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Th1 biased response to a novel *Porphyromonas gingivalis* protein aggravates bone resorption caused by this oral pathogen

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Abstract

In previous studies we showed that biasing the immune response to *Porphyromonas gingivalis* antigens to the Th1 phenotype increases inflammatory bone resorption caused by this organism. Using a T cell screening strategy we identified eight *P. gingivalis* genes coding for proteins that appear to be involved in T-helper cell responses. In the present study we characterized the protein, encoded by *PG_1841* gene and evaluated its relevance in the in bone resorption caused by *P. gingivalis* because subcutaneous infection of mice with this organism resulted in the induction of Th1 biased response to the recombinant PG1841 antigen molecule. Using an immunization regime that strongly biases toward the Th1 phenotype followed by challenge with *P. gingivalis* in dental pulp tissue, we demonstrate that mice pre-immunized with rPG1841 developed severe bone loss compared with control immunized mice. Pre-immunization of mice with the antigen using a Th2 biasing regime resulted in no exacerbation of the disease.

These results support the notion that selected antigens of *P. gingivalis* are involved in a biased Th1 host response that leads to the severe bone loss caused by this oral pathogen.

1. Introduction

The optimal clearance of pathogens requires selective activation of a particular cellular or humoral immune response, which in many cases can also be critical to the clinical outcome of the disease. In the oral cavity, *Porphyromonas gingivalis* is a consensus oral pathogen that has been implicated in periodontal and periapical diseases in humans and other species [1,2,3,4]. In all of these models, infection with *P. gingivalis* elicits a cell-mediated Th1 type response characterized by increased production of IFN- γ , IL-12 and TNF α , and leads to increased inflammation and bone destruction [5,6]. Similarly, high levels of Th1 cytokines

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in gingival tissues and mononuclear cells, and gingival crevicular fluid are associated with increased periodontal disease progression [7,8,9]. We recently showed that dental pulp infection with *P. gingivalis* causes extensive inflammation and bone destruction and is associated with a strong Th1 response, characterized by increased intra-lesional production of IFN γ and IL-1 [6].

To further characterize the molecular pathogenesis of *P. gingivalis*-induced inflammation and bone destruction, we used T cell expression cloning to identify *P. gingivalis* proteins that induce Th1 type T cell responses during infection [10]. Eight protein candidates were identified using this methodology, including three hypothetical proteins. In the present work we evaluated the involvement of one of these hypothetical proteins (encoded by the *PG_1841* gene) induces a potent Th1 response during infection, which is associated with periapical bone destruction caused by *P. gingivalis*.

2. Materials and methods

2.1. Animals

BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). The mice were maintained under specific pathogen-free conditions and used at 6–12 weeks of age. The Institutional Animal Care and Use Committee of Forsyth Institute approved all experiments.

2.2. High-level expression and affinity purification of recombinant *P. gingivalis* PG1841 protein

Oligonucleotide PCR primers were designed to amplify the full-length sequence of *P. gingivalis* PG1841 (a hypothetical protein) using as template genomic DNA of the virulent W83 strain. The following oligonucleotide primers were used for PG1841: Forward, (5'-CATATG TGGGCTATTGTGGAGGGC-3'; reverse, 5'-GGATCCTCACGCTTGGATTCGGTCATGATC-3'. The forward primers contained an *NdeI* restriction site preceding the ATG initiation codon (underlined) followed by sequences derived from the gene (italic). The reverse primer contained a *BamHI* restriction site followed by a stop codon. The resultant PCR product was digested with *NdeI* and *BamHI* and subcloned into a pET14b expression vector similarly digested with *NdeI* and *BamHI* for directional cloning. Ligated pET14b, which has in its construct a 6xHis chain in the N-terminus of the molecule, was subsequently used to transform *E. coli* BL-21 (DE3)pLysS host cells (Novagen, Madison, WI) for expression. rPG1841 was purified from 500ml of IPTG induced batch cultures by affinity chromatography using the one step QIAexpress Ni-NTA Agarose matrix (QIAGEN, Chatsworth, CA) as described [11]. The yields of recombinant protein were 25–35mg per liter of induced bacterial culture, and purity was assessed by SDS-PAGE, followed by Coomassie blue staining. Endotoxin contamination was removed using immobilized polymyxin B (Detoxi-Gel – Pierce, Rockford, IL) followed by passage over a ProteoSpin column (Norgen-Biotek Corp, St. Catharines, Ontario, Canada). Endotoxin levels of purified rPG1841 was <100 EU/mg protein as indicated by the Limulus Amebocyte Lysate assay (BioWhittaker, Walkersville, MD).

2.3. Rabbit antiserum

Purified rPG1841 (200 μ g) was emulsified with incomplete Freund's adjuvant (IFA) and injected at multiple subcutaneous (s.c.) sites into one female New Zealand rabbit. The rabbit was given two s.c. boosters (200 μ g antigen in IFA) four weeks apart. One week after the final boost, the rabbit was sacrificed and serum was collected and stored at -70°C.

2.4. Western blot analysis

Antigens were separated by 4–20% SDS-PAGE and transferred to PVDF membranes, followed by blocking with 5% non-fat dried milk and 0.1% (v/v) Tween 20 in TBS. The blots were probed with either pre-immune rabbit serum or with the specific rabbit antiserum followed by incubation with goat anti-rabbit IgG/Steptavidin HRP conjugate (BD Biosciences PharminGen, San Diego, CA). Reaction was detected with the ECL Western blotting system (GE Healthcare Ltd., Buckinghamshire, UK) according to standard protocols [12].

2.5. Bacteria and antigen preparation

P. gingivalis W83 (ATCC# BAA-308TM), was grown in Mycoplasma Broth (Sigma, St. Louis, MO, Saint Louis, MO) medium under anaerobic conditions (80% N₂, 10% H₂, 10% CO₂), harvested, and suspended in pre-reduced, anaerobic sterilized Ringer's solution (PRAS) under an inert (N₂) atmosphere. The bacteria were washed three times with PBS and suspended to a concentration of ~10⁹ microorganisms/ml followed by five cycles of freeze/thaw in liquid nitrogen to lyse of the bacterial cells. Lysed cells were centrifuged at 10,000×g to obtain a soluble *P. gingivalis* antigen preparation (*Pg* lysate). The antigen preparation was concentrated with an Amicon 3 Centriprep concentrator (Beverly, MA) to yield a protein concentration of 3 mg/ml as determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL).

2.6. IgG isotype ELISA

Mice were pre-bled before inoculation with *P. gingivalis* and four weeks after challenge. Sera were stored at –70°C until assay. The specific serum IgG isotype antibody response was measured by conventional enzyme-linked immunoadsorbent assay (ELISA). Wells of ELISA plates (Costar, Cambridge, MA) were coated with *Pg* lysate at a concentration of 10 µg/ml and at a concentration of 2 µg/ml for rPG1841. Sera were added at two fold serial dilutions followed by addition of biotinylated isotype-specific secondary antibodies (rabbit anti-mouse IgG1 or IgG2a; BD Biosciences PharminGen, San Diego, CA). Wells were then incubated with streptavidin-conjugated horseradish peroxidase (SAV-HRP) (Zymed) after which substrate and chromogen were added and absorbance was read on an ELISA plate reader (Dynatech, Chantilly, VA) at 490nm.

2.7. Immunization with recombinant protein

Groups (n=10 each) of BALB/c mice (6 weeks old) were immunized with 5 µg of rPG1841 mixed with 50 µg of alum (Rehydragel HPA, Brekeley Heights, NJ) and 50 µg of CpG (Coley Pharmaceutical Group Inc., Wellesley, MA) in the footpad and s.c. in the dorsum of the lumbar area on days 0 and 18. Mice were bled before immunization and 4 weeks later for assessment of IgG1/IgG2a antibody response as previously described [13].

2.8. Pulpal infection with *P. gingivalis*

Mice were anesthetized with ketamine HCl (80 mg/kg) and xylazine (10 mg/kg), and were placed on a jaw retraction board. The dental pulp of mandibular first molars was exposed using an electric dental handpiece with a no. 1/4 round bur, under a surgical microscope (MC-M92; Seiler, St. Louis, Mo.). The exposure site was approximately 1.5 times the diameter of the bur. Exposed dental pulps were infected with live *P. gingivalis* W83. The bacteria were grown and harvested as described above, and were suspended in PRAS containing 2% methylcellulose at 10¹⁰ cells/ml. At the time of pulp exposure (day 0), exposed dental pulps were directly inoculated with 5 µl of the suspension, and the suspension was introduced into the root canals using #06 endodontic files. The access cavities were sealed with a dental composite resin (Zenith, Englewood, NJ).

2.9. Micro-computed tomography

Mice were sacrificed by CO₂ asphyxiation 14 days after pulpal infection. Mandibles were isolated and the left hemimandibles were fixed in fresh 4% PFA in PBS, and subjected to microcomputed tomography (mCT) after defleshing soft tissues. A cone-beam-type tomograph (SMX-225CT, Shimadzu Corporation, Kyoto, Japan) was employed. Acquired stacks for each sample contained approximately 120 serial micro-tomographic slices at an increment of 32µm. The stacks were re-sliced using Image software (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD) to obtain the 'pivot' section, which included the mesial and distal roots of the mandibular first molar and that exhibited a patent distal root canal apex. The periapical radiolucency surrounding the distal root was determined by Adobe Photoshop CS (Adobe Systems Inc., San Jose, CA) with the use of a standard template superimposed on the periapical region of the distal root, and the lesion was quantified with Image software. The images were analyzed by three examiners in double blind fashion, and the results were expressed in mm² of the mean +/- S.D.

2.10. Histology

Bone blocks containing periapical tissue were decalcified and embedded in paraffin. Serial sections, 6µm thick, were cut and stained with H&E.

2.11. Statistical analysis

Statistical analysis was performed using ANOVA followed by Tukey's multiple comparison tests (INSTAT Software, GraphPad, San Diego, California). All values were considered significantly different at $p < 0.05$.

3. Results

3.1. Expression, purification, and characterization of a truncated recombinant *P. gingivalis* protein encoded by the *PG_1841* gene

In a previous report, using a T cell expression cloning strategy we identified several *P. gingivalis* proteins that are immunodominant antigens during infection of mice with this microorganism [10]. To begin to characterize the involvement of these molecules in periapical bone destruction caused by *P. gingivalis*, the protein encoded by the *PG_1841* gene was selected for further studies because the deduced amino acid sequence of this protein has a suggestive signal peptide sequence, and therefore was possibly a membrane bound or secreted protein.

To express rPG1841, primers designed to amplify a DNA fragment that excluded the first 135bp of the gene. This procedure was essential to circumvent difficulties in expressing the full length gene coding for long hydrophobic signal sequences at the N-terminus of the protein. Oligonucleotide primers were designed and used to amplify by PCR the DNA encoding the truncated PG_1841 gene sequence from *P. gingivalis* genomic DNA. The obtained DNA was initially cloned into Topo® TA cloning vector (Invitrogen) and subsequently sequenced. No PCR mutations were observed. The truncated cloned gene was sub-cloned into the pET14b expression vector, which was used to transform (DE3) pLysS competent cells for expression. The pET14b vector has been designed to express 6xhistidine residues at the N-terminal of the recombinant protein for ease of purification by affinity chromatography over Ni-NTA matrices. To verify the expression of the hypothetical protein PG1841 by *P. gingivalis*, Western blot analysis was performed with antibodies developed against the recombinant protein. The results indicate that rPG1841 is detectable, and migrates at slightly lower molecular weight position (32 kDa) than the predicted 34.9 kDa MW of the full-length native protein (Fig. 1). The smaller size of the recombinant molecule

corresponds exactly to the expected MW of the truncated protein in that it is 45 amino acids shorter than the native molecule, has a 6x His tag, a 3 amino acids linker sequence and a 6 amino acids of the thrombin site sequence.

To categorically define that the amino acid sequence of purified recombinant protein corresponded to that of the predicted amino acid sequence of the hypothetical PG1841 protein, amino acid sequence was performed by mass spectroscopy. The recombinant protein was run on a 4–20% gradient gel and the single band seen in the gel was excised and in gel tryptic digested followed by LC-MS/MS analysis for identification. PG1841 protein sequences were identified with high confidence through 6 tryptic peptide amino acid sequence regions. These sequences had 100% identity with the deduced amino sequence of the PG_1841 gene. These regions were amino acid residues: 61–84, SRSHPVYLFPGADPEQVVAAGR; 85–107, LKAEVVHEDYPLIFMGVTPDIHR; 185–199, GDQDGRELEVEMVER; 241–254, ADAMVEGAFAMINR; 266–279, YINREEDLGLPGLR; and 283–293, LSYPAILLPK. Taken together, these results clearly point to the purified recombinant molecule as genuine *P. gingivalis* protein.

To ascertain that the PG1841 is a representative molecule of *P. gingivalis* that is present in various clinical isolates of this pathogen, genomic DNA was prepared from the following isolates ATCC33277, A7436, H66 and 381 and then tested by PCR for the presence of Pg_1841. Figure 2 shows that specific PCR products were obtained for all four isolates. In addition, the PCR products matched the exact molecular size of the PCR product obtained for the reference strain Pg W83. These results indicate that Pg_1841 is uniformly distributed among different *P. gingivalis* isolates, thus validating this molecule for biological and immunological studies.

3.2. Antibody response to purified recombinant proteins in mice challenged with viable *P. gingivalis*

To verify that PG1841 was expressed by *P. gingivalis* during infection *in vivo*, BALB/c mice were initially inoculated in the footpad and subcutaneously with approximately 10^8 CFU of viable *P. gingivalis* two times, two weeks apart. Anti-PG1841 antibody responses were evaluated two weeks after the second inoculation by ELISA, using specific anti-mouse IgG1 and IgG2a isotype antibodies. IgG1 and IgG2 isotypes of immunoglobulins are surrogates of Th2 and Th1 immune phenotypes, respectively [14]. Both IgG1 and IgG2a specific anti-PG1841 antibodies were present in the sera of inoculated mice (Fig. 3). However, the IgG2a antibody response was clearly stronger than the IgG1 response. These results suggest that infection of mice with *P. gingivalis* results in a specific immune response to PG1841 thus indicating that this protein is indeed expressed *in vivo* during infection, and therefore validates the antigen discovery approach we employed. In addition the results suggest that infection with *P. gingivalis* preferentially induces a Th1 biased response to PG1841.

3.3. Selective induction of Th1/Th2 biased immune response to *P. gingivalis* antigen PG1841

To evaluate the role of Th biased responses to the purified antigen in modulating *P. gingivalis*-stimulated bone resorption, groups of mice were injected twice, 2 weeks apart with rPG1841 formulated in either Th1- or Th2-inducing adjuvant. Twenty eight days after immunization, sera were collected and the titer of specific IgG isotype antibodies determined by ELISA. Fig. 4 shows that mice inoculated with rPG1841 plus Th1 biased adjuvant developed elevated levels of IgG2a, compared to those injected with the antigen formulated in a Th2 biasing adjuvant. In contrast, mice immunized with PG1841 in a Th2 biasing adjuvant produced high titers of specific IgG1 antibodies and no IgG2a to rPG1841.

Sera from all mice obtained prior to immunization did not react with the antigen (not shown). These results indicate that the immune response of mice to rPG1841, similar to other previously described antigens [10], can be readily modulated towards Th1 or Th2 using delivery in the appropriate adjuvant.

3.4. Effects of immunization with rPG1841 on periapical bone resorption

To evaluate the association of the specific immune response to the rPG1841 antigen with bone resorption caused by *P. gingivalis*, groups of mice (n=8) were immunized with rPG1841 formulated with Th1 or Th2 biasing adjuvant. As controls, mice were not immunized or immunized with each adjuvant alone. Mice were immunized twice, 2 three weeks apart. Three weeks after the second immunization, mice were subjected to surgical exposure of the dental pulp in the first mandibular molars followed by inoculation with live *P. gingivalis*. Two weeks thereafter, the mice were sacrificed and bone resorption was evaluated by micro-computed tomography (mCT). As shown in Fig. 5 & 6, significant bone loss occurred surrounding the roots of infected teeth only in mice previously immunized with rPG1841 formulated with the Th1 biasing adjuvant. In contrast, animals immunized with the antigen in a Th2 biasing adjuvant had no influence in the outcome of the bone loss caused by *P. gingivalis*, and was comparable to that found in adjuvant only immunized and infected animals. Taken together, these results point to a specific ability of certain *P. gingivalis* antigens to exacerbate bone loss caused by infection with this oral pathogen.

3.5. Histopathology of periapical lesions in mice immunized with rPG1841

To evaluate the inflammation present in the infrabony lesions, histopathological studies were performed on mandibular bone samples from mice previously immunized with *P. gingivalis* antigens formulated with Th1 or Th2 adjuvant. The results are illustrated in Fig. 7, and show that a large inflammatory reaction occurred in mice immunized with rPG1841 formulated with the Th1 biasing adjuvant. Of particular interest was the presence in these lesions of pronounced infiltration of mononuclear cells as well as many multinucleated osteoclasts found associated with resorption lacunae in bone adjacent to the granulomas. In contrast, the periapical inflammation observed in mice immunized with rPG1841 formulated with Th2 biasing adjuvant was characterized by very sparse distribution of mononuclear cells; few osteoclasts were seen in these lesions.

4. Discussion

CD4⁺ T-cell mediated immunity to infection induced by microorganisms is modulated by helper cells of the Th1 or Th2 type. The two cell types are distinguished by their roles in regulating the immune system, via the cytokines they produce. We and others have shown that infection with live *P. gingivalis* predominantly elicits a Th1-type response in experimental animals and in humans [15]. In addition, adoptive transfer experiments have indicated an active role for Th1 cytokines in periodontal disease exacerbation, and some studies have shown that Th2 cytokines may be protective [9,16]. However, other studies suggest that this association is not consistently observed [17,18,19]. The induction of polarized T cell responses, and their association with infection-induced pathology or disease-protective mechanisms has led to the development of novel vaccines and immunotherapeutics [20,21].

In this work we tested the hypothesis that inflammatory bone resorption in periapical periodontitis caused by *P. gingivalis* is associated with CD4⁺ T cell mediated immune responses specific for certain antigens of this microorganism. We previously reported the identification of eight *P. gingivalis* antigens associated with T cell responses elicited by this oral pathogen [10]. Of the eight genes, *PG_1841* was chosen for further study, in an attempt

to verify the relevance of individual antigens of *P. gingivalis* to the pathogenesis of periapical and periodontal bone resorption caused by this organism. *PG_1841* was an interesting candidate for detailed analysis because it encodes a protein with a signal peptide sequence, and therefore was likely to represent a protein that is either actively secreted or membrane bound. In either case PG1841 would likely be readily available for recognition by the immune response during infection.

Our *in vivo* experiments indicated that PG1841 was expressed by *P. gingivalis* during infection, and was recognized by the immune response as indicated by the induction of antibody particularly of the IgG2a isotype. Interestingly, our former observation with another *P. gingivalis* recombinant antigen (rPG1729) indicated that the antibody immune response of infected animals to the latter antigen encompassed both IgG1 and IgG2a isotypes [10]. Therefore, these data were important in establishing that the T cell expression screen was valid, and was in fact identifying possibly Th1 antigens that are actively produced by *P. gingivalis* during infection. This was of particular importance for PG1841 which was previously considered a 'hypothetical' protein of *P. gingivalis*.

The possible role of PG1841 protein in the inflammatory process caused by *P. gingivalis* was evaluated using a mouse model of periapical bone loss. In this model mice were immunized with rPG1841 in combination with Th1 biasing and Th2 biasing protocols followed by pulpal infection with *P. gingivalis*. To achieve a Th1 biased response the adjuvant of choice has been IL-12 or IL-12-inducing molecules such as CpG oligodeoxynucleotides [22]. CpG oligodeoxynucleotides act as adjuvants that switch on Th1 immunity [23]. For Th2 stimulation, alum has traditionally been the most commonly used adjuvant. We and others have shown that adding alum to the IL-12/CpG formulation augments the magnitude of the immune response, without interfering with the Th1 biasing property of the adjuvant [13,24].

In these experiments the immunization protocols used generated specific and polarized Th1 or Th2 immune responses to rPG1841. Immunization of mice with rPG1841 formulated with alum alone resulted, as expected, in high titers of specific IgG1 and no IgG2a antibody. In contrast, immunization with rPG1841 in CpG plus alum resulted in high titers of specific IgG2a antibodies as well as significant levels of IgG1. These patterns of IgG isotypes are generally accepted as reliable surrogates of Th2 and Th1 responses respectively [14]. It is important to note that IgG1 was believed in the past to be a surrogate of the Th2 response, because IL-4 was known to promote immunoglobulin class switching to IgG1. However, recent evidence [25] demonstrates that IgG1 antibodies are divided in two distinct sub-families of molecules, one that is dependent on IL-4 (Th2 associated) and another that is dependent on IL-12 and IFN- γ (Th1 associated). Therefore, the generation of high titers of IgG1 antibody can only be interpreted as a surrogate of a polarized Th2 response in the absence of IgG2a antibody (which was the case for immunization with rPG1841 plus alum alone). In contrast, because the class switch to IgG2a is solely dependent on IFN- γ , high titers IgG2a antibody, whether or not associated with IgG1 antibody has been generally accepted as a good surrogate of a typical Th1 response.

The consequence pre-immunization with rPG1841 in the Th1 biasing adjuvant was severe bone resorption, as analyzed by micro-CT and histological studies. In contrast, mice immunized with rPG1841 using the Th2 biasing protocol developed minimal bone resorption similar to that observed for non-pre-immunized but *P. gingivalis* challenged mice. These results strongly indicate that a Th1 response to selective *P. gingivalis* antigens is involved in increased alveolar bone resorption caused by infection with this organism, and confirm our previous findings with a complex mixture of *P. gingivalis* antigens [6].

Although we and others have established that the immune response to *P. gingivalis* antigens leads to the bone destruction caused by this pathogen, little is known about the specific bacterial antigens that are involved in this process. Previous studies have demonstrated that systemic immunization of rats with purified recombinant *P. gingivalis* hemagglutinin B (rHag B) in complete Freund's adjuvant (CFA) resulted in the induction of a mixed Th1/Th2 systemic response, as reflected by the serum IgG subclass specific to rHag B [26]. Interestingly rHag B protected rats against experimental periodontal bone loss induced by infection with *P. gingivalis* had a more polarized Th2 response. Therefore, albeit performed in a different animal species these results indirectly support our findings. However, more recent observations corroborate directly with our results. In these latter studies O'Brien-Simpson et al [27] reported that immunization with *P. gingivalis* proteinase and adhesin epitopes protected mice against experimental periodontitis. Protected animals developed a strong systemic *P. gingivalis*-specific IgG1 response, and a higher ratio of IL-4 to IFN- γ by lymph node cells in response to antigen stimulation in vitro, consistent with the development of a predominant Th2 response. In contrast mice with disease produced an inverse pattern of cytokines. Taken together, these studies with other antigens of *P. gingivalis* are consistent with our findings and support the concept that Th1 responses to *P. gingivalis* are destructive, whereas Th2 responses are protective against oral inflammatory bone loss.

The concept of Th1/Th2 immune response involvement in disease pathology has recently been re-evaluated in the context of vaccine development and immunologic intervention [20,21]. In *Leishmaniasis*, Th1 responses are protective and Th2 response are disease promoting, which is opposite to the *P. gingivalis*, paradigm. Paradoxically, Leishmanial antigens that induced strong Th1 (disease protection) cytokine responses during the natural evolution of the disease usually did not induce protection if they were used in Th1-inducing vaccination protocols. However, antigens that induced a strong Th2 (disease promoting) response were highly protective, if used in vaccination protocols that induce a strong Th1 response to them [13,28]. Using this counter-intuitive strategy, a highly protective anti-*Leishmania* vaccine was developed and has been successfully tested in a Phase I clinical trial (FDA Office of Vaccines, BB-IND #10116).

The novel hypothesis that emerges from this work is that subverting the infectious agent's ability to elicit the disease promoting phenotype can be an efficient strategy in vaccine development against microbial pathogens [20,21]. In the context of the present work, a successful immunotherapeutic candidate to halt the inflammation mediated by *P. gingivalis* will be a protein that during infection induces a potent Th1 response (disease promoter), when used in a vaccination formulation that induces a potent and antigen specific Th2 response. The present findings suggest that PG1841 is such a candidate, based on its ability to induce a potent Th1 response during natural infection with *P. gingivalis*, and the ability to modulate the response to Th2 using an appropriate adjuvant (in this case alum). It is possible that alum alone may not completely bias to sufficiently strong, highly polarized Th2 response, as is the case with the response induced by the Th1 adjuvant CpG. Further modulation procedures may be required to promote Th2 biasing, for example by including Th2 cytokines or anti-IL12 in the adjuvant.

In any case, molecules such as PG1841 can be important tools for the development of vaccines and immunotherapeutics, as well as for a better understanding at the molecular level the pathology caused by this important oral pathogen.

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References

1. Baker PJ, Evans RT, Roopenian DC. Oral infection with *Porphyromonas gingivalis* and induced alveolar bone loss in immunocompetent and severe combined immunodeficient mice. *Arch Oral Biol.* 1994; 39:1035–1040. [PubMed: 7717884]
2. Haffajee AD, Socransky SS, Dzink JL, Taubman MA, Ebersole JL. Clinical, microbiological and immunological features of subjects with refractory periodontal diseases. *J Clin Periodontol.* 1988; 15:390–398. [PubMed: 3165398]
3. Holt SC, Ebersole J, Felton J, Brunsvold M, Kornman KS. Implantation of *Bacteroides gingivalis* in nonhuman primates initiates progression of periodontitis. *Science.* 1988; 239:55–57. [PubMed: 3336774]
4. Socransky SS. Relationship of bacteria to the etiology of periodontal disease. *J Dent Res.* 1970; 49:203–222. [PubMed: 4313844]
5. Stashenko P, Jandinski JJ, Fujiyoshi P, Rynar J, Socransky SS. Tissue levels of bone resorptive cytokines in periodontal disease. *J Periodontol.* 1991; 62:504–509. [PubMed: 1920018]
6. Stashenko P, Goncalves RB, Lipkin B, Ficarella A, Sasaki H, Campos-Neto A. Th1 immune response promotes severe bone resorption caused by *Porphyromonas gingivalis*. *Am J Pathol.* 2007; 170:203–213. [PubMed: 17200194]
7. Ebersole JL, Taubman MA. The protective nature of host responses in periodontal diseases. *Periodontol 2000.* 1994; 5:112–141. [PubMed: 9673165]
8. Gemmell E, Seymour GJ. Immunoregulatory control of Th1/Th2 cytokine profiles in periodontal disease. *Periodontol 2000.* 2004; 35:21–41. [PubMed: 15107056]
9. Takeichi O, Haber J, Kawai T, Smith DJ, Moro I, Taubman MA. Cytokine profiles of T-lymphocytes from gingival tissues with pathological pocketing. *J Dent Res.* 2000; 79:1548–1555. [PubMed: 11023273]
10. Goncalves RB, Leshem O, Bernards K, Webb JR, Stashenko PP, Campos-Neto A. T-cell expression cloning of *Porphyromonas gingivalis* genes coding for T helper-biased immune responses during infection. *Infect Immun.* 2006; 74:3958–3966. [PubMed: 16790769]
11. Skeiky YA, Ovendale PJ, Jen S, Alderson MR, Dillon DC, Smith S, Wilson CB, Orme IM, Reed SG, Campos-Neto A. T cell expression cloning of a *Mycobacterium tuberculosis* gene encoding a protective antigen associated with the early control of infection. *J Immunol.* 2000; 165:7140–7149. [PubMed: 11120845]
12. Skeiky YA, Alderson MR, Ovendale PJ, Lobet Y, Dalemans W, Orme IM, Reed SG, Campos-Neto A. Protection of mice and guinea pigs against tuberculosis induced by immunization with a single *Mycobacterium tuberculosis* recombinant antigen, MTB41. *Vaccine.* 2005; 23:3937–3945. [PubMed: 15917115]
13. Campos-Neto A, Porrozzzi R, Greeson K, Coler RN, Webb JR, Seiky YA, Reed SG, Grimaldi G Jr. Protection against cutaneous leishmaniasis induced by recombinant antigens in murine and nonhuman primate models of the human disease. *Infect Immun.* 2001; 69:4103–4108. [PubMed: 11349082]
14. Stevens TL, Bossie A, Sanders VM, Fernandez-Botran R, Coffman RL, Mosmann TR, Vitetta ES. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature.* 1988; 334:255–258. [PubMed: 2456466]
15. Hourri-Haddad Y, Soskoine WA, Shapira L. Immunization to *Porphyromonas gingivalis* enhances the local pro-inflammatory response to subcutaneous bacterial challenge. *J Clin Periodontol.* 2001; 28:476–482. [PubMed: 11350512]
16. Eastcott JW, Yamashita K, Taubman MA, Harada Y, Smith DJ. Adoptive transfer of cloned T helper cells ameliorates periodontal disease in nude rats. *Oral Microbiol Immunol.* 1994; 9:284–289. [PubMed: 7808770]
17. Bartova J, Kratka-Opatrna Z, Prochazkova J, Krejsa O, Duskova J, Mrklas L, Tlaskalova H, Cukrowska B. Th1 and Th2 cytokine profile in patients with early onset periodontitis and their healthy siblings. *Mediators Inflamm.* 2000; 9:115–120. [PubMed: 10958385]

18. Lappin DF, MacLeod CP, Kerr A, Mitchell T, Kinane DF. Anti-inflammatory cytokine IL-10 and T cell cytokine profile in periodontitis granulation tissue. *Clin Exp Immunol.* 2001; 123:294–300. [PubMed: 11207661]
19. Manhart SS, Reinhardt RA, Payne JB, Seymour GJ, Gemmell E, Dyer JK, Petro TM. Gingival cell IL-2 and IL-4 in early-onset periodontitis. *J Periodontol.* 1994; 65:807–813. [PubMed: 7990015]
20. Campos-Neto A. What about Th1/Th2 in cutaneous leishmaniasis vaccine discovery? *Braz J Med Biol Res.* 2005; 38:979–984. [PubMed: 16007269]
21. Reed SG, Campos-Neto A. Vaccines for parasitic and bacterial diseases. *Curr Opin Immunol.* 2003; 15:456–460. [PubMed: 12900279]
22. Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J Exp Med.* 1997; 186:1623–1631. [PubMed: 9362523]
23. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci USA.* 1996; 93:2879–2883. [PubMed: 8610135]
24. Jankovic D, Caspar P, Zweig M, Garcia-Moll M, Showalter SD, Vogel FR, Sher A. Adsorption to aluminum hydroxide promotes the activity of IL-12 as an adjuvant for antibody as well as type 1 cytokine responses to HIV-1 gp120. *J Immunol.* 1997; 159:2409–2417. [PubMed: 9278332]
25. Faquim-Mauro EL, Coffman RL, Abrahamsohn IA, Macedo MS. Cutting edge: mouse IgG1 antibodies comprise two functionally distinct types that are differentially regulated by IL-4 and IL-12. *J Immunol.* 1999; 163:3572–3576. [PubMed: 10490948]
26. Katz J, Black KP, Michalek SM. Host responses to recombinant hemagglutinin B of *Porphyromonas gingivalis* in an experimental rat model. *Infect Immun.* 1999; 67:4352–4359. [PubMed: 10456874]
27. O'Brien-Simpson NM, Pathirana RD, Paolini RA, Chen YY, Veith PD, Tam V, Ally N, Pike RN, Reynolds EC. An immune response directed to proteinase and adhesin functional epitopes protects against *Porphyromonas gingivalis*-induced periodontal bone loss. *J Immunol.* 2005; 175:3980–3989. [PubMed: 16148146]
28. Afonso LC, Scharton TM, Vieira LQ, Wysocka M, Trinchieri G, Scott P. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major*. *Science.* 1994; 263:235–237. [PubMed: 7904381]

Figure 1. Western blot analysis of PG1841 in *P. gingivalis*

P. gingivalis cells were lysed with SDS, separated by 4–20% SDS-PAGE and transferred to PVDF membrane. The presence of PG1841 protein in the lysate was probed with a specific rabbit anti-rPG1841 antiserum diluted at 1/2,000 (A) or as control with serum obtained from the same rabbit before immunization at the same dilution (B). *Lanes:* 1, purified truncated rPG1841; 2, native *P. gingivalis* W83 antigens. Numbers on left indicate MW of the markers in kDa. The standard MW curve was obtained for the 4–20% gel (C). Arrow and arrow head point to the calculated MW of truncated recombinant protein (32 kDa) and of the expected MW of native molecule (35 kDa) respectively.

Figure 2. Presence of *Pg_1841* in various strains of *P. gingivalis*

Genomic DNAs were obtained from the following strains of *P. gingivalis*: ATCC33277, A7436, H66, 381 and control W83. PCR settings were set according to optimized conditions obtained for genomic DNA of *P. gingivalis* W83 using the primers designed to clone the *PG_1841* gene (Materials and Methods). Lane 1, *P. gingivalis* W83 genomic DNA; lane 2, *P. gingivalis* ATCC 33277 genomic DNA; lane 3, *P. gingivalis* A7436 genomic DNA; lane 4, *P. gingivalis* H66 genomic DNA; and lane 5, *P. gingivalis* 381 genomic DNA. Numbers on the left are the numbers of base pairs of markers of the DNA ladder.

Figure 3. IgG1 and IgG2a isotype-specific antibody response of mice challenged with viable *P. gingivalis*

Sera were obtained from mice before inoculation (Pre) and after two subcutaneous injections (one month apart) with 10^8 CFU of viable *P. gingivalis* (Post). Four weeks following the second inoculation the animals were bled and sera were obtained. Anti-PG1841 antibody responses (IgG1 and IgG2a isotypes) were tested and compared to total whole bacterial cell lysate values by ELISA using a specific HRP-labeled goat anti-mouse immunoglobulin isotypes. Sera were diluted at 1/20.

Figure 4. IgG1 and IgG2a isotype-specific antibody response of mice subcutaneously immunized with recombinant proteins rPG1841 in Th1 and Th2 bias adjuvant

Mice were immunized twice with the recombinant antigens formulated with either Alum (Th2 adjuvant) or Alum plus CpG (Th1 adjuvant), and anti-PG1841 antibody responses (IgG1 and IgG2a isotypes) were tested by ELISA using a specific HRP labeled goat anti-mouse immunoglobulin isotypes. Sera were obtained from mice 2 weeks following the second immunization.

Figure 5. Micro-Computed Tomography (mCT) images of teeth from mice challenged with viable *P. gingivalis*

Groups of BALB/c mice (n=8) were immunized with *P. gingivalis* rPG1841 as described in legend to Fig. 3 followed by challenge in the root canal with $\sim 10^8$ viable bacteria. A–E are representative mCT images obtained from mice of the following groups: (A), immunization with recombinant protein + alum + CpG; (B), immunization with recombinant protein plus alum; (C), immunization with recombinant protein + saline; (D), immunization with saline + alum + CpG; (E), immunization with saline + alum;. Note the extensive bone resorption (arrows) in mouse immunized with PG1841 plus Alum + CpG (Th1 adjuvant) compared to all other groups. These patterns were consistently observed in all mice of each group. The SD of the means of areas of bone loss in each group was in all cases <10%.

Figure 6. Quantitative micro-computed tomography (mCT) analysis of bone loss caused by *P. gingivalis* in immunized mice with rPG1841 formulated either in Th1-biased or Th2-biased adjuvants

Areas of periapical bone loss were calculated from the micro-computed tomography images and are expressed in mm² of increment of the radiolucent area, which is expressed as the difference between the radiolucent areas observed in animals immunized with rPG1841 plus adjuvant subtracted from the radiolucent areas observed in animals immunized with adjuvant alone. Groups of BALB/c mice (n=8) were immunized with *P. gingivalis* rPG1841 formulated in either CpG + Alum (Th1 protocol) or with Alum alone (Th2 protocol) followed by challenge in the root canal with $\sim 10^8$ viable bacteria. The immunization groups were: CpG + alum (Th1 control); CpG + Alum + rPG1841 (Th1 immunization); Alum alone (Th2 control); Alum + rPG1841 (Th2 immunization). Radiolucent areas observed in animals (n=8) not immunized but challenged with *P. gingivalis* was 0.088 ± 0.012 mm² (not shown). Bars represent the mean $\pm$ SE.

Figure 7. Histology of periapical tissues of mice pre-immunized with PG1841 and challenged with viable *P. gingivalis*

Adult BALB/c female mice were immunized with the recombinant antigen followed by infection with *P. gingivalis* in the root canals as described in Material and Methods. Animals were sacrificed and mandibles decalcified. Sections (6 μ) were obtained from the distal root of the first mandibular molar and H&E stained. (A) Inflammation at the periapical site of mice immunized with rPG1841 plus Th1 bias adjuvant and infected with *P. gingivalis*; note the intense mononuclear cell infiltration (asterisk) and the presence of several osteoclasts in bone lesions (arrows); (B) mice immunized with PG1841 plus Th2 adjuvant. Mononuclear cellular infiltration is markedly less intense than in A and fewer osteoclasts are detected (arrow). Magnification, 40x.