Booster immunization with a fractional dose of Prevnar 13 affects cell-mediated immune response but not humoral immunity in CD-1 mice

Rose-Marie Catalioto a,*, Claudio Valentia, Francesca Belluccia, Cecilia Cialdai a, Maria Altamura b, Laura Digilio c, Andrea Ugo Enrico Pellacanid, Stefania Meini d

a Experimental Pharmacology Department, Menarini Ricerche S.p.A., Via dei Sette Santi 1, I-50131 Florence, Italy
b Corporate Preclinical Development for New Technologies, Antiinfectives and Non-oncological Drugs, A. Menarini NewTech S.r.l., Via dei Sette Santi 1, I-50131 Florence, Italy
c Independent Consultant, Italy
d Menarini Ricerche S.p.A., Via dei Sette Santi 1, I-50131 Florence, Italy

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ABSTRACT
Achieving durable protective immunity following vaccination is dependent on many factors, including vaccine composition and antigen dose, and it has been investigated for various types of vaccines. Aim of the present study was to investigate the overall immune response elicited by two different booster doses in CD-1 mice, by exploiting the largely used 13-valent pneumococcal conjugate vaccine Prevnar 13® (PCV13). Immunization was performed by two primary doses of PCV13 two weeks apart, and a full or fractional (1/5) booster dose on week 10. Serotype-specific antibody titer, avidity, and opsonophagocytic activity were evaluated one week later, and compared to cell-mediated immunity (CMI) responses determined as the frequency of cytokines producing splenocytes by in vitro recall with the antigens (carrier protein and polysaccharides). Data showed that regardless of the booster dose, a comparable humoral response was produced, characterized by similar amounts of serotype-specific antibodies, with analog avidity and opsonophagocytic properties. On the other hand, when CMI was evaluated, the presence of CRM197-specific IL-5 and IL-2 producing cells was evident in splenocytes from mice immunized with the full dose, while in those immunized with the fractional booster dose, IFN-γ producing cells responsive to both protein and polysaccharide antigens were significantly increased, whereas the number of IL-5 and IL-2 positive cells remained unaffected. Overall the present findings show that PCV13 humoral response in mice is associated to a Th2 predominant response at the full booster dose, while the fractional one favors a mixed Th1/Th2 response, suggesting an important role of CMI besides measurement of functional protective antibodies, as an additional and important key information in vaccine development.

1. Introduction
Achievement of high quality and long-lasting immune responses giving durable protective immunity following vaccination is determined by several factors and vaccine composition is of particular importance. Indeed, the amount of antigens per dose influences the magnitude of host immune response as well as protection efficacy. Although increasing the antigen dose is expected to lead to improved immune response, the immunogenicity and safety of reduced antigen dose in primary and boost administrations has been investigated for various types of vaccines including combined Tetanus Diphtheria and Acellular Pertussis (Tdap), influenza, and yellow fever vaccines [1, 2, 3], indicating that reduced doses may show immune responses equivalent to that of standard ones. The possibility to reduce the antigen dose could be exploited to produce combination vaccines and to improve reactogenicity as in the case of Tdap [4], or to overcome limitation in vaccine manufacturing capacity to avoid vaccine shortages and reduce total costs.

For glycoconjugate vaccines such as Prevnar 13® (13-valent pneumococcal conjugate vaccine, PCV13), indicated for the prevention of pneumonia infections and disease burden caused by Streptococcus pneumoniae, dose sparing has been evaluated by reducing the number of vaccine doses and modifying intervals between doses [4, 5]. PCV13 is composed of purified capsular saccharides of S. pneumoniae conjugated to an immunogenic protein carrier, the cross-reactive material 197
immunogenicity in terms of serotype-specific differences in the production of functional antibodies [10, 11]. More recently, it has also been shown that even reducing the number of priming doses, but also presence and timing of a booster vaccination [9]. Similar efficacy in reducing pneumonia symptoms in children with pneumococcal pneumonia and emphysema was reported for different schedules, supported by WHO recommendations, regarding the number of priming and booster doses and timing of immunization showing no differences in the production of functional antibodies [10, 11]. More recently, it has also been shown that even reducing the number of primary doses (followed by a booster) does not compromise the long-term immunogenicity in terms of serotype-specific IgG concentration and opsonophagocytic activity (OPA) [12], thus confirming that immunogenicity of the PCV booster dose is relatively independent from the number of priming doses.

Other than the prime-boost regimen, and the number and intervals among doses, also the amount of antigen contained in the priming versus boosting doses is an important determinant of immune response. Some reports have demonstrated that not always higher antigen doses, for both priming or boosting, are the most effective, both in human clinical trials and animal models [13, 14]. A greater antibody protection and affinity maturation has been shown to improve by a delayed fractional booster dose following administration of a malaria vaccine [15]. To our knowledge, preclinical studies in mice to evaluate the immunogenic properties of PCVs, have focused on the influence of mice strain, vaccine composition, routes of immunization and intervals on antibody levels and functionality [16, 17, 18, 19, 20], but no reports have investigated the effect of the booster dose, in both humoral and cell-mediated immune response. In the present study, the immunogenic responses produced by PCV13 in a murine model were investigated using two different boosting dose regimens, i.e. the full booster dose was compared to a fractional dose, both following two priming administrations with the full dose. Immune responses were evaluated in terms of antibody titer, avidity, and opsonophagocytic activity, and compared to cell-mediated immune responses by determining the frequency of cytokine (IFN-γ, IL-2, IL-4, IL-5)-producing splenocytes after in vitro recall with both protein or polysaccharide antigens or polyclonal activation. Results show that the use of a fractional booster dose of PCV13 vaccine, although not modifying both qualitatively and quantitatively the humoral response, however did have an impact on CMI responses, favoring the shift from a Th2 into a mixed Th1/Th2 profile, thus suggesting that this aspect should be carefully explored during the development of experimental vaccine candidates.

2. Methods

2.1. Mouse immunization and blood sample collection

All animal experiments were performed in strict accordance with the European guidelines on the protection of animals used for scientific purpose (Directive 2010/63/EU) and Italian legislation (D.lgs. 26/2014). The protocol was approved by the Institutional Animal Welfare Body (Organismo Preposto al Benessere degli Animali, OPBA, Menarini Ricerche SpA). Six week old female CD-1 mice were purchased from Charles River Laboratories (Charles River, Lecco, Italy), and housed in the animal facility with food and water available ad libitum. Mice were randomly assigned to treatment groups using a random number generator provided by QuickCalcs web site (GraphPad, https://www.graphpad.com/quickcalc/) and immunized s.c., 2 weeks apart, with 2 priming clinical doses (or standard herein) of Prevnar 13® (Wyeth Pharmaceuticals, UK) (2.2 μg per polysaccharide except serotype 6B, 4.4 μg) formulated with 125 μg AlPO4 adjuvant in 5 mM succinic acid pH 5.8 in saline. The standard dose was selected on the basis of previous investigations aimed to prove the immunogenicity of Prevnar 13® and other PCVs [20, 21, 22, 23]. On week 10 primed animals received the standard dose of vaccine, as in the primary series, or a fractional dose (0.5 μg per polysaccharide except serotype 6B, 1 μg). Mice (n = 5) immunized with adjuvant alone were used as control animals (naïve). On week 11 blood was collected by terminal intracardiac puncture in tubes containing clotting activator (Sarstedt, Numbrecht, Germany). After 30 min at RT, the clot was pelleted by centrifugation (2000 x g, 10 min, 4 °C), and collected sera decompplemented and stored at –80 °C.

2.2. Serotype-specific enzyme-linked immunosorbent assay (ELISA)

The level of IgG antibodies specific for S. pneumoniae capsular polysaccharides (PnPs ELISA) was quantified by determining endpoint titres according to the WHO standard ELISA [24] (www.vaccine.uab.edu). Briefly, medium binding 96-well microplates (Microlon® - Greiner Bio-One, Milano, Italy) were incubated for 5 h at 37 °C with PnPs (ATCC/LGC Standards, Milano, Italy) at appropriate concentration: 1.25 μg/ml for PnPs 4, 5, 7F, 9V, 14; 2.5 μg/ml for PnPs 3; 5 μg/ml for PnPs 1, 6B, 18C, 23F; and 10 μg/ml for PnPs 6A, 19A, 19F. For assay, 3-fold serial dilutions (1:10 to 1:21870) of serum samples were performed in buffer containing 10 μg/ml of pneumococcal CPS Multi (Statens Serum Institut, Copenhagen, DK) and added to antigen coated plate for 2 h at RT. The antigen-antibody complex was detected by incubating plates first with alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (ab49678, Abcam, Cambridge, UK,) diluted 1:40000 (except for 23F, 1:10000) in antibody buffer for 2 h at RT, then with p-nitrophenyl phosphate in substrate buffer for another 2 h period at RT in the dark. The enzyme reaction was stopped by adding 3M NaOH, and absorbance read at 405 nm, with 690 nm as a reference wavelength, by a microtiter plate reader (Synergy HT BioTek, Winooski, USA). Between each step, plates were washed with PBS-0.05% Tween 20. IgG titers against each PnPs were determined by plotting the logarithm of OD values against the logarithm of the inverse of serum sample dilution (Log-Log plot). The reciprocal of the linearly interpolated dilution corresponding to the lower limit of detection, LOD, expressed as 2-fold the average of blank values, was used to determine the antibody titer.

2.3. Antibody avidity assay

The avidity of PnPs-specific IgG was investigated by employing the ammonium thiocyanate (NaSCN) elution ELISA method. For each serotype, titration curves were performed to select the appropriate concentration of chaotropic agent inducing the dissociation of the antigen–antibody complexes following indications of previous reports [25, 26, 27]. Seven 2.5-fold serial dilutions (from 1:60 to 1:14648) of selected mouse antisera with antibody titer against Prevnar 13 serotypes ranging from high (sample 1) to medium/low (sample 2 and 3) were dispensed in PnPs-coated plates (six technical replicates each) and incubated at RT for 2 h. After washing, all wells corresponding to six complete serum dilution curves received 50 μl of assay buffer (control) or NaSCN at 0.3, 0.5, 0.65, 0.8, or 0.95 M concentration. The plates were incubated at RT, for 15 min, and then processed for anti-PnPs IgG detection (as described in paragraph 2.2). The PnPs-specific IgG titer, in the absence (control) or presence of NaSCN, was obtained by interpolating the serial dilution corresponding to the LOD (Table 1), and the NaSCN-induced percent reduction of IgG titer as compared to control was calculated (Table 2) and plotted (Figure 1) to determine the lowest concentration of NaSCN reaching approximately the maximum antibody titer reduction in all three samples. Selected concentrations of NaSCN for each serotype were: 0.5 M (PnPs 1, 3, 4, 5, 6A, 6B, 7F, 9V, 19F, and 23F), 0.65 M (PnPs 18C and 19A), or 0.8 M (PnPs 14). Serial dilutions (from 1:60 to 1:36621) of serum samples were dispensed into PnPs-coated plates and incubated at RT for 2 h. Plates were then washed and
incubated for 15 min at RT in the absence or presence of NaSCN. Thereafter, plates were treated as described above (paragraph 2.2) to detect the antigen–antibody complexes resistant to NaSCN treatment. Results are expressed as avidity index (AI), reflecting the percentage of antibodies that remained bound to the antigens after NaSCN elution. AI was calculated for each serotype by dividing the IgG titer determined in the presence of NaSCN by the IgG titer in the absence of NaSCN and multiplying by 100 [25].

2.4. Bacterial strains and culture

Streptococcus pneumonia serotypes to be used in the opsonophagocytic killing assay (OPA) were obtained from Statens Serum Institut (Copenhagen, DK). Freeze-dried bacteria were reconstituted at 37 °C in Todd-Hewitt-Yeast (THY) broth containing 5 g/l yeast extract (Biotec, Grosseto, Italy) and spreaded into blood agar plates (Biotec, Grosseto, Italy). Colonies that developed after 24 h of incubation at 37 °C in a 5% CO₂ incubator, were cultured in suspension in 50 ml THY broth at 37 °C until they reach exponential growth phase. Bacteria were then pelleted, resuspended in THY medium with 16% glycerol, and stored at –80 °C.

2.5. Opsonophagocytic killing assay

The functionality of antibodies against Streptococcus pneumoniae was determined by a standardized OPA using human leukemia HL-60 granulocytes as described in detail at [28]. HL-60 cells (ATCC CCL240) were differentiated into phagocytes by treatment for five days with 0.8% N,N-dimethylformamide (Sigma). Serial dilutions of serum samples (from 1:10 to 1:238419) were dispensed into 96-well microplates, with bacteria strain, HL-60 granulocytes (at a 400:1 effector-to-target cell ratio), and baby rabbit complement (Cedarlane) for the development of opsonophagocytic process. Thereafter, the content of the wells was spotted onto THY extract agar plate for the growth of surviving bacteria as colony forming unit (CFU), that were counted by using a high resolution photo scanner (ProcScan, Synbiosis, Cambridge, UK) and the colony counting software, NICE (National Institute of Immunology, New Delhi, India). For each serotype, selected concentrations of NaSCN to be used in antibody avidity assay (Figure 2B) are highlighted in bold.

### Table 1. Pneumococcal serotype-specific IgG titers of selected mouse antisera with antibody levels against Prevnar 13 serotypes ranging from high (sample 1) to medium/low (sample 2 and 3) determined in the absence (control) or presence of NaSCN at the indicated concentrations.

| NaSCN [M] | Antibody titer of serotype-specific anti-PnPS IgG |
|-----------|--------------------------------------------------|
|           |  1   |  3     |  4    |  5 |  6A |  6B |  7F |  9V |  14 |  18C |  19A |  19F |  23F |
| Sample 1  |  0.95 | 1214  | 554  | 37810 | 387 | 2264 | 119 | 436 | 8321 | 8741 | 435 | 3058 | 5518 | 12372 |
|           |  0.8  | 1434  | 620  | 41162 | 448 | 2611 | 123 | 507 | 8932 | 12675 | 534 | 3335 | 7294 | 13569 |
|           |  0.65 | 2394  | 767  | 48917 | 756 | 3375 | 126 | 737 | 10145 | 16882 | 692 | 4420 | 13761 | 15683 |
|           |  0.5  | 3501  | 1327 | 60995 | 879 | 4560 | 128 | 769 | 9608 | 19289 | 865 | 5070 | 17091 | 18193 |
|           |  0.3  | 7168  | 2091 | 9869  | 164 | 152  | 900 | 117 | 4403 | 1081 | 212 | 5773 | 1205 | 326   |
|           |  0  | 21534 | 11734 | 101814 | 5280 | 22302 | 1402 | 1715 | 14819 | 29147 | 1813 | 12203 | 2972296 | 30263 |

Table 2. Percent reduction of pneumococcal serotype-specific IgG titers induced by the indicated concentrations of NaSCN as compared to control (0 M NaSCN) calculated from data in Table 1.

| NaSCN [M] | Antibody titer % reduction compared to control |
|-----------|------------------------------------------------|
|           |  1 |  3 |  4 |  5 |  6A |  6B |  7F |  9V |  14 |  18C |  19A |  19F |  23F |
| Sample 1  |  0.95 | 94 | 95 | 63 | 93 | 90 | 92 | 75 | 44 | 71 | 76 | 75 | 100 | 59   |
|           |  0.8  | 93 | 95 | 60 | 92 | 88 | 91 | 70 | 40 | 58 | 71 | 73 | 100 | 55   |
|           |  0.65 | 89 | 93 | 52 | 86 | 85 | 91 | 57 | 32 | 44 | 62 | 64 | 100 | 48   |
|           |  0.5  | 84 | 89 | 40 | 83 | 80 | 91 | 55 | 35 | 36 | 52 | 58 | 99 | 40   |
|           |  0.3  | 67 | 82 | 22 | 75 | 70 | 90 | 43 | 26 | 17 | 30 | 34 | 98 | 26   |
| Sample 2  |  0.95 | 90 | 93 | 37 | 74 | 86 | 94 | 39 | 54 | 42 | 91 | 85 | 87 | 91   |
|           |  0.8  | 89 | 92 | 34 | 70 | 87 | 93 | 40 | 50 | 38 | 90 | 79 | 82 | 91   |
|           |  0.65 | 85 | 90 | 25 | 61 | 86 | 89 | 35 | 40 | 27 | 88 | 67 | 78 | 90   |
|           |  0.5  | 84 | 87 | 20 | 52 | 83 | 84 | 39 | 34 | 23 | 84 | 56 | 75 | 89   |
|           |  0.3  | 75 | 78 | 17 | 30 | 76 | 64 | 29 | 16 | 10 | 67 | 35 | 56 | 83   |
| Sample 3  |  0.95 | 89 | 96 | 18 | 84 | 24 | 85 | 14 | 75 | 30 | 78 | 45 | 98 | 10   |
|           |  0.8  | 89 | 95 | 21 | 82 | 23 | 83 | 18 | 67 | 23 | 76 | 44 | 97 | 12   |
|           |  0.65 | 87 | 95 | 16 | 75 | 18 | 76 | 12 | 57 | 13 | 71 | 34 | 95 | 12   |
|           |  0.5  | 84 | 92 | 14 | 69 | 14 | 66 | 26 | 53 | 18 | 58 | 27 | 91 | 12   |
|           |  0.3  | 66 | 88 | 12 | 52 | 8 | 51 | 16 | 37 | 10 | 48 | 22 | 83 | 10   |
Institute of Standards and Technology, US). The percent killing values (ratio between the number of CFU/spot detected in test samples and the number of CFU/spot in wells without serum sample) were plotted against the inverse of serum sample dilution on logarithmic scale, and fitted with a four-parameter logistic curve to determine the opsonic index (OI), i.e., the serum dilution corresponding to 50% killing. Acceptance criteria were that non-specific killing, i.e., killing of bacteria in the absence of test serum, was \( \leq 70\% \), and the maximum CFU/spot was \( \leq 210 \) and \( \geq 180 \).

### 2.6. Preparation of mouse splenocytes

Mice were euthanized, spleens were removed and processed individually to prepare single cell splenocyte suspensions [29]. Spleens were minced under aseptic conditions and subsequently pressed gently using the plunger of a syringe through a cell strainer (70 μm mesh, Becton Dickinson/Falcon) into a 50 ml Falcon tube and flushed with PBS. Splenocytes were washed by centrifugation and erythrocytes were lysed by using the red blood cell lysis buffer (eBioscience Invitrogen/Thermo Fisher, 00–4333) according to manufacturer’s instructions. After further washing steps, the cell pellet was resuspended in RPMI 1640 medium (10% heat-inactivated FBS, 1 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin), counted and checked for viability using the trypan blue exclusion assay and the countess cell counter. Splenocytes preparations were finally resuspended in 90% FBS, 10% DMSO and cryopreserved in liquid nitrogen until use [30]. During thawing, splenocytes suspensions were treated with CTL Anti-Aggregate Wash™ medium (CTL-AA-005, Cellular Technology Limited Europe) to prevent cell loss through aggregation [31].

### 2.7. FluoroSpot assessment of cytokine producing cells

The number of cytokines producing cells was determined by using the murine fluorescent IFN-γ/IL-4, IL-2, and IL-5 ImmunoSpot® Test Kits from Cellular Technology Limited Europe. Briefly, splenocyte preparations from individual mice were resuspended in CTL-Test™ medium plus 2 μg/mL anti-CD28 (clone: 37.51; eBioscience™ ThermoFisher) at a density of \( 3 \times 10^6 \) cells/ml. 100 μl of the suspension was plated into a 96-well FluoroSpot plate coated with capture antibody and containing 100 μl of the appropriate stimulus. Medium alone was used as a negative control whereas a solution of PMA/ionomycin (81 nM/1.34 μM) (eBioscience™ cell stimulation cocktail, Invitrogen-Thermofisher) was used as a positive control. Other stimuli included: CRM197 (ab188505, Abcam, Cambridge, UK) and PnPS mixture (2.2 μg/ml each serotype, except 6B 4.4 μg/ml). After 22–24 h incubation at 37 °C in a CO2 incubator, FluoroSpot plates were run and developed according to the manufacturer’s protocol. Cytokine positive spots were imaged, analyzed, and counted.

![Figure 1. Selection of the optimal concentration of NaSCN inducing the dissociation of antigen–antibody complexes. The percent reduction of IgG titer by NaSCN as compared to control (0 M NaSCN) calculated for selected mouse antisera with antibody levels against Prevnar 13 serotypes ranging from high (sample 1) to medium/low (sample 2 and 3) (Table 2) was plotted to determine the lowest concentration of NaSCN reaching approximately the maximum antibody titer reduction in all three samples.](image)
using an ELISPOT reader (Immunospot S6 universal reader) equipped with the ImmunoSpot software version 7.0.15.1 (Cellular Technology Limited Europe). The number of cells secreting Th1 cytokines (IL-2, IFN-γ) or Th2 cytokines (IL-4 or IL-5) was quantified and expressed as Spot-Forming Units (SFU) per $3 \times 10^5$ splenocytes.

2.8. Statistical analysis

Data and statistical analyses were performed by using the Prism software (version 7.03, GraphPad, La Jolla, CA, USA). IgG titer and OI were expressed as geometric mean and 95% confidence interval (GMT ± 95% C.I.). Differences in IgG or OPA titers between immunization groups were assessed after logarithmic transformation of data, two-way ANOVA analysis of variance and Sidak’s multiple comparison tests. Avidity data were expressed as the median with interquartile range (IQR). Independent sample Mann–Whitney U test was used to assess differences in AI values between vaccine groups, and one-sample t test analysis was performed to classify serotypes as low (<40%) or high (>60%) avidity. ELISPOT data were expressed as mean ± standard error of mean (S.E.M.), and datasets analyzed by One-Way or Two-Way ANOVA followed by multiple comparisons as described in Figure legends. Differences were considered significant at p values < 0.05 and were indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001; ns, not significant.

3. Results

3.1. Effect of the fractional booster dose on PCV13 humoral immune responses

3.1.1. Serotype-specific IgG levels and avidity

The effect of the booster dose on humoral immunity was first investigated by measuring antibody level and avidity against each pneumococcal serotype. Results showed that PCV13 administered at standard human dose induced high antibody titers with immunogenicity and avidity varying based on polysaccharide nature. Capsular-specific IgG GMTs ranged from 806 (129–5033, 95% CI) for serotype 23F to 39128 (8139–188106, 95% CI) for serotype 6B ($p < 0.05$) (Figure 2A). Serum...
levels of IgG against serotype 23F were also significantly lower than those obtained for serotype 1 (GMT = 8998, 2333–34707, 95% CI) (p < 0.01) and for serotypes 4, 9V and 19A, GMT = 21398 (12406–36906, 95% CI), 17303 (7579–39502, 95% CI) and 14406 (3556–58352, 95% CI) (p < 0.05), respectively. IgG against serotypes 9V and 14 displayed a high avidity index with median value of 83% (IQR 82.5%), while a low avidity index was observed for IgG against serotypes 3, 6B and 19A with median values of 10% (IQR 8–14%), 8% (IQR 6.5–27.5%), and 18% (IQR 8.5–30%) (Figure 2B) and for serotypes 1 and 19F, AI = 11% (IQR = 9.5–13.5%) and 8.5–19.5%, respectively. However, no significant difference could be observed when comparing IgG levels and avidity index between standard and fractional booster dose groups for any of the 13 serotypes.

### 3.1.2. Serotype-specific opsonophagocytic activity

Opsonization assay was used to determine the functional antibody titers of sera against the S. Pneumoniae serotypes present in PCV13 [32]. Sera from CD-1 mice immunized with a standard dose of PCV13 provided effective opsonophagocytic killing of bacteria with serotype-specific opsonic index titers (OI GMTs) that ranged from 17.5 (6.8–32.3, 95% CI) for serotype 1 up to 643.3 (202–1267, 95% CI) for serotype 14 (Figure 3). Indeed the OI titers of both serotypes 1 and 3 were significantly lower than that obtained for serotypes 4 and 14 (p < 0.01). Again OI values of sera from mice treated with the standard or the fractional booster dose did not significantly differ for any of the 13 serotypes.

### 3.2. Effect of the fractional booster dose on PCV13 cellular immune responses

#### 3.2.1. Frequency of cytokine producing splenocytes in response to PMA-ionomycin

The effect of the booster dose on Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-5) cytokine response was first investigated in splenocytes cultures exposed to the polyclonal stimulus, PMA/ionomycin. FluoroSpot analysis showed the presence, in naïve mice, of a high number of IFN-γ and IL-2 producing cells responsive to PMA/ionomycin, a lower number of IL-4 positive cells while IL-5 producing cells are poorly represented (Figure 4A). By difference in mice receiving 2.2 μg booster dose of PCV13, the number of IL-4 and IL-5 producing cells increased in response to PMA/ionomycin from 223 ± 15 and 41 ± 7 SFU/3 × 10^5 cells in naïve mice to 364 ± 28 (p < 0.01) and 222 ± 43 (p < 0.0001) SFU/3 × 10^5 cells in immunized mice, respectively (Figure 4A). Decreasing the booster dose to 0.5 μg did not affect the response to PMA/ionomycin. The number of cells positive for both IFN-γ/IL-4 was also determined since T cell may co-produce these cytokines during differentiation [33]. Stimulation of splenocytes by PMA/ionomycin revealed the presence of a small population of cells producing both IFN-γ and IL-4 that was not affected by immunization.

Globally, immunization increased the number of cytokine positive cells from 1113 ± 80 SFU/3 × 10^5 cells in naïve mice (Figure 4B) to 1472 ± 60 (p < 0.01, Figure 4C) and 1590 ± 44 (p < 0.001, Figure 4D) SFU/3 × 10^5 cells for the standard and fractional dose group, respectively. Immunization, but not the booster dose, also changed the splenocytes population responsive to PMA/ionomycin, with a diminution of IFN-γ and IL-2 positive cells and a parallel increase of IL-5- and IL-4-secreting cells frequency (Figure 4B–D).

#### 3.2.2. Frequency of cytokines producing splenocytes in response to antigens

Antigen-specific CMI was investigated by defining the profile of cytokine-producing cells after stimulation of splenocytes with the carrier protein CRM197 or with capsular PS. Results showed that the number of cytokine positive cells was low (IL-4) or almost undetectable (IFN-γ, IL-2 and IL-5) when splenocytes from naïve mice were exposed to both CRM197 or PS antigens, being not different from that obtained with cells exposed to medium alone (Figure 5A–D). However, in cells obtained from immunised mice, an increase in the frequency of CRM197 responsive IL-2 (Figure 5A) and IL-5 (Figure 5B) producing cells was evidenced. After treatment with CRM197, the number of IL-2 positive cells raised to 43.8 ± 10.4 and 41.5 ± 14.2 SFU/3 × 10^5 cells (p < 0.0001) and the number of IL-5 positive cells increased to 25.9 ± 2.9 and 23.8 ± 7.6 SFU/3 × 10^5 cells (p < 0.001) for the 0.5 μg and 2.2 μg booster dose group, respectively. For both IL-2 and IL-5, no statistically significant differences in the response to CRM197 was observed between booster dose groups. Moreover, capsular PS were not without effect.

As concerns IFN-γ, both CRM197 and PnPs induced a significant increase in the number of cytokine producing cells up to 67.8 ± 34.4 (p < 0.01) and 77.6 ± 34.3 SFU/3 × 10^5 (p < 0.001) for splenocytes obtained from the 0.5 μg booster dose group (Figure 5C). No statistically significant effect of CRM197 or PnPs was observed in mice boosted with the standard dose of PCV13. Differently from what was observed with IFN-γ, no statistically significant effect of CRM197 or PnPs on IL-4 production was detected for both naive or immunized mice (Figure 5D). CRM197 or PnPs had no effect on the number of IFN-γ/IL-4 producing cells that remained undetectable (data not shown).

On the whole, the number of cells responsive to CRM197 was 79 ± 24 and 129 ± 38 SFU/3 × 10^5 cells (p < 0.01) in the standard and fractional booster dose group, respectively (Fig. 5E–F). Qualitatively, the splenocytes population from mice receiving the standard booster dose is composed by one half of IL-2 positive cells (51%), the remaining half being divided into IL-5 (28%) and IFN-γ (21%) positive cells (Figure 5E). By difference, in the fractional dose group the percentage of IFN-γ positive cells is higher (44%), while the frequency of IL-2 (33%) and IL-5 (19%) positive cells is reduced (Figure 5F). Moreover this population also contains PnPs responsive cytokines producing cells, 85 ± 34 SFU/3

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*Figure 3. Opsonophagocytic activity of anti-PnP IgG responses following two different booster doses of PCV13. CD-1 mice were immunised as previously described, receiving either a full or a fractional booster dose of Prevnar 13 (n = 5 per group). Serum was taken for analysis of opsonophagocytic ability of anti-PnP (measured as OPA titer) 7 days after the booster immunisation. Opsonophagocytic killing assay titers against each serotype were determined using a standard protocol. Data are expressed as serotype-specific OPA geometric mean titers (GMT) ± 95% CI. Blue and red color symbol represent low and high OI GMT serotypes, respectively.*
× 10^5 splenocytes, that is almost exclusively composed of IFN-γ-positive cells (90%).

4. Discussion

The immune response to PCV13 was evaluated in CD-1 mice receiving two primary doses of vaccine followed by a standard or fractional booster dose. Results mainly indicate that the administration of the fractional booster dose does not affect at all the humoral response, whereas it shifts the CMI from a Th2 predominant towards a mixed Th1/Th2 response.

Under the present experimental immunization conditions a robust antibody response was generated for all serotypes, with no difference between the different booster dosing. Some differences among serotype-specific antibody titers and functional properties were evident for some serotypes, but in general obtained results were in agreement with previous observations in this and other mouse models, such as lower IgG serotypes, but in general obtained results were in agreement with previous observations in this and other mouse models, such as lower IgG levels accounting for reduced immunogenicity for serotype 23F administered alone or as part of a multivalent PnPs conjugate vaccine [16, 18, 20, 21], and low avidity and opsonophagocytic activity for serotypes 1 and 3, that well match with the immunogenic properties reported for a PCV15 [20].

In addition to humoral responses, CMI is an important feature of PCVs, and the present study shows that cell-mediated immune responses to individual vaccine antigens are differently affected by the booster dose, with carrier-specific IL-5 and IL-2 producing cells observed in mice boosted with the standard dose, and antigen-specific IFN-γ positive cells, in addition to IL-5 and IL-2, in animals that received the fractional boost, indicating that CRM197 stimulated spleen cells can result in a substantial cytokine secretion typical of a Th1/Th2 response. Similarly, preclinical studies with PCV15 showed that only a reduced immunization dose could induce the highest CRM197-specific IFN-γ and IL-5 production in murine splenocytes [20].

Moreover, present results indicate that although the reduction of the booster dose does not affect the splenocytes response to polyclonal stimulation (response to PMA and ionomycin), the pattern of cytokine secreting cells, following the challenge with the vaccine antigens, was modified such as the cells secreting cytokines shifted from a predominant Th2 into a mixed Th1/Th2 response. A dual Th1/Th2 in vitro response to carrier protein, or its pooled peptides, has been reported in Balb/c mice following pneumococcal vaccination with PCV7, PCV13, or PCV15 [20, 34, 35], as evidenced by enhanced splenocytes proliferation and release of several cytokines including IFN-γ, IL-2, IL-5 and IL-13. Present data show that the splenocytes population from naive mice is composed of IFN-γ- and IL-2-producing cells and a lower percentage of IL-4-producing cells, responsive to PMA/ionomycin and unresponsive to CRM197 or PS. Data also indicate that IL-5-producing cells are poorly represented in naive mice splenocytes stimulated with PMA/ionomycin, whereas the number of Th2 cells, represented by the higher frequency of cells producing IL-5 and IL-4, was consistently increased in splenocytes from mice immunized with PCV13 at either booster doses. Conversely, the presence of Th1 cells producing IFN-γ in response to both protein and polysaccharide antigens was unmasked only in splenocytes from mice immunized with the fractional booster dose.

Diversification of T cell response could bring several benefits to the overall immune response due to the distinct functions exerted by Th1 and Th2 linked cytokines. Whereas IL-5 is involved in supporting the growth and differentiation of B cells into antibody-secreting plasma cells [36], Th1 linked cytokines drive both immune cells differentiation and proliferation but also macrophage activation. Thus, IL-2 is involved in promoting the differentiation and proliferation of many immune cell types, both in autocrine and paracrine manner, and determines the activation of T cells into Th1 or Th2 cells for pathogen clearance and/or generation of memory T cells and NK cells [37]. On the other hand, IFN-γ can drive macrophage activation towards a proinflammatory phenotype, meanwhile enhancing their phagocytic ability and cytolytic potential by the secretion of pro-inflammatory cytokines and mediators [38]. The antigen dose is an important parameter that may influence T cell subsets activation and differentiation, leading to T cell population heterogeneity [39, 40, 41, 42], and a possible explanation for an inverse relationship between priming and/or boosting dose of vaccine and T cell reactivity might be that recurrent immunizations and boosting with high-dose antigen, conducted with the aim of achieving maximum antibody

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**Figure 4.** Detection of PMA/ionomycin responsive cytokine-secreting splenocytes following immunization with PCV13. CD-1 mice were immunised with two primary doses and a booster dose of Prevnar 13 as previously described. Spleens were collected one week after booster immunization with 2.2 or 0.5 μg vaccine (n = 5 mice per group) and single cell splenocytes cultures were prepared from individual mice. For assessment of cytokine production, cells were plated in Fluorospot plates and incubated with PMA/ionomycin for 24 h. Following Fluorospot analysis, cytokine-secreting cells were detected as spots forming units (SFU) per 3×10^5 splenocytes for each cytokine. Cells incubated with medium alone were used to determine background responses for each cytokine population and subtracted from PMA/ionomycin-specific responses. A) The data are represented as mean ± S.E.M. Two-way ANOVA followed by Tukey's multiple comparisons test showed significant differences between immunized and naive mice (***p < 0.0001, *p < 0.01). B-D) Cell populations were also summarized as pie charts, to show the proportion of PMA/ionomycin responsive cells that produced IFN-γ, IL-2, IL-4, or both IFN-γ and IL-4. Each population is defined by one color and the percentage is indicated. The size of the responding population (total number of cells secreting cytokines) is indicated for each mice group by a numerical value below each pie.
titers, may led to the selective exhaustion of a population of vaccine-induced T cells [20, 39, 40].

Examples of low antigen dose inducing higher number of IFN-γ positive T cells, and a diverse relationship in IgG, have been described in preclinical studies with a tetanus toxoid vaccine [43], and a low vaccine booster dose was associated with the higher capacity by HIV antigens to induce cytokine (IFN-γ) release [44]. In the case of tuberculosis immunization, a low protein dose was associated with the selective higher frequency of polyfunctional T cells showing an increased production of IFN-γ as compared to the T cells from animals immunized with a higher antigen dose [13]. However, the relationship between the antigen dose and the quality of an immune response generated upon immunization is poorly understood, and the molecular mechanisms responsible for the determination of the Th1/Th2 phenotype are not clearly defined, and further work certainly needs to be done to clarify this issue [45].

Overall, present findings indicate that, in a CD-1 mouse model, it is possible to convert a Th2 predominant response into a mixed Th1/Th2 one, by reducing the PCV13 booster dose, without affecting the quantity and functionality of produced antibodies. Due to the different and complementary role of Th1/Th2 cytokines in mediating immune protection, present results suggest that, besides measuring the presence of functional protective antibodies, determining the effect of antigen dose on CMI can provide a necessary information to optimize the development of an experimental vaccine candidate. Moreover, the principle that a low dose immunization regimen can give at least a similar reactogenity, both in terms of antibody and cell immune responses, is becoming very timely, as recently shown with the ChAdOx1 nCoV-19 vaccine (AZD1222) [46,47] suggesting that lower doses might be exploited to reach a broad immunization coverage in case of vaccine shortage.

Declarations

Author contribution statement

Rose-Marie Catalioto: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Claudio Valenti, Francesca Bellucci, Cecilia Cialdai: Performed the experiments; Analyzed and interpreted the data.
Stefania Meini: Conceived and designed the experiments.
Maria Altamura, Laura Digilio, Andrea Ugo Enrico Pellacani: Conceived and designed the experiments.
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References
[1] RJ. Engler, MB. Nelson, MM. Kloet, M.J. VanRaden, CY. Huang, Cox, et al., Half-vs full-dose trivalent inactivated influenza vaccine (2004-2005): age, dose, and sex effects on immune responses, Arch. Intern. Med. 168 (2008) 2405–2414.
[2] A.C. Campi-Azevedo, P. de Almeida Estevam, J.G. Coelho-dos-Reis, V. Peruhype-Magalhães, G. Villela-Rezende, P.F. Quaresma, et al., Subdoses of 31TD yellow fever vaccine elicit equivalent virological/immunological kinetics timeline, BMC Infect. Dis. 14 (2014) 391.
[3] P. Kosounov, B. Warzicht, Y. Hutangalag, T. Borkirk, P. Koulalaraka, H.L. Bock, et al., Reactogenicity and immunogenicity of reduced antigen content diphtheria-tetanus-acellular pertussis vaccine (dTap) administered as a booster to 4–6 year-old children primed with four doses of whole-cell pertussis vaccine, Vaccine 21 (2003) 4194–4200.
[4] CG. Whitney, MM. Farley, J. Hadler, J.L. Harrison, N.M. Bennett, R. Lynfield, et al., Decline in invasive pneumococcal disease after the introduction of protein-poly saccharide conjugate vaccine, N. Engl. J. Med. 348 (2003) 1737–1746.
[5] NJ. Andrews, PA. Waight, P. Barber, E. Pearce, L. Roalfe, M. Zancolli, et al., Serotype-specific effectiveness and correlates of protection for the 13-valent pneumococcal conjugate vaccine: a postlicensure indirect cohort study, Lancet Infect. Dis. (2014) 839–846.
[6] AJ. Sucher, EB. Chaitine, M. Nelson, BJ. Sucher, Prevnar 13, the new 13-valent pneumococcal conjugate vaccine, Ann. Pharmacother. 45 (2011) 1516–1524.
[7] T. Trück, A. Thompson, B. Morales, A. Clutterbuck, M. Voysey, E. Clarke, et al., Fractional third and fourth dose of RTS, S/AS01 malaria candidate vaccine: a phase 2a controlled human malaria parasite infection and immunogenicity study, Hum. Vaccines Immunother. 13 (2017) 1169–1176.
[8] RI. Moreno, JS. Sampson, S. Romero-Stei ner, B. Wong, SE. Johnson, E. Ades, G.M. Carlone, A murine model for the study of immune memory in response to pneumococcal conjugate vaccination, Vaccine 22 (2004) 3099–3105.
[9] Z. Polonskaya, S. Deng, A. Sarkar, L. Kain, M. Comellas-Aragonès, McKay, et al., T cell control the generation of -...
[42] N.J. Tubo, M.K. Jenkins, TCR signal quantity and quality in CD4+ T cell differentiation, Trends Immunol. 35 (2014) 591–596.

[43] N.H. Overgaard, T.M. Froig, J.T. Jakobsen, S. Buss, M.H. Andersen, G. Jungersen, Low antigen dose formulated in CAF09 adjuvant favours a cytotoxic T-cell response following intraperitoneal immunization in Gottingen minipigs, Vaccine 35 (2017) 5629–5636.

[44] R. Billeskov, Y. Wang, S. Solaymani-Mohammadi, B. Frey, S. Kulkami, P. Andersen, et al., Low antigen dose in adjuvant-based vaccination selectively induces CD4 T cells with enhanced functional avidity and protective efficacy, J. Immunol. 198 (2017) 3494–3506.

[45] P. Bretscher, On analyzing how the Th1/Th2 phenotype of an immune response is determined: classical observations must not be ignored, Front. Immunol. 10 (2019) 1234.

[46] M.N. Ramasamy, A.M. Minassian, K.J. Ewer, A.L. Flaxman, P.M. Folegatti, D.R. Owens, et al., Safety and immunogenicity of ChAdOx1 nCoV-19 vaccine administered in a prime-boost regimen in young and old adults (COV002): a single-blind, randomised, controlled, phase 2/3 trial, Lancet 396 (10267) (2020) 1979–1992.

[47] M. Voysey, S.A.C. Clemens, S.A. Madhi, L.Y. Weckx, P.M. Folegatti, P.K. Aley, et al., Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: an interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK, Lancet 397 (2021) 99–111.