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Th17-Mediated Cross Protection against Pneumococcal Carriage by Vaccination with a Variable Antigen

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ABSTRACT Serotype-specific protection against *Streptococcus pneumoniae* is an important limitation of the current polysaccharide-based vaccines. To prevent serotype replacement, reduce transmission, and limit the emergence of new variants, it is essential to induce broad protection and restrict pneumococcal colonization. In this study, we used a prototype vaccine formulation consisting of lipopolysaccharide (LPS)-detoxified outer membrane vesicles (OMVs) from *Salmonella enterica* serovar Typhimurium displaying the variable N terminus of PspA ($\alpha1\alpha2$) for intranasal vaccination, which induced strong Th17 immunity associated with a substantial reduction of pneumococcal colonization. Despite the variable nature of this protein, a common major histocompatibility complex class (MHC-II) epitope was identified, based on *in silico* prediction combined with *ex vivo* screening, and was essential for interleukin-17 A (IL-17A)-mediated cross-reactivity and associated with cross protection. Based on 1,352 PspA sequences derived from a pneumococcal carriage cohort, this OMV-based vaccine formulation containing a single $\alpha1\alpha2$ type was estimated to cover 19.1% of strains, illustrating the potential of Th17-mediated cross protection.

KEYWORDS intranasal vaccination, protein antigens, *Streptococcus pneumoniae*, PspA, colonization, Th17, broad protection, *Salmonella* outer membrane vesicle (OMV), antigen surface display, autotransporter Hbp

Respiratory bacterial infections remain a major cause of severe morbidity and mortality worldwide in both infants and adults (1, 2). Vaccination is considered one of the most cost-effective strategies to reduce the global burden of infectious diseases, the associated health care costs, and the risk of emerging antibiotic-resistant strains (3). Vaccination against *Streptococcus pneumoniae* is implemented in different parts of the world. However, about 1 million children still die of pneumococcal disease every year (1, 2). Pneumococcal polysaccharide conjugate vaccines are designed based on epidemiological data from the Western world. These vaccines protect against serotypes that are the most prevalent and most frequently associated with severe invasive disease. They induce serotype-specific protection against 13 of the 97 identified serotypes that are circulating worldwide (4). This is an important limitation, and during the last decade, nonvaccine variants of *S. pneumoniae* that cause severe invasive disease have

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emerged (5–7). The phenomenon of strain or serotype replacement and the remaining high disease burden in developing countries highlight the need for alternative vaccination strategies against *S. pneumoniae*.

Currently, there is much attention for the development of novel, broadly protective, protein-based vaccines (8–13). The best-studied protein vaccine target in *S. pneumoniae* is pneumococcal surface protein A (PspA), which is a virulence factor that binds lactoferrin and interferes with complement-mediated opsonization (14, 15). PspA is expressed by most pneumococcal strains and consists of an N-terminal alpha-helical coiled-coil domain, a proline-rich region (PRR), and a choline binding domain. PspA is classified into three different families and six different clades based on the clade-defining region (CDR), located at the C-terminal site of the alpha-helical coiled-coil domain (16). Previously, we showed that vaccination with recombinant *Salmonella* outer membrane vesicles (OMVs) displaying the N-terminal part of the coiled-coil domain ($\alpha1\alpha2$ fragment) of PspA at the surface confers strong protection against pneumococcal colonization (17). The reduction in bacterial density strongly correlated with nasal interleukin-17A (IL-17A) levels, a response critical for the clearance of colonizing pneumococci (18, 19). The extent to which variable proteins are able to induce broadly protective Th17 memory was hitherto not known. In this study, we found that protection against pneumococcal colonization, raised by vaccination with OMVs expressing $\alpha1\alpha2$, was dependent on IL-17 signaling. We further explored the cross-reactive Th17-mediated potential of the $\alpha1\alpha2$ fragment, the most immunogenic but variable region of PspA.

RESULTS

IL-17A is required for PspA $\alpha1\alpha2$-induced reduction in pneumococcal colonization. In a previous study, we observed a strong reduction in pneumococcal colonization following intranasal vaccination of mice with the $\alpha1\alpha2$ fragment of PspA, but not with other PspA fragments, presented on the surface of attenuated *Salmonella* OMVs by using autodisplay (17). In that study, we used OMVs with wild-type lipopolysaccharide (LPS). Since our anticipated pneumococcal vaccine formulation is intended for use in humans, we created an LPS-detoxified OMV production strain by inactivating the *msbB* gene, which results in the synthesis of LPS that lacks the myristic acid moiety of lipid A.

Modification and detoxification were confirmed by negative-ion electrospray ionization–Fourier transform (ESI-FT) mass spectrometry and human peripheral blood mononuclear cell (PBMC) stimulation, respectively, showing that the Δ*msbB* mutant has reduced levels of hexacylated lipid A and increased levels pentacylated lipid A (see Fig. S1A in the supplemental material), which led to reduced cytokine production (Fig. S1B). The inactivation of *msbB* had no effect on the heterologous expression and display of PspA $\alpha1\alpha2$ on *Salmonella* OMVs (Fig. S1C). Importantly, vaccination of mice with Δ*msbB* OMVs decorated with PspA $\alpha1\alpha2$ resulted in a significant reduction of intranasal colonization compared to the control carrying no antigen, i.e., the Δ*msbB* OMV HbpD (Fig. 1A). This reduction in colonization was associated with high levels of IL-17A in the nasal tissue, reminiscent of the effects using wild-type OMVs (Fig. 1B).

To determine whether protection induced by Δ*msbB* OMVs decorated with PspA $\alpha1\alpha2$ was Th17 mediated, IL-17R-deficient mice were vaccinated and challenged. No protection was measured in mice deficient for IL-17R, which strongly indicates that protection raised by PspA $\alpha1\alpha2$ displaying OMVs requires Th17 signaling (Fig. 1C).

Novel PspA classification based on $\alpha1\alpha2$ to predict cross protection. Although the CDR is used to classify PspA proteins, the $\alpha1\alpha2$ region is responsible for PspA-mediated protection (Fig. 2A). Therefore, we propose an alternative classification of PspA based on the $\alpha1\alpha2$ sequence, allowing prediction of cross protection. PspA sequences from a sequenced collection of 349 invasive clinical strains, isolated from patients with pneumococcal bacteremia, were aligned and classified based on the $\alpha1\alpha2$ region. Four sequences were excluded because the $\alpha1\alpha2$ fragment was (partly) undetectable due to sequencing errors. To explore the variability of the $\alpha1\alpha2$ fragment

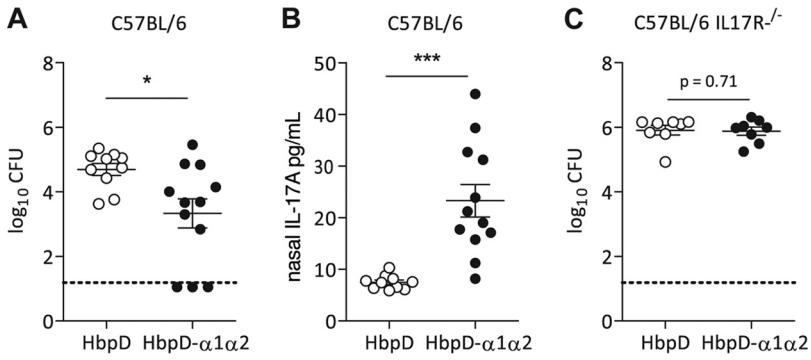

FIG 1 OMV-induced reduction in pneumococcal colonization is IL-17A dependent. Mice were vaccinated with control OMVs (HbpD) (open circles) or OMVs displaying $\alpha1\alpha2$ (HbpD-$\alpha1\alpha2$) (black circles) derived from a *Salmonella* Typhimurium Δ*msbB* strain. Mice were intranasally infected with the pneumococcal TIGR4 strain (homologous challenge). (A and C) Bacterial colonization (log CFU) of the nasal cavities of C57BL/6 (A) and IL-17R$^{-/-}$ (C) mice was measured at 3 days postinfection. (B) The IL-17A concentration in the nasal tissue of vaccinated C57BL/6 mice was measured. Data represent results for individual mice, and bars indicate group means ± standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

between pneumococcal strains, sequences were aligned by using MEME, resulting in the identification of six variable regions (VRs) (Fig. 2B; see also Table S2 in the supplemental material). Of note, not all the respective VRs were present in each pneumococcal strain. The majority of clinical isolates expressed VR2 (98.6%), VR3 (99.7%), and VR4 (98.4%). A selection of strains had VR5 (27.3%) between VR4 and VR2. Either VR1 (33.9%) or VR6 (51.1%) was present after VR2. Based on $\alpha1\alpha2$ fragment homology, the 345 strains were divided into 35 different groups, or $\alpha1\alpha2$ subtypes, of which all strains within each group had identical $\alpha1\alpha2$ sequences (Table S2). The vaccine construct of TIGR4 $\alpha1\alpha2$ contained these VRs in the following order: VR3, VR4, VR5, VR2, and VR1 (Table S2).

Based on this new classification, we searched for a common epitope predicting IL-17A-inducing potential leading to protection against pneumococcal colonization. NetMHCII was used to predict T-cell epitopes present in TIGR4 $\alpha1\alpha2$. Three regions in TIGR4 $\alpha1\alpha2$ had peptides with a predicted 50% inhibitory concentration (IC_{50}) value lower than or around 100 nM, amino acids 41 to 61 in VR3 (Fig. 2C, blue), amino acids 151 to 171 in VR2 (red), and amino acids 180 to 199 in VR2 and VR1 (green), with the highest affinity (lowest IC_{50} value) for EKKYEEAKAKADTAK (dashed blue bar), VRAVVV PEPNALAET (dashed red bar), and AKRKYDYATLKVALA (dashed green bar) (Fig. 2C). For all peptides, homologues were found in other Pneumococcal Bacteremia Collection Nijmegen (PBCN) strains (Table 1; see also Table S2 in the supplemental material).

Predicted major histocompatibility complex class II (MHC-II) epitope in $\alpha1\alpha2$ correlates with *ex vivo* vaccine-induced Th17 responses. To empirically determine whether the *in silico*-predicted epitope was involved in the induction of IL-17A, we developed an *ex vivo* screening assay. Three days after pneumococcal challenge, splenocytes were isolated from mice immunized with control OMVs or OMVs displaying TIGR4 PspA $\alpha1\alpha2$. Splenocytes were restimulated with bacterial cells from 35 different PBCN strains, representing the 35 $\alpha1\alpha2$ subtypes. After 3 days, IL-17A levels in cell supernatants were measured by an enzyme-linked immunosorbent assay (ELISA). Splenocyte restimulation of TIGR4 $\alpha1\alpha2$-immunized mice showed cross-reactivity with multiple pneumococcal strains. TIGR4 and PBCN268 contain an identical $\alpha1\alpha2$ fragment, explaining the high IL-17A level (Fig. 3A), which can be considered a validation of this method. Consistent with the NetMHCII-predicted peptide AKRKYDYATLKVALA, restimulation with pneumococcal strains PBCN064, PBCN461, PBCN266, PBCN321, and PBCN016 also showed a significant increase in IL-17A concentrations, to the same magnitude as that with TIGR4. In contrast, restimulation of splenocytes from control mice (immunized with OMV/HbpD but infected with TIGR4) showed extremely low to undetectable IL-17A levels (Fig. 3B).

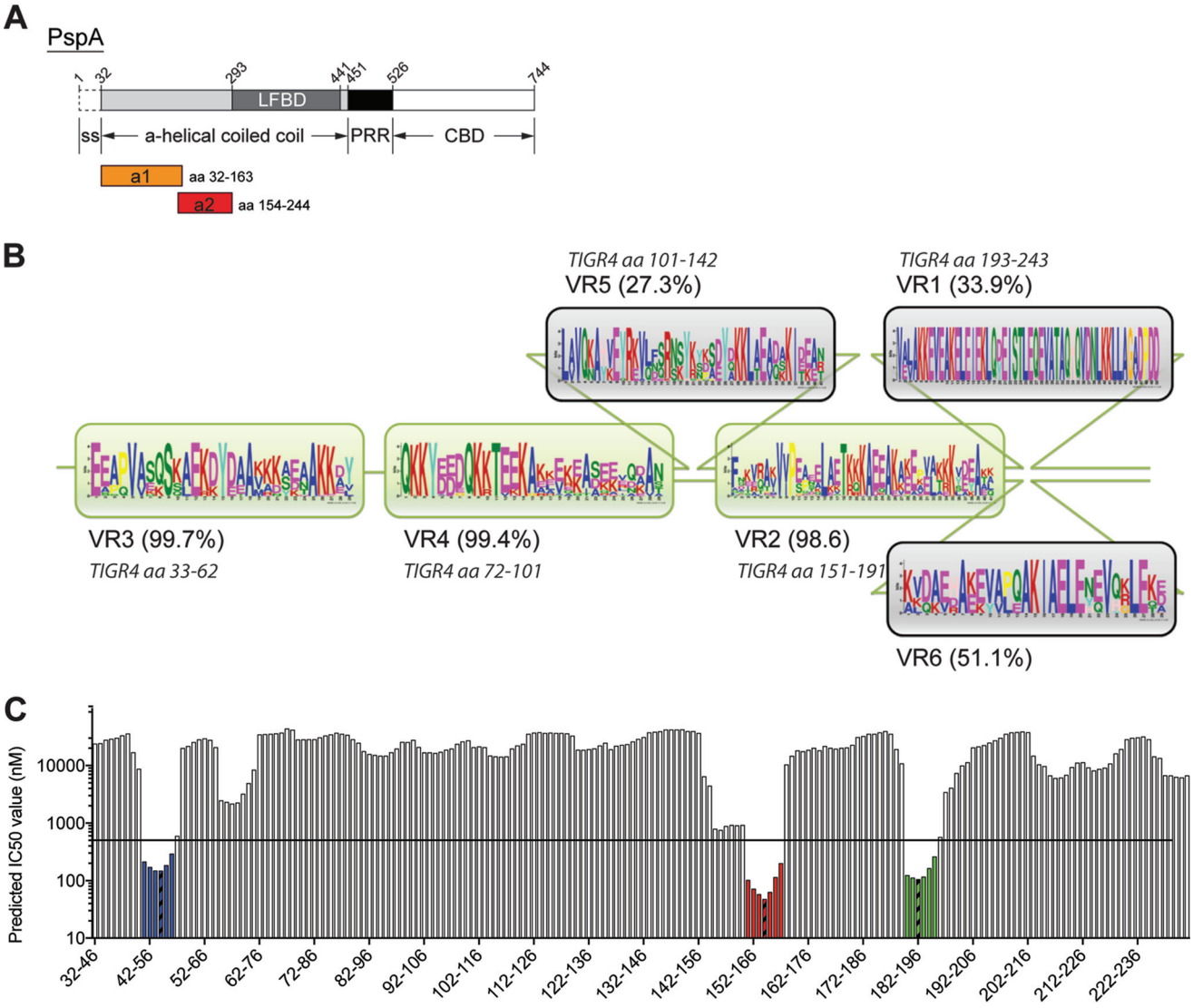

FIG 2 Vaccine $\alpha1\alpha1$ fragment. (A) Schematic overview of TIGR4 PspA $\alpha1\alpha2$, adapted from Vaccine vol. 33, K. Kuipers et al., *Salmonella* outer membrane vesicles displaying high densities of pneumococcal antigen at the surface offer protection against colonization, p. 2022–2029, Copyright 2015, with permission from Elsevier (17). The slightly overlapping $\alpha1$ and $\alpha2$ fragments (overlap of 9 amino acids [aa]) were selected to cover the α-helical coiled-coil domain. The lactoferrin binding domain (LFBD), the proline-rich region (PRR), and the choline binding domain (CBD) of PspA were not included in the vaccine. SS, signal sequence. (B) Alignment of the $\alpha1\alpha2$ domains derived from 345 sequenced pneumococcal invasive isolates. Based on amino acid identity, MIME analysis subdivided the $\alpha1\alpha2$ vaccine region into six variable regions (VR1 to VR6). Some VRs appeared more conserved and were alternately expressed by the clinical isolates. Variable domains are numbered according to their sequence conservation, as determined by the E value. The percentage of VR expression among pneumococcal isolates was calculated. The presence of the VR is shown as percentages. VRs 3, 4, 5, 2, and 1 were present in TIGR4; VR3, VR4, and VR5 are part of $\alpha1$; and VR2 and VR1 are part of $\alpha2$, and the positions of the amino acids are indicated. (C) NetMHCII was used to predict T-cell epitopes present in TIGR4 $\alpha1\alpha2$. The binding affinity is expressed as IC_{50} values (*y* axis). The different 15-mer peptides with a 1-amino-acid overlap are indicated on the *x* axis from amino acids 32 to 244, spanning the whole $\alpha1\alpha2$ domain. The region within the TIGR4 $\alpha1\alpha2$ domain showed a predicted IC_{50} value of around or lower than 100 nm, indicating strong binding affinity, including amino acids 41 to 61 (blue), 151 to 171 (red), and 180 to 199 (green), with the lowest values being measured for EVRAVVVPEPNALAE (dashed red bar) and AKRKYDYATLKVALA (dashed green bar).

Peptide oligomer overrepresentation analysis of the $\alpha1\alpha2$ sequences yielding high IL-17A concentrations revealed a sequence epitope of 41 amino acids shared by these pneumococcal isolates (see Fig. S2A and S2B in the supplemental material), which consisted of the AKRKYDYATLKVALA peptide (shown in boldface type in Fig. S2B). In addition, we found that the reactivation of vaccine-induced Th17 memory responses was influenced by both capsule thickness and PspA expression levels, whereas the serotype background had no significant effect on *ex vivo* IL-17A levels (Fig. 4A to C). This may explain the differences in IL-17A levels measured upon stimulation with different strains.

TABLE 1 MHC-II peptides

Peptide sequence	Positions in PspA	IC_{50} (nM)	Other PBCN strains with homologous sequence
EKKYEEAKAKADTAK	44–58	148.0	PBCN036, PBCN118, PBCN200, PBCN356, PBCN461, PBCN477
VRAVVVPEPNALAET	153–167	47.9	PBCN023, PBCN118
AKRKYDYATLKVALA	182–196	105.7	PBCN006, PBCN016, PBCN064, PBCN266, PBCN321, PBCN461 (with a single mutation, AKRKYDYATLKVVLA)

***Ex vivo* Th17 responses predict *in vivo* nasal IL-17A levels.** Next, we assessed whether the IL-17A levels measured *ex vivo* can be linked to *in vivo* vaccine-induced IL-17A responses in the nasal cavity. Based on *ex vivo* Th17 screening (Fig. 3A), we expected that pneumococcal strains that produced high *ex vivo* IL-17A levels would also cause *in vivo* vaccine-induced IL-17A responses. To this end, we selected heterologous strains inducing high and low concentrations of IL-17A after restimulation ("heterologous high-IL-17A" strains PBCN266 and PBCN321 and "heterologous low-IL-17A" strains PBCN200 and EF3030). Mice were immunized with either control OMVs (HbpD; control) or OMVs expressing TIGR4 $\alpha1\alpha2$ (HbpD-$\alpha1\alpha2$) and were subjected to challenge with homologous (TIGR4) or heterologous (PBCN266, PBCN321, PBCN200, and EF3030) strains. Homologous challenge with TIGR4 led to a significant increase in nasal IL-17A levels in $\alpha1\alpha2$-vaccinated animals (Fig. 5A), corresponding to the above-described observations (Fig. 1B). Combining the data for the individual strains showed that heterologous high-IL-17A strains induced significantly increased levels of nasal IL-17A following vaccination, unlike heterologous low-IL-17A strains (Fig. 5F). Levels of nasal IL-17A induced by individual strains can be found in Fig. 5.

We next explored whether the activation of nasal IL-17A responses correlated with protection, i.e., reduction of pneumococcal colonization. Vaccination with OMVs ex-

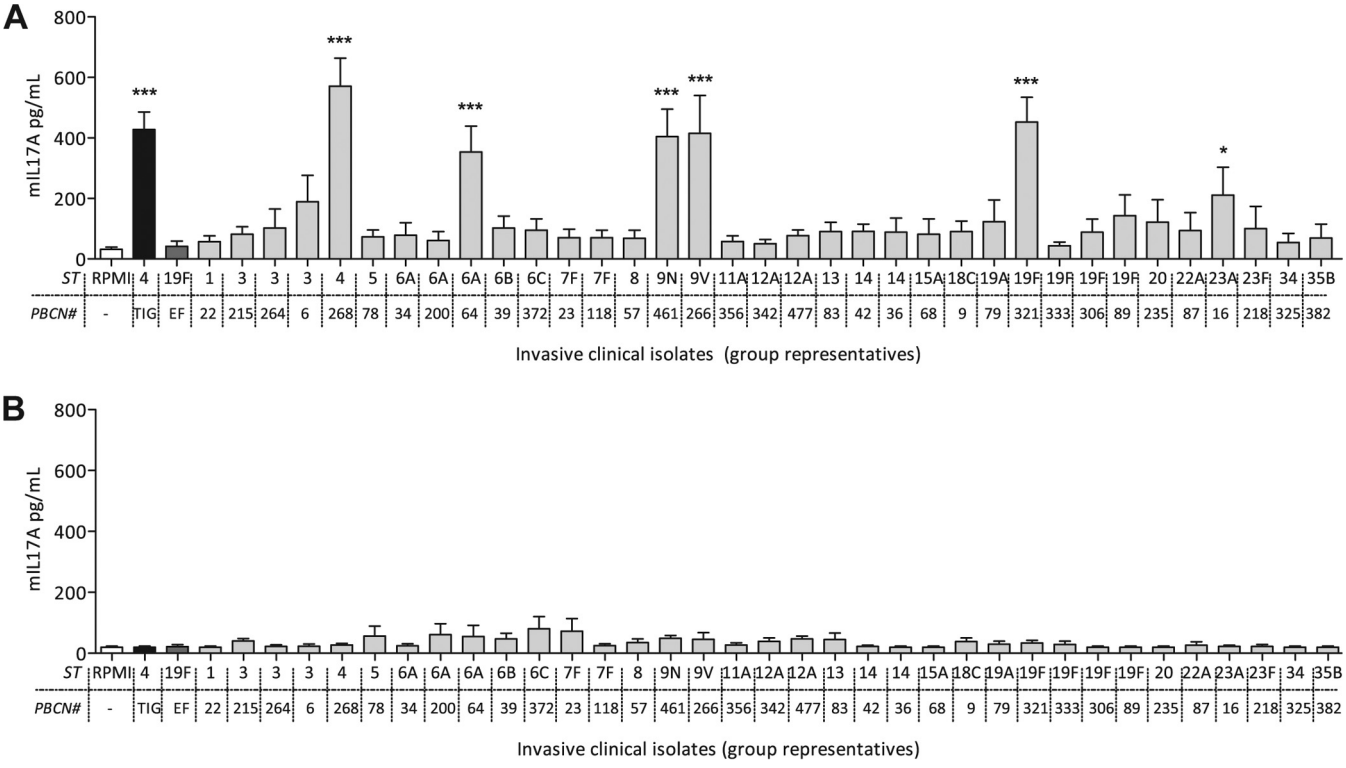

FIG 3 *Ex vivo* cross-reactivity of Th17 memory cells. At 3 days postinfection, spleens were harvested from mice that were vaccinated with Δ*msbB* OMVs expressing $\alpha1\alpha2$ (Hbp-$\alpha1\alpha2$) (A) or with control OMVs (Hbp) (B). Single-cell suspensions were obtained and stimulated with selected pneumococcal isolates (based on 35 $\alpha1\alpha2$ subtypes) for 3 days. The IL-17A concentration in the cell supernatant was measured. IL-17A values represent data for 10 mice (Hbp-$\alpha1\alpha2$) (A) and 4 mice (Hbp) (B) and are shown as means ± standard errors of the means. Clinical strains, including one representative of each $\alpha1\alpha2$ subtype, were ordered based on serotype (ST). TIG, TIGR4; EF, EF3030. *, $P < 0.05$; ***, $P < 0.001$.

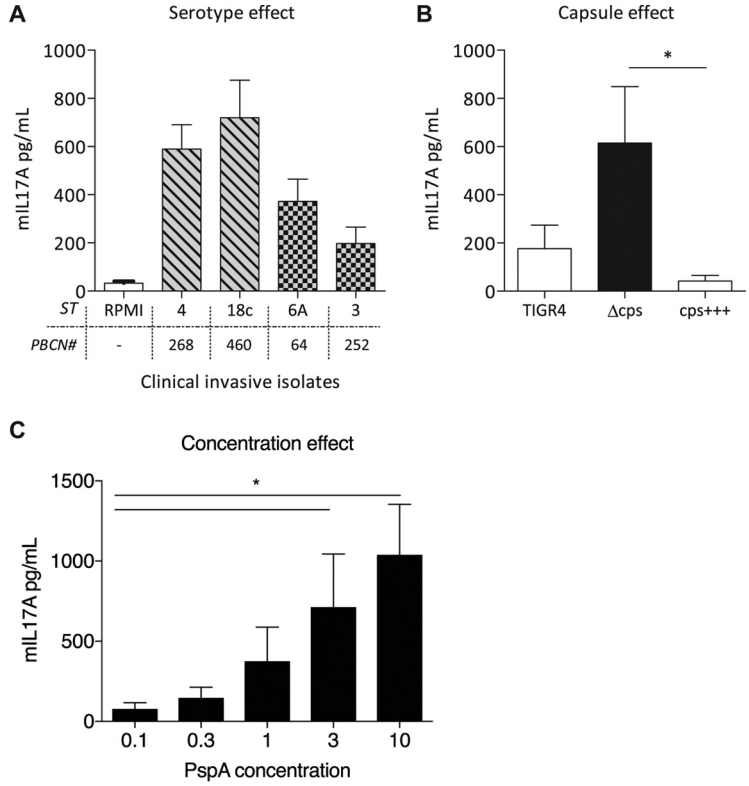

FIG 4 Influence of bacterial factors on *ex vivo* IL-17A levels. At 3 days postinfection, spleens were harvested from mice that were vaccinated with Δ*msbB* OMVs expressing $\alpha1\alpha2$ (Hbp-$\alpha1\alpha2$). Splenocytes were stimulated with selected pneumococcal strains, TIGR4 isogenic strains, or purified PspA for 3 days to assess the effect of serotype (A), capsule (B), and the PspA protein concentration (C). (A) PBCN268 and PBCN460 share identical $\alpha1\alpha2$ regions but belong to different serotypes. This is similar for PBCN64 and PBCN252. (B and C) Splenocytes were exposed to wild-type TIGR4, a nonencapsulated TIGR4 strain (Δ*cps*), and a TIGR4 strain overexpressing capsule (cps^{+++}) (B), and a dose-dependent effect of PspA (0.1, 0.3, 1, 3, and 10 μg/ml) was determined (C). The IL-17A concentrations in the cell supernatant of restimulated splenocytes derived from 5 to 9 mice were measured and are shown as means ± standard errors of the means. *, $P < 0.05$; ns, not significant.

pressing $\alpha1\alpha2$ strongly protected against homologous challenge with TIGR4. A significant reduction in pneumococcal colonization was also observed for the heterologous high-IL-17A strains selected by the *ex vivo* screen (Fig. 6). No protection was observed for the heterologous low-IL-17A strains (Fig. 6). Protection data per individual strain can be found in Fig. S3 in the supplemental material. Together, these results illustrate the predictive capacity of *ex vivo* IL-17A for *in vivo* nasal IL-17A following vaccination. Simultaneously, these findings demonstrate the degree to which vaccine-induced IL-17A is cross-reactive with heterologous strains.

Prevalence of the TIGR4 $\alpha1\alpha2$ subtype among human carriage strains. To gain insight into the potential effect of a vaccine consisting of the TIGR4 $\alpha1\alpha2$ subtype on pneumococcal carriage in the human population, we performed *in silico* analyses of the prevalence of the 35 $\alpha1\alpha2$ subtypes in a large carriage cohort. Of 1,352 isolates, the 35 $\alpha1\alpha2$ subtypes that we identified accounted for 89.3% of the strains (Table 2). These PspA $\alpha1\alpha2$ variants could not be determined for 144 isolates (Table 3).

Subsequently, we calculated the prevalence of pneumococcal strains that could potentially be covered by a vaccine comprising TIGR4 $\alpha1\alpha2$, because the significantly high IL-17A responses measured in our *ex vivo* screen predict protection. Based on the PspA sequences derived from the large carriage collection presented in Table 1, an OMV-based vaccine comprising only one $\alpha1\alpha2$ type (TIGR4) was estimated to cover 19.1% of the 1,352 carriage strains.

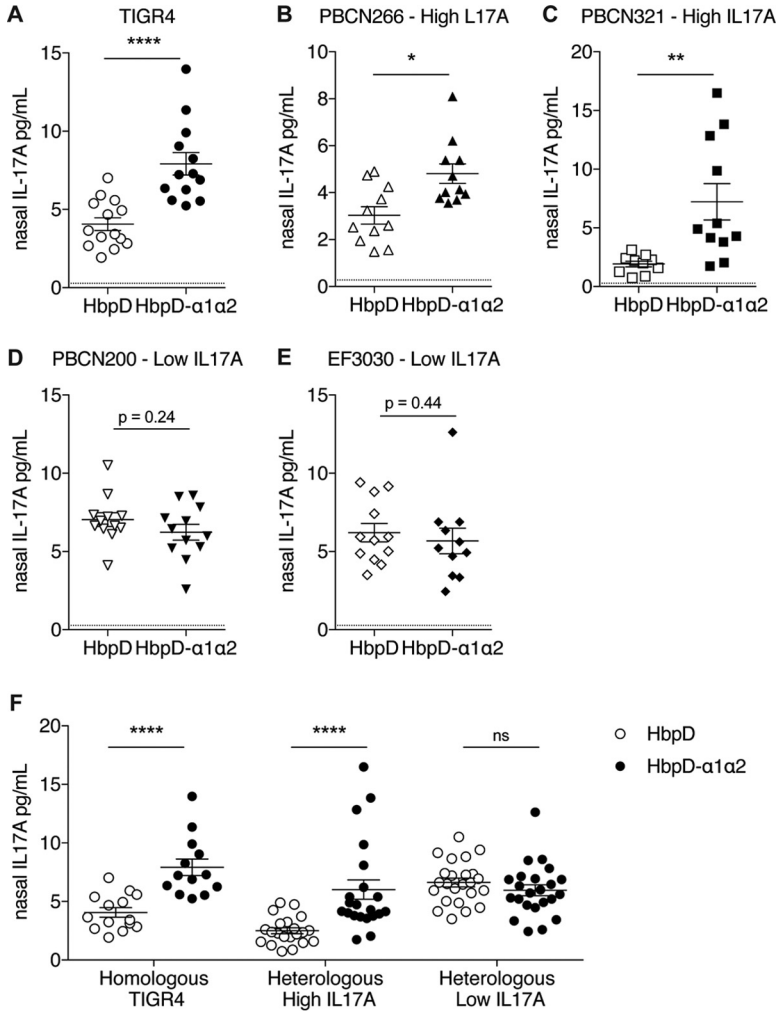

FIG 5 Nasal IL-17A concentrations measured after homologous and heterologous challenge infections. Mice were vaccinated with empty OMVs (Hbp) or OMVs expressing $\alpha1\alpha2$ (Hbp-$\alpha1\alpha2$). Mice were intranasally infected with the homologous strain (TIGR4) or with heterologous high-IL-17A strains (PBCN266 and PBCN321) and heterologous low-IL-17A strains (PBCN200 and EF3030). IL-17A levels in nasal tissue were assessed following infection with TIGR4 (A), PBCN266 (B), PBCN321 (C), PBCN200 (D), and EF3030 (E). IL-17A levels in nasal tissue were pooled for heterologous high-IL-17 (PBCN266 and PBCN321) and low-IL-17A (PBCN200 and EF3030) strains (F). Data represent results for individual mice, and bars indicate group means ± standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

DISCUSSION

Generally, immunizations with variable, often highly immunogenic proteins offer strong but limited cross protection (11, 17, 20, 21). Whether variable proteins are suitable antigens to induce cross-reactive Th17-mediated protection has not been investigated thus far. In a previous study, we demonstrated that vaccination with an OMV-based vaccine comprising the variable $\alpha1\alpha2$ fragment of PspA led to the induction of IL-17A and strong protection against pneumococcal colonization with a homologous strain (17). In the present study, we set out to explore the ability to induce Th17-mediated cross protection with an OMV-based vaccine. The variable $\alpha1\alpha2$ domains were classified into 35 subtypes by using a large cohort of sequenced strains. The results obtained by *in silico* prediction of common MHC-II epitopes were confirmed by *ex vivo* screening. We confirmed that an epitope located within the $\alpha2$ regions of TIGR4 was also present in different clinical isolates, which correlated with the *ex vivo* production of IL-17A. Interestingly, the presence of this epitope strongly correlated with the induction of nasal IL-17A and with a reduction of pneumococcal colonization.

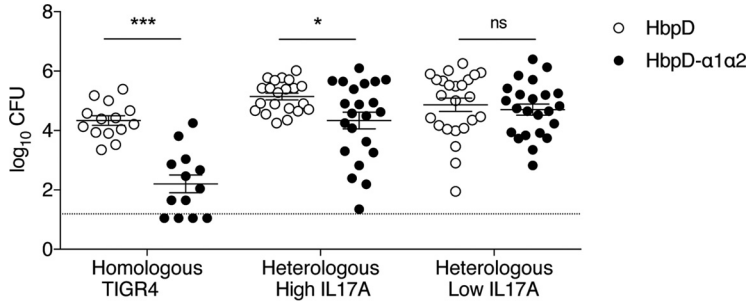

FIG 6 Pneumococcal colonization measured after homologous and heterologous challenge infections. Mice were vaccinated with empty OMVs (Hbp) or OMVs expressing $\alpha1\alpha2$ (Hbp-$\alpha1\alpha2$). Mice were intranasally infected with the homologous strain (TIGR4) or with heterologous high-IL-17A strains (PBCN266 and PBCN321) and heterologous low-IL-17A strains (PBCN200 and EF3030). IL-17A levels in nasal tissue and pneumococcal colonization were assessed at 3 days postinfection. Results were pooled for heterologous high-IL-17A (PBCN266 and PBCN321) and low-IL-17A (PBCN200 and EF3030) strains (F). Data represent results for individual mice, and bars indicate group means ± standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Reactivation of Th17 memory responses measured both *ex vivo* and *in vivo* may, however, be influenced by antigen and capsule expression. In the *ex vivo* Th17 cross-reactivity screening, IL-17A concentrations were reduced when splenocytes were exposed to lower PspA concentrations or to a highly encapsulated pneumococcal strain

TABLE 2 Prevalence of PspA $\alpha1\alpha2$[a]

$\alpha1\alpha2$ variant	PBCN ID	Protein ID	Total no. of isolates carrying variant (n = 1,352)	% of isolates carrying variant
ND	NA	NA	144	10.7
1	6	10050_2#2_00383	16	1.2
2	9	10050_2#4_00581	35	2.6
3	16	10050_2#8_00268	48	3.6
4	22	10050_2#11_00768	0	0.0
5	23	10050_2#12_01906	25	1.8
6	34	10050_2#20_01349	5	0.4
7	36	10050_2#21_00326	122	9.0
8	39	10050_2#22_01317	11	0.8
9	42	10050_2#24_00225	8	0.6
10	57	10050_2#30_00292	36	2.7
11	64	10050_2#35_00095	31	2.3
12	68	10050_2#37_01815	22	1.6
13	78	10050_2#41_00777	1	0.1
14	79	10050_2#42_00670	115	8.5
15	83	10050_2#45_00391	1	0.1
16	87	10050_2#48_00214	103	7.6
17	89	10050_2#50_00198	47	3.5
18	118	10050_2#71_00865	2	0.1
19	200	9953_7#38_01834	2	0.1
20	215	9953_7#47_00982	82	6.1
21	218	9953_7#49_01131	31	2.3
22	235	9953_7#58_00025	15	1.1
23	264	9953_7#79_01103	0	0.0
24	266	9953_7#81_00909	110	8.1
25	268	9953_7#82_01423	35	2.6
26	306	10071_4#18_00006	3	0.2
27	321	10071_4#30_00648	29	2.1
28	325	10071_4#32_00112	23	1.7
29	333	10071_4#40_00911	2	0.1
30	342	10071_4#46_00491	30	2.2
31	356	10071_4#56_01432	135	10.0
32	372	10071_4#71_00979	18	1.3
33	382	10071_4#80_00989	59	4.4
34	461	10208_2#57_01836	6	0.4
35	477	10396_8#26_00179	0	0.0

[a]The 1,352 isolates are carriage isolates from Massachusetts. ND, not determined (i.e., in these strains, no $\alpha1\alpha2$ sequence related to the 35 PBCN $\alpha1\alpha2$ types was found); NA, not applicable.

TABLE 3 Serotype distribution of nondetermined PspA $\alpha1\alpha2$ variants

Serotype	No. of isolates of serotype	% of isolates of serotype
23B	36	25.0
NT	19	13.2
23A	12	8.3
35B	12	8.3
15BC	10	6.9
6C	10	6.9
17F	8	5.6
3	7	4.9
21	6	4.2
37	4	2.8
19A	3	2.1
23F	3	2.1
35F	3	2.1
10A	2	1.4
11A	2	1.4
19F	2	1.4
34	1	0.7
16F	1	0.7
18C	1	0.7
22F	1	0.7
6B	1	0.7
Total	144	100.0

(see Fig. S3B and S3C in the supplemental material). Similarly, others have shown that *ex vivo* splenocyte-derived IL-17A produced by whole-cell-vaccine-immunized animals slightly differed between different isogenic capsule switch strains (22). In that same study, vaccination with a whole-cell vaccine followed by homologous challenge induced Th17 memory responses in the spleens of mice (22). Consistent with these data, we show that vaccination with an $\alpha1\alpha2$ peptide of PspA promoted Th17 memory responses in mouse splenocytes. Our study showed that it is possible to screen for Th17-mediated vaccine cross-reactivity with a simple method, thereby preventing the need for large animal studies.

Vaccination with OMVs expressing TIGR4 $\alpha1\alpha2$ led to a clear reduction in pneumococcal colonization in homologous challenge infection but was less pronounced after heterologous challenge infection with heterologous high-IL-17A strains PBCN266 and 321 (Fig. 6 and Fig. S3B and S3C). Hence, the observed production of equal levels of IL-17A in the *ex vivo* screen for these strains does not automatically result in similar degrees of protection. This might be due to the fact that the concentration of nasal IL-17A was slightly lower in mice infected with heterologous strains than in mice infected with the homologous challenge strain. Also, the kinetics of nasal IL-17A in response to pneumococcal infection may differ between homologous and heterologous strains. This could not be determined in the present study, as the IL-17A concentration was measured at only a single time point, i.e., 3 days postinfection. Vaccine-induced IgG or other pneumococcal virulence factors, including capsule or PspA expression, may also contribute to the difference in the magnitude of colonization reduction seen among TIGR4, PBCN266, and PBCN321. Also, the clinical strains might have an increased capacity to colonize the murine nasopharynx, compared to TIGR4, which may result in delayed clearance of these strains, as measured at 3 days postinfection.

A nonspecific reduction in bacterial colonization following vaccination with empty OMVs was observed for TIGR4 (Fig. S4A). This reduction in CFU was associated with an increased nasal IL-17A level (Fig. S4B), suggesting that IL-17A may contribute to the nonspecific decrease in pneumococcal loads. Mucosal adjuvants such as cholera toxin subunit B (CTB) and cholera toxin (CT) were also described to reduce pneumococcal colonization in a nonspecific manner, although the underlying mechanisms may differ (23, 24). The nonspecific increase in the IL-17A concentration might be derived from

innate cells present in the nasal mucosa, including macrophages, NKT cells, ILC3 cells, and $\gamma\delta$ T cells, which directly respond to *S. pneumoniae* infection upon recognition (25, 26).

The $\alpha1\alpha2$ fragment grafted onto the OMVs represents the majority of the N-terminal region of PspA (17) and is an interesting vaccine target based on existing studies of both mice and humans. Multiple studies implicate the coiled-coil N-terminal domain in cross-reactivity with and cross protection against pneumococcal strains (27–33). Importantly, the PRR that potentially cross-reacts with human myosin is not part of the $\alpha1\alpha2$ fragment of PspA (34). In mice and humans, MHC-II T-cell epitopes are localized in this particular fragment of PspA (33, 35–39). Mucosal $CD4^+$ $IL\text{-}17^+$ T cells were detected in the human upper respiratory tract and are likely to play a role in protection against bacterial colonization in humans, similar to what we have observed in our mouse studies (40). Whether an OMV-based vaccine shown to reduce pneumococcal colonization will also prevent pneumococcal disease and transmission remains to be studied.

The classification based on the $\alpha1\alpha2$ types from 345 invasive clinical isolates from The Netherlands appears to be consistent with those from other regions of the world, as suggested by analyses of the large carriage cohort. One exception is formed by serotype 23B, which was not present in the cohort of 345 invasive clinical isolates. Nineteen (25%) of the undeterminable PspA $\alpha1\alpha2$ variants were nontypeable pneumococci, which play a limited role in invasive disease (41). However, almost half (46.2%) of the serotype 23B isolates had undeterminable $\alpha1\alpha2$ variants, suggesting the existence of a subpopulation with a divergent $\alpha1\alpha2$ genotype. Altogether, we observed high coverage (89.3%) of our studied $\alpha1\alpha2$ variants in carriage isolates of this large cohort.

The method for Th17 cross-reactivity screening, as described in the present study, can be applied to pneumococci collected in any region of the world as well as for screening of cross-reactive Th17 responses by other pneumococcal proteins. This is particularly interesting, as currently, global efforts are being undertaken to sequence thousands of pneumococcal strains before and after vaccine introduction in developing countries (42).

In conclusion, OMV-induced protection was dependent on IL-17 immunity. A common "IL-17A-associated" epitope, important for cross protection, was identified in the variable $\alpha1\alpha2$ region. The capacity to evoke *in vivo* protection can easily be measured by *ex vivo* Th17 screening, which allows the optimal selection of combined protein antigens to increase vaccine coverage.

MATERIALS AND METHODS

Bacterial strains. Pneumococcal laboratory strains and human clinical isolates belonging to the Pneumococcal Bacteremia Collection Nijmegen (PBCN) were grown in Todd-Hewitt broth supplemented with yeast extract until mid-log phase was reached and were stored at −80°C (43, 44). Pneumococcal stocks, used for mouse challenge infections with 10^6 CFU in 10 μl phosphate-buffered saline (PBS), were prepared as described previously (17).

A description of the methods used for the cultivation of *Salmonella enterica* serovar Typhimurium and *Escherichia coli*, for the construction of the Δ*msbB* OMV production strain (Table S1), and for the methods used to confirm the detoxification of LPS using mass spectrometry and human PBMC stimulation can be found in the supplemental material.

OMV vaccine production. Unless stated otherwise, *S.* Typhimurium SL3261 Δ*tolRA* OMVs displaying HbpD or HbpD-$\alpha1\alpha2$ derived from the *S. pneumoniae* TIGR4 background were isolated as previously described (17). To isolate OMVs derived from the SL3261 Δ*tolRA* Δ*msbB* strain, cells harboring HbpD or HbpD-$\alpha1\alpha2$ expression vectors were grown until an optical density at 660 nm (OD_{660}) of ~0.02 was reached, at which time 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to induce the expression of HbpD-$\alpha1\alpha2$. Growth was continued overnight, after which OMVs were isolated from the culture medium as described previously (17).

Alignment and classification of $\alpha1\alpha2$ domains. Genome sequences and serotypes were obtained as described previously by Cremers et al. (43, 44). PspA-encoding genes were identified from the genome sequences by aligning the conserved signal sequences, the CDRs, and the conserved C-terminal domains against all protein-encoding sequences of the genomes by using blastp (see Table S2 in the supplemental material) (45). Conserved protein domains in the $\alpha1\alpha2$ PspA coding sequence were detected by aligning the protein sequence between the signal sequence and the PRR with MEME using default settings (46). Variable domains were numbered according to their sequence conservation, as determined

by the E (expectation) value. Overrepresented peptides were detected by associating the number of unique amino acid 13-mers in the $\alpha1\alpha2$ region with the level of IL-17 expression after splenocyte stimulation.

Methods for the prediction of MHC-II epitopes in the $\alpha1\alpha2$ domains are described in the supplemental material.

Mouse immunization and challenge. Mouse immunization and challenge were performed as previously described and as described in detail in the supplemental material (17). Animal experimentation was performed with the approval of the Radboud University Medical Center Committee for Animal Ethics (RU-DEC2014-206).

Mouse splenocyte isolation and stimulation. Harvested spleens were temporarily stored in RPMI-GlutaMAX (Life Technologies) with 10% fetal calf serum (FCS; Greiner Bio-One) and 1% penicillin-streptomycin and transferred through a 70-μm cell strainer (BD Falcon) to obtain single-cell suspensions. Erythrocytes (RBCs) were lysed by using RBC lysis buffer (eBioscience), after which cells were thoroughly washed. Splenocytes (5×10^5 cells/well) were added to round-bottom 96-well plates (Nunc) and mixed with 5×10^5 CFU of the pneumococcal cultures at a multiplicity of infection (MOI) of 1 in an end volume of 200 μl. Cells were incubated at 37°C in 5% CO_2 for 72 h. The supernatant was harvested by centrifugation at 450 $\times$ *g* and stored at −20°C until further analysis.

IL-17A analysis. Mouse IL-17A concentrations in the supernatants collected after restimulation of the splenocytes were measured by using the commercial Mouse IL-17A ELISA Max Standard kit (BioLegend, ITK Diagnostics) according to the manufacturer's instructions. The detection limit was 31.25 pg/ml.

IL-17A production in mouse nasal samples was determined with a cytometric bead array (Becton Dickinson) according to the manufacturer's instructions, using the Mouse Enhanced Sensitivity buffer kit in combination with the Enhanced Sensitivity Flex set for IL-17A (Becton Dickinson). Concentrations were calculated by using Soft Flow FCAP Array v1.0 (Becton Dickinson).

Vaccine coverage prediction. To determine the prevalence of PspA $\alpha1\alpha2$ variants and thereby the projected vaccine coverage, available sequences from pneumococcal isolates collected from a large carriage cohort were used. In total, 1,352 isolates, including 33 serotypes, were evaluated to determine their $\alpha1\alpha2$ genotype. Detailed methods are described in the supplemental material.

Statistical analyses. All statistical analyses were performed by using GraphPad Prism version 5.0 (GraphPad Software). Human cytokine data were analyzed by using a Wilcoxon signed-rank test. For bacterial recovery data, the Grubbs outlier test was used to test for significant outliers. Mann-Whitney *t* tests for two groups were used for comparisons of vaccine-induced reductions in pneumococcal colonization and nasal IL-17A immune responses. *Ex vivo* Th17 screening data were compared to the RPMI medium control using univariate analysis of variance with Dunnett's posttest in SPSS.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00281-17.

SUPPLEMENTAL FILE 1, PDF file, 2.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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J.L. is chief scientific officer at and W.S.P.J. is (in part) employed by Abera Bioscience, which aims to exploit the outer membrane vesicle platform for vaccine development.

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