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Bordetella pertussis Induces Interferon Gamma Production by Natural Killer Cells, Resulting in Chemoattraction by Respiratory Epithelial Cells

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Background. Whooping cough is caused by infection of the airways with *Bordetella pertussis* (Bp). As interferon gamma (IFN-γ) is essential for protective immunity against Bp, we investigated how IFN-γ is induced by Bp or the virulence antigens filamentous hemagglutinin adhesin, pertactin, or pertussis toxin, and how IFN-γ contributes to local immune responses in humans.

Methods. Peripheral blood mononuclear cells (PBMCs) from healthy donors and/or respiratory epithelial cells were stimulated with soluble antigens or inactivated intact Bp and the presence or absence of blocking antibodies or chemokines. Supernatants and cells were analyzed for IFN-γ and chemokine production, and lymphocyte migration was tested using epithelial supernatants.

Results. The soluble antigens failed to induce IFN-γ production, whereas inactivated Bp induced IFN-γ production. Natural killer (NK) cells were the main source of IFN-γ production, which was enhanced by interleukin 15. Epithelial–PBMC co-cultures showed robust IFN-γ–dependent CXCL9 and CXCL10 production by the epithelial cells following stimulation with IFN-γ and Bp. The epithelial-derived chemokines resulted in CXCR3-dependent recruitment of NK and T cells.

Conclusions. Inactivated Bp, but not antigens, induced potent IFN-γ production by NK cells, resulting in chemoattraction of lymphocytes toward the respiratory epithelium. These data provide insight into the requirements for IFN-γ production and how IFN-γ enhances local immune responses to prevent Bp-mediated disease.

Keywords. whooping cough; *B. pertussis*; IFN-γ; CXCL10; CXCR3; NK; respiratory epithelium; cell migration.

Bordetella pertussis (Bp) is the causative bacterium for whooping cough. Bp colonization is mainly observed on the ciliated columnar epithelium of the respiratory tract. Adhesion molecules such as pertactin (Prn) and filamentous hemagglutinin adhesin (FHA) bind to the bronchial epithelium, which aids colonization [1, 2]. Although FHA binding to cilia is important for infection, the production of pertussis toxin (PT) is especially associated with disease. Because of their importance in virulence, these antigens are incorporated in component vaccines.

Studies in both humans and mice indicate the protective role of interferon gamma (IFN-γ) [3, 4]. IFN-γ–producing Th1 cells are observed in patients following infection and are implicated in clearing infections with Bp [5]. Natural killer (NK) and IFN-γ knockout mice fail to clear Bp, indicating their importance [3, 6, 7]. Although IFN-γ–producing Th1 cells are being induced by an infection, the development of Th1-mediated immunity has been reported to take weeks in mice. This indicates that the early-phase responses of the host to Bp, for instance by NK cells, may therefore be essential to contain an infection in the early stages of infection [3]. IFN-γ–enhanced clearance of Bp by macrophages likely benefits from rapid induction of IFN-γ production by innate T cells or NK cells [8]. NK cells and innate T cells are reported to contribute to controlling respiratory infections and may therefore also contribute to protective immunity to Bp [9, 10].

The airway epithelial cells comprise an essential first line of defense. This defense consists of a mechanical barrier, the production of antimicrobial factors, and by orchestrating downstream immune responses following infection through the production of chemokines and cytokines [11]. Respiratory epithelial cells express pattern recognition receptors that aid in the recognition of Bp and the recruitment of different immune cells in order to control an infection [12]. Epithelial cells respond to IFN-γ, and production of IFN-γ in the airways thus likely influences how epithelial cells regulate downstream immune responses [13]. This cross-talk is essential for the recruitment

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of cells required for early-phase immune defense to control infecting microbes [14, 15].

Although animal studies indicate the importance of early innate IFN-γ production in protective immunity to Bp infections, how early IFN-γ may protect in humans is incompletely understood. Such insight is also relevant in light of diminished cellular and IFN-γ responses induced by the acellular component vaccine compared to the previously used whole-cell Bp vaccine or natural infection. Therefore, we aimed at studying the production of IFN-γ at the early stage of stimulation with Bp antigens or intact Bp using human cell models. Because Bp infects epithelial cells that respond to IFN-γ, we investigated the implications of IFN-γ production for the response of epithelial cells to Bp exposure.

MATERIALS AND METHODS

Peripheral Blood Mononuclear Cells, Cell Purification, and Epithelial Cell Culture

Peripheral blood was collected in heparin vacuum tubes (BD Biosciences) from anonymous healthy donors who provided informed consent following the guidelines set by the Dutch government. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll plaque PLUS (GE Healthcare) density centrifugation. CD14 cells were depleted using CD14 microbeads (Miltenyi Biotec). PBMCs resuspended (10^7 cells/100 µL) in cold MACS buffer (phosphate-buffered saline [PBS], 0.5% bovine serum albumin [BSA], 2 mM ethylenediaminetetraacetic acid [EDTA]) were added to the beads and incubated at 4°C while rolling for 30 minutes. Then, cells were washed and added to LS columns (3 mL), washed 3 times, and harvested.

A549 and Calu-3 cells were obtained from ATCC. The cells were maintained in Dulbecco's modified Eagle's medium containing L-glutamine, sodium pyruvate, and 10% fetal bovine serum (FBS); 50 000 cells were added to cell culture inserts (PIHA01250, Millipore) and allowed to form confluent monolayers in submerged cultures. PBMCs and NK cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (1% FBS), which was also used for co-cultures with epithelial cells and the epithelial supernatant transfer migration experiments. In co-culture experiments, the epithelial cells were changed to IMDM + 1% FBS medium the day before. Epithelial cells on cell culture inserts and PBMCs were both stimulated with no physical contact between epithelial cells and PBMCs.

Bacteria and Antigens

The *Bordetella pertussis* isolates B0213, B0558, B1377 (before 2000), B2914, B3115, B3896, B4393, B4418, and B1917 (majority of studies) were cultured on Bordet-Gengou agar plates supplemented with 15% sheep blood (BD Biosciences) for 4 days at 35°C. Bacteria were harvested and colony-forming units determined by plating. Bacteria were heat-inactivated at 56°C for 30 minutes, and heat inactivation was confirmed by plating. Cytokine production by Bp was tested using a concentration range from 0.004 to 8 µg/mL, and 2 µg/mL was selected for further stimulation as interleukin 6 (IL-6) and interleukin 10 (IL-10) reached a plateau using this concentration, whereas tumor necrosis factor alpha (TNF-α), IFN-γ, and interferon alpha (IFN-α) did not plateau up to 8 µg/mL, indicating robust stimulation at a submaximal concentration of antigen. Bacteria were added to the cells at 2 µg/mL or a multiplicity of infection equivalent of 4. Inactivated PT (1 µg/mL) and FHA (2 µg/mL) were obtained from Kaketsuken and Prn (2 µg/mL) from the Dutch Institute of Public Health and the Environment, and lipopolysaccharide (LPS) (LPS-EK, 100 ng/mL) and cytosine phosphodiester guanine (CpG) (oligodeoxynucleotides (ODN) M362, 10 µg/mL) were obtained from InvivoGen.

Recombinant Proteins and Antibodies

Recombinant human IFN-γ, CXCL10, CCL3, and CX3CL1 were obtained from Peprotech and CXCL9 from BioLegend. Low endotoxin azide-free blocking antibodies for CXCR3 and IFN-γR and isotype control were obtained from BioLegend.

Detection of Cytokines

Supernatants were analyzed using the Legendplex human inflammatory panel (Biolegend). Supernatants were diluted in assay buffer and incubated in polypropylene V-shaped 96-well plates in equal volumes of beads, assay buffer, and detection antibody for 2 hours while shaking at room temperature in the dark. Then streptavidin–phycoerythrin (PE) was added and incubated for an additional 30 minutes, washed in access volumes of wash buffer, and acquired on a fluorescence activated cell sorting (FACS; BD Canto II).

Flow Cytometry

For intracellular staining, brefeldin A was added to the cells and incubated for 4 hours. The cells were placed on ice, harvested, and stained for surface markers for 30 minutes at 4°C in FACS buffer (PBS + 0.5% BSA and 2 mM EDTA). After washing (FACS buffer) the cells were fixed and permeabilized (BD FACS lysing and permeabilization solution 2, BD Biosciences) for 10 minutes. Cells were washed and incubated (30 minutes, 4°C) with antibodies for intracellular staining, washed and acquired using the BD Fortessa X-20 system, and analyzed using FlowJo software (version 10, BD).

The following antibodies were used for flow cytometry: CD16-BV421, CD56-PE-Cy5, CD3-Pacific Blue, CD4-BV786, CD8-AF700, CD161-BV605, and vα7.2-PE-Cy7 from BioLegend; IFN-γ-PE, IP-10-PE, and granzyme B–AF647 from BD Biosciences; and interleukin 15 (IL-15)–APC from Invitrogen.

Data Analyses

Data were entered into GraphPad Prism 8 software. Figures show mean values and standard error of the mean. Statistically significant differences were tested using 1-way analysis of

variance (ANOVA) with Dunnett post hoc testing, or 2-way ANOVA with Sidak post hoc testing.

RESULTS

Cytokine Production by Antigens and Intact *B. pertussis*

To study how the soluble antigens FHA, Prn, or PT or intact Bp induce cytokine production, PBMCs from healthy donors were stimulated for 20 hours and supernatants analyzed [16]. FHA did not induce production of TNF-α, IL-6, or IL-10 (Figure 1A). PT induced high levels (around 6000 pg/mL, similar to Bp) of TNF-α, but little IL-6 and IL-10. Prn induced moderate levels of TNF-α, IL-6, and IL-10. Some (around 100 pg/mL) IFN-α, an antiviral cytokine also involved in inducing Th17 immunity to Bp in mice [8], was observed following stimulation with PT or Bp. FHA failed to induce IFN-γ production, intermediate levels were induced by Prn and PT (around 500 pg/mL), and the highest levels by inactivated Bp (1000 pg/mL).

Although antigens and inactivated Bp were added at similar concentrations, less IFN-α was induced by PT and Prn, and less IFN-γ was induced by FHA, PT, and Prn compared to intact Bp (Figure 1B). As this may be because Bp is a complex mixture of antigens and Toll-like receptor (TLR) ligands, PBMCs were co-stimulated with antigens and TLR ligands. Addition of CpG induced increased production of IFN-α, whereas no additional production was observed when FHA was added, and production was inhibited by PT and Prn (Figure 1C). LPS and FHA together increased IFN-γ production, and cytosine phosphodiester guanine and synthetic acylated lipopeptide increased the production of IFN-γ in combination with PT. None of the 3 TLR ligands tested increased interferon production induced by Prn.

IL-15 Contributes to the Induction of IFN-γ Production

Macrophages and monocytes bind to Bp-FHA and are a major source of the pleiotropic cytokine IL-15 that potently induces IFN-γ production and contributes to respiratory immunity [17–20]. The expression of IL-15 on monocytes was increased following exposure to Bp (Figure 2A). Stimulation of PBMCs with Bp resulted in increased IL-15 on the membranes of lymphocytes (Supplementary Figure 1). Addition of IL-15 to Bp-stimulated PBMCs indeed results in synergistic production of IFN-γ. The production of IFN-γ was inhibited in the presence of IL-15R–blocking antibodies, but not in the presence of isotype control antibodies (Figure 2B).

IFN-γ Is Produced by NK Cells and Mucosal-Associated Invariant T Cells

To identify the cellular origin of the produced IFN-γ, PBMCs were stimulated and stained for CD4 and CD8 T cells, NK cells, and mucosal-associated invariant T cells (MAIT) ($CD3^+CD161^+TCRv\alpha7.2^+$) and IFN-γ (Supplementary Figure 1). Little IFN-γ expression was observed in CD4 or CD8 T cells after stimulation, which slightly increased when IL-15 was added (Figure 2C). Of the MAIT and NK cells, 15% and 23%, respectively, produced IFN-γ, significantly more than CD4 and CD8 T cells (Supplementary Figure 2). Intracellular staining confirmed that soluble antigens of Bp, even when all 3 were combined, do not induce IFN-γ production (Figure 2D). The addition of IL-15 did not induce significant production of IFN-γ following stimulation with single antigens or the combination of Bp antigens. However, when LPS was added, NK and MAIT cells produced more IFN-γ (Supplementary Figure 3).

Monocytes Regulate IFN-γ Production

As monocytes are a source of IL-15 and may contribute to NK and MAIT cell activation [21], the effect of depleting $CD14^+$ monocytes on IFN-γ production was evaluated. Depletion of monocytes resulted in significantly decreased levels of TNF-α, IL-6, and IL-10 following stimulation with Bp or Bp and IL-15 (Figure 3A). However, the production of IFN-γ was increased in PBMCs stimulated in the absence of monocytes (Figure 3B) by both NK and MAIT cells (Figure 3C), but not CD4 and CD8 T cells (Supplementary Figure 4). Because depletion of monocytes resulted in increased levels of IFN-γ and concentrations of the regulatory cytokine IL-10 were decreased, we tested whether the increased IFN-γ production may be caused by the production of IL-10 by monocytes. Indeed, when cells were stimulated with Bp and IL-15 in the presence of an IL-10R blocking antibody, increased percentages of IFN-γ-producing NK and MAIT cells were observed (Figure 3D).

NK Cells Produce IFN-γ Independent of Other Cells

As NK cells are the main source of IFN-γ (Figure 4A) and do not need monocytes to produce IFN-γ, we tested whether purified NK cells could produce IFN-γ. Purified NK cells (untouched, Supplementary Figure 5) co-stimulated with IL-15 produced some IFN-γ when stimulated with Prn and IL-15, but more profound IFN-γ production was observed when NK cells were stimulated with Bp and IL-15 (Figure 4B; Supplementary Figure 6). LPS or CpG alone induced IFN-γ production by purified NK cells co-stimulated with IL-15, which was inhibited when FHA was added. Further analyses of the NK cells showed that the highest proportion of $IFN\text{-}\gamma^+$ cells are $CD16^+$ and the highest levels of IFN-γ were observed in $CD16^-$ NK cells (Figure 4C). Granzyme B levels were largely unaffected by stimulation with Bp (Figure 4C). Stimulation with 8 genetically distinct isolates confirmed that the induction of IFN-γ production by Bp-exposed NK cells is a general phenomenon (Figure 4D). Comparison of multiple Bp isolates and donors indicate that the variation between donors is larger than the variation between Bp isolates (Supplementary Figure 7).

Epithelial Cells Enhance IFN-γ Production

As Bp is a noninvasive pathogen that adheres to respiratory epithelial cells, we studied how co-culturing and co-stimulating epithelial cells on transwell inserts with PBMCs (contact-free)

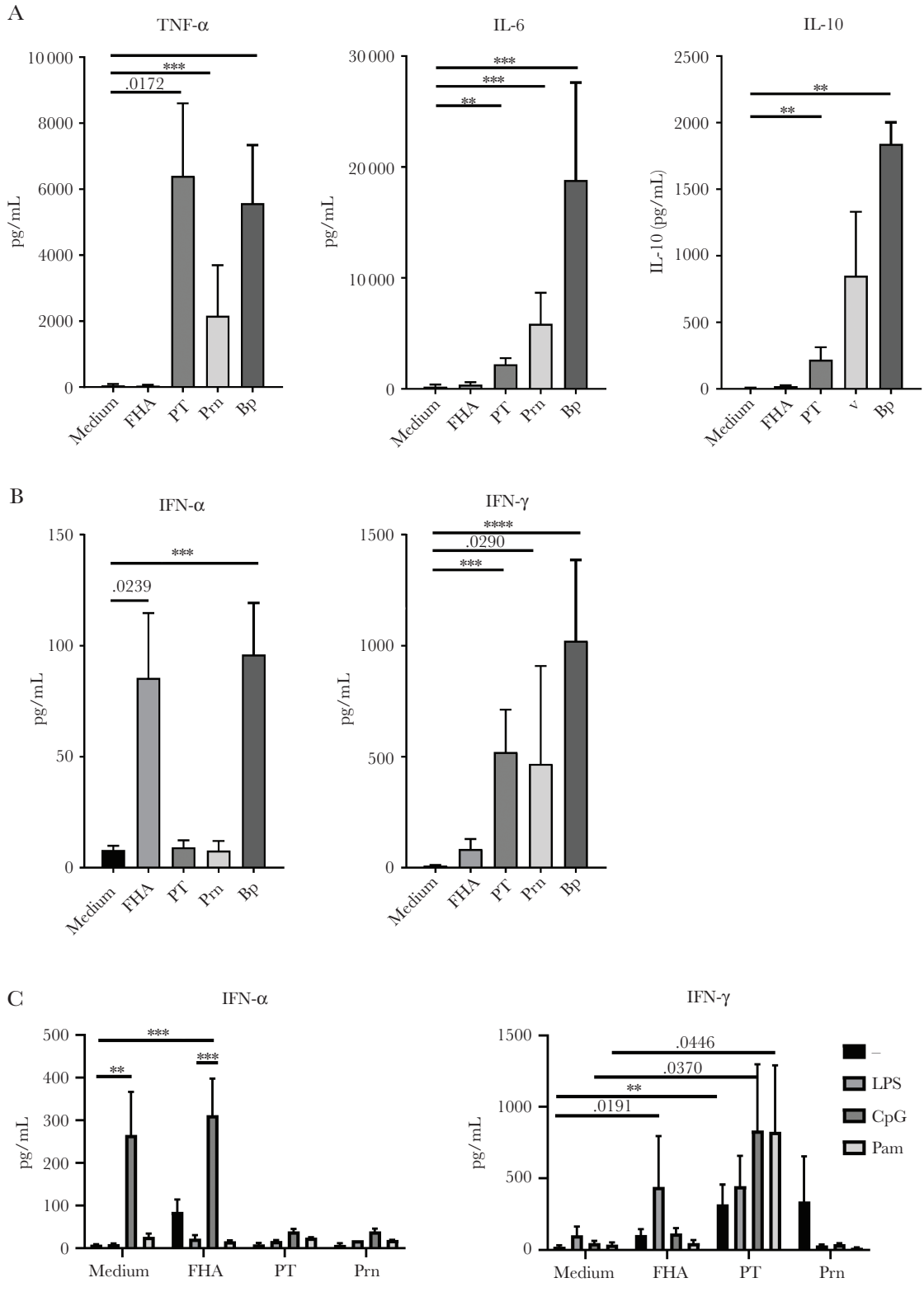

Figure 1. Induction of interferon production following stimulation with *Bordetella pertussis* (Bp). Peripheral blood mononuclear cells (PBMCs) from healthy donors were stimulated for 20 hours with Bp, and tumor necrosis factor α, interleukin 6, and interleukin 10 (*A*) or interferon (*B*) levels in supernatants were determined. *C*, PBMCs were stimulated with Bp antigens in the absence or presence of the Toll-like receptor ligands lipopolysaccharide, cytosine phosphodiester guanine, or synthetic acylated lipopeptide, and interferon production was measured. Statistical test results were obtained using 1-way analysis of variance (ANOVA) with Dunnett post hoc test (*A* and *B*) or 2-way ANOVA and Sidak post hoc test (*C*). $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. Abbreviations: Bp, *Bordetella pertussis*; CpG, cytosine phosphodiester guanine; FHA, filamentous hemagglutinin adhesin; IFN-α, interferon alpha; IFN-γ, interferon gamma; IL-6, interleukin 6; IL-10, interleukin 10; LPS, lipopolysaccharide; Pam, synthetic acylated lipopeptide; Prn, pertactin; PT, pertussis toxin; TNF-α, tumor necrosis factor alpha.

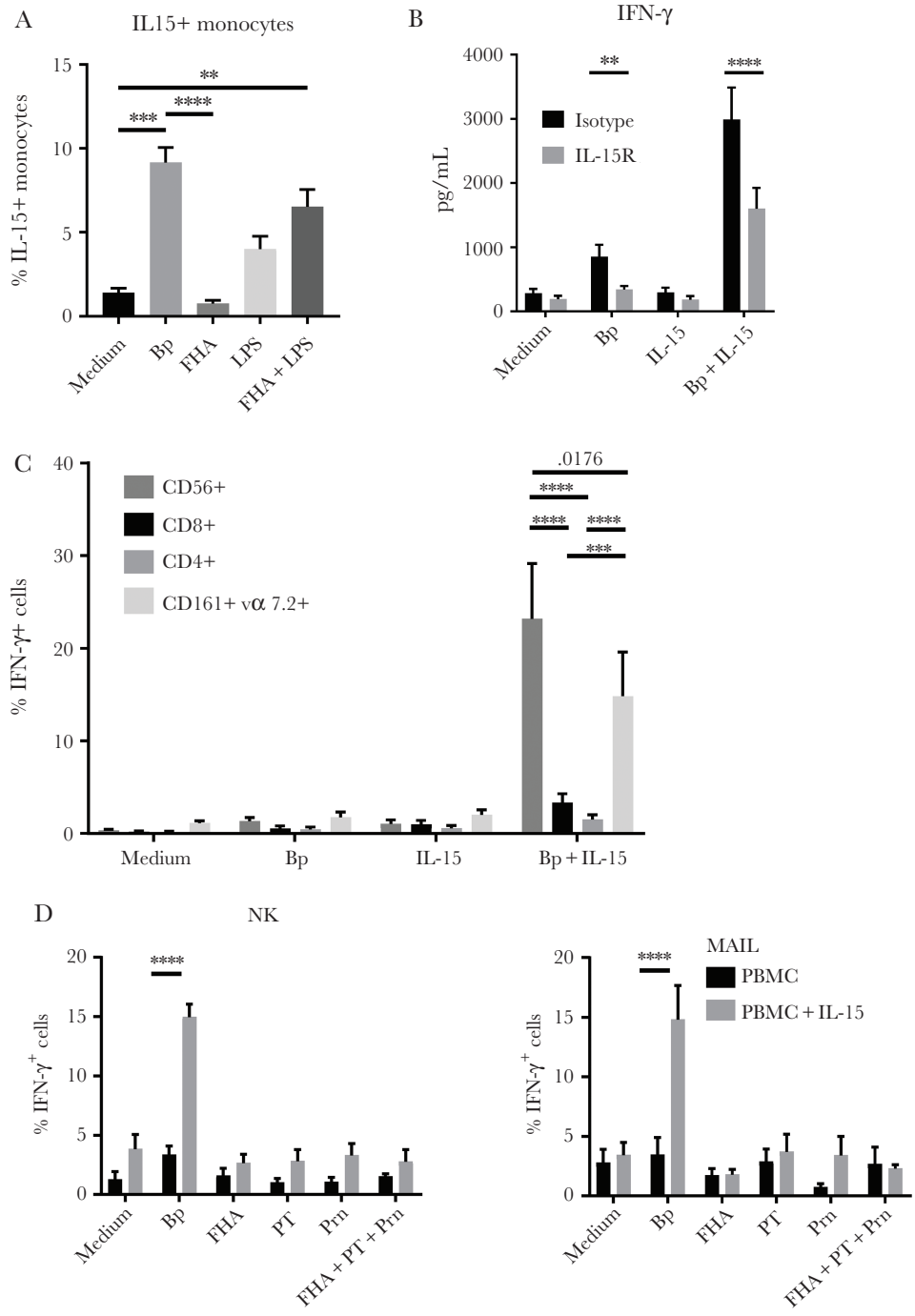

Figure 2. Role of interleukin 15 (IL-15) in inducing interferon gamma (IFN-γ) production. *A*, Monocytes were stained for membrane expression of IL-15 following stimulation for 20 hours. *B*, Peripheral blood mononuclear cells (PBMCs) were stimulated with *Bordetella pertussis* (Bp) and/or IL-15 in the presence or absence of an IL-15R blocking antibody or isotype control for 20 hours, and IFN-γ production was measured. *C*, PBMCs were stimulated with Bp and/or IL-15 for 16 hours, followed by 4 hours of stimulation in the presence of brefeldin A and cells harvested for intracellular IFN-γ staining. *D*, PBMCs were stimulated with Bp antigens as in (*C*), and natural killer and mucosal-associated invariant T cells were analyzed for IFN-γ production. Statistical test results were obtained using 1-way analysis of variance (ANOVA) with Dunnett post hoc test (*A*) or 2-way ANOVA and Sidak post hoc test (*B–D*). $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. Abbreviations: Bp, *Bordetella pertussis*; FHA, filamentous hemagglutinin adhesin; IFN-γ, interferon gamma; IL-15, interleukin 15; LPS, lipopolysaccharide; MAIT, mucosal-associated invariant T cell; NK, natural killer; PBMC, peripheral blood mononuclear cell; Prn, pertactin; PT, pertussis toxin.

influenced IFN-γ production. The presence of Bp-stimulated A549 or Calu-3 cells resulted in increased production of IFN-γ (Figure 5A). Respiratory epithelial cells did not affect levels of IFN-α (Supplementary Figure 8). In the presence of epithelial cells, increased expression of IFN-γ was observed in NK cells (Figure 5B).

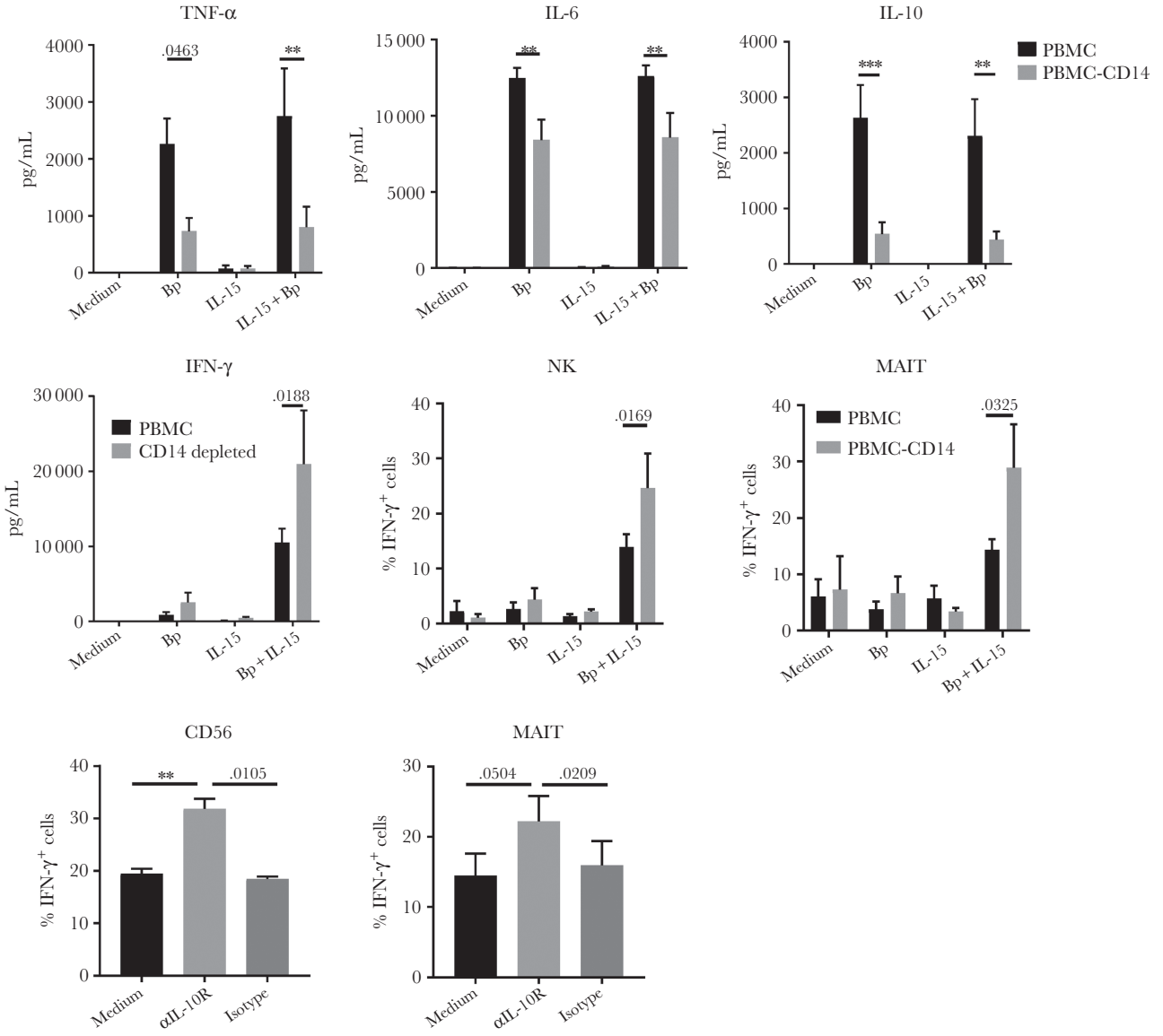

Figure 3. Establishing the role of CD14 monocytes in interferon gamma (IFN-γ) production. Peripheral blood mononuclear cells (PBMCs) were depleted of $CD14^+$ monocytes or not, and then stimulated with *Bordetella pertussis* and/or interleukin 15 before interferon alpha, interleukin 6, and interleukin 10 (IL-10) (*A*) or IFN-γ (*B*) levels were measured. *C*, Following stimulation, PMBCs or monocyte-depleted PBMCs were assessed for intracellular IFN-γ production by mucosal-associated invariant T cells (MAIT) and natural killer (NK) cells as in Figure 2C. *D*, As CD14 depletion resulted in increased IFN-γ production and IL-10 levels were decreased in monocyte-depleted cultures, IFN-γ production by NK and MAIT cells was determined in PBMCs stimulated in the presence of an IL-10R blocking antibody or isotype control. Statistical test results were obtained using 1-way analysis of variance (ANOVA) with Dunnett post hoc test (*D*) or 2-way ANOVA and Sidak post hoc test (*A*–*C*). $^{**}P < .01$, $^{***}P < .001$. Abbreviations: Bp, *Bordetella pertussis*; IFN-γ, interferon gamma; IL-6, interleukin 6; IL-10, interleukin 10; IL-15, interleukin 15; MAIT, mucosal-associated invariant T cell; NK, natural killer; PBMC, peripheral blood mononuclear cell; TNF-α, tumor necrosis factor alpha.

Production of IFN-γ–Inducible Chemokines by Respiratory Epithelial Cells

To study the implications of enhanced IFN-γ production, we analyzed co-culture supernatants for the IFN-γ–inducible chemokines CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC). Synergistically increased production of CXCL9 and CXCL10 was observed when PBMCs were stimulated with Bp in the presence of Calu-3 cells (Figure 5C).

Analysis of the cellular origin of CXCL10 showed that Calu-3 cells are a major source of CXCL10 following stimulation with Bp (Figure 5D). This upregulation of CXCL10 production was observed to a much lesser extent in NK and MAIT cells, CD4 T cells, and CD8 T cells (Supplementary Figure 9). Stimulation of co-cultured Calu-3 epithelial cells and PBMCs also resulted in the upregulation of the activation markers CD86 and HLA-DR (MHCII) on Calu-3 cells.

The Role of IFN-γ in Inducing CXCL9 and CXCL10 Production

To study how CXCL9-CXCL11 is induced, Calu-3 cells were stimulated with Bp and/or IFN-γ. IFN-γ efficiently induced CXCL9-11 production, which was further increased when Bp was

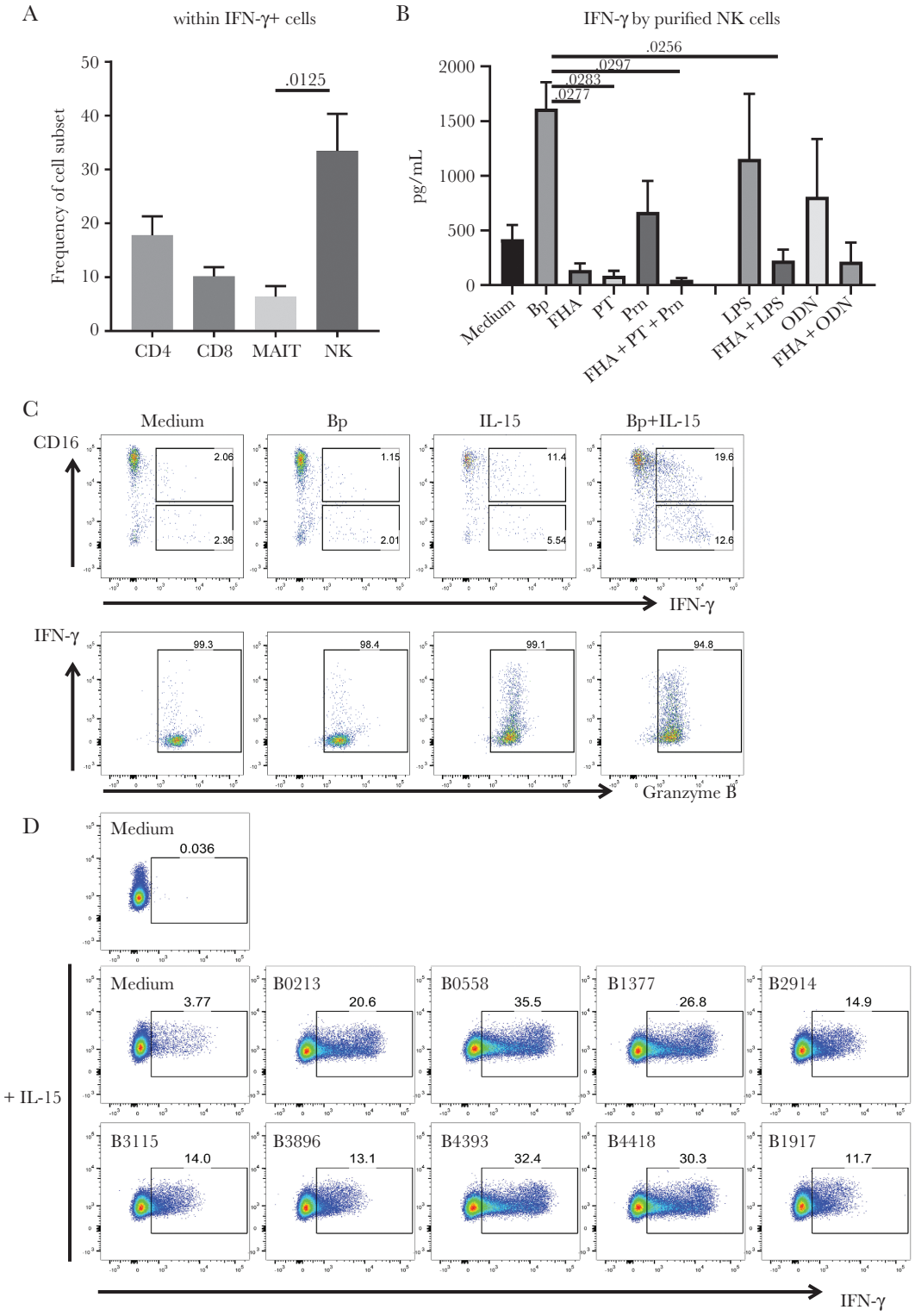

Figure 4. Stimulation of purified natural killer (NK) cells. *A*, The percentages of different cell types within the interferon gamma (IFN-γ)–producing cell population following stimulation with *Bordetella pertussis* (Bp) and interleukin 15 (IL-15) were determined. *B*, IFN-γ production by purified NK cells from 5 donors stimulated with intact Bp or Bp antigens in the presence of IL-15 and/or Toll-like receptor ligands lipopolysaccharide and oligodeoxynucleotides. *C*, Sorted NK cells were stained for CD16 and intracellular granzyme B and IFN-γ following stimulation. *D*, To confirm that our observations were not limited to the B1917 Bp isolate, 8 additional isolates of Bp were selected and added to NK cells, and NK cells were stained for IFN-γ production. Statistical test results were obtained using 1-way analysis of variance with Dunnett post hoc test. Abbreviations: Bp, *Bordetella pertussis*; FHA, filamentous hemagglutinin adhesin; IFN-γ, interferon gamma; LPS, lipopolysaccharide; MAIT, mucosal-associated invariant T cell; NK, natural killer; ODN, oligodeoxynucleotides; Prn, pertactin; PT, pertussis toxin.

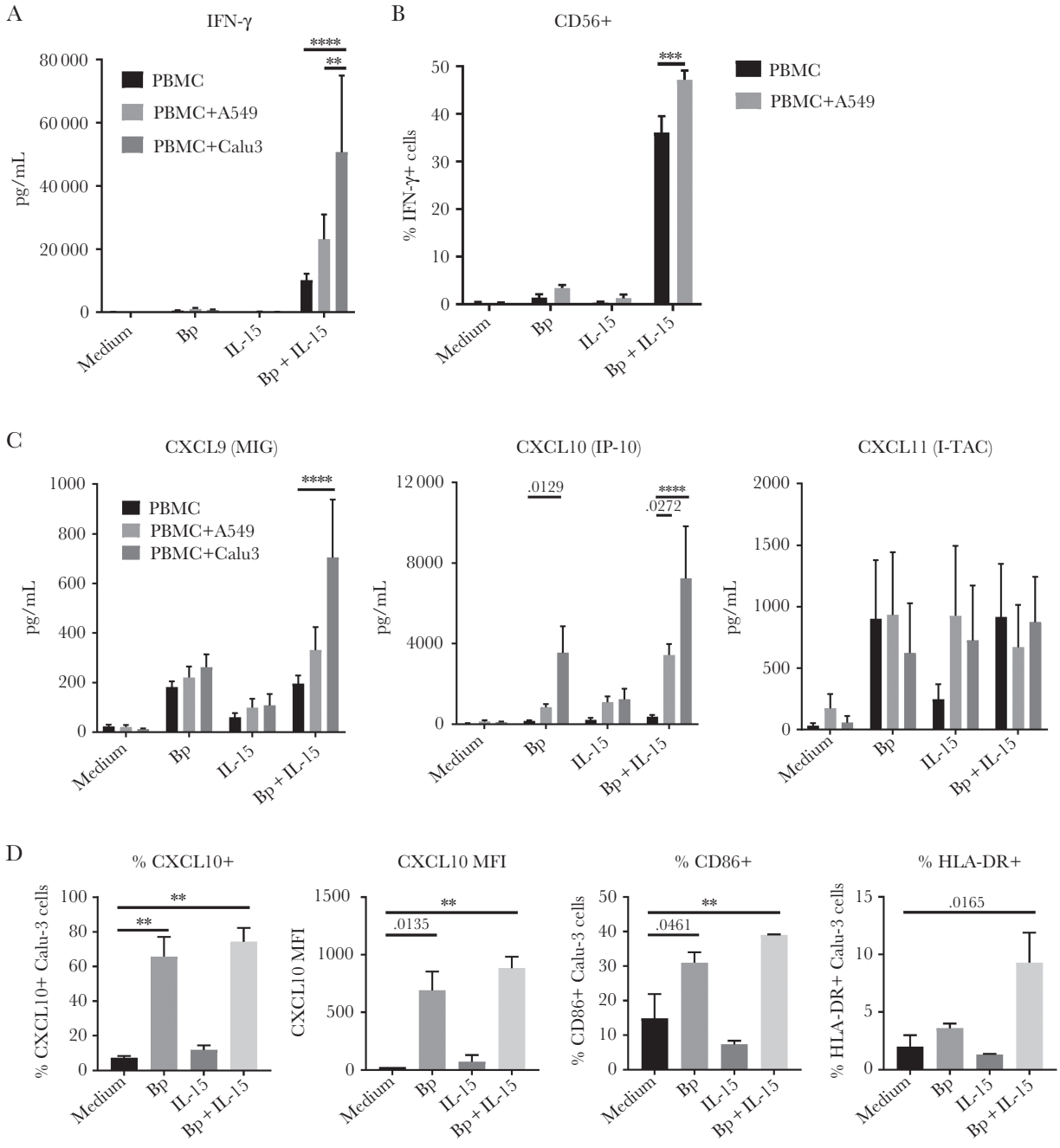

Figure 5. Interferon gamma (IFN-γ) in peripheral blood mononuclear cell (PBMC)–epithelial co-cultures. *A*, IFN-γ production following stimulation with inactivated *Bordetella pertussis* and/or interleukin 15 was measured in PBMCs co-cultured with A549 or Calu-3 epithelial cells. *B*, Percentage of IFN-γ–positive natural killer cells stimulated in the presence of A549 cells for 20 hours. *C*, Production of IFN-inducible chemokines in the supernatants of PBMCs co-cultured with epithelial cells. *D*, Calu-3 cells from stimulated PBMC co-cultures were stained for intracellular CXCL10 (IP-10) and membrane expression of CD86 and MHCII (HLA-DR). Statistical test results were obtained using 2-way analysis of variance (ANOVA) and Sidak post hoc test (*A–C*) or 1-way ANOVA with Dunnett post hoc test (*D*). $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. Abbreviations: Bp, *Bordetella pertussis*; IFN-γ, interferon gamma; IL-10, interleukin 10; IL-15, interleukin 15; I-TAC, interferon–inducible T cell alpha chemoattractant; MFI, mean florescence intensity; MIG, monokine induced by gamma; PBMC, peripheral blood mononuclear cell.

also present (Figure 6A). To a lesser extent, CCL3 was induced by IFN-γ, whereas CCL2 was induced by Bp, but not by IFN-γ.

Next, PBMCs were co-cultured with Calu-3 cells and stimulated with Bp and/or IL-15 in the absence or presence of control antibodies or blocking antibodies for the receptor for CXCL9-CXCL11 (CXCR3) or IFN-γ (IFN-γR), respectively. Blocking the chemokine receptor did not change the levels of CXCL9-CXCL11 (Figure 6B). Blocking the receptor for IFN-γ significantly reduced the concentration of secreted CXCL9 and CXCL11, but not CXCL10 and CCL3 (Figure 6B; Supplementary Figure 10). These data indicate that in the presence of Bp, IFN-γ is responsible for inducing the production of these CXCL chemokines.

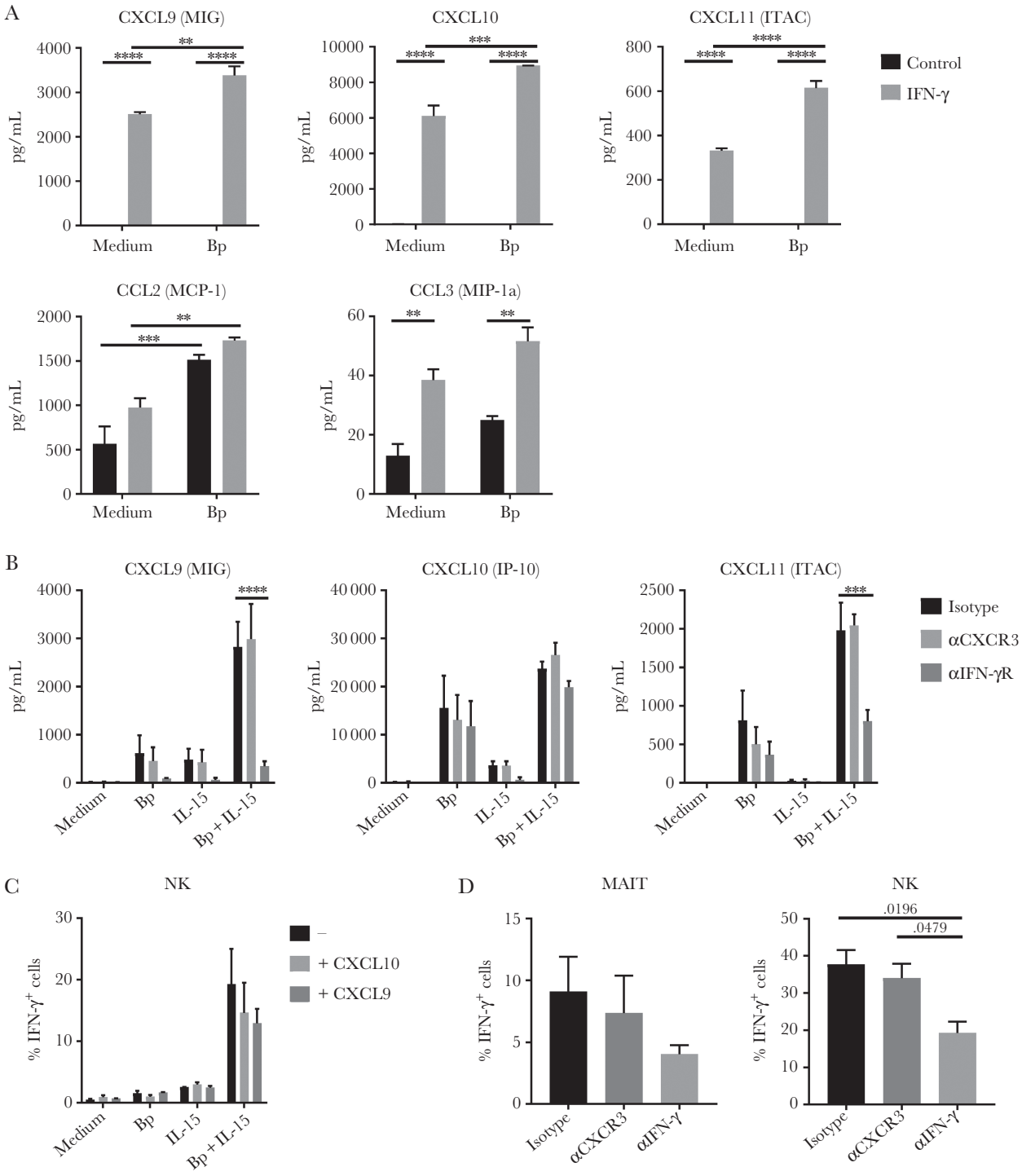

Figure 6. Role of IFN-γ in the production of chemokines. *A*, Production of CXCL9-11, CCL3, and CCL2 by Calu-3 epithelial cells stimulated with *Bordetella pertussis* and/or interferon gamma (IFN-γ). *B*, Analysis of CXCL9-11 in the cell culture supernatants of peripheral blood mononuclear cell (PBMC)–Calu-3 co-cultures in the presence of blocking antibodies for IFN-γR and CXCR3 (receptor for CXCL9-CXCL11). *C*, PBMCs were stimulated in the absence or presence of CXCL9 or CXCL10 and natural killer (NK) cells were analyzed for IFN-γ production. *D*, To confirm that chemokines do not alter IFN-γ production by NK cells, PBMCs were stimulated in the presence of an isotype control, CXCR3-blocking antibody, or IFN-γR-blocking antibody, and IFN-γ production by NK and mucosal-associated invariant T cells was determined. Statistical test results were obtained using 2-way analysis of variance (ANOVA) and Sidak post hoc test (*A–C*) or 1-way ANOVA with Dunnett post hoc test (*D*).***P* < .01, ****P* < .001, *****P* < .0001. Abbreviations: Bp, *Bordetella pertussis*; IFN-γ, interferon gamma; IL-15, interleukin 15; I-TAC, interferon–inducible T cell alpha chemoattractant; MAIT, mucosal-associated invariant T cell; MCP-1, monocyte chemoattractant protein 1; MIP-1α, macrophage inflammatory protein 1 alpha; MIG, monokine induced by gamma; NK, natural killer.

Chemokines Do Not Alter NK Cell Activation

As significant levels of CXCL9 and CXCL10 were observed in the supernatants of PBMC–Calu-3 co-cultures, PBMCs were stimulated in the absence or presence of CXCL9 or CXCL10. Neither co-stimulation with these chemokines (Figure 6C) nor blocking the receptor for CXCL9 and CXCL10 in PBMC Calu-3 co-cultures altered the number of IFN-γ–producing cells (Figure 6D). Also, chemokines did not alter expression of the activation marker of NK cells, CD107 (Supplementary Figure 11). However, blocking the receptor for IFN-γ reduced the percentage of IFN-γ–producing NK cells (Figure 6D). In addition, CCL3 and CX3CL1 (not analyzed in the supernatants, however reported to target NK cells) did not alter IFN-γ production by NK cells (Supplementary Figure 12). These data show that IFN-γ itself enhanced IFN-γ production through binding to its receptor.

Epithelial Chemokines Recruit Lymphocytes

Because epithelial-derived chemokines induced by Bp did not regulate IFN-γ production by NK cells, we investigated whether stimulation of respiratory epithelial cells resulted in cell migration. Supernatants of Calu-3 stimulated with Bp and/or IFN-γ (Figure 7A) increased migration of lymphocytes, which was significant for NK cells (Figure 7B). We next investigated whether the observed migration of cells depended on factors produced by Calu-3 cells and was mediated by CXCR3. Therefore, Bp and IFN-γ themselves or supernatants from Calu-3 stimulated with Bp and IFN-γ were added to the basolateral compartment, and PBMCs were added apically in the presence or absence of isotype control or anti-CXCR3 (receptor for CXCL9-CXCL11) antibodies. Very little cell migration was observed when Bp and IFN-γ were added, indicating that migration required factors produced by the Calu-3 cells (no Calu-3 cells, Figure 7C). Compared to the isotype control antibody, reduced numbers of lymphocytes incubated with anti-CXCR3 antibodies migrated toward the basolateral compartment. These data implicate a role for CXCL9, CXCL10, and/or CXCL11 in recruiting lymphocytes to the epithelium following exposure to Bp.

DISCUSSION

The aim of this study was to investigate how intact Bp or soluble virulence antigens induce the production of IFN-γ to modulate local immune responses through the epithelium. Inactivated Bp resulted in robust IFN-γ production by NK cells, whereas the soluble antigens of Bp, also used in vaccines, induced IFN-γ production at much lower levels. Despite the potential source of IL-15, monocytes were not required for the production of IFN-γ, which could be induced by stimulated purified NK cells directly. IFN-γ induced the production of the chemokines CXCL9 and CXCL10 by the respiratory epithelium. These chemokines did not alter activation of NK cells but induced migration of CD4 T cells, CD8 T cells, and NK cells toward the epithelium, at least partly, through the chemokine receptor CXCR3.

Although some CD4 T-helper cells and CD8 cytotoxic T cells produced IFN-γ, the majority of the IFN-γ produced upon stimulation with Bp originated from NK cells, whereas MAIT cells were also consistently involved in the production of IFN-γ. These data are consistent with literature showing a role for NK and MAIT cells in respiratory infections and, specifically, protective responses to Bp [3, 9]. Induction of IFN-γ production was observed following stimulation with 9 different clinical isolates encompassing the years 1950–2016 and with varying genetic traits for Prn, PT, and FHA [22, 23], indicating that production of IFN-γ by NK cells following exposure to Bp is a general phenomenon. Whereas stimulation with inactivated bacteria resulted in robust IFN-γ production, soluble antigens failed to induce similar levels of IFN-γ even in the presence of additional TLR co-stimulation. This may be explained by the cross-linking of receptors on host cells that result in more robust activation in NK cells [24], the complexity of multiple cell receptors that are activated, or a combination of the 2. This observation my also shed light on the differences in longevity of cellular immunity induced by acellular vaccines compared to the whole-cell vaccines, especially since NK cell–derived IFN-γ promotes the differentiation of Th1 cells [3] (Supplementary Figure 13). These observations could also contribute to the evaluation of acellular vaccines currently under development to assess the degree of Th1 cell immunity.

Monocytes and macrophages are a major source of IL-15, a pleiotropic cytokine involved in protective mucosal responses [20, 25, 26]. Although IL-15 promotes the production of IFN-γ, the presence of monocytes was not required for IFN-γ production; rather, the depletion of monocytes resulted in increased IFN-γ production. This increased IFN-γ production could be caused by the absence of IL-10 after depletion of monocytes, which is in line with a study in infants that indicated a suppressive role for monocyte-derived IL-10 in IFN-γ responses following stimulation with Bp [27]. Epithelial cells could form an additional source of IL-15 in vivo, which may also contribute to the enhanced IFN-γ production in PBMC–epithelial co-cultures [18, 26].

Bordetella pertussis carriage and infection are typically observed at the laryngeal and ciliated columnar epithelium of the conducting airways [28, 29]. Here we show that epithelial cells enhance the production of IFN-γ. Calu-3 cells enhanced IFN-γ production even more than A549 cells, which may be explained by the origin of the cells; Calu-3 cells are bronchial cells that are a natural target for Bp, whereas A549 cells are type II alveolar epithelial cells. IFN-γ induced the production of the IFN-γ–inducible chemokines CXCL9 and CXCL10, but not of CXCL11, by the epithelium. Blocking the IFN-γR inhibited the secretion of CXCL9 and CXCL11, but not CXCL10, indicating, together with other data, that activation routes for the chemokines

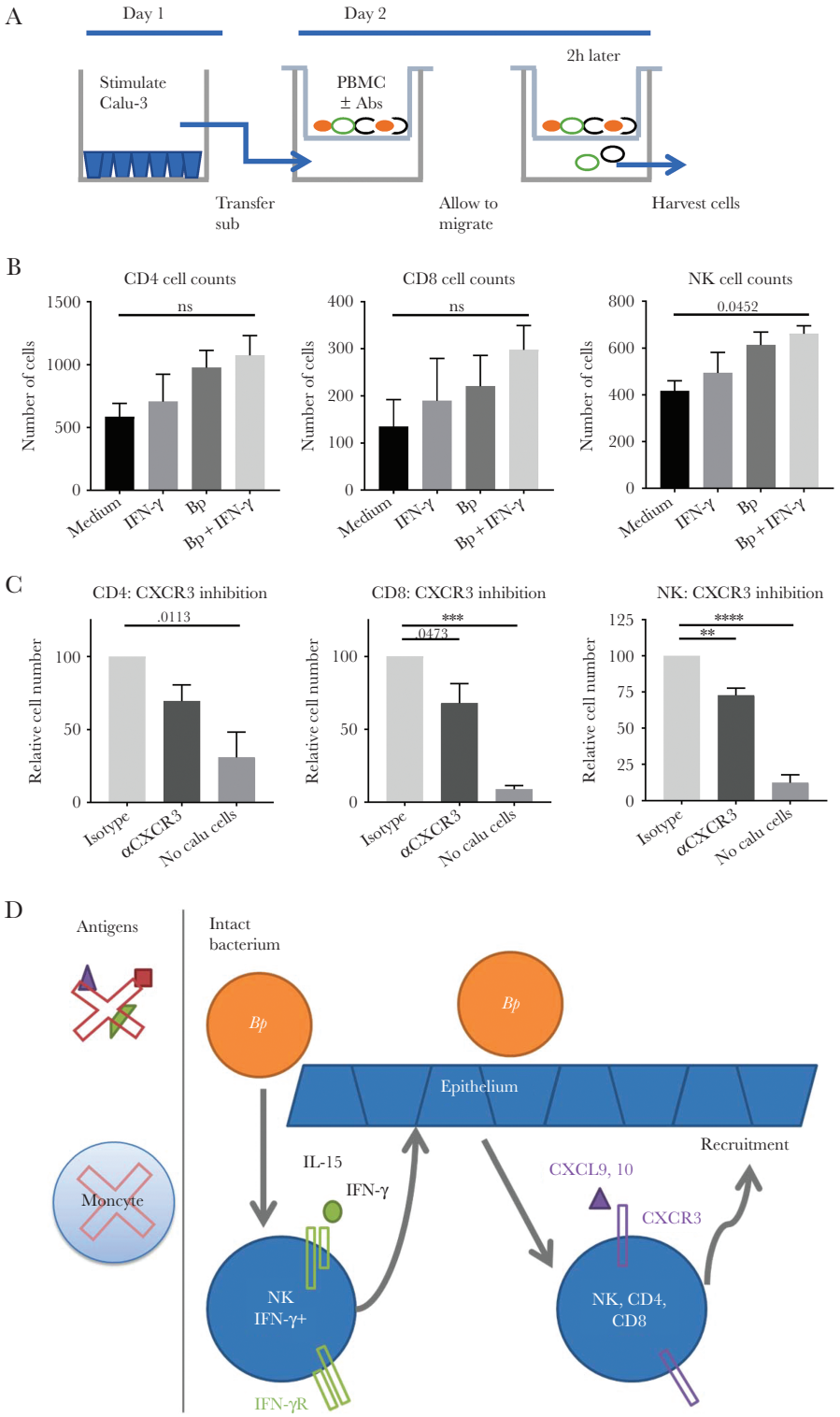

Figure 7. Lymphocyte migration induced by epithelial cells. *A*, Calu-3 cells are stimulated overnight with interferon gamma (IFN-γ) and/or *Bordetella pertussis* (Bp). The next day peripheral blood mononuclear cell (PBMCs) were allowed to settle onto polyester 3.0 µm cell culture inserts, Calu-3 cell culture supernatants were transferred and cells were allowed to migrate through the cell culture insert for 2 hours. *B*, Migrated cells were harvested from the basolateral compartments, and following staining, CD4 and CD8 T cells and natural killer (NK) cells were enumerated by flow cytometry. *C*, As increased migration was induced by Calu-3 cell culture supernatants, migration by IFN-γ and Bp directly (in the absence of Calu-3 cells) or the involvement of IFN-γ–inducible chemokines by blocking the receptor CXCR3 on PBMCs was investigated. *D*, Graphical summary: Intact inactivated Bp induced IFN-γ production, by NK cells, while soluble antigens fail to activate NK cells. Monocytes are dispensable for IFN-γ by NK cells. IFN-γ production was enhanced through IFN-γR, but not CXCR3, and induced the production of chemokines by epithelial cells. The chemokines produced by the epithelium recruit NK and T cells, at least partly through CXCR3 on the PBMCs. Statistical test results were obtained using 1-way analysis of variance with Dunnett post hoc test (*B* and *C*). $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. Abbreviations: Abs, antibodies; Bp, *Bordetella pertussis*; IFN-γ, interferon gamma; IL-15, interleukin 15; NK, natural killer; ns, not significant; PBMC, peripheral blood mononuclear cell.

CXCL9-CXCL11 can vary. These chemokines did not alter the levels of IFN-γ, but induced migration of NK cells and CD4 and CD8 T cells toward the epithelial-derived chemokines. These data suggest that IFN-γ production by NK cells contributes to the recruitment of other immune cells, including T cells, by the respiratory epithelium in vivo. Such recruitment of immune cells may be essential to protect against progression of infection with Bp and thereby prevent disease. Although we used respiratory epithelial cell lines to resemble the in vivo setting more closely, it needs to be noted that these data need to be confirmed using normal human bronchial epithelial cells.

Relatively low proportions of IFN-γ–producing T cells were measured. However, adaptive specific Th1 and Th17 cells are still essential to entirely clear an infection with Bp, particularly when the abundance of bacteria is low. IFN-γ has been shown to enhance phagocytosis by myeloid cells. Our results point to an additional role of IFN-γ production to recruit lymphocytes to the site of infection. The NK responses described here, although occurring fast after infection, last a relatively short time. The activation of MAIT cells and recruitment of adaptive T cells may be essential for immunity at the later stages of infection due to longer survival and extension of the window of IFN-γ production.

In conclusion, we show that Bp induces robust IFN-γ production by MAIT and NK cells. Purified proteins used in the acellular vaccine fail to induce IFN-γ production, in contrast to intact bacteria. We show that in humans, NK cell–derived IFN-γ contributes to protective local immune responses to Bp through initiating chemokine production by epithelial cells, resulting in recruitment of other immune cells. These results expand our knowledge on immune mechanisms to Bp infection and could contribute to improved design of preventive strategies including vaccination.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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References

1. Wilson R, Read R, Thomas M, et al. Effects of *Bordetella pertussis* infection on human respiratory epithelium in vivo and in vitro. Infect Immun **1991**; 59:337–45.
2. Bassinet L, Gueirard P, Maitre B, Housset B, Gounon P, Guiso N. Role of adhesins and toxins in invasion of human tracheal epithelial cells by *Bordetella pertussis*. Infect Immun **2000**; 68:1934–41.
3. Byrne P, McGuirk P, Todryk S, Mills KH. Depletion of NK cells results in disseminating lethal infection with *Bordetella pertussis* associated with a reduction of antigen-specific Th1 and enhancement of Th2, but not Tr1 cells. Eur J Immunol **2004**; 34:2579–88.
4. Warfel JM, Merkel TJ. *Bordetella pertussis* infection induces a mucosal IL-17 response and long-lived Th17 and Th1 immune memory cells in nonhuman primates. Mucosal Immunol **2013**; 6:787–96.
5. Ryan M, Murphy G, Gothefors L, Nilsson L, Storsaeter J, Mills KH. *Bordetella pertussis* respiratory infection in children is associated with preferential activation of type 1 T helper cells. J Infect Dis **1997**; 175:1246–50.
6. Mills KH, Barnard A, Watkins J, Redhead K. Cell-mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. Infect Immun **1993**; 61:399–410.
7. Ross PJ, Sutton CE, Higgins S, et al. Relative contribution of Th1 and Th17 cells in adaptive immunity to *Bordetella pertussis*: towards the rational design of an improved acellular pertussis vaccine. PLoS Pathog **2013**; 9:e1003264.
8. Wu V, Smith AA, You H, et al. Plasmacytoid dendritic cell-derived IFNα modulates Th17 differentiation during early *Bordetella pertussis* infection in mice. Mucosal Immunol **2016**; 9:777–86.
9. Meierovics A, Yankelevich WJ, Cowley SC. MAIT cells are critical for optimal mucosal immune responses during in vivo pulmonary bacterial infection. Proc Natl Acad Sci U S A **2013**; 110:E3119–28.
10. Meierovics AI, Cowley SC. MAIT cells promote inflammatory monocyte differentiation into dendritic cells during pulmonary intracellular infection. J Exp Med **2016**; 213:2793–809.
11. Bals R, Hiemstra PS. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. Eur Respir J **2004**; 23:327–33.
12. Whitsett JA, Alenghat T. Respiratory epithelial cells orchestrate pulmonary innate immunity. Nat Immunol **2015**; 16:27–35.
13. Kampf C, Relova AJ, Sandler S, Roomans GM. Effects of TNF-alpha, IFN-gamma and IL-beta on normal human bronchial epithelial cells. Eur Respir J **1999**; 14:84–91.
14. Belcher CE, Drenkow J, Kehoe B, et al. The transcriptional responses of respiratory epithelial cells to *Bordetella pertussis*

reveal host defensive and pathogen counter-defensive strategies. Proc Natl Acad Sci U S A **2000**; 97:13847–52.
15. Schijf MA, Lukens MV, Kruijsen D, et al. Respiratory syncytial virus induced type I IFN production by pDC is regulated by RSV-infected airway epithelial cells, RSV-exposed monocytes and virus specific antibodies. PLoS One **2013**; 8:e81695.
16. Bart MJ, Zeddeman A, van der Heide HGJ, Heuvelman K, van Gent M, Mooi FR. Complete genome sequences of *Bordetella pertussis* isolates B1917 and B1920, representing two predominant global lineages. Genome Announc **2014**; 2:e01301–14.
17. Neely GG, Epelman S, Ma LL, et al. Monocyte surface-bound IL-15 can function as an activating receptor and participate in reverse signaling. J Immunol **2004**; 172:4225–34.
18. Regamey N, Obregon C, Ferrari-Lacraz S, et al. Airway epithelial IL-15 transforms monocytes into dendritic cells. Am J Respir Cell Mol Biol **2007**; 37:75–84.
19. Strutt TM, Dhume K, Finn CM, et al. IL-15 supports the generation of protective lung-resident memory CD4 T cells. Mucosal Immunol **2018**; 11:668–80.
20. Umemura M, Nishimura H, Hirose K, Matsuguchi T, Yoshikai Y. Overexpression of IL-15 in vivo enhances protection against *Mycobacterium bovis* bacillus Calmette-Guérin infection via augmentation of NK and T cytotoxic 1 responses. J Immunol **2001**; 167:946–56.
21. Kroes MM, Mariman R, Hijdra D, et al. Activation of human NK cells by *Bordetella pertussis* requires inflammasome activation in macrophages. Front Immunol **2019**; 10:2030.
22. Mooi FR, van Loo IH, van Gent M, et al. *Bordetella pertussis* strains with increased toxin production associated with pertussis resurgence. Emerg Infect Dis **2009**; 15:1206–13.
23. Clark TA. Changing pertussis epidemiology: everything old is new again. J Infect Dis **2014**; 209:978–81.
24. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. Blood **2006**; 107:159–66.
25. Di Sabatino A, Calarota SA, Vidali F, Macdonald TT, Corazza GR. Role of IL-15 in immune-mediated and infectious diseases. Cytokine Growth Factor Rev **2011**; 22:19–33.
26. Hocke AC, Lampe MP, Witzenrath M, et al. Cell-specific interleukin-15 and interleukin-15 receptor subunit expression and regulation in pneumococcal pneumonia—comparison to chlamydial lung infection. Cytokine **2007**; 38:61–73.
27. Dirix V, Verscheure V, Goetghebuer T, et al. Monocyte-derived interleukin-10 depresses the *Bordetella pertussis*-specific gamma interferon response in vaccinated infants. Clin Vaccine Immunol **2009**; 16:1816–21.
28. Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. Proc Natl Acad Sci U S A **2014**; 111:787–92.
29. Guevara C, Zhang C, Gaddy JA, et al. Highly differentiated human airway epithelial cells: a model to study host cell-parasite interactions in pertussis. Infect Dis (Lond) **2016**; 48:177–88.