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Protease Inhibitors Do Not Affect Antibody Responses to Pneumococcal Vaccination

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HIV$^+$ subjects on optimal antiretroviral therapy have persistently impaired antibody responses to pneumococcal vaccination. We explored the possibility that this effect may be due to HIV protease inhibitors (PIs). We found that in humans and mice, PIs do not affect antibody production in response to pneumococcal vaccination.

Despite available vaccines, *Streptococcus pneumoniae* remains the most common cause of bacterial pneumonia worldwide (1). The bacterium is surrounded by capsular polysaccharides (PSs), which are major determinants of virulence and immunogenicity (2). The existing vaccines against *S. pneumoniae*, the 23-valent PS vaccine (PPV) and 13-valent PS-conjugated vaccine (PCV), contain PSs of 23 and 13 serotypes of *S. pneumoniae*, respectively. Anti-PS antibodies induced by both vaccines provide serotype-specific protection against invasive pneumococcal disease (IPD) (2–5). PPV contains pure PS, while PCV has PS conjugated to a protein carrier (CRM197), which enhances immunogenicity (2). However, a major public health problem is that both vaccines have poor efficacy in the adult populations at high risk for developing IPD, including HIV$^+$ patients on antiretroviral therapy (ART) (6–15). Our research group is currently investigating the possible causes for poor vaccine efficacy in HIV$^+$ individuals on ART.

HIV$^+$ subjects have a 35-fold increased burden of IPD compared with age-matched uninfected controls despite ART (15, 16). Pneumonia remains a leading cause of hospitalization among HIV$^+$ subjects, and *S. pneumoniae* is the most common identified bacterial pathogen (16). HIV$^+$ patients on optimal ART have reduced antibody responses to both pneumococcal vaccines (17, 18). The reasons for this defect in immune function of HIV$^+$ patients who have virologic control are not completely understood. There has been a major emphasis on studying the role of persistent immune activation due to chronic subclinical viral replication (19), but an unexplored hypothesis is that the defect in B cells found in HIV$^+$ patients represents a side effect of the long-term use of certain antiretrovirals, particularly protease inhibitors (PIs).

Recent data indicate that PIs can cause immunological side effects (20–23). PIs constrain HIV replication by binding the HIV aspartyl-proteases and blocking proteolytic cleavage of HIV protein precursors, including Gag and Pol polyproteins, but they can also affect human cellular proteases at pharmacological concentrations (20). PIs reduce dendritic cell (DC) production of cytokines important in adaptive immunity (interleukin-12 [IL-12] and IL-15) and impair DC surface expression of key molecules for antigen presentation (CD86, CD36, CD1d, and CD209) *in vitro* (21). In mice infected with lymphocytic choriomeningitis virus (LCMV), PIs inhibit tumor necrosis factor alpha (TNF-α) production and proteasome activity and interfere with major histocompatibility complex (MHC) class I presentation, thereby reducing cytotoxic T lymphocyte responses (22). PIs may impair host defense as they increase LCMV viral load after LCMV infection *in vivo* and promote hepatitis B virus replication (22, 24). Finally, PIs also inhibit proliferation and induce apoptosis in human B cell lines (23).

There are numerous trials of pneumococcal vaccine efficacy and immunogenicity in HIV-infected individuals (5, 13, 15, 17, 25). However, no trial has addressed the question of whether different types of antiretroviral therapy (e.g., PIs versus non-PIs) affect pneumococcal vaccine efficacy. In addition, the effects of PIs on B cell responses against pneumococcal vaccines are not clear. We hypothesized that PIs impair antibody responses to pneumococcal vaccines. We focused on antibody responses to PPV since human samples from a clinical trial were available and this vaccine is still recommended and widely used in HIV$^+$ patients (15, 17). We determined the effects of the PI ritonavir on quantitative and qualitative B cell responses to PPV by measuring PPV-specific B cell frequencies, serum antibody levels, and opsonophagocytic killing activity (OPA), an *in vitro* assay that measures the ability of vaccine-induced antibodies to facilitate opsonization and killing of *S. pneumoniae* by human phagocytes (26, 27).

PIs do not impair antibody responses to PPV in mice. As mice are excellent models of the human immune response to pneumococcal vaccines (1), we used this model to assess whether PIs affect antibody responses to pneumococcal vaccination *in vivo*. We focused on ritonavir as it is the most commonly used PI (28). We administered ritonavir (20 to 30 mg/kg body weight) (Selleckchem) or vehicle (30% polyethylene glycol 400 [PEG 400], 5% Tween 80, and 5% propylene glycol) by intraperitoneal injections to 6- to 8-week-old C57BL/6 mice daily for 15 days. On the second day of ritonavir or vehicle treatment, mice were intraperitoneally

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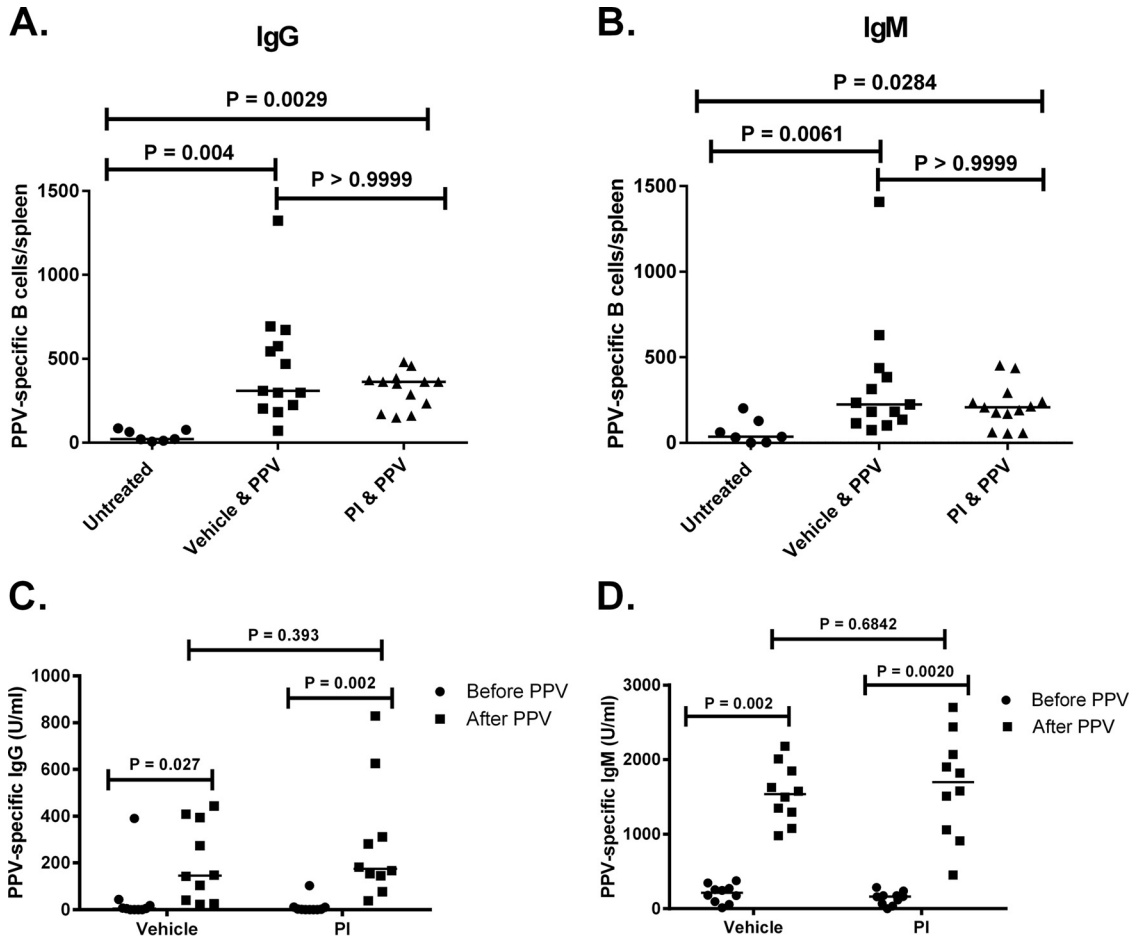

FIG 1 PIs do not impair IgG/IgM antibody production against PPV in mice. Splenocytes from mice treated with PI (ritonavir) or vehicle were collected and processed to measure PPV-specific B cells producing IgG (A) or IgM (B) using ELISpot. Data points represent the numbers of animals (13 mice per group). Seven untreated mice were used as control for ELISpot. In addition, ELISA was performed to measure serum levels of PPV-specific IgG (C) or IgM (D) at baseline and at day 15 post-PPV vaccination (10 mice per group). The Kruskal-Wallis test (ELISpot), Wilcoxon signed-rank test (ELISA, before PPV versus after PPV) and Mann-Whitney test (ELISA, vehicle after PPV versus ritonavir after PPV) were performed. Medians are shown. $P < 0.05$ was considered significant. Three independent experiments are shown.

injected with 100 μl of PPV (Pfizer) diluted 1:10 in phosphate-buffered saline (PBS). This method, including similar ritonavir doses and exposure time, has been used previously to show that ritonavir and other protease inhibitors markedly inhibit the proliferation of B cell lines *in vitro* (23) and impair cytotoxic T lymphocyte activity and T cell expansion against lymphocytic choriomeningitis virus (LCMV) infection in mice (22, 23, 29, 30). Most memory B cells that respond against *S. pneumoniae* are generated in the spleen (31). To determine if the numbers of PS-specific B cells were reduced after PI exposure, spleens were processed using a 40-μm-pore-size cell strainer (Falcon) and splenocytes were collected in RPMI medium (Lonza) to perform enzyme-linked immunospot (ELISpot) analysis 15 days after PPV immunization (32), the critical period for B cell expansion and antibody production (33). B cells were incubated in 96-well plates coated with PPV overnight at 37°C with 5% CO_2. After incubation, B cells were washed away, and plates were incubated with either biotin–anti-IgG (Biolegend) or biotin–anti-IgM (Biolegend) and developed using streptavidin (BD Biosciences) and 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIPD) (Sigma). The frequencies of B cells that produced PPV-specific IgG and IgM antibodies were quantitated manually. Spleens from untreated and unvaccinated mice were used as the control ($n = 7$). There was a significant increase in the numbers of PPV-specific B cells producing IgG and IgM antibodies in mice vaccinated with PPV ($n = 13$) versus unvaccinated/untreated mice (Fig. 1A and B). However, no significant differences were found in the numbers of B cells producing PPV-specific antibodies in the groups treated with ritonavir versus those treated with vehicle (13 mice per group) (Fig. 1A and B). These results indicate that ritonavir does not impair PPV-specific B cell frequencies postvaccination. We performed the enzyme-linked immunosorbent assay (ELISA) (Alpha Diagnostics International) to assess the serum concentrations of PPV-specific IgG and IgM antibodies in mice treated with ritonavir or vehicle before and after PPV immunization (10 mice per group). There was a significant increase in PPV-specific IgG and IgM serum levels 15 days following PPV vaccination, but no differences were found between mice treated with ritonavir and those treated with vehicle (Fig. 1C and D). Finally, we evaluated whether ritonavir affected OPA against *S. pneumoniae*. We collected mouse sera before and

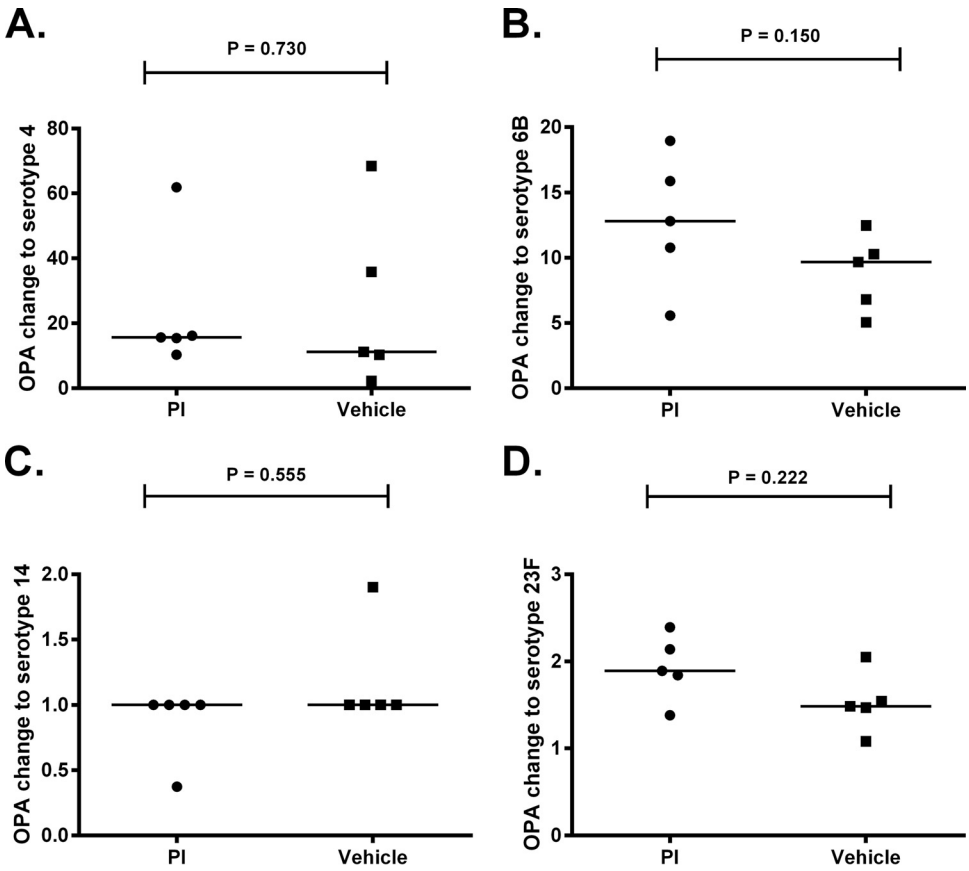

FIG 2 PIs do not affect opsonophagocytic killing activity (OPA) against *S. pneumoniae* in mice. OPA assays against vaccine serotypes of *S. pneumoniae* (4, 6B, 14, and 23F) were performed on mouse sera obtained at baseline and at day 15 post-PPV vaccination (5 mice per group). Medians are shown. OPA change was calculated as the OPA measured with serum obtained at day 15 divided by that with serum obtained at baseline. The Mann-Whitney test was performed. $P < 0.05$ was considered significant.

after PPV vaccination and compared OPAs against several vaccine serotypes of *S. pneumoniae* (4, 6B, 14, and 23F) between mice treated with ritonavir and those treated with vehicle. We did not find differences in OPAs between mice treated with ritonavir and those treated with vehicle (Fig. 2A to D). Although this could have been due to the small number of animals tested (5 mice per group), the lack of any trend toward a difference and our human data related to OPA suggest that testing additional animals would not find differences. Taken together, our results indicate that the PI ritonavir does not affect quantitative and qualitative antibody responses to PPV in mice. All experiments involving mice were performed in

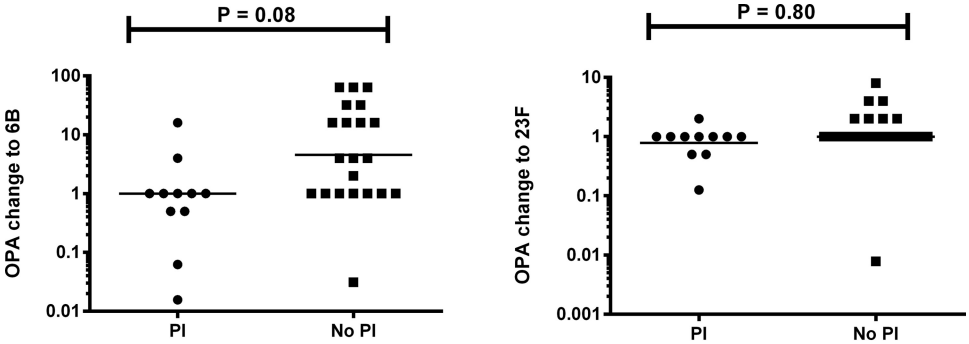

FIG 3 PIs do not affect opsonophagocytic killing activity (OPA) against *S. pneumoniae* in humans. OPA assays against *S. pneumoniae* of serotypes 6B and 23F were performed on human serum samples obtained before and after PPV vaccination. OPA change was calculated using serum obtained 1 month after PPV divided by serum obtained before PPV vaccination. Eleven subjects were on PI, and 21 subjects were on non-PI antiretrovirals (No PI). Medians are shown. *P* values were calculated and adjusted for age, $CD4^+$ T cell counts, and viral loads at the time of vaccination, using linear regression analysis. $P < 0.05$ was considered significant.

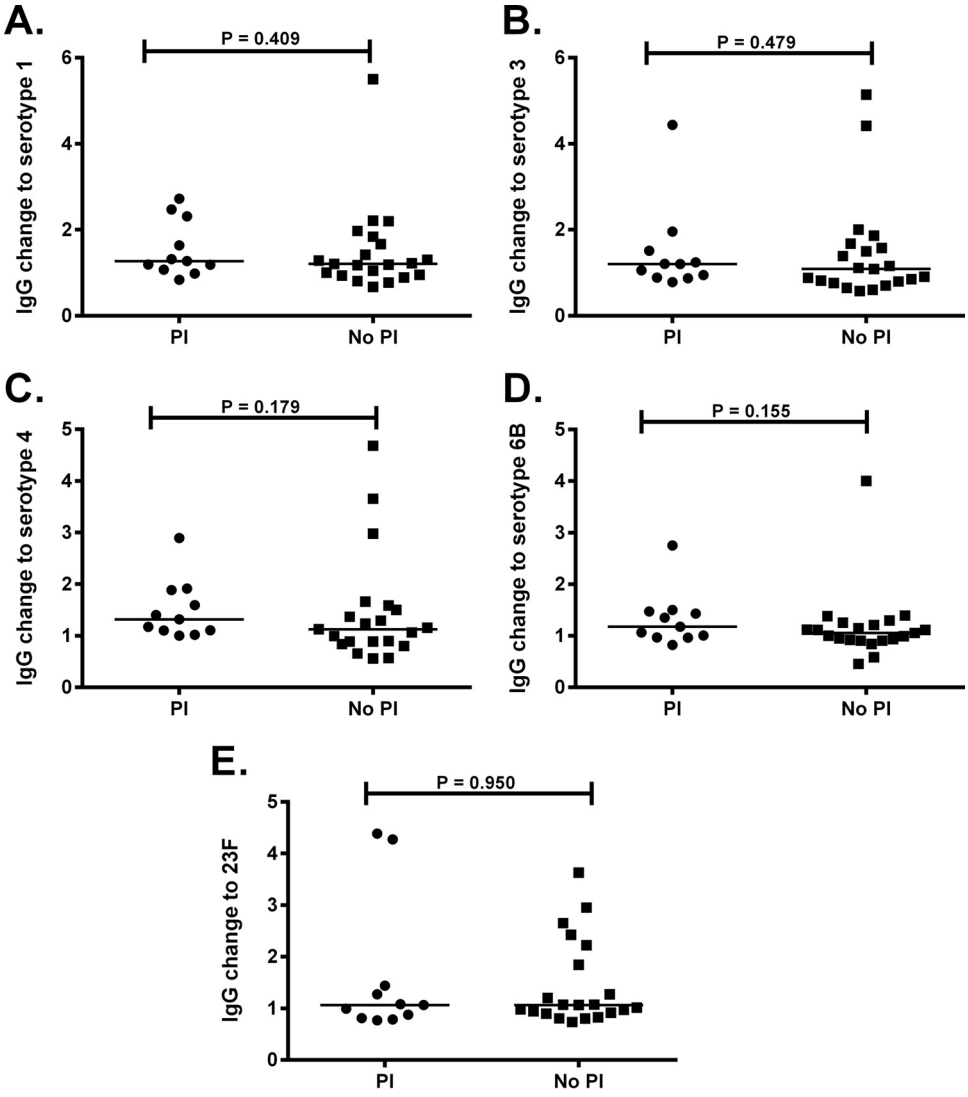

FIG 4 PIs do not impair IgG antibody production against PS in humans. ELISA was performed using human sera to detect the presence of IgG antibodies against the PS serotypes 1, 3, 4, 6B, and 23F, which are included in pneumococcal vaccines. IgG change was calculated as the serum levels of IgG obtained 1 month after PPV vaccination divided by IgG levels obtained before vaccination. Eleven subjects were on PI, and 21 subjects were on non-PI antiretrovirals (No PI). The Mann-Whitney test was performed. Medians are shown. $P < 0.05$ was considered significant.

compliance with protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

PIs do not decrease OPA and antibody production to PPV in humans. As PIs may impair human proteases (22) and other enzymatic targets (23) but not murine enzymes, we assessed the effects of PIs in humans using pre- and postvaccine samples from a clinical trial in which we evaluated the immunogenicity of PPV in 36 HIV$^+$ subjects on ART and 36 HIV$^+$ subjects who were not on ART (17). All subjects had CD4$^+$ counts of >200 cells/μl. For this study, we specifically focused on the 36 HIV$^+$ subjects who were on ART. Of the 36 HIV$^+$ subjects on ART, 11 were on PI and 21 were on non-PI-based regimens. Three subjects were not included in this study because they were noncompliant with their ART. One month after PPV vaccination, the subjects treated with PIs ($n = 11$) did not have reduced OPAs against vaccine serotypes 6B and 23F compared with those treated with regimens not containing PIs ($n = 21$) (Fig. 3). The results were adjusted for age, CD4$^+$ T cell counts, and viral load. There was no difference between PI-treated ($n = 11$) and non-PI-treated ($n = 21$) subjects in postvaccine serum IgG or IgM titers against PS contained in PPV using ELISA (Fig. 4). Five out of 11 (45.5%) subjects from the PI group and 7 out of 21 (33.3%) from the non-PI group responded to at least one PS vaccine ($P = 0.7$, Fisher's test). We used the standard definition of responders: ≥2-fold increase in PS-specific IgG 1 month postvaccination with an absolute concentration of at least 1 μg/ml. Of note, the prevaccine serum concentrations of PS-specific IgG were similar between the groups. The prevaccine median serum concentrations of 6B-specific IgG were 1.18 μg/ml (interquartile range [IQR], 0.97 to 1.48) and 1.06 μg/ml (IQR, 0.92 to 1.23) in the PI group and non-PI group, respectively ($P = 0.16$, Mann-Whitney test). The prevaccine median serum concentrations of 23F-specific IgG were 1.07 μg/ml (IQR, 0.81 to 1.44) and 1.06 μg/ml (IQR, 0.91 to 2.03) in the PI group and non-PI group, respectively ($P = 0.95$, Mann-Whitney test). Overall, PIs

do not affect the capacity of postvaccine serum to opsonize *S. pneumoniae* or affect antibody production against PPV in humans.

Most unvaccinated adults have been previously exposed to pneumococcal antigens by colonization or prior infections during childhood (1). The vast majority of the HIV$^+$ subjects in our study had detectable serum levels of pneumococcus-specific IgG before PPV vaccination, indicating that they had been exposed to pneumococcal antigens previously and that the post-PPV responses that we measured were recall responses. The presence of PS-specific memory B cells and PS-specific antibodies after PPV vaccination is associated with protection against IPD (34–36). HIV$^+$ patients on ART remain at high risk for developing IPD (37), and the efficacy of existing pneumococcal vaccines is suboptimal in this patient group (37, 38). We found that PIs do not impair PS-specific antibody production and OPA following PPV immunization in humans and mice, indicating that PIs do not play a causal role in the persistent B cell dysfunction observed in HIV$^+$ patients on ART (18) and in the increased incidence of pneumococcal pneumonia observed among these patients (16–18). The results provide reassurance about using PIs in humans and support investigation of other mechanisms, such as the impact of subclinical viral replication in antiretroviral-treated HIV$^+$ patients or the effects of non-PI antiretrovirals on B cell responses.

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