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A Novel Outbred Mouse Model to Study Lung Memory Immunological Response Induced by Pertussis Vaccines

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ABSTRACT
Lung tissue resident memory (TRM) T cells can provide rapid and effective protective immunity against respiratory pathogens such as Bordetella pertussis. We assessed an outbred CD1 mouse model and i.m. immunization to study vaccine-induced immune memory, using pertussis vaccines as an example. The phenotypes of cells from the lungs of CD1 mice that had been primed with either i.m. whole-cell B. pertussis (wP), acellular B. pertussis (aP) vaccines or buffer (unvaccinated) and challenged with B. pertussis were determined using flow cytometry and immunohistology. We observed a rapid and high increase of CD4+ T cells expressing TRM markers by flow cytometry, supported by immunohistology observations, in lungs from wP-immunized mice. Priming mice with wP vaccine induced a more potent CD4+ response in lungs following B. pertussis challenge than priming with aP vaccine, although both were less potent than that observed in primoinfected mice. We also observed for the first time, to our knowledge, that CD8+ and γδ+ TRM-like T cell responses were induced in lungs of wP-primed mice postinfection. This novel outbred CD1 mouse model with i.m. immunization that enabled us to study vaccine-induced B. pertussis–specific memory T cells in lungs could be useful for evaluating candidate parenteral vaccines against B. pertussis or others pulmonary pathogens. ImmunoHorizons, 2020, 4: 762–773.

INTRODUCTION
Mouse models are useful for dissecting the role of different elements of the innate and adaptive immunity and are therefore used in vaccine development, although they do not reproduce entirely all symptoms of human diseases, including coughing (1). The most widely used mouse strains in biomedical research are isogenic, or inbred. Their genetic homogeneity is thought to minimize phenotypic or trait variability, and therefore improve reproducibility, although a recent study did not find evidence of greater trait stability in inbred mice (2). However, inbred strains cannot always replicate genetic variations to disease or response to vaccines like those seen in human populations.

Whooping cough (pertussis) is a vaccine-preventable respiratory infectious disease caused by Gram-negative bacteria, Bordetella pertussis. Initially, whole-cell B. pertussis (wP) and then acellular B. pertussis (aP) vaccination programs drastically reduced the incidence of pertussis worldwide, however a resurgence of B. pertussis infections has been reported recently in many countries (3–5). Waning immunity after pertussis vaccination is one plausible explanation (6, 7). Studies on cellular immune response in humans, baboons, and mice have consistently demonstrated distinct immune response profiles induced by wP and aP vaccines. Immunization with wP vaccines, as well as natural B. pertussis infection, induce specific Th1 and Th17 responses, whereas aP vaccines predominantly induce Th2-type responses (8–11). Also, there is some evidence the protection against B. pertussis infection induced by aP vaccines does not last long as that induced by wP vaccines, which is considered to be one of the potential reasons for resurgence of the disease (12, 13).

There is a growing body of evidence that tissue resident memory (TRM) T cells play a central role in many different...
pathogenic infections, including whooping cough, by coordinating host lung immunity (14–16). An inbred BALB/c mouse model was used to demonstrate that CD4+ TRM T cells, that accumulated in the lungs following B. pertussis infection, were significantly expanded through local proliferation following reinfection, with rapid bacterial clearance (17). These CD4+ TRM T cells, which were shown to be B. pertussis–specific secreted IL-17 and IFN-γ after in vitro restimulation with inactivated bacteria. Similarly, Ag-specific TRM T cells observed in mice lungs infected with B. pertussis proliferated rapidly after reinfection of convalescent mice, with an associated IL-17 production (18). It has been shown that i.p. immunization of inbred C57BL/6 mice with two doses of wP vaccine at a 4-wk interval, but not aP vaccine, induced Ag-specific CD4+ TRM T cells in their lungs (19). The wP-induced CD4+ TRM T cells were shown to confer protection when transferred to naive irradiated mice, in the same way as CD4+ TRM T cells induced by previous natural infection. Thus, induction of B. pertussis–specific CD4+ TRM T cells might partly explain the longer-lasting protection observed for wP vaccines compared with aP vaccines (19–22).

The objective of the current study was to develop a relevant model of lung memory immune response in outbred mice that could be used to investigate lung memory immune responses to vaccines during preclinical development, using wP and aP vaccines to test the model because they have distinct immune response profiles (8–11). Unlike previous studies that used inbred mice and administration routes that are not used in humans, the model we developed was in an outbred mouse strain using administration routes that are used in humans as recommended by the World Health Organization for vaccine development (23).

We, therefore, initially tested two immunization routes commonly used for pertussis vaccines (i.e., s.c. and i.m.). The lung memory immune response was evaluated by assessing TRM T cells responses following challenge with B. pertussis in lungs of outbred mice following vaccination using flow cytometry and localization of the TRM T cell responses using immunofluorescence staining on frozen lung sections.

MATERIALS AND METHODS

Mice

Outbred female CD1 mice (Charles River Laboratory, Saint Germain sur l’Arbresle, France) were used at 9 wk at day 0 (D0) for the infection-induced immunity mouse model and 5 wk old at D0 for the vaccine-induced immunity mouse model. All animal experiments were carried out in accordance with European guidelines and approved by French Ministry of Higher Education, of Research and Innovation and Sanofi Pasteur’s ethics committee.

Infection-induced lung immunity model

A group of CD1 mice were infected with B. pertussis at D0 and, after recovering, were reinjected at day 41 (D41) (the reinjected or convalescent group), another group received casamino acid buffer at D0 and were infected with B. pertussis at D41 (primoinfected group), and another group mice received casamino acid buffer at D0 and D41 (naive group). On D43, day 47 (D47), and day 57 (D57) mice in each group were sacrificed and their lungs harvested to perform cell cytometry and immunofluorescence staining.

The B. pertussis suspensions used for the infection were prepared from a culture of the World Health Organization reference B. pertussis strain 18323 (provided by the U.S. Food and Drug Administration) grown on Bordet-Gengou agar (Difco) supplemented with 20% defibrinated sheep blood (Sanofi Pasteur, Alba La Romaine, France) at 36°C in 5% CO2 for 24 h. A theoretical dose of 5 × 10^6 CFUs was instilled intranasally in a volume of 30 μl in mice anesthetized by i.m. injection of Imalgene (ketamine 60 mg/kg; Merial SAS) and Rompun (Xylazine 4 mg/kg; Bayer). The naive mice received 30 μl of casamino acid buffer under the same conditions.

A nonsense challenge with a suspension of the Pseudomonas aeruginosa, strain PA103 (ATCC29260) was performed to assess the specificity of the results (nonsense challenge group). P. aeruginosa was selected as a nonsense challenge because it also causes respiratory infections. The challenge suspensions were prepared directly from a frozen stock of bacteria grown overnight in tryptic soy broth (no. 41146; Biomerieux). A theoretical dose of 7.5 × 10^5 CFUs in 30 μl was instilled intranasally in mice, anesthetized as above, who had previously been infected with B. pertussis or had received casamino acid buffer (nonsense challenge group and nonsense naive group, respectively).

Vaccines and vaccination schedule

Two commercialized vaccines diphtheria, tetanus, and wP (DTwP) (D.T.COQ/D.T.P; Sanofi Pasteur), DTaP (DAPTACEL; Sanofi Pasteur), and a novel modified Tdap formulation (mTdap) containing a lower dose of diphtheria, denoted by the lowercase d, than the previous vaccines and genetically detoxified pertussis toxin (10 μg), pertactin (5 μg), filamentous hemagglutinin (5 μg), and Fimbriae 2.3 (FIM2.3; 7.5 μg) were used.

Mice were primed via s.c. and i.m. routes, as indicated in each experiment, with one fifth of a human dose of DTwP, or with one fifth of a human dose of DTaP (50 μl in both hind legs). The DTwP-primed mice received an i.m. booster of one fifth of a human dose of DTaP (50 μl in both hind legs) of DTwP, and the DTaP-primed mice received mTdap containing 0.66 mg/ml aluminum hydroxide (AlOOH) alone. The third dose, when given, was mTdap in both groups. The different vaccination schemas were chosen because it has been observed that two doses of DTwP are needed to induce a Th1/Th17–orientated response in the spleen (N. Rouleau, C. Panel, E. Proust, Y. Liu, and M. Chabaud-Riou, unpublished, internal data observed on splenocytes), whereas the first dose of DTaP induces a Th2-orientated response. Hence in DTaP-primed mice, the second and third doses were with the booster vaccine (mTdap), which has a lower Ag concentration, in contrast with the DTwP-induced mice that needed a second dose of DTwP before the booster vaccine. Control mice received i.m. injections of TBS under the same conditions.
**Preparation of lung cell suspensions**

To differentiate between circulating and tissue-resident cells, 1.8 μg of fluorescent-labeled pan-lymphocyte marker (CD45.2, anti-mouse CD45-BV786 Ab, no. 564225; BD Biosciences) was administered i.v. to mice 10 min before euthanasia and harvesting of lungs, as described previously (24–26). The circulating T cells were thus labeled in vivo and could be readily excluded from the flow cytometry analysis, whereas the T cells within tissues were protected from in vivo labeling.

Mice were euthanized under Imalgen and Rompun anesthesia (80 mg/kg ketamine and 16 mg/kg xylazine) with 1 ml of a 50% Dolethal solution by i.p. injection before lung harvest. After harvest, the lungs were washed in sterile PBS to eliminate the external blood, and 0.1 ml of an enzymatic solution (no. 130-095-927; Miltenyi Biotec) was injected in the lobes using an insulin syringe and the lungs transferred to gentleMACS C tubes containing 2.5 ml of enzymatic solution, supplemented with brefeldin A (5 μg/ml) when intracellular cytokine staining was performed. The lungs were cut into small pieces using the gentle MACS Octodissociator before being incubated for 30 min at 37°C and mechanically disrupted. The samples were then passed through a 70-μm cell strainer followed by RBC lysis. Finally, the viable mononuclear cells were isolated using Histopaque 1083 solution (no. 1083-1; Sigma-Aldrich) and counted using a Guava Muse Cell Analyzer (Luminex). We observed that more than 80% of the cells were viable cells (data not shown).

**Determination of bacterial load kinetics**

The course of bacterial infection was followed by assessing the number of bacteria (in log10 CFU per lung) in the lungs at specified time points. For this, undiluted and serially diluted lung cell suspensions were plated on Bordet-Gengou agar supplemented with 20% sheep’s blood (six drops, 25 μl each) and incubated for 72 h at 36°C, and the number of colonies recorded.

**T cell phenotyping by flow cytometry**

T lymphocytes were distinguished from the other cells by their expression of CD3. Among these CD3+ T cells, CD4+, CD8+ and γδ T cells were selected by the expression of CD4, CD8a, and γδ TCR, respectively. The phenotypes of CD4+ and CD8+ T cells were investigated to distinguish between central memory T-like cells that were CD44+ and CD62L and effector memory T (TEff)-like cells that were CD44+ and CD62L-. CD4 and CD8 T Eff-like T cells express CD69 and, optionally, CD103 (CD69+, CD103+) and γδ T Eff-like T cells both CD69 and CD103 (CD69+, CD103-). The TH1 and Th17-immune orientation of cells was documented with intracellular staining of IFN-γ and IL-17A, respectively.

The isolated lung cells, prepared as described above, were washed in PBS and incubated with 1/100 dilution of Zombie NIR (no. 423105; BioLegend). The cells were then washed, blocked with TrueStain (Fc Block CD16/CD32 equivalent, no. 101320; BioLegend), and surface stained for 20 min at 4°C with a mixture of Abs composed of CD3-BV786 (no. 100231; BioLegend), CD4-Viogreen (no. 130-109-413; Miltenyi Biotec), CD69-Vioblue (no. 130-103-949; Miltenyi Biotec), CD103-Viobright 515 (no. 130-111-609; Miltenyi Biotec), TCRγδ-PerCP-Vioblue (no. 130-109-754; Miltenyi Biotec), CD8a-PE-Vio615 (no. 130-109-251; Miltenyi Biotec), CD62L-PE-Vio770 (no. 130-112-649; Miltenyi Biotec), and CD44-AF700 (no. 103026; BioLegend).

For detection of intracellular cytokines, the cells were fixed with Cytofix/Cytoperm (no. 554714; BD) followed by staining with IFN-γ–allophycocyanin (no. 130-109-723; Miltenyi Biotec) and IL-17A–PE (no. 130-111-857; Miltenyi Biotec). Fluorescent-labeled Abs without one specific Ab isotype or with a nonspecific Ab isotype were used as controls. Flow cytometry was performed on an LSR Fortessa flow cytometer, and data were acquired using Diva software (BD Biosciences). The results were analyzed using FlowJo software.

**Immunofluorescent staining of lung sections**

The mice were euthanized with a 1-ml dose of Dolethal (diluted to one half) after blood sampling and before lung harvest. The harvested lungs were fixed at +4°C in a 1% paraformaldehyde solution overnight and then placed in sucrose gradients (10, 20, 30%) at +4°C for 2 h each. Lung tissues were transferred to OCT (Optimum cutting temperature compound, no. 12678646; Thermo Fisher Scientific), placed in the OCT chamber to freeze, and were then stored at −80°C.

Just before use, the frozen specimens were processed with a cryostat to obtain 10- to 12-μm-thick sections that were placed on slides and kept at −20°C. After permeabilization, Fc receptors were blocked with anti-mouse CD16/CD32 (no. 553142; Becton-DickinsonFrance) 1/50 in (NaH2PO4, Na2HPO4, Trizma hydrochloride, BSA Fract V, Triton solution) and FCS for 30 min. The slides were incubated with a mixture of conjugated CD4 (AF647-labeled anti-mouse CD4, no. 100530; BioLegend), CD103 (BV421-AF405–labeled anti-mouse CD103, no. 562771; BD Pharmingen), and CD69 (AF555-labeled anti-mouse CD69, no. AH09045818; CliniScience) Abs for 1 h at room temperature and in darkness. The stained lung sections were photographed with a fluorescence microscope AxioObserver 7 (Zeiss) at ×20 magnification and the pictures analyzed with ImageJ.

**Statistical analysis**

The results were expressed as the mean ± SEM. Differences between groups were assessed using one-way ANOVA. All analyses were done on SAS v9.4. The p values < 0.05 were statistically significant.

**RESULTS**

**B. pertussis challenge in convalescent CD1 mice induced a rapid accumulation of CD4+ T cells associated with a Th1/Th17 profile in lungs**

To characterize the infection-induced lung immunity mouse model, mice received buffer solution at D0 followed by *B. pertussis* at D41 (primoinfected group), or they were infected with *B. pertussis* twice at D0 and D41 (convalescent group). In addition, an uninfected group received buffer solution at D0 and D41 (Fig. 1A). In the
FIGURE 1. Bacterial load and accumulation of CD4+ T cells in lungs of primoinfected and convalescent mice.

(A) Schema of the model: one group of six mice received casamino acid buffer on D0 and was infected with B. pertussis on D41 (primoinfected group); one group of six mice was infected with B. pertussis on D0 and reinfected on D41 (convalescent group); and one group of three mice received casamino acid buffer on D0 and D41 (uninfected group). The lungs from two mice in the primoinfected and convalescent groups and one mouse in the uninfected group were harvested on D43, D47, and D57, respectively, for determination of bacterial load, T cell phenotyping and immunofluorescence labeling. (B) Kinetics of bacterial load per lung (log_{10} CFUs ± SEM) on D43, D47, and 57 in the three groups. (C) Immunofluorescence labeling of lung sections from mice in the uninfected, primoinfected, and convalescent groups with AF647-labeled anti-mouse CD4 Abs on D43 (i.e., 2 d postchallenge), showing clustering of CD4+ T cells in convalescent mouse lungs but not in primoinfected or uninfected mice lungs. The staining shown is representative of samples from three or four mice. T cell phenotyping by flow cytometry (dots represent individual results and the bar represents the mean for each group, showing a statistically significant increase in (D) CD4+ among CD3+ T cells, in (E) CD4+−IFN-γ among CD4+ T cells, and in (F) CD4+ IL-17 T cells in convalescent mice on D43, 2 d postchallenge compared with primoinfected mice. NB, the scales for the Y-axis are different.

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primoinfected group, the bacterial load peaked 2 d postinfection (D43) and declined down to 16 d postinfection (D57) but remained above the limit of detection, whereas in the convalescent group, the bacterial load was eliminated rapidly (Fig. 1B).

The immunofluorescence staining showed clustering of CD4+ T cells in the lungs of the convalescent mice 2 d postreinfection, but none were observed in the lungs of primoinfected or control mice (Fig. 1C). On 2 d postreinfection (D43), the frequency of CD4+ T cells among the CD3+ T cells was significantly higher in reinfected mice than in primoinfected mice (74 versus 56%, p < 0.001) (Fig. 1D). The frequencies of IFN-γ- and IL-17–producing CD4+ T cells among the CD4+ T cells were also statistically significantly higher in the convalescent mice than in primoinfected mice (p = 0.002 and p < 0.001, respectively).

**Convalescence from a primoinfection increased frequency of CD4 T cells expressing TRM markers after homologous, but not nonsense bacterial challenge**

As immunity induced by *B. pertussis* infection is reported to induce lung TRM CD4+ T cells that might confer long-lasting protection, we verified that our CD1-mouse model could be used to demonstrate local memory recall responses consistent with that reported in the literature (17). Our data showed that the CD4+ T cells exhibiting TRM markers, called TRM-like CD4+ T cells, were already present at 2 d postinfection (D43) at higher frequencies in reininfected mice than in naive mice (Fig. 2). At 2 d postinfection the frequencies of TRM-like phenotype CD4+ T cells were similar in both the *B. pertussis*– and the *P. aeruginosa*–challenged groups because the mice in both groups were convalescent after a first infection with *B. pertussis* (Fig. 2). Fig. 1 shows a significant reduction of bacteria by 2 d postreinfection, which could be related to the increase in pertussis-specific TRM up to day 6 after homologous challenge, which was not seen after the nonsense challenge with *P. aeruginosa*. In mice reinfected with *B. pertussis*, there was a statistically significant increase in TRM-like CD4+ T cells, compared with the naive mice, which peaked at 6 d postreinfection (D47). In contrast, in mice reinfected with *P. aeruginosa* no increase in CD4+ TRM-like T cells was observed, only a slow decrease over time.

Taken together, our data from the infection-induced lung immunity model demonstrated that TRM CD4+ T cells were induced by *B. pertussis* infection and they were still present at significant level 43 d later. These cells were specifically recalled and proliferated rapidly following a homologous challenge but not a nonsense challenge. Moreover, our results showed that the experimental conditions used were suitable for studying local immune memory response and that we could, therefore, use them for the vaccination model.

**i.m. immunization with wP induced higher TRM-like T cell responses in lungs following B. pertussis challenge than s.c. immunization**

To assess if the two administration routes used for human vaccination induced different responses in mice, two groups of eight mice were immunized with DTwP via the i.m. or s.c. route on D0 and day 42 (D42) (Fig. 3A). Three mice received an injection of casamino acid buffer on D0 and D42 (nonvaccinated group). All mice were then challenged with *B. pertussis* on D57 and their lungs harvested 2 d postchallenge (day 59). CD4+ and CD8+ TEM-like T cells were statistically significantly more frequent among the total populations of CD4+ and CD8+ T cells at 2-d postchallenge in the i.m.-vaccinated group, but not the s.c.-vaccinated group, compared with the nonvaccinated group (Fig. 3B). The frequencies of CD4+, CD8+, and TCRγδ TRM-like T cells among the total CD4+, CD8+, and TCRγδ T cells at D2 postinfection in the i.m.-vaccinated mice were statistically higher in the nonvaccinated group mice (Fig. 3C). The frequency of CD4+ TEM-like cells, but not the CD8+ and TCRγδ TRM-like T cells, was statistically significantly higher in the s.c.-vaccinated mice compared with the nonvaccinated mice (Fig. 3C, left-hand panel). These results demonstrated that there was an early increase of TEM-like and TRM-like T cells in lungs, which was higher in the i.m.-vaccinated mice; i.m. vaccination was therefore used in the subsequent experiments.

![FIGURE 2. Specificity of response to *B. pertussis*.](https://doi.org/10.4049/immunohorizons.2000094)
Memory T cell profile in lungs of mice primed with i.m. DTwP or DTaP and boosted with DTwP/mTdap or mTdap/mTdap following challenge with B. pertussis

Mice received i.m. vaccination with DTwP (n = 24) or DTaP (n = 24) on D0 and were boosted on D42 and day 81 (D81) with either DTwP/mTdap or mTdap/mTdap and then challenged with B. pertussis on day 134 (D134) (Fig. 4A). The lungs from six mice in each group were harvested on day 129 (5 d prechallenge) and then from six mice in each group at 2 d, 6 d, and 7 wk postchallenge (i.e., day 136 [D136], day 140 [D140], and day 192, respectively) (Fig. 4A). Nonvaccinated mice that had recovered from a B. pertussis infection on D80 were also challenged with B. pertussis on D134 and their lungs harvested at the same time points in six mice (convalescent group). A group of mice received three i.m. injections of casamino acid buffer on D0, D42, and D81 and were then challenged and their lungs harvested on day 129, D136, D140, and day 192 (nonvaccinated group). The bacterial load in the lungs declined rapidly 2 d postchallenge (D136) in the
convalescent groups as well as in the wP- and aP-immunized group, whereas it remained high in the nonvaccinated controls (Fig. 4B).

The frequencies of CD4+, CD8+, and γδ T RM-like T cells in the reinjected group were statistically significantly higher than in the nonvaccinated group (Fig. 4C). The frequency of CD4+ T RM-like
T cells peaked 6 d postchallenge (D140), and for the CD8\(^+\) and γδ TRM-like T cells, the peak was 2 d postchallenge (D136). The gating strategy used is shown in Supplemental Fig. 1.

The mice that had been primed with DTwP and boosted with DTwP/mTdap showed a rapid and significant increase in CD4\(^+\) TRM-like T cells by D2 postchallenge, which was maintained up to day 6 before declining to baseline level at 7 wk postchallenge. A significantly higher proportion of these CD4\(^+\) TRM-like T cells coexpressing CD103 and CD69 was observed in the convalescent group compared with the DTwP/DTwP/mTap-vaccinated group, which primarily induced TRM cells expressing CD69 but not CD103 (data not shown). There was also an increase in CD8\(^+\) and γδ TRM-like cells that was significant only on D2 postchallenge. In contrast, a small and transient increase in CD4\(^+\) TRM-like T cells D2 postchallenge (p < 0.05 compared with controls), with no significant increases in CD8\(^+\) and γδ TRM-like T cells, was observed in the mice immunized with DTaP/mTdap/mTdap. In both the DTwP/DTwP/mTdap- and DTaP/mTdap/mTdap-immunized groups, we detected small but statistically significantly higher frequencies of CD4\(^+\) TRM-like, but not CD8\(^+\) or γδ TRM-like T cells, 5 d prior to challenge compared with nonimmunized control mice.

**Immunization with a DTwP vaccine promotes apparition of T cell clusters containing CD4\(^+\) T cells expressing TRM-like phenotype**

Because residency is an important feature of TRM cells, we demonstrated that the CD4\(^+\) T cells expressing TRM-like phenotypes were localized within the lung tissue, using immunofluorescence staining of lung sections collected 7 d postchallenge from mice immunized as shown in Fig. 4A. We observed a few CD69\(^+\) CD4\(^+\) T cells in the lungs of nonvaccinated infected mice but none in the naive mice lungs (Fig. 5A, 5B). In reinfected mice, clusters of CD69\(^+\) and CD103\(^+\) stained cells were observed colocalized with CD4\(^+\) T cells near the airways, around the bronchi, demonstrating that these T cells were located near the site of pathogen entry (i.e., via the airways into the lungs 7 d postchallenge) (Fig. 5C). In reinfected mice and in mice immunized with DTwP, immunofluorescence analyzes of tissue sections cut from mouse lung samples revealed an

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**FIGURE 5. Localization of clusters of CD4\(^+\) T cells expressing TRM-like phenotypes in mice lungs collected 7 d postchallenge using immunofluorescence staining.**

In each panel, the white square on the left-hand image indicates the location of the magnified area shown on the right-hand side. The cells were labeled with triple-staining (gray, anti–CD4–AF647; red, anti–CD69–AF555; purple, anti–CD103–BV421) and green is autofluorescence. Red arrows indicate double-positive CD4\(^+\)/CD69\(^+\) cells. Purple arrows indicate double-positive CD4\(^+\)/CD103\(^+\) cells. (A) Nonvaccinated naive mouse; (B) nonvaccinated primoinfected mouse; (C) convalescent-reinfected mouse; (D) DTwP/DTwP/mTap–vaccinated mouse; (E) DTaP/mTdap/mTdap–vaccinated mouse. The photos are representative of staining seen in samples from three or four mice.
abundance (between 12 and 25) of CD4+ T cells clusters, some of which were too dense to enable the total CD4+ T_{RM}-like cells to be counted. In primoinfected mice and in mice immunized with DTaP, immunofluorescence analyses revealed fewer CD4+ T cells clusters (between 2 and 12).

In challenged DTwP-primed mice, CD69 and CD103 CD4+ T cells were observed in cluster near blood vessels and around bronchi (Fig. 5D). Most cells were double-stained, CD69+/CD4+, or CD103+/CD4+, with a few triple-stained cells. In contrast, in DTaP-primed mice, CD4+ T cells were distributed throughout the lung parenchyma in low numbers, with some forming small clusters near blood vessels and bronchi (Fig. 5E). Some of these cells were double-stained with CD69+ but only a few were double-stained with CD103+. These observations highlight different CD4+ T cells and CD4+ T_{RM}-like T cell responses to B. pertussis challenge between mice with infection-induced immunity and those with DTwP or DTaP vaccine-induced immunity.

**DISCUSSION**

Animal infection challenge models are needed to test human vaccine candidates and vaccination schedules in preclinical studies. In this study, we demonstrated that an outbred CD1 mouse challenge model for B. pertussis lung memory immunity could be useful for assessing both infection-induced lung memory immunity in reinjected mice that had recovered from a previous infection and, more importantly, vaccine-induced lung memory immunity in mice that were challenged with B. pertussis. In this approach, we combined the identification of CD4+, CD8+, and γδ T_{RM}-like T cells by flow cytometry of cells harvested from lungs, with immunofluorescence staining of frozen lung sections. This enabled the phenotype of the memory T cell subsets to be determined, to provide evidence of their residency and to document their localization within the lung tissue.

The flow cytometry results from our study showed the outbred CD1 mice could be used to study lung T cell phenotype profiles from infection-induced immunity. We showed that B. pertussis infection-induced immunity was associated with the generation of T_{RM} T cells during the primary infection, which then proliferate rapidly after reinfection with B. pertussis, as has been reported previously (17). In addition, the results provide evidence that the proliferation of T_{RM} T cells in the lungs was pertussis-specific because infection with a P. aeruginosa strain (nonsense challenge) did not induce their proliferation. We deduced that proliferation had occurred from the increased frequency of the cells, but this would need to be confirmed by comparing BrdU/5-ethyl-2’-deoxyuridine (EdU) incorporation in the lung T_{RM} and circulating spleen cells.

In the vaccine-induced immunity model, we observed a statistically significant high increase of CD4+ T_{RM} T cells in lungs harvested from DTwP-primed mice (DTwP/DTwP/mTdap) after B. pertussis challenge, whereas in mice primed with DTaP and boosted with mTdap (DTaP/mTdap/mTdap), we observed only a low and transient increase of these cells 2 d postchallenge. However, it was not possible to study cytokine production with our vaccine-induced immunity model because of the low cell count and the associated low cytokine concentration. In addition, because the mice are outbred, it was not possible to induce proliferation by coculturing them with APCs from other mice.

The immunofluorescence results showed many CD4+ T cell clusters localized mainly near the major blood vessels in the reinjected and vaccinated groups. Some of these CD4+ T cells coexpressed the associated resident-memory markers CD69 or CD103, in agreement with the flow cytometry results. The ability of aP and wP pertussis vaccines to induce such differences in T_{RM} T cell responses was described previously in another experimental C57BL/6 inbred mouse model (19). In this model, i.p. immunization with an aP vaccine (two 1/50 human doses at 4 wk intervals) was reported not to increase respiratory T_{RM} CD4+ T cells, compared with control mice, in contrast to the increase observed after immunization with a wP vaccine.

We also observed a transient and significant increase in CD8+ T_{RM}-like T cells in mice immunized with i.m. DTwP 2 d postchallenge with B. pertussis, but this was not maintained at 6 d postchallenge. Using this model, we demonstrated for the first time, to our knowledge, that a wP vaccine can induce CD8+ T_{RM} T cells. Because B. pertussis has been reported to persist intracellularly within human macrophages in vitro, pertussis-specific CD8+ T_{RM}-like T cells could thus, contribute to protection against this pathogen but this needs to be explored further (27). Compared with T_{RM}-like T cell populations in other tissues, lung T_{RM}-like T cells generally have a limited longevity although repeated Ag exposure has been reported to extend the longevity of lung CD8+ T_{RM} T cells in a C57BL/6 mouse influenza model (28–31). Finally, we observed an increase of γδ T_{RM}-like T cells in mice immunized with i.m. DTwP, demonstrating that it was possible to measure an expanded memory cell response with this mouse model and that the model could, therefore, be used to study the overall memory cell responses for new candidate vaccines.

Our data suggest that the balance between the cellular and humoral responses are different in convalescent mice, those vaccinated with wP, and those vaccinated with aP vaccines, implying that there three distinct mechanisms of protection. Additionally, IgG1 and IgG2a titers are lower in sera from convalescent mice than sera from vaccinated mice, which could result in less mucosal Ig being present in the lung initially following challenge, which could explain the slower bacterial clearance in convalescent mice. Although correlates of protection for pertussis vaccines have not been well defined, it has been suggested that Abs play a critical role in mediating protection (32). This is further supported by the immunity observed after the transfer of pertussis-specific maternal Abs to newborns (33). However, several murine studies have demonstrated an important role for CD4+ Th1/Th17 cells in long-lasting protection; therefore, these are often considered as critical effectors for novel pertussis vaccines (20, 34). Vaccination with DTaP/mTdap has been shown to induce high concentrations of anti–pertussis toxin (PT) neutralizing Abs, as measured by Chinese hamster ovary test (C. Panel, Y. Liu, N. Reveneau, and M. Chabaud-Riou, unpublished work).
results). Anti-PT neutralizing Abs, whether actively induced or passively administered have been reported to confer protection against lethal infection in mice (35, 36).

The presence of CD4+ and CD8+ TRM T cells have been reported in human and mice lungs (37, 38). The CD8+ TRM T cells are preferentially localized within specific niches of regenerated tissue following lung injury (termed repair-associated memory depots), whereas the CD4+ TRM T cells are localized in the airways or around B cell follicles (26, 39). In our study, we observed that mice primed with either DTwP or DTaP and then boosted with mTdap were protected against B. pertussis lung colonization, despite no increase in CD4+ TRM-like T cells in aP-vaccinated mice. This also supports the plausibility of distinct mechanisms of protection between DTwP and DTaP against lung colonization, particularly that humoral responses might play a more important role in DTaP-induced protection. DTaP/mTdap vaccination elicited similar titers of pertactin-, filamentous hemagglutinin-, and FIM2,3-specific IgG1 and IgG2a Abs as DTwP/DTwP and higher PT-specific IgG1 (E. Proust, N. Rouleau, Y. Liu, and M. Chabaud-Riou, unpublished results), consistent with the lower PT content of DTwP (40). Consistent with these results, the number and size of T cell clusters in immunofluorescent-stained lung sections from mice immunized with DTaP vaccine 7 d postchallenge were not higher than in lung sections from naive mice and CD69+ and CD103+ CD4+ T cells were also absent in both. Moreover, whereas we observed an increase of CD4+ TRM T cells in nonvaccinated mice long after the challenge, nothing changed in DTaP-immunized mice. This suggests that DTaP vaccination could even interfere with the natural infection-acquired memory immunity, maybe because of faster Ab-mediated clearance of the bacteria, which could have prevented an effective cellular response.

In human and animal lungs, several cell surface markers have been associated with TRM cells, including the upregulation of CD49a, CD69, and CD103 (30, 41, 42). However, some markers currently used to identify TRM-like cells, particularly CD103, have been reported to be differentially expressed between and within tissues, in particular, in lung tissue (43, 44). In our study, CD103 expression by TRM-like T cells was different following natural infection or vaccination. For instance, natural infection with B. pertussis induced a significantly higher proportion of CD4+ TRM-like coexpressing CD103 in addition to CD69 compared with vaccination, which primarily induced T RM cells expressing CD69 but not CD103. In mice, CD69+ is expressed by the majority of CD4+ and CD8+ TRM-like T cells located in multiple sites (24, 28, 44, 45). Although CD69 is also a marker of early activation of T cells, its expression has been associated with T RM T cell phenotype in human and mouse lungs (46). The functional requirement for CD69 in the generation and maintenance of CD8+ T RM T cells has been reported to depend on the tissue (47). In some tissues, such as the small intestine, it seems to have no detectable role, whereas in others, such as the kidney, it is critical. This suggests that the functional relevance of CD69 varies considerably and so should be used cautiously as a marker for CD8+ TRM-like T cells, at least. However, the expression of CD69 does not affect CD4+ T RM T cell formation unlike for CD8+ T RM T cells (48). Although, it is reassuring to have consistent results from two different techniques for studying T RM T cells, such as those used in our study, other techniques could be used to assess different aspects of the vaccine-induced local memory response (e.g., single-cell RNA sequencing of the lung cells could be used to determine the transcriptomic profile of the T RM-like and T RM T cells) (46). This technique, single-cell RNA sequencing has recently been reported used to show that the onset, kinetics, and longevity of immune responses induced by DTwP and DTaP priming followed by Tdap boosting in a Göttingen minipig model are very similar to the human responses (C. Vaure, V. Gregoire-Barou, V. Courtois, E. Chautard, C. Degletagne, and Y. Liu, submitted for publication).

Both the immunization route and the vaccine formulation are important parameters to be considered in the development of safe and effective pertussis vaccines that will be able to induce T RM-like T cells in lungs and nasal tissue (49, 50). Although the mechanism for the generation of the T RM T cells is still unclear, evidence suggests that the route of priming is crucial for the induction of a strong humoral response (49). Systemic immunization routes have been shown to induce local cell-mediated immunity, for example, in the intestine (51). Recirculation of dendritic cells or early effector T cells through different lymphoid organs, where they may access a variety of homing signals could contribute to the dissemination of effector T cell responses (52, 53). Nevertheless, our study results demonstrate that parenteral immunization of mice, at least with a wP vaccine, elicited an effective distant mucosal response with generation of lung T RM T cells. Recent studies, consistent with our findings, demonstrated similar effective mucosal memory response following s.c. parenteral vaccination in the genital tract and nasal tissue (54, 55). Furthermore, in this outbred mouse model of memory immunity, we observed that vaccination by the i.m. route induced a higher level of T RM-like T cells in the lungs than vaccination by the s.c. route, in compliance with World Health Organization guidance for human vaccine development, which states that the vaccination route to be used in animal testing should be the same as that to be used in humans (23).

Unlike previous models using inbred mice and vaccination routes that are not used in humans, we demonstrated the advantages of using a murine immunogenicity model, which combined outbred mice and i.m. vaccine administration, for candidate vaccine selection. This approach should increase the probability of success for the selected candidate vaccine in human trials.

We have presented the characteristics of a novel model of lung memory immunity in outbred CD1 mice using the example of wP and aP pertussis vaccines administrated by the parenteral route. Using this model, we showed that reinfection with B. pertussis in mice, that have recovered from a previous infection, induced pertussis-specific CD4+ T cells in lungs, as has already been reported in inbred mice. We also confirmed that priming mice with wP vaccine induced a more potent CD4+ T RM-like T cell response in lungs following B. pertussis challenge than priming with aP vaccine, although both were less potent than the natural
primoinfection. In addition, this is the first demonstration, to our knowledge, that the i.m. vaccine administration leads to CD4+ TRM T cell response, as well as CD8+ γδ T RM-like T cell responses in lungs following challenge. Thus, this model can be used for further assessments of lung memory immunity induced by novel pertussis parenteral vaccines and could be considered for assessing lung memory immunity induced by other vaccines, such as influenza and pneumococcal vaccines.

DISCLOSURES

All authors are employed by Sanofi Pasteur.

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