinomycotina subphylum of the Ascomycota which colonize the lungs of mammals. *Pneumocystis jirovecii*, the species colonizing humans, can cause pneumonia (PCP) in hosts with a suppressed immune system, most commonly due to HIV infection. At present, PCP is the second most frequent life-threatening invasive fungal infection worldwide. [1] Although antibiotics are available to both prevent and treat PCP, these antibiotic regimens entail both cost and drug toxicity. A vaccine to prevent PCP would be the most cost effective solution to this worldwide fungal pathogen. Target vaccine populations would include early HIV infected subjects (before significant depletion of CD4+ lymphocytes) and planned immunosuppressive chemotherapy patients (bone marrow transplant, cancer chemotherapy). To date no successful vaccine has been developed against *Pneumocystis jirovecii*.

We here report the application of a chemical labelling technique to identify surface proteins of *P. murina* as potential vaccine targets. This technique has been previously applied to yeast [2] and to *Pneumocystis* in a prior report from our laboratory. [3] Using this technique we have identified and characterized a putative surface peptidase, which we have designated SPD1. SPD1 is immunogenic in mice and has protective efficacy as a vaccine against experimental *Pneumocystis* pneumonia.

MATERIALS AND METHODS

Mice

Specific-pathogen-free (SPF) female BALB/c mice were purchased at 6–7 weeks of age from Charles River Breeding Labs (Wilmington MA) and *Rag2^{-/-}Il2rg^{-/-}* mice from Taconic Farms Inc. (Hudson, NY). All animals were housed in filter-topped cages in an isolation room at the Louisiana State University Health Science Center Animal Care Facility. All caging procedures and surgical manipulation were done under sterile conditions. Mice were fed autoclaved food and sterile water was provided *ad libitum*. The animals were kept in the facility for at least 2 days prior to the beginning of treatment. All experiments were approved by the Institutional Animal Care and Use Committee of LSUHSC (protocol No. 2939).

***P. murina* Inoculation**

P. murina organisms for inoculation were isolated from lung homogenates from chronically infected *Rag2^{-/-}Il2rg^{-/-}* mice. In brief, lungs were aseptically removed and frozen in 1 ml phosphate-buffered saline (PBS) at 70°C. Frozen lungs were homogenized in 10 ml of PBS, forced through a sterile cell strainer, and pelleted at 500 g for 10 min at 4°C. The number of Giemsa-stained *P. murina* cysts was quantified microscopically as previously described. [4] Recipient mice were anesthetized with isoflurane and suspended by their front incisors, the tongue was extended out by forceps and a 100 ul inoculum containing 2×10^5 cysts was injected into the trachea using a pipette. This method does not deposit inoculum into the stomach.

Purification of *P. murina*

P. murina organisms were collected from lung bronchoalveolar lavage (BAL) fluid of *Rag2^{-/-}Il2rg^{-/-}* mice chronically infected with *P. murina* for 10 weeks. The lavage fluid was sedimented at $3000 \times g$ for 15 min at 4°C and the entire pellet was suspended in 10 ml autoMACS rinsing solution (Cat #: 130-091-222, Miltenyibiotec, San Diego, CA), vortexed, and centrifuged again at $3000 \times g$ for 15 min at 4°C. The pellet was suspended in 180 ul MACS rinsing solution and 20 ul of mouse CD45 microbeads (Cat #: 130-052-301, Miltenyibiotec, San Diego, CA). The sample was incubated with the beads for 15 min at 4°C, then washed with 2 ml rinsing buffer. Place LD Column in the magnetic field of a suitable MACS separator, and rinsing column with 2 ml of rinsing buffer. Apply cell suspension on to the column and collect unlabeled cells which pass through and wash column with 1ml rinsing buffer. The total effluent which contain purified *P. murina* organisms and host leukocytes were depleted from the sample by magnetic separation with an MACS separator. *P. murina* organisms were separated from the unlabeled fraction by centrifugation at $3000 \times g$, 15 min, 4°C. To identify PC organisms in the purified sample, the pellet was suspended in phosphate-buffered saline (PBS), diluted 1:5 with phosphate-buffered saline (PBS) and stained with modified Giemsa stain (Diff-Quick; Baxter). This method results in greater than 90% purity of cysts and trophozoites forms by microscopic examination.

Identification of *P. murina* surface proteome by chemical labeling

Suspended live *P. murina* cells were labeled by sulfo-NHS-LC-biotin (Pierce, Rockford, IL) that does not penetrate beyond the cell surface and does not cross cell membranes. [5] The labeled proteins were then denatured, enzymatically cleaved by trypsin (Promega, Madison, WI) to yield a mixture of peptides that were analyzed by LC-MS/MS. Tandem mass spectra of labeled peptides were acquired on a LTQ mass spectrometer (Thermo Electron, San Jose, CA). The labeled peptides were identified by comparing their tandem mass spectra against the *P. murina* protein sequence database (*Pneumocystis murina* Sequencing Project, Broad Institute of Harvard and MIT [<http://www.broadinstitute.org/>]) and by manual verification. The tandem mass spectra of peptides were processed by two database search engines, PEAKS Studio (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) and BioWorks 3.3 (Thermo Electron, San Jose, CA). Those proteins that contain peptide fragments labeled

by sulfo-NHS-LC-biotin can be assigned as associated with the cell surface and exposed to the extra-cellular environment.

ELISA Reactivity of Mouse and Monkey Serum to SPD1 Peptides

Overlapping peptides covering the SPD1 sequence were synthesized by GenScript (Piscataway, NJ). ELISA methodology for these peptides is provided as SUPPLEMENTARY MATERIAL. Mouse serum was obtained from normal mice following inoculation with *P. murina*. Monkey serum was provided by Dr. Karen Norris from macaques co-housed with *Pneumocystis*-infected animals.

ELISA Reactivity of Monkey Serum to SPD1 antigen (N-230 and C450)

To determine whether murine SPD1 antigen is cross reactive with monkey *Pneumocystis*, recombinant N-230 and C-450 (see below) were dissolved at 5ug/ml in 0.1M Na₂CO₃ and 0.1 M NaHCO₃ (PH 9.5) to coat 96-well ELISA plates. Serum was tested from monkeys exposed to monkey *Pneumocystis* and from control, unexposed monkeys (Dr. K. Norris, Univ Pittsburgh). Serum samples were diluted 1: 500 with PBS containing 1% BSA added to each well, and incubated at 4° C overnight followed by Goat anti-Monkey-IgG-HRP diluted at 1:2000 (Accurate Chemical) and incubated 1 hour at room temperature. The plates were developed with SureBlue™ TMB Microwell Peroxidase Substrate and absorbance at 450 determined.

Immunogens and Immunization Strategy

Because full-length SPD1 was too large for insect virus expression, we elected to make protein fragments corresponding to the N-terminal (N-230) and C- terminal (C-450) portions of SPD1. N-230 & C-450 were expressed in a Baculovirus by GenScript (Piscataway, NJ). Recombinant proteins were fused with an N-His tag. Purity was >75%. Expression data for these expressed proteins is provided as Supplemental Figure S3. The recombinant proteins were tested as antigenic targets for vaccine development. Female 6–8 week old BALB/c mice were vaccinated 3 times at 3 week intervals with 5 ug/mouse of N-230 or C450 plus adjuvant MF-59 (InvivoGen, San Diego, CA) by subcutaneous injection. Control mice were given PBS buffer or adjuvant MF-59 only. Serum samples were collected 2 weeks after the 3rd immunization and specific *Pneumocystis* IgG antibody measured by ELISA. Three weeks after the 3rd immunization, CD4 cells were depleted with 100 ug anti-CD4 antibody GK1.5 by i.p. injection. This dose of GK 1.5 results in 98–99% depletion of CD4+ T cells [5]. 2 days later, the mice were challenged with 2×10^5 *P. murina* organisms by intraoral inoculation. CD4 depletion continued weekly. Spleen cells, lung cells, and serum were collected at 1, 4 and 6 weeks after *Pneumocystis* challenge for flow cytometry and serum ELISA. Infection burden was assayed as real-time PCR of ribosomal RNA from the right lung (see below).

Serological Assays

Serum samples were collected 2 weeks after the 3rd vaccination before *Pneumocystis* challenge and then at 1, 4 and 6 weeks after challenge. ELISA plates were coated with 250ng of N-230 or C-450 recombinant protein/well in carbonate buffer pH 9.5 with

overnight incubation. Control wells contained no antigen or a lung homogenate from uninfected, unimmunized mice. After washing and blocking, serum samples diluted 1: 1000 were added to each well. Plates were further incubated with secondary AP-conjugated goat anti-mouse IgG antibody diluted 1: 1500 and developed using AP-yellow one component microwell substrate (SurModics BioFX). Absorbance at 450 nm was determined.

Flow cytometry

B cells and memory B cells were identified by flow cytometry. Lung and spleen cells were collected from immunized or control mice at 4 and 6 weeks after PC challenge and suspended at 1×10^6 cells per well. Cells were stained with PE-eFluor 610 anti-mouse CD45R antibody (eBioscience, San Diego, CA) to measure total B cell number and with anti-mouse CD45R, anti-mouse CD73 (PE-Cyanine7), anti-mouse CD273 (PE) and anti-mouse CD80 (APC) (eBioscience, San Diego, CA) to assay memory B cells. After 2 washes, cells were fixed with 1% paraformaldehyde. Cytometry data were acquired using an LSR II flow cytometer (BD Biosciences, San Jose, CA), and analyzed using FlowJo software (Tree Star, Ashland, OR).

RNA isolation and real-time RT-PCR for *P. murina* rRNA

RNA was isolated from the right lung tissue using TRIzol reagent. (Invitrogen, Grand Island, NY), reverse transcribed, and real time quantitative PCR of PC ribosomal RNA to determine *P. murina* lung burden was performed as previously described. [4]

Statistical Analysis

All data are presented as means $\pm$ standard errors of the means (SEM) and analyzed with GraphPad Prism 6 (La Jolla, CA). The student *t* test used to determine the significance of difference between sample means. Significant differences were accepted at *p* values < 0.05 .

RESULTS

Identification of SPD1

Using our chemical labeling technique, we identified a previously uncharacterized protein that matched the translation (Figure 1) of PNEG_01848's full length cDNA (*Pneumocystis murina* Sequencing Project, Broad Institute of Harvard and MIT [<http://www.broadinstitute.org/>]) This surface protein contains conserved peptidase M16 and peptidase M16 C domains [7,8] (Supplemental Figure S1) thus we designate it as Surface Peptidase 1 (SPD1). The putative SPD1 (sequence derived from full-length cDNA of PNEG_01848) contains 1068 amino acids with a MW of 126.4Kda. It should also be noted that SPD1 sequence (Figure 1, based on the original release of PNEG_01848 cDNA from Broad Institute) is identical to EMR09658.1 (submitted to GenBank by Broad Institute on January 25, 2013) and XP_007873819.1 (NCBI reference sequence based on resubmission from Broad Institute on June, 9 2015), our identification of the protein is unambiguous. The n-terminus (in peptide segment A³⁴TTATLLTENIKKPEGDHYSYR⁵⁵, A³⁴ is labeled by sulfo-NHS-LC-biotin) and c-terminus (in peptide segment N¹⁰⁵⁰SLKLSPVPLPVMKWSSYY¹⁰⁶⁸) of matured SPD1 were identified along with 12 other exposed regions of this protein. The 15 exposed regions of SPD1 are among a pool of

labeled peptide segments derived from *P. murina*. This pool of labeled peptide segments also includes segments (about half of the total number) belonging to *Pneumocystis* major surface glycoproteins known to be on the cell surface. [9] This indicates that the chemical labeling approach was targeting the cell surface.

Reactivity of SPD1 (N230, C450) with Immune Mouse and Macaque Serum

Because mouse and monkey sera showed reactivity to peptides from both the C terminus and N terminus regions of SPD1 (Supplemental Figure S2), fragments of the exposed n-terminal (N230) and c-terminal (C450) matured SPD1 protein (Figure 1) were expressed for further study. SPD1 N230 and C450 were coated on a plate and incubated with serum from control mice who had cleared a *Pneumocystis* challenge (PC serum) or from rhesus macaques (1791, 3713, and R697) following co-housing with a *Pneumocystis*-infected animal. As shown in Figure 2, convalescent mouse sera showed reactivity by ELISA to both N230 and C450 as did immune sera from three rhesus macaques exposed to *Pneumocystis*. These data also support some cross reactivity between murine SPD1 and nonhuman primate *Pneumocystis*.

Antibody Response after SPD1 Immunization

Mice were immunized 3 times with either N230 or C450 along with adjuvant as described in MATERIALS AND METHODS. Two weeks after the 3rd vaccination, sera from immunized mice were tested for antibodies to either N230 or C450 in ELISA (Figure 3). Both N230 (Panel A) and C450 (Panel B) immunization generated a significant IgG antibody response to the respective immunogen ($p < 0.0001$ compared to MF alone). Sera from mice immunized with N230 or C450 also showed IgG antibody response to *P. murina* by ELISA (Supplemental Figure S4)

Antibody Responses of SPD1-Immunized Mice Challenged with *Pneumocystis*

Antibodies to both N230 (Panel A) and C450 (Panel B) persisted in the *Pneumocystis* challenged animals up to 6 weeks post challenge, in spite of continued depletion of CD4⁺ lymphocytes (Supplemental Figure S5). Serum IgG levels were significantly higher ($p < 0.0001$) in comparison to MF alone for both N230 and C450 at 1, 4, and 6 weeks after PC challenge.

B-Cell Responses to SPD1 Immunization

Total B cell numbers were calculated in both spleen and lung of mice immunized with either N230 or C450 followed by *Pneumocystis* inoculation. The results are shown in Figure 4. Both SPD1 fragments induced a significant increase in B cell numbers in lung tissue (Panel A) and to a lesser extent in spleen (Panel B). B cell numbers were significantly elevated in comparison to MF only mice at 1, 4, and 6 weeks in lung tissue but only at 1 and 4 weeks in spleen..

Memory B-cell Responses to SPD1 Immunization

In a similar manner, numbers of B cells with a memory phenotype were calculated in both spleen and lung of mice immunized with either N230 or C45 followed by *Pneumocystis*

inoculation (Figure 5). Both SPD1 fragments induced a significant increase in memory B cell numbers in lung (Panel A) and in spleen tissue (Panel B). This increase in memory B cell numbers was statistically significant at 1, 4 and 6 weeks after *Pneumocystis* challenge.

Protective Efficacy of SPD1 Immunization against *Pneumocystis* Pneumonia

Protection against infection was studied by measuring clearance of *Pneumocystis* inoculated into the lungs of mice immunized with either N230 or C450. Normal mice clear *Pneumocystis* experimental infection. To assay SPD1 as a vaccine and to better simulate immunosuppression similar to HIV, immunized mice were depleted of CD4 T-cells prior to inoculation of *Pneumocystis* and weekly up to the time of sacrifice. (Analysis of lung CD4+ cells showed that at 6 weeks after *Pneumocystis* challenge, lung tissue from control mice contained 23±2% CD4+ cells, while lung tissue from vaccinated mice and adjuvant-treated mice contained less than 2% CD4+ cells.) Lung tissue burdens from vaccinated mice are shown in Figure 6. Immunization with either N230 or C450 permitted clearance of infection in spite of depletion of CD4+ T-lymphocytes. This was statistically significant at 1,4, and 6 weeks post infectious challenge in comparison to animals immunized with adjuvant alone (MF), CD4+ control mice, and mice depleted of CD4 cells but receiving no immunization (control). Immunization with C450 appeared to be more effective at protection against infection than N230, showing no detectable organisms in lung tissue at 6 weeks after challenge in comparison to persistent infection in the CD4-depleted no vaccine and adjuvant alone controls.

DISCUSSION

Our studies identify a putative surface protein of *P. murina* that is immunogenic, inducing B cell and antibody responses in mice along with protection against infection in the setting of CD4 deficiency. This protein (Broad Institute gene accession no. PNEG_01848), designated as SPD1, has potential for development as a vaccine against *Pneumocystis jirovecii*, an important opportunistic fungal pathogen of humans.

Much remains to be learned about SPD1. Live cells labeled by sulfo-NHS-LC-biotin followed by proteomic analysis, the technique that was employed to identify SPD1, is known for bias to surface exposed abundant proteins with enriched unmodified primary amine groups (i.e, lysine sidechains, n-termini of proteins/peptides). Even though contamination from intracellular proteins cannot be totally ruled out, such protein contamination is highly likely to be masked by known abundant cell surface proteins (such as major surface glycoproteins) and host proteins in the sample (data not shown). We know that SPD1 is an abundant protein on the surface of the organism in the later stage of infection as the *P. murina* cells labeled for LC-MS/MS analysis were collected 10 weeks after first inoculation. It is not clear if SPD1 is expressed on the *P. murina* cell surface in the early stage of infection. Protection data (Figure 6) show that fungal burden increases from week 1 to week 4, in spite of detectable antibodies in immunized animals (Supplemental Figure S2). This may reflect a lag time necessary to generate antibody-induced phagocytosis and killing of proliferating organisms. Or it is possible that expression of SPD1 on the cell

surface only occurs in the later stage of infection, triggering generation of further antibodies, recruitment of innate immune cells, and clearance of infection at week 6 (Figure 6).

Protein size constraints led us to synthesize SPD1 in two fragments, an N terminal (N230) and a C terminal fragment (C450). Both fragments are immunogenic and show protective efficacy as a vaccine with C450 being more effective. Earlier comparison of the exposed regions of SPD1 to *Pneumocystis jirovecii* (isolate SE8) polypeptide sequences [10] lead to two unnamed partial protein products, gi|430814576|emb|CCJ28211.1| and gi|430814577|emb|CCJ28212.1|. Both of the unnamed partial protein products have a peptidase M16 C domain (Supplemental Figure S1). [7] Since the combination of the two unnamed partial protein products has similar domain structure to that of SPD1, it is likely that gi|430814576|emb|CCJ28211.1| and gi|430814577|emb|CCJ28212.1| belong to one gene product homologous to SPD1. With the recently available annotated genome of *Pneumocystis jirovecii* (isolate RU7)[7]), the unnamed protein products gi|430814576|emb|CCJ28211.1| and gi|430814577|emb|CCJ28212.1| were found to match to the cDNA of T551_00903 (Broad Institute accession number). Ma et al also pointed out that *P. murina* protein PNEG_01848 (named SPD1 by us in this report) and its *P. jirovecii* ortholog T551_00903 belong to a sub-cluster in the family of M16 peptidase, which also includes two *S. cerevisiae* genes Ste23 (YLR389C) and Ax11 (YPR122W). Studies on the protein product of Ste23 suggest that such protein(s) are peptidases that could cleave insulin b chain (but not intact insulin), and process yeast P2 a-factor mating propheromone [11] Phylogentic study also indicates that *P. jirovecii* gene T551_03640 is the other orthologue of *S. cerevisiae* gene Ste23 in addition to T551_00903. Based on the similarity at amino acid level between *P. murina* SPD1 and *P. jirovecii* protein T551_00903 (as confirmation of Ma et al's result by us in this report), we suggest to name the protein of T551_00903 as PJ_SPD1, and the protein of ortholog T551_03640 as PJ_SPD2. Additional studies on SPD proteins in *Pneumocystis jirovecii* and in *P. murina* need to be performed to identify a functional correlate to *P. murina* SPD1 in the human *Pneumocystis* species.

Sequence analysis suggests that SPD1 is a peptidase in M16 family [8]but the substrate for this peptidase is unknown as is its purpose in the life processes of *P. murina*. The SPD1 we identified is derived from a mixture of living *Pneumocystis* organisms. It remains to be determined whether SPD1 expression is confined to the trophozoite or ascus forms. We also do not know whether SPD1 can serve as a target for antibody binding to opsonize internalization and killing by phagocytic cells. Work to answer many of these questions for SPD1 is underway in our laboratory.

The host immune response to *Pneumocystis* involves both T and B cell immunity along with antibody production. [12] Antibodies produced may function as opsonins to facilitate killing by alveolar macrophages. [13,14] Our data suggest that SPD1 induces potent B cell, antibody, and presumably T cell responses when used as a vaccine with the MF59 adjuvant. MF59 is a squalene-based adjuvant, licensed for use in human influenza vaccines in Europe in 1997. [15,16] Preliminary data from our laboratory show that SPD1 also has protective efficacy as a DNA vaccine (data not shown). It should be noted that while we here show that SPD1 vaccination increases B cell and memory phenotype B cell numbers in vaccinated

animals, we have not yet shown that this translates into increases in antigen (SPD1) specific B cell numbers in lung tissue.

There are limitations to the approach and methodology employed in our study. We have tested SPD1 coupled with the MF59 adjuvant. We have yet to test this vaccine without adjuvant or with additional adjuvants. Local (pulmonary) vaccination is also a logical vaccination approach to be explored. Although C450 vaccination resulted in no detectable *Pneumocystis* at 6 weeks after challenge, we can only claim partial protection against infection for SPD1 vaccination at this time. Demonstration of full or complete protection from infection will require longer animal housing experiments, varying doses of the *Pneumocystis* challenge, and varying doses of the vaccine.

There are other *Pneumocystis* vaccine candidates in addition to SPD1. The major surface glycoprotein (MSG) of *Pneumocystis* elicits antibody and lymphocyte proliferative responses in animals and humans with *Pneumocystis* infection. [9,17,18] However, experiments with an MSG-based vaccine showed no protection against infection [19], perhaps due to the antigenic variation. [3] A 55 KD *Pneumocystis* vaccine has been developed in rats. [20] A *Pneumocystis* surface protease, kexin, has also shown promise [18,21–23] and work is in progress in our laboratory to further develop this vaccine. HIV-infected humans who are hospitalized with *Pneumocystis* pneumonia develop serum antibody responses to components of both MSG and kexin in the recovery period. [24] To date there is no vaccine approved for use in humans to prevent or treat *Pneumocystis* pneumonia.

In summary, we here report a novel surface protein of *Pneumocystis murina* that is capable of inducing systemic and pulmonary immune responses and provides protection against infection in immunocompromised mice challenged with *Pneumocystis*. These studies have promise for the future development of a vaccine against this important human pathogen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

<i>P. murina</i>	<i>Pneumocystis murina</i>
PC	<i>Pneumocystis</i> (species not specified)

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HIGHLIGHTS

- A novel cell surface peptidase, SPD1, on *Pneumocystis murina* is identified
- Immunization with SPD1 induces antibody and B cell responses
- Immunization with SPD1 protects CD4 lymphocyte-deficient mice from *Pneumocystis* infection

1 MWSYFIFFSL IFLKTESHI IOHNKTDHIL YKRATTATLL TENIKKPEGD HYSYRLIQLS NGLKTLISY
 71 PDSLSAGAAL DVKVGHFSDP DDIPGVAHFC EHLLFMGTTK YPGEDDFSEY LLTHAGTYNA YTSTEDTNYF
 141 FSVEPKSEK ALDMFSQLEI SPLILKRSVE REAHAVDLEH KKNLQNDAWR LEQLEKSISN PKSPYSKFGT
 211 GDYVTIVENT RKKGIDIASV VSKFFSTYYS SNLMKLVIFS SESIDNLEKL AVKYFTDIPN KNLEVPKFTE
 281 KPFGADVIGK QYWKPIADS NSIELVFPID TQRMFYKSSP SDYLRHFLDH RSYGSVFYTL NQRGWITDIS
 351 VYSEYIVSET DALRINIGLT EIGLDNYEDI LGLVFQFLRM LRDNTPNEDY FKDLIKIDDV SFRFKDQPKL
 421 MSYASHLASV MQLPYIEDVD LLRSSYLSEY DHKQFAKLLS VLRDDNYFLT ITSKTKPGYW DLREPWYGSE
 491 YKVDVFSQTL LEKTRKDDLN ISLKFPEKNI FIPELFIRAN VSTHKTKPT LMYNDTRLRY WYKEDDTFSI
 561 PKTFISALIK IPDYSSSPFE TAHSKIYLDL LINHVSTQHY NAIVAGYSLT ISAIDIGIYL SIYGFSDKIL
 631 LLLDKVIESM RLYTPSLASF MSSRQKLYLM YEYDKVSMPY SKVSQVSLAF HDPLYWPSSE MLYAINSTTY
 701 EDVQVFHSRR FSSVFTEVLA VGLLSNSTKD LLEIYFKALS PKKQYPYQIL PSRSYIFKEG SNYIYEMSLI
 771 NPQEVNSAIL YSLQVGSSE SKLIALLNVL FSLIRSRVEN QLRTEEQLSY VVRVSSYTST ALMSFFFVLQ
 841 SLRDPFYLEQ RINAFLYRFA AFIDTITDEQ LKSVVNTLLP LFSGRYTSLP KESGVYIASI VSGFYDFDIR
 911 KKIYRSLKGL TKEDLKDYFY NYFYPDSATR KKLSIHLKSQ TLESVSVRDF SPRRLQYYFK CNGLDISLED
 981 LSDLIESSHL LVDFEKALKK FFTNKYPHKD VSEIVSQAFK YLKELYTKLK KDAIKNYDAQ HFTDLTTFKN
 1051 SLKLSPVPLP VMKWSSYY

Figure 1.

Putative sequence of SPD1 (Broad Institute accession no. PNEG_01848). Peptide segments (in red letters) were identified by chemical labeling and LC-MS based proteomic approach and supposed to be the exposed regions of the protein. N-terminal of matured SPD1 was determined by the labeling of A³⁴ in peptide “A³⁴TTATLL TENIKKPEGD HYSYR⁵⁵”. C-terminal of SPD1 was also confirmed by the detection of peptide “N¹⁰⁵⁰SLKLSPVPLP VMKWSSYY¹⁰⁶⁸”. Signal peptide of protein SPD1 is underlined. Fragment **SPD1-N230** (AA 34-262) contains the experimentally identified N-terminus of the matured protein, and fragment **SPD1-C450** (AA 627-1068) contains the C terminus. SPD1-N230 and C450 are selected as targets for vaccination

Figure 2.

Control mice and simian/human immunodeficiency virus (SHIV)-infected rhesus macaques exposed to *Pneumocystis* have serum IgG antibodies to SPD1 N230 and C450. Serum from control mice challenged with *Pneumocystis* (PC serum) and from three rhesus macaques containing high titers of antibody to PC kexin (1791, 3613, and R697) were tested by ELISA for IgG antibodies to SPD1 N230 and C450. –serum represent sera from mice that were not immunized. Data represent mean $\pm$ SD, n=6 for mice, n=3 for nonhuman primates.

Figure 3A.

Figure 3B.

Figure 3.

Antibody responses to SPD1 after immunization with SPD1. Mice were immunized with N230 or C450 plus MF59 adjuvant or adjuvant alone 3 times at 3 week intervals. –control mice were not immunized. +control mice represent sera from mice that cleared a PC challenge. Two weeks after the last immunization, serum was tested for IgG antibodies to the respective immunogen. Panel A. N230 immunization. Panel B. C450 immunization. Results are compared to animals vaccinated with adjuvant alone and to serum from unexposed mice (–control) and from mice who cleared an experimental challenge with *Pneumocystis* (+control). Note that control mice show an antibody response to SPD1 but that vaccination with SPD1 produces a greater serum IgG response. Data represent mean $\pm$ SD, n=10.

Figure 4A.

Figure 4B.

Figure 4.

Tissue B cell responses after SPD1 immunization and challenge with *Pneumocystis*. Mice were immunized with N230 + MF59 adjuvant or C450 + MF59 adjuvant and then challenged with *Pneumocystis*. Some mice received adjuvant alone (MF) or no vaccine (CD4+ control and control). CD4 depletion was started 3 weeks after the last immunization (2 days prior to PC challenge) and continued weekly, except for the CD4+ control mice. Total B cells were assayed by flow cytometry at 1,4, and 6 weeks post PC challenge. Panel A. Lung B cell responses (1,4,6 weeks), Panel B. Spleen B cell responses (1, 4, 6 weeks). Data represent mean $\pm$ SD, n=10 at each time interval.

Figure 5A.

Figure 5B.

Figure 5.

Tissue memory B cell responses after SPD1 immunization and challenge with *Pneumocystis*. Mice were immunized with N230 + MF59 adjuvant or C450 + MF59 adjuvant and then challenged with *Pneumocystis*. Some mice received adjuvant alone (MF) or no vaccine (CD4+ control and control). CD4 depletion was started 3 weeks after the last immunization (2 days prior to PC challenge) and continued weekly, except for the CD4+ control mice. Memory B cells (CD45R+, CD73+, CD80+, CD273+) were assayed by flow cytometry at 1, 4, and 6 weeks post PC challenge. Panel A. Lung memory B cell responses (1, 4, 6 weeks), Panel B. Spleen memory B cell responses (1, 4, 6 weeks). Data represent mean $\pm$ SD, n=10.

Figure 6.

Clearance of infection in SPD1 immunized mice. Mice were immunized with N230 + MF59 adjuvant or C450 + MF59 adjuvant and then challenged with *Pneumocystis*. Some mice received adjuvant alone (MF) or no vaccine (CD4+ control and control). CD4 depletion was started 3 weeks after the last immunization (2 days prior to PC challenge) and continued weekly, except for the CD4+ control mice. Lung tissue burden of *Pneumocystis* was assayed by real time PCR at 1, 4, and 6 weeks after infectious challenge. Data represent mean $\pm$ SD, n=10/experimental group, experiment repeated $\times$ 1.