Suppression of mucosal Th17 memory responses by acellular pertussis vaccines enhances nasal *Bordetella pertussis* carriage

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**INTRODUCTION**

Pertussis, also known as whooping cough, is a highly contagious respiratory disease, mainly caused by *Bordetella pertussis* (Bp). The severe manifestations of pertussis occur in young children, but pertussis also affects adolescents, adults, and the elderly. After the implementation of whole-cell pertussis vaccines (wPVs) combined with diphtheria- and tetanus-toxoid-based vaccines, referred to as DTP vaccines, in the 1950s and 1960s, the disease burden markedly lessened. However, concerns about the safety of wPV led to the replacement of DTP vaccines with diphtheria-tetanus acellular pertussis vaccines (aPVs) in most industrialized countries in the 2000s. Less reactogenic than wPV, aPVs contain one to five purified Bp antigens and are highly effective in preventing pertussis disease.

Despite high vaccination coverage, pertussis is now re-emerging in several areas of the world. Possible reasons that may contribute to this resurgence include improved diagnosis, the emergence of antigenic variants that escape vaccine-mediated immunity, suboptimal and fast waning immunity, especially after aPV vaccination, and the inability of the current vaccines to prevent infection and transmission of Bp.

Improving our understanding of pertussis pathogenesis and how vaccines may modulate Bp interactions with its host is needed to enhance whooping cough control. Although mice do not develop the characteristic cough associated with the disease, immunization with wPV was associated with the induction of Th1 and Th17 immune responses and ensuing neutrophil recruitment.

**RESULTS**

aPV immunization prolongs nasal carriage of Bp in Th2-prone mice.

To evaluate the relative protection conferred by wPV and aPV in the upper and lower respiratory tracts of mice, 6-week-old C57BL/6 and BALB/c mice were immunized twice subcutaneously with 1/10 human dose of Infanrix (aPV) or ShanS (wPV) vaccine at a 4-week interval or left unvaccinated as controls. Compared to Infanrix (aPV), the bacterial burden in the noses and lungs of the mice was determined by counting colony-forming units (CFUs) on the homogenized organs.
mice were injected intravenously with an anti-CD45-PE antibody. Protection was also evident when the mice were infected with a nasal colonization by Bp in C57BL/6 mice20,24. We, therefore, investigated whether the Th2-prone BALB/c mice might be less efficient than C57BL/6 mice in inducing these cells in the nasal tissues upon Bp infection. To identify TRM cells in the nasal tissues, mice were injected intravenously with an anti-CD45-PE antibody (CD45siv), enabling us to distinguish infiltrated resident cells from circulating immune cells. Following the intranasal challenge, up to ~30% of immune cells in the noses of BALB/c mice were lymphocytes expressing CD3 (Fig. 2a). Among the CD3+ T cells, the proportion of CD69+ CD4+ T cells doubled 14 d.p.c. (Fig. 2a). These CD69+ CD4+ T cells up-regulated CD44 and CD103 expression over time after infection (Fig. 2a), indicating that BALB/c mice were able to induce TRM cells in the nasal tissue after Bp infection. However, compared to C57BL/6 mice, expression of these TRM markers was somewhat delayed in BALB/c mice (Fig. 2b). Nonetheless, at 14 d.p.c., C57BL/6 and BALB/c mice shared a similar expression pattern of both markers (Fig. 2b).

Measurement of IL-17- and/or IFN-γ-producing cells after stimulation with phosphor myristate acetate (PMA) and ionomycin in the presence of brefeldin A showed that similar to TRM cells from C57BL/6 mice, TRM cells from BALB/c mice were activated quickly after challenge and expressed mainly IL-17 (Fig. 2c). However, only 14% of TRM cells from BALB/c mice versus 32% of TRM cells from C57BL/6 mice expressed IFN-γ or both IL-17 and IFN-γ 14 d.p.c. (Fig. 2c). Thus, BALB/c mice are not intrinsically unable to induce nasal IL-17-producing CD4+ TRM cells upon infection with Bp.

aPV immunization impairs the induction of nasal Th1 and Th17 TRM cells in BALB/c mice upon Bp infection

As Bp infection leads to the induction of Th1 and Th17 TRM cells in the nasal tissues of BALB/c mice, we examined the effect of aPV or wPV immunization on Bp infection-induced TRM cells in the noses of these mice. Whereas upon Bp infection an increase in both CD103+ CD4+ and CD103+ CD4+ TRM cells was detectable among CD69+ CD4+ T cells in the nasal tissues of BALB/c mice from 7 d.p.c. on and peaking at 14 d.p.c., prior aPV vaccination completely abolished the induction of these cells (Fig. 3a, upper panels). Representative examples of CD103+ CD4+ T cell enrichment among CD69+ CD4+ -resident T cells between 7 and 28 d.p.c. are shown in Fig. 3a (lower panels). In contrast, immunization with wPV did not inhibit the induction of CD4+ TRM cells upon Bp infection, but instead resulted in earlier induction of the CD103+ CD4+ TRM cells that were significantly enriched 7 d.p.c. compared to non-vaccinated mice (Fig. 3a).

In addition, the CD103+ CD4+ TRM cells from aPV-immunized mice were poorly activated, as <20% of CD103+ TRM cells from aPV-immunized mice expressed IL-17 or IFN-γ upon stimulation with PMA and ionomycin 7 or 28 d.p.c., whereas most of (>50%) of the stimulated CD103+ CD4+ TRM cells from control and wPV-immunized mice produced IL-17 and/or IFN-γ 28 d.p.c. (Fig. 3b). In IL-17-IFN-γ- cells were also enriched in the CD103+ CD4+ TRM cell subset of control and wPV-immunized mice. No IL-17-IFN-γ- TRM cells were detected in aPV-immunized mice. Absolute counts of IL-17+ TRM and IFN-γ+ TRM cells show that IL-17+ TRM cells predominated in wPV-immunized mice (Fig. 3c, d). IL-17+ CD103+ TRM cells were significantly enriched in wPV-immunized mice compared to control mice 7 d.p.c., while at later time points after infection, the numbers of IL-17+ CD103+ TRM cells tended to be higher in control mice than in wPV-immunized mice (Fig. 3c). A similar observation was made for the IFN-γ-producing CD103+ CD4+ TRM cells (Fig. 3d). Thus, early protection conferred by wPV against nasal colonization of BALB/c mice was associated with an rapid expansion of IL-17+ CD103+ CD4+ TRM and to a lesser extend IFN-γ- CD103+ CD4+ TRM cells. In contrast to wPV, aPV immunization totally inhibited the induction of these cells in the nasal tissues of Bp-infected BALB/c mice. A similar trend was also observed for IL-17+ CD103+ CD4+ TRM and for IFN-γ+ CD103+ CD4+ TRM cells.

To examine whether the difference between the aPV and wPV effect on the inhibition of TRM cell induction in the nose of BALB/c mice might be related to differences in serum antibody responses to the two vaccines, we measured serum IgG titers to whole B. pertussis cell extracts in aPV- and in wPV-immunized mice before and after...
B. pertussis challenge. Both before and after the challenge, wPV and aPV induced high levels of anti-B. pertussis serum antibodies (Supplementary Fig. 2). These titers were slightly, but significantly higher in wPV-immunized than in aPV-immunized mice, making it unlikely that the difference in nasal TRM cell induction was due to a blunting effect by aPV-induced serum antibodies.

IL-17 is essential for the control of nasal but not of lung infection by Bp

As aPV immunization appeared to prevent natural clearance of nasal Bp colonization and to inhibit the induction of IL-17- and/or IFN-γ-producing CD4+ TRM cells in the noses of Bp-infected BALB/c mice, we investigated the relative role of IL-17 and IFN-γ in controlling nasal carriage of Bp. IL-17 knockout (KO) mice and IFN-γ KO mice were infected nasally with Bp, and lung and nasal colonization was followed over time. Whereas both KO mice cleared the lung infection as quickly as the control mice, regardless of the initial bacterial burden (Fig. 4a, left panel and Supplementary Fig. 3a, left panel), IL-17-deficient mice were unable to control nasal infection by Bp and carried the infection for at least 56 d.p.c. without a trend of decrease in bacterial load (Fig. 4a, right panel and Supplementary Fig. 3a, right panel).

**Fig. 2** Bp-infected BALB/c mice develop Th17 TRM cells in the nose. Six-week-old C57BL/6 mice and BALB/c mice were infected with 10⁶ CFU of B1917GR. Nasal immune cells were collected at indicated time points post challenge (D0–D28). Ten minutes before euthanasia, mice were intravenously injected with anti-CD45-PE antibody enabling us to discriminate TRM cells from circulating cells. TRM cells express CD44, CD69, and/or CD103. Cell activation was assessed by nonspecific ex vivo stimulation and intracellular staining of IL-17 and IFN-γ. Cells were fixed and permeabilized to perform intracellular staining of IL-17 and IFN-γ. Numbers indicate percentages of events in each square. a Representative graph showing the accumulation of CD103+CD44+TRM cells in the nose of BALB/c mice at indicated time points after Bp challenge. b Representative graph comparing the pattern of expression of CD44 (left panels) and CD103 (right panels) in the nose of C57BL/6 (black) and BALB/c mice (gray) at indicated time points after Bp challenge. Numbers indicate mean fluorescence intensities and dotted lines indicate the cut-off between positive and negative cells. c Representative graph showing the proportion of IL-17- and/or IFN-γ-producing CD4+ TRM cells in the nose of C57BL/6 (left panels) and BALB/c mice (right panels) at indicated time points after Bp challenge. Numbers indicate percentages of events in each square.
IFN-γ deficiency only slightly affected Bp clearance compared to control mice.

We then assessed the effect of aPV and wPV immunization on lung and nasal clearance of Bp in IL-17 KO mice compared to control mice. With a moderate challenge dose, either vaccine provided protection against lung colonization in IL-17 KO mice similar to the control mice (Supplementary Fig. 3b, c, left panels, respectively), while protection conferred by aPV and wPV was similar to the control mice (Supplementary Fig. 3b, c, left panels, respectively). As expected, neither vaccine was able to provide protection against nasal colonization compared to non-transferred mice, as at 7 d.p.c. the bacterial load in the noses of KOt and KO mice were similar (Fig. 5b). After the second transfer given 7 d.p.c., the bacterial load in the noses of KOt mice started to decrease at a rate similar to that of C57BL/6 control mice, indicating that the transfer of Bp-induced CD4+ TRM cells at indicated time points after Bp challenge. Results shown are geometric means ± SD. n = 6 per group. Kruskal–Wallis tests were performed to compare aPV (*) and wPV (**) immunized mice to control mice. Only significant differences are indicated. *P < 0.05; **P < 0.001.

Lack of CD4+ TRM cell induction results in lack of neutrophil recruitment

In an attempt to identify the effector cells involved in nasal clearance, we examined the nasal influx of neutrophils upon Bp infection. CD45+ Ly-6G+ neutrophils infiltrated in the noses of Bp-infected C57BL/6 mice 14 d.p.c. were compared to non-infected mice (Fig. 5c, d). No significant increase in neutrophils was observed in infected IL-17 KO mice, whereas transfer of nasal CD4+ TRM cells from Bp-infected C57BL/6 mice resulted in significant neutrophil recruitment in the noses of IL-17 KO mice.
neutrophils (Fig. 5d). Overall, the data suggest that upon Bp infection nasal CD4+ T_em cells induce the recruitment of neutrophils in the noses, we immunized BALB/c mice with aPV or wPV and examined the recruitment of nasal neutrophils over time. While Bp infection nasal CD4+ T_em cells induce the recruitment of neutrophils by releasing IL-17 and thereby enhance bacterial clearance.

To determine whether the inability of aPV-immunized BALB/c mice to clear the nasal infection by Bp was also due to their failure in recruiting neutrophils in the noses, we immunized BALB/c mice with aPV or wPV and examined the recruitment of nasal neutrophils over time. While Bp infection nasal CD4+ T_em cells induce the recruitment of neutrophils in the noses, this influx was totally abolished in aPV-immunized, but not in wPV-immunized mice (Fig. 5e, f). The recruitment of other nasal CD3− CD11b+ immune cells was not impaired in aPV-immunized mice (Supplementary Fig. 4).

aPV immunization allows extracellular persistence of Bp in the noses of BALB/c mice

As in vitro data suggest that Bp can invade and survive within eukaryotic cells25 and can also adopt a biofilm lifestyle allowing persistent colonization in mice26, we assessed the impact of aPV immunization on intracellular versus extracellular nasal Bp carriage. aPV- or wPV-immunized BALB/c mice were nasally infected with Bp B1917GR, and nasal homogenates (NWs) and homogenates were assessed for CFU counts at different time points after challenge (Fig. 6a). The nasal homogenates were treated with collagenase D and DNase I for tissue and biofilm disruption26,27. After treatment, the cells were harvested by centrifugation and extracellular bacteria were counted by CFU measurements of the supernatants. To measure the amounts of intracellular bacteria, the cells were washed and incubated with polymyxin B in order to kill potentially remaining extracellular bacteria. Cells were then lysed and intracellular bacteria were quantified by CFU measurements.

Approximately 10- to 100-fold more bacteria were found in the nasal homogenates compared to the NWs at each time point, suggesting that most bacteria were tightly associated with the nasal epithelium (Fig. 6b). In contrast to control mice and wPV-immunized mice, the bacteria remained associated with the nasal epithelium in aPV-immunized mice for at least up to 56 d.p.c., whereas both vaccines reduced the bacterial load in the NWs (Fig. 6b). Virtually no intracellular bacteria were detected 3 h.p.c. (day 0), whereas substantial amounts of intracellular bacteria were detectable at 7 d.p.c., especially in control mice and in aPV-immunized mice (Fig. 6c). Intracellular bacteria were eventually cleared in all three groups 28 d.p.c. (Fig. 6c).

**DISCUSSION**

Several reasons have been proposed to explain the resurgence of pertussis, especially in countries using aPV, although many studies have shown a good efficacy of aPV against pertussis disease. These reasons include the rapid waning of protective immunity conferred by aPV28 compared to wPV29,30 and the inability of
aPv, as well as of wPV to prevent Bp infection and pertussis transmission. Using mouse models, we show here that in addition to the failure of immunization with aPV or wPV to prevent against nasal colonization aPV immunization resulted in substantially prolonged nasal Bp carriage in Th2-prone BALB/c mice when compared to non-immunized mice, while it was protective against lung colonization. These findings are consistent with observations in a non-human primate model showing that aPV immunization, while protecting against disease, caused prolonged carriage of Bp-challenged baboons. We found that aPV-induced prolonged nasal carriage in BALB/c mice was paralleled with vaccine-mediated inhibition of nasal IL-17- and/or IFN-γ-producing CD103⁺ CD4⁺ T_Rm cells induced by Bp infection. Recently, Wilk et al.⁰ showed that, in contrast to wPV immunization, aPV immunization fails to expand T_Rm cells in the lungs and noses and provided evidence that T_Rm cells promote bacterial clearance in mouse lungs by adoptive transfer of CD4⁺ T_Rm cells in irradiated mice or by treating mice with FTY720 during the immunization phase, which prevents migration of T and B cells from lymph nodes to the circulation¹⁹,²⁰. We found here that aPV not only fails to induce IL-17⁺ and/or IFN-γ⁺ CD4⁺ T_Rm cells, but even prevents the induction of these cells in the nasal tissue of Bp-infected BALB/c mice, while they were strongly induced in non-vaccinated mice upon Bp challenge, peaking at 14 d.p.c., and were maintained for

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**Fig. 5** Bp-specific nasal CD4⁺ T_Rm cells protect IL-17 KO mice against nasal colonization and induce recruitment of neutrophils. **a** Schematic of the transfer protocol. C57BL/6 mice were infected nasally (i.n.) with 10⁶ CFU of B1917GR. CD4⁺ T_Rm cells were purified from noses of infected C57BL/6 mice 14 d.p.c. A total of 10⁶ CD45iv⁻CD45⁺ CD4⁺⁺ CD69⁺ CD4⁺ T_Rm cells were transferred intravenously (i.v.) to IL-17 KO mice 10 h.p.c. and 7 d.p.c. (blue arrows). Control IL-17 KO and C57BL/6 mice received PBS (black arrows). CFU in the nasal tissues was counted at indicated time points post challenge. Nasal cells were collected 14 d.p.c. and analyzed by flow cytometry (FACS). **b** C57BL/6 (in gray), IL-17 KO (KO, in purple), and transferred IL-17 KO mice (KO_t, in blue) mice were infected with 10⁵ CFU of B1917GR (open bars) or left uninfected (gray bars), and absolute numbers of neutrophils per nose were measured 14 d.p.c. Numbers indicate percentages of events in each square. **c** Representative graphs showing neutrophil (CD45iv⁻ CD45⁺ Ly-6G⁺) recruitment in the nose of C57BL/6 (left panels), IL-17 KO (KO, middle panels), and transferred IL-17 KO mice (KO_t, right panel) mice 14 days after infection with 10⁵ CFU of B1917GR. Neutrophil recruitments of non-infected and infected mice are depicted in the upper (gray) and lower (red) panels, respectively. Numbers indicate percentages of events in each square. **d** C57BL/6 (in gray), IL-17 KO (KO, in purple), and transferred IL-17 KO mice (KO_t, in blue) were infected with 10⁵ CFU of B1917GR, and CFU numbers were counted at indicated time points. **e** General findings are consistent with observations in a non-human primate model showing that aPV immunization, while protecting against disease, caused prolonged carriage of Bp-challenged baboons. We found that aPV-induced prolonged nasal carriage in BALB/c mice was paralleled with vaccine-mediated inhibition of nasal IL-17- and/or IFN-γ-producing CD103⁺ CD4⁺ T_Rm cells induced by Bp infection. Recently, Wilk et al.¹⁹ showed that, in contrast to wPV immunization, aPV immunization fails to expand T_Rm cells in the lungs and noses and provided evidence that T_Rm cells promote bacterial clearance in mouse lungs by adoptive transfer of CD4⁺ T_Rm cells in irradiated mice or by treating mice with FTY720 during the immunization phase, which prevents migration of T and B cells from lymph nodes to the circulation¹⁹,²⁰. We found here that aPV not only fails to induce IL-17⁺ and/or IFN-γ⁺ CD4⁺ T_Rm cells, but even prevents the induction of these cells in the nasal tissue of Bp-infected BALB/c mice, while they were strongly induced in non-vaccinated mice upon Bp challenge, peaking at 14 d.p.c., and were maintained for...
Fig. 6 aPV immunization prevents clearance of extracellular, tightly adhering Bp in the noses of BALB/c mice. a Schematic of experimental procedures. aPV- (in yellow in b, c), wPV-immunized (in blue in b, c) or control BALB/c mice (Ctrl, in gray in b, c) were challenged with 10⁶ CFU of B1917GR and euthanized at indicated time points post challenge. NWs were collected before nose harvest to count recruitment, suggesting that Th17 TRM cells control the outcome of nasal colonization by Bp, as IL-17 KO mice were unable to clear nasal tissue, the latter were the main cells involved in the control of nasal colonization by Bp, as IL-17 KO mice were unable to clear nasal Bp infection, while able to control lung infection as well as control mice. Furthermore, adoptive cell transfer of nasal CD4⁺ TRM cells isolated from Bp-infected mice conferred protection against nasal colonization. Thus, aPV immunization prevents nasal Bp clearance in Th2-prone mice by preventing the natural induction of nasal IL-17⁺CD103⁺CD4⁺ TRM cells upon Bp infection.

IL-17 deficiency was associated with failure to recruit neutrophils upon Bp infection, and adoptive transfer of Th17 T RM cells to IL-17 KO mice was associated with accelerated neutrophil recruitment, suggesting that Th17 T RM cells control the outcome of the infection by enhancing neutrophil recruitment in the noses of Bp-infected mice. Through releasing of IL-17, Th17 T cells are known to attract neutrophils to the site of infection via CXC chemokines and to modulate neutrophil granulopoiesis via the induction of endogenous granulocyte colony-stimulating factor and stem cell factor. Consistent with a protective role of neutrophils in immunized or convalescent mice, neutropenia severely impairs clearance. Furthermore, Bp clearance from mouse lungs after the transfer of antibodies from convalescent mice was abrogated by neutrophil depletion or Fcy receptor deletion, suggesting that neutrophils mediate the killing of Bp in the lungs by opsonic phagocytosis. We recently showed that passive transfer of serum from aPV-immunized mice or mice immunized with the live attenuated BPZE1 vaccine conferred protection in the lungs of severe combined immunodeficiency mice but not in the nose, suggesting that serum immunoglobulins (Igs) do not efficiently protect in the nose. Instead, by using knockout models and passive transfer of mucosal Igs, it was demonstrated that mucosal IgA induced by BPZE1 contributes to nasal protection as well as IL-17, possibly through the induction of Th17 T RM cells. We found that Bp was present in three locations in the noses of BALB/c mice. A minority was loosely attached to the nasal epithelium and could easily be recovered in NWs. A small fraction was found in intracellular compartments and the vast majority of Bp organisms was tightly attached to the nasal epithelium. Both wPV and aPV immunization diminished the loosely attached bacteria, suggesting that they may have been cleared by transuded wPV- or aPV-induced serum antibodies. Immunization with aPV slightly prolonged the presence of intracellular bacteria in the noses of BALB/c mice. The strongest effect of aPV immunization was seen on the Bp organisms that were tightly attached to the nasal epithelium. Both wPV and aPV immunization diminished the loosely attached bacteria, suggesting that they may have been cleared by transuded wPV- or aPV-induced serum antibodies. Immunization with aPV slightly prolonged the presence of intracellular bacteria in the noses of BALB/c mice. The strongest effect of aPV immunization was seen on the Bp organisms that were tightly attached to the nasal epithelium. Both wPV and aPV immunization diminished the loosely attached bacteria; suggesting that they may have been cleared by transuded wPV- or aPV-induced serum antibodies. Immunization with aPV slightly prolonged the presence of intracellular bacteria in the noses of BALB/c mice. The strongest effect of aPV immunization was seen on the Bp organisms that were tightly attached to the nasal epithelium.

The localization of CD103⁺CD4⁺ T RM cells in the nasal epithelium positions them well to act as the first line of defense at subsequent exposure to Bp. While CD103⁺ T RM cells interact with epithelial cells through E-cadherin, CC44⁺ T cells may be retained in the underlying extracellular matrix. The greatest differences in T RM cell numbers and activation between aPV-immunized BALB/c mice and control mice were observed in the CD103⁺ subset of T RM cells. In addition, early accumulation of CD103⁺ T RM cells by wPV-primed mice compared to control mice was associated with a decrease in nasal bacterial burden at early
After 40 h of growth, the bacteria were harvested by scraping the plates
vaccines.

expression of antimicrobial genes by epithelial cells, further
IL-17/IFN-γ these cytokines are known to enhance phagocytosis. In addition,
may also be relevant for older Th2-prone individuals. The aPV

10% debrinated sheep blood, and 10^5 μg/ml gentamicin as described21. After electroporation, gentamicin-resistant derivatives were checked by
acquire gentamicin resistance as described43, thereby yielding B1917GR. B1917 was electroporated with the pFUS2-BctA1 suicide plasmid to
B1917, a pertactin-producing strain, came from the RIVM collection (Bilthoven, The Netherlands). For counter selection purposes, B1917 was electroporated with the pFUS2-BctA1 suicide plasmid to acquire gentamicin resistance as described43, thereby yielding B1917GR. After electroporation, gentamicin-resistant derivatives were checked by
PCR to verify the site of insertion of the pFUS2-BctA1 suicide vector in the bacterial genome. Bacteria were cultured at 37 °C on Bordet-Gengou (BG) agar (Difco Bordet-Gengou Agar Base), supplemented with 1% glycerol, 10% defibrinated sheep blood, and 10 μg/ml gentamicin, as described43. After 40 h of growth, the bacteria were harvested by scraping the plates and resuspended in PBS at the density of 5 x 10^6 or 5 x 10^7 CFU/ml. Whole B. pertussis cell lysates were prepared and used for antibody determination as described45.

Animals and ethical statement
BALB/c and C57BL/6 mice were purchased from Charles Rivers France. IL-17 and IFN-γ KO mice were kindly provided by F. Trottein (Institut Pasteur de Lille). All the animal experiments were carried out in accordance with the guidelines of the French Ministry of Research, and the protocols were approved by the Ethical Committees of the Region Nord Pas de Calais and the Ministry of Research (agreement number APAFIS 

Protection experiments
Mice were immunized subcutaneously with 1/10 human dose of aPV (Inflanrix, GlaxoSmithKline) or wPV (Shans, Shantha Biotechnics, India) and boosted 4 weeks later with the same dose. Control mice were injected with PBS. Eight weeks after the first immunization, mice were challenged intranasally with 10^6 or 10^7 CFU of virulent B1917GR. Mice were euthanized at different times post challenge, that is 3 h.p.c. and 7, 14, 28, and 56 d.p.c. NWs were performed with stainless steel feeding tubes (24 ga x 25 mm, Phymep). One milliliter of PBS was slowly injected through the trachea and the NW was collected. Lungs and noses were harvested and homogenized for CFU measurement by plating 10-fold serial dilutions onto BG agar plates containing 10 μg/ml gentamicin as described44.

Cell isolation for CFU tests
Infected BALB/c mice were euthanized at different times post challenge (3 h.p.c. and 7, 28, and 56 d.p.c.). NWs were collected prior to nose harvest to assess extracellular CFU in the nasal fluid. Nasal tissue was scraped from the nasal cavity, cut into small pieces, and digested with collagenase D (0.6 mg/ml; Roche) and DNase I (20 U/ml; Sigma-Aldrich) for 40 min at 37 °C. The nasal tissue was flushed several times and then passed through a cell strainer to obtain a single-cell suspension. Cells were pelleted and the supernatant was conserved to assess extracellular CFU. Cells were washed twice and then incubated for 1 h with 100 μg/ml polymyxin B sulfate (Sigma) to eliminate extracellular bacteria46. Cells were washed twice and then lysed with 0.1% saponin (Sigma). CFU counting was performed on cell lysates by plating 10-fold serial dilutions onto BG agar plates containing 10 μg/ml gentamicin to estimate the number of intracellular bacteria.

Cell isolation for flow cytometry analysis
Ten minutes before euthanasia, mice were injected intravenously with 10 μg of anti-CD45-PE antibody from eBioscience (30-F11, catalog#12-0451-82) to allow for the distinction of circulating T cells (CD45-PE+) and resident T cells or infiltrated immune cells (CD45-PE-). Nasal tissue was treated as described above. Red blood cells were lysed using ACK lysing buffer (Gibco). Isolated cells were stimulated with 50 ng/ml phorbol myristate (InvivoGen) and 500 ng/ml ionomycin (Sigma) in the presence of 5 μg/ml brefeldin A (Sigma) for 4 h at 37 °C. Cells were incubated with Dead Cell Stain Kit LIVE/DEAD Aqua (Invitrogen) first as recommended by the supplier, then with Fe block (BD Biosciences, dilution 1:50), followed by surface staining with the following fluorochrome-conjugated antibodies (dilution 1:400): CD45-APC (30-F11, catalog#559864), CD69-FITC (H1.2F3, catalog#11-0691-82), CD8-AF700 (53-6.7, catalog#56-0081-82), CD3-APC-eF780 (17A2, catalog#47-0302-82) from eBioscience, CD44-BV605 (IM7, catalog#103047), CD4-BV785 (RM5-4, catalog#100552) from BioLegend, and CD103-PE-CF594 (M290, catalog#565849) from BD Biosciences. For the detection of intracellular cytokines, cells were fixed and permeabilized using a FoxP3 transcription factor staining buffer set (eBioscience), incubated with normal rat serum (eBioscience) (1:50), and stained with the following antibodies: IL-17-V450 (TC11-18H10, catalog#560522, dilution 1:200) from BD Biosciences and IFN-γ-FITC-Y5 (XM12, catalog#25-7319-41, dilution 1:400) from eBioscience. Fluorescence minus one sample were used as controls. The gating strategy for the identification of 17^+ Tm cells is shown in Supplementary Fig. 5a. Fluorescence-activated cell sorting samples were acquired on an LSR Fortessa using the BD Diva Software (BD Biosciences) and analyzed using the FlowJo Software (v10, TreeStar). Neutrophils were identified with Ly-6G-PE-CF594 (1A8, catalog#562700, dilution 1:400) and CD11b-BV605 (M1/70, catalog#563015, dilution 1:400) antibodies from BD Biosciences (see Supplementary Fig. 5b). The total number of cells was calculated by multiplying the number of registered events by the ratio of CD45-APC+ cells over the absolute number of immune cells in the nasal tissue, determined by counting live immune cells in a hemocytometer.

Adoptive transfer of CD4^+ Tm cells from the nose
CD4^+ Tm cells were purified from noses of C57BL/6 mice infected with 10^7 CFU of B1917GR 14 d.p.c. Cells were treated as described above. CD45^+ CD44^+ CD69^+ CD4^+ T cells were sorted using BD FACS Aria II sorter (BD Biosciences). Purity of the isolated cells was checked prior to transfer (Supplementary Fig. 6). A total of 10^6 cells were transferred intravenously to IL-17 KO mice 10 h.p.c. and 7 d.p.c.

Statistical analyses
Statistical analyses were performed by non-parametric T tests, Kruskal–Wallis, or one-way analysis of variance tests and Mann–Whitney tests using the GraphPad Prism software. P values < 0.05 were considered significant.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.
DATA AVAILABILITY
The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary Materials. All other relevant data are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS
V.D. and C.L. designed the experiments; V.D., J.C., H.B.-L.R., A.T., and A.-S.D. performed the experiments; modified gentamicin-resistant Bp was constructed by L.C. V.D., A.T., and J.C. analyzed the data; V.D. and C.L. wrote the manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION
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