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Bordetella Type III Secretion and Adenylate Cyclase Toxin Synergize to Drive Dendritic Cells into a Semimature State

Jason A. Skinner, Annette Reissinger, Hao Shen, and Ming H. Yuk

Bordetella bronchiseptica establishes persistent infection of the murine respiratory tract. We hypothesize that long-term colonization is mediated in part by bacteria-driven modulation of dendritic cells (DCs) leading to altered adaptive immune responses. Bone marrow-derived DCs (BMDCs) from C57BL/6 mice infected with live B. bronchiseptica exhibited high surface expression of MHCII, CD86, and CD80. However, B. bronchiseptica-infected BMDCs did not exhibit significant increases in CD40 surface expression and IL-12 secretion compared with BMDCs treated with heat-killed B. bronchiseptica. The B. bronchiseptica type III secretion system (TTSS) mediated the increase in MHCII, CD86, and CD80 surface expression, while the inhibition of CD40 and IL-12 expression was mediated by adenylate cyclase toxin (ACT). IL-6 secretion was independent of the TTSS and ACT. These phenotypic changes may result from differential regulation of MAPK signaling in DCs. Wild-type B. bronchiseptica activated the ERK 1/2 signaling pathway in a TTSS-dependent manner. Additionally, ACT was found to inhibit p38 signaling. These data suggest that B. bronchiseptica drive DC into a semimature phenotype by altering MAPK signaling. These semimature DCs may induce tolerogenic immune responses that allow the persistent colonization of B. bronchiseptica in the host respiratory tract. The Journal of Immunology, 2004, 173: 1934–1940.

Bordetella are Gram-negative bacteria capable of colonizing the respiratory tracts of mammals. Three highly related Bordetella species are B. pertussis, B. parapertussis, and B. bronchiseptica. B. bronchiseptica expresses the broadest host range, infecting most mammals, including mice (2). B. parapertussis infects both sheep and humans, while B. pertussis only infects humans (3). Infection by B. bronchiseptica leads to permanent asymptomatic colonization of the respiratory tract, whereas acute B. pertussis infection usually ends with the clearance of B. pertussis from the host. The mouse model of B. bronchiseptica infection and persistence is especially useful to study Bordetella-host colonization because it allows the study of Bordetella in a natural host (4). Expression of most known Bordetella virulence genes is regulated by the BvgAS two-component regulatory system (5). In the Bvg<sup>+</sup> virulent phase, all Bordetella express a nearly identical array of virulence factors, which include adhesions such as filamentous hemagglutinin (FHA) and toxins such as adenylate cyclase toxin (ACT). In addition to these common virulence factors, the BvgAS system positively regulates the expression of pertussis toxin in B. pertussis and a type III secretion system (TTSS) in B. bronchiseptica (6, 7).

Intranasal inoculation of B. bronchiseptica into mice leads to the persistent colonization of the nasal cavity and trachea, despite the development of humoral and cellular immune responses (8). Notably, B. bronchiseptica deficient in type III secretion are cleared more readily from the trachea of C57BL/6 mice and elicit higher anti-Bordetella Ab titers, suggesting a role for type III secretion in persistent colonization (9). Type III secretion is used by a number of Gram-negative pathogens to inject effector molecules directly into eukaryotic cells to alter host cell function (10–12). We hypothesize that the B. bronchiseptica TTSS interacts with immune cells responsible for the development of adaptive host immune responses, and this interaction leads to prolonged persistence in the trachea.

Dendritic cells (DCs) are professional APCs that direct both cellular and humoral immune responses (13). DCs reside at mucosal surfaces in an immature state, and are highly phagocytic, resulting in the efficient capture of foreign Ags. DCs express an array of pattern recognition receptors capable of recognizing pathogen-associated molecular patterns displayed by pathogens. TLRs are the prototypical pattern recognition receptors capable of detecting the presence of an invading pathogen. Upon activation, DCs mature and migrate from the periphery to the local lymph nodes, where they direct adaptive immune responses. DC maturation is associated with a phenotypic switch characterized by a decrease in Ag capture and an increase in Ag presentation. The surface expression of peptide-loaded MHCII complexes (signal 1) and costimulatory CD40, CD80, and CD86 molecules (signal 2) is significantly increased upon DC maturation. Additionally, mature DCs secrete cytokines (signal 3) that influence the polarization of naive T cells upon stimulation (13). The integration of all three signals affects the nature and intensity of the final adaptive immune response. Although there are reports of DC maturation by other Gram-negative pathogens, including Salmonella (14), Neisseria (15), and Yersinia spp (16), little is known about the interaction of Bordetella with DCs. Recent work has begun to elucidate these interactions. Gueirard et al. (17) have shown that B. bronchiseptica triggers the early delivery of DCs to local lymph nodes following intranasal infection. Purified ACT from B. pertussis has been
shown to dominantly inhibit Th1-polarizing IL-12 cytokine secretion from LPS-activated human monocyte-derived DCs (18). Purified ACT was recently reported to augment LPS-induced IL-6 secretion and inhibit IL-12 production from bone marrow-derived DCs (BMDCs), while enhancing the cell surface expression of CD80, CD86, and MHCII and inhibiting CD40 (19). Ag-specific CD4+ T cell clones generated from mice infected with Ag and ACT exhibited cytokine profiles characteristic of Th2 or type 1 regulatory cells (19). Likewise, purified FHA from *B. pertussis* also inhibits IL-12 and stimulates IL-10 production from DCs. Subsequently, these DCs are capable of directing naive T cells into a type 1 regulatory subtype (20). Given these results, we hypothesize that *B. bronchiseptica* may affect DC phenotype and function via the expression of bacterial virulence factors.

We tested this hypothesis by first examining the effects of live bacteria on BMDC maturation. We assayed BMDCs for the surface expression of stimulatory (signal 1) and costimulatory (signal 2) molecules and secretion of T cell-polarizing cytokines (signal 3). Next, we assayed mutant bacteria defective for different virulence factors to determine the possible roles these factors have in shaping DC maturation. Finally, we assayed the MAPK pathways of BMDCs for activation dependent on the differential expression of these factors by infecting bacteria.

Our results show that wild-type *B. bronchiseptica* can up-regulate the surface expression of MHCII, CD80, and CD86 beyond that induced by heat-killed bacteria or saturating amounts of LPS. However, there was no significant up-regulation of CD40 surface expression and a minimal increase in IL-12 secretion by BMDCs infected with wild-type bacteria. The increased surface expression of MHCII and CD86 was dependent on the *B. bronchiseptica* TTSS, whereas the up-regulated surface expression of CD80 was dependent on the differential expression of BMDCs for activation dependent on the differential expression of these factors by infecting bacteria.

Results

**BMDc preparation and infection**

BMDCs were generated using methods reported by Lutz et al. (23). Briefly, bone marrow from C57BL/6 mice was cultured in RPMI 1640 supplemented with 10% FBS (HyClone, Logan, UT), 2-ME (Sigma-Aldrich, St. Louis, MO), 2 mM l-glutamine (Invitrogen Life Technologies, Carlsbad, CA), 100 U/ml penicillin, 10 µg/ml streptomycin (Invitrogen Life Technologies), and 20 ng/ml murine rGM-CSF (PeproTech, Rocky Hill, NJ) for 10 days. Day 10 BMDCs were washed and resuspended to 2 x 10^6 DC/ml in growth medium containing 20 ng/ml GM-CSF. Log phase wild-type, type III mutant, or ACT mutant bacteria were added to BMDCs (multiplicity of infection (MOI) = 1–50). Alternatively, BMDCs were treated with 50 µg/ml LPS (Escherichia coli serotype 0127:B8) (Sigma-Aldrich) or left untreated. Then 4 x 10^5 BMDCs were aliquoted into a 96-well plate. BMDCs were cultured in vitro at 37°C and 5% CO_2. Gentamicin (Sigma-Aldrich) was added at indicated time points to kill extracellular bacteria.

**Phenotypic analysis of infected BMDCs**

Following addition of gentamicin, BMDCs were cultured in vitro at 37°C and 5% CO_2 for a total of 18 h postinfection. Then BMDCs were collected for phenotypic analysis. DCs were stained with FITC-labeled Abs specific for murine Iaα clone (A3-12), PE-labeled CD86 clone (GL1), CD40 Abs, and allophycocyanin-labeled CD11c clone (HL3) Ab for 30 min at 4°C in the presence of unlabelled FcRII clone (2.4G2) Ab to block Fc receptor binding. To assay intracellular cytokine levels, PE-labeled IL-6 clone (MP5-20F3) and allophycocyanin-labeled IL-12p40/p70 clone (C15.6) Abs were used to stain BMDCs treated with Golgiplug for 3 h and permeabilized using the BD Pharmingen Cytofix/Cytoperm kit. Stained cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA) and FlowJo version 4.0 (TreeStar, San Carlos, CA).

**Annexin V/7-aminocinomycin D (7-AAD) cell death assay**

One hour postinfection, BMDCs were assayed for the externalization of phosphatidylserine by incubating cells with FITC-annexin V (BD Pharmingen) and assayed for cell viability by 7-AAD staining (BD Pharmingen) for 15 min at room temperature. Cells were immediately analyzed by FACS.

**Analysis of MAPK pathway activation**

BMDCs were infected, as described above, with the following modifications. Cells were serum deprived in RPMI for 2 h before infection. BMDCs were infected with bacteria at MOI = 10. Total incubation time for bacterial infection was 20 min for ERK 1/2 and 70 min for p38 at 37°C and 5% CO_2. Infections were terminated with PBS containing 1 mM Na_3VO_4 on ice. Cells were then processed according to a previously described method (24) using Fix & Perm reagents (CalTag Laboratories, Burlingame, CA). Abs were diluted in PBS containing 0.5% FCS, 1 mM EDTA, 3% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, and protease inhibitor mixture tablet (Roche, Indianapolis, IN). Primary phosphospecific polyclonal Abs to ERK 1/2 and p38 were purchased from Cell Signaling Technology. Alexa Fluor 647-conjugated anti-rabbit Abs were purchased from Molecular Probes (Eugene, OR). Stained cells were immediately analyzed by FACS.

Materials and Methods

**Bacteria and mice**

*B. bronchiseptica* strains RB50 (wild type), WD3 (∆hscN), and RB58 (∆cyaA) were all previously described (7, 21, 22). Before each experiment, a single colony was inoculated in Stainer-Scholte liquid medium and cultured overnight at 37°C with aeration. Before infection, bacteria were subcultured 1/20 into fresh Stainer-Scholte medium, grown at 37°C for 2.5–3 h. Heat-killed bacteria were obtained by incubation at 65°C for 30 min. C57BL/6 mice aged 6–10 wk were obtained from the National Cancer Institute (Frederick, MD) and housed in insulator cages and cared for in accordance with Institutional Animal Care and Use Committee-approved protocols at the University of Pennsylvania School of Medicine animal facility.

**Reagents and Abs**

All Abs and kits were acquired from BD Pharmingen (San Diego, CA), except polyclonal phosphospecific ERK 1/2 and p38 Abs from Cell Signaling Technology (Beverly, MA). The mAbs were directly labeled with FITC, PE, or APC. In all experiments, nonspecific binding of mAbs to FcγRIII/II was blocked with unlabeled FcγRIII/II Ab. Cells were left unblocked when the surface expression of FcγRIII/II was assayed with a FITC-labeled FcγRIII/II Ab.
with heat-killed bacteria. However, live bacteria induced CD40 surface expression only minimally in infected BMDCs compared with cells treated with heat-killed bacteria. Live *B. bronchiseptica* also induced significantly less IL-12 production in infected BMDCs than cells treated with heat-killed bacteria. Therefore, live *B. bronchiseptica* can induce BMDCs into a unique phenotypic state, with up-regulation of only specific factors (MHCII, CD86, and CD80, but not CD40 or IL-12) in these cells, and this may have functional implications for DC signaling to the adaptive immune system.

### Induction of cytotoxicity in BMDCs by *B. bronchiseptica* is dependent on a functional TTSS

Previous studies have shown that wild-type *B. bronchiseptica* can induce cytotoxicity in a variety of cultured cells (7, 9, 25). We examined the size and granularity (via forward/side scatter) of BMDCs infected with live wild-type *B. bronchiseptica* and observed rapid alterations in these parameters, consistent with signs of cytotoxicity (Fig. 2A). The altered expression of stimulatory and costimulatory molecules described in the previous section is dependent on the stringent gating and analyses of only live cells. BMDCs shifted outside of the live gate exhibit significant down-regulation of surface expression of all tested surface markers (data not shown). The *B. bronchiseptica* TTSS is required for induction of cytotoxicity in macrophages and epithelial cells (7, 9, 25). When we compared the altered size and granularity profiles of BMDCs infected by either wild-type or type III secretion-defective *B. bronchiseptica*, there was only a minimal change in these parameters in the BMDCs infected with the type III secretion mutant compared with cells infected with wild-type bacteria (Fig. 2A). We used annexin V/7-AAD staining to confirm the cytotoxic effect caused by wild-type bacteria and to validate the use of the size/granularity parameters for identifying and gating on live infected BMDCs (Fig. 2B). At 2 h postinfection, BMDCs infected by wild-type bacteria exhibited an elevated level of the annexin V+/7-AAD+ population compared with cells infected by the type III secretion mutant (Fig. 2B). The annexin V+/7-AAD+ population is indicative of cells with a cytotoxic phenotype, and this population correlates with that observed under the parameters of altered size and granularity. There appear to be more live cells quantitatively in the annexin V+/7-AAD analysis compared with the size/granularity measurements (e.g., at the 2-h time point presented in Fig. 2B), but we have specifically chosen a more stringent gating window for qualifying live cells in the size/granularity parameters to exclude all dead cells at the expense of excluding some viable cells. Treatment of the BMDCs with heat-killed wild-type bacteria or LPS did not lead to significant cytotoxicity compared with uninfected cells in this time period. These observations show that *B. bronchiseptica* can induce cytotoxicity in BMDCs via a type III secretion-dependent mechanism. Additionally, we did not detect an increase in the annexin V+/7-AAD+ early apoptotic population in wild-type infected BMDCs, indicating that most type III secretion-mediated cell death probably occurs via a nonapoptotic pathway. This is consistent with previous findings that the *B. bronchiseptica* TTSS induces caspase 1-independent necrosis in macrophages (25). More importantly, there is still a significant fraction of BMDCs infected by wild-type bacteria that did not show signs of cytotoxicity, and these cells exhibited the unique phenotype that was described in the previous section. The observed differences in BMDCs infected by either wild-type or mutant bacteria are not due to any variation in the uptake of the bacteria by the BMDCs, as gentamicin protection assays indicate that there is no difference in
bacterial cell uptake between wild-type and TTSS-defective bacteria by the host cells (data not shown).

The B. bronchiseptica TTSS mediates the up-regulated surface expression of stimulatory and costimulatory molecules

To determine the bacterial factors that may be responsible for the modulation of maturation of live BMDCs, we examined a panel of B. bronchiseptica strains that are each deficient in a single virulence factor, in their interactions with BMDCs. Infection of BMDCs by the mutant strain that is deficient in type III secretion (that did not lead to increased cytotoxicity, as described in the previous section) also did not lead to significant up-regulation of MHCII or CD86 in the infected cells (Fig. 3). Comparison of the live gated populations of BMDCs infected by either wild-type or type III secretion mutant bacteria shows that only wild-type bacteria induced significant increases in MHCII and CD86 surface expression. Furthermore, FcγRIII/II surface expression was decreased in BMDCs infected by wild-type bacteria, but not in cells infected by the type III secretion mutant. As positive controls, either LPS or heat-killed bacteria were able to stimulate the BMDCs to increase MHCII and CD86 surface expression, while decreasing FcγRIII/II surface expression. These results indicate that the rapid and efficient up-regulation of CD86 and MHCII by B. bronchiseptica in BMDCs is dependent on a functional TTSS.

Up-regulation of CD86 surface expression by B. bronchiseptica is independent of cell death

It is possible that the increased surface expression of MHCII and CD86 in the live population of BMDCs may be the result of live BMDCs phagocytosing cellular components from surrounding dead cells. To investigate this possibility, the MOI of both wild-type and type III secretion mutant were increased, because type III secretion mutant bacteria are capable of inducing cytotoxicity in vitro at high MOI. After 4 h of infection, the cytotoxic effect of type III secretion mutant bacteria was approximately equal to wild-type infection at 2 h, in terms of ratio of live to dead cells. However, the live BMDCs in the wild-type infected culture expressed significantly higher levels of CD86 (Fig. 4) and MHCII (data not shown). These results suggest that the TTSS-dependent stimulation of CD86 and MHCII surface expression by wild-type B. bronchiseptica is independent of activation signals from dead cells in vitro.

B. bronchiseptica ACT suppresses CD40 surface expression and IL-12 cytokine production

In addition to up-regulating the surface expression of MHCII and CD86, LPS-treated BMDCs also increased the surface expression of CD40 and secretion of IL-12, as expected (Fig. 5). Upon infection by wild-type B. bronchiseptica, the CD40 surface expression and IL-12 secretion of infected BMDCs remained low (Fig. 5).
Previous reports indicated that purified ACT (a nontype III secreted factor) from *B. pertussis* inhibited IL-12 production from LPS-activated human monocyte-derived DCs (18). Recently, *B. pertussis* ACT was shown to down-regulate CD40 on BMDCs (19). We investigated whether ACT expression by live *B. bronchiseptica* is required for the suppression of IL-12 production and/or CD40 surface expression. A *B. bronchiseptica* strain deficient in ACT stimulated BMDCs to produce significantly higher quantities of IL-12 than BMDCs infected with wild-type bacteria (Fig. 5A). Likewise, ACT-deficient bacteria stimulated BMDCs to significantly increase the surface expression of CD40 compared with wild-type *B. bronchiseptica* (Fig. 5B). There was no significant difference in the production of IL-6 in BMDCs infected by any of these strains of *B. bronchiseptica* (Fig. 5A). Infection of BMDCs with a type III secretion, ACT double mutant yielded CD40 and IL-12 expression similar to that of the ACT single mutant (data not shown).

In addition to reporting the suppressive effects of ACT on CD40 and IL-12 expression by BMDCs, Ross et al. (19) also reported the ACT-dependent up-regulation of MHCII, CD86, and CD80 by BMDCs. However, our results show that infection of BMDCs with...
live *B. bronchiseptica* defective in ACT expression increased the surface expression of MHCII, CD86, and CD80 (Fig. 5C). Our results show that in the context of live bacterial infection, *B. bronchiseptica* ACT inhibits the expression of specific factors that mediate internal DC signaling. The activation of specific factors by the *Bordetella* TTSS and suppression of these and other factors by ACT may thus result in the generation of DCs with unique characteristics to modulate the host adaptive immune responses.

**The *B. bronchiseptica* TTSS and ACT differentially regulate MAPK signaling pathways**

MAPK pathways are involved in the regulated surface expression of stimulatory and costimulatory molecules on DCs (26–29). Therefore, we investigated the differential activation of MAPK pathways by wild-type and mutant bacteria. BMDCs infected by wild-type *B. bronchiseptica* showed a significant increase in the population of phosphorylated (activated) ERK 1/2 compared with cells infected by the type III secretion mutant (Fig. 6A). However, BMDCs infected by wild-type or type III secretion mutant bacteria did not show significant increases in p38 phosphorylation, whereas cells infected by the ACT deletion mutant showed increased phosphorylation of p38 (Fig. 6B). These results show that the TTSS is required for the activation of the ERK 1/2 MAPK pathway and that ACT is required for the inhibition of the p38 MAPK pathway. Therefore, the observed immunomodulatory phenotypes described above may be the result of differential MAPK activation mediated by the *B. bronchiseptica* TTSS and ACT.

**Discussion**

Differentiation of naïve T cells into Th1 effector cells requires DCs expressing a highly regulated DC1 phenotype (13, 30). DC maturation status and secreted cytokine profile are important components of this phenotype. Pathogen-driven modulation of DC maturation may be one mechanism used by microorganisms to evade host immune responses and establish persistent infection. In this study, we investigated the effects of live *B. bronchiseptica* on DC maturation and what role known *Bordetella* virulence factors play in shaping DC maturation. Compared with LPS-stimulated BMDCs, live *B. bronchiseptica*-infected BMDCs exhibited higher surface expression of MHCII, CD86, and CD80. Conversely, *B. bronchiseptica*-infected BMDCs exhibited lower surface expression of CD40 and decreased IL-12 secretion compared with LPS-treated cells. The up-regulation of MHCII, CD86, and CD80 activation markers was dependent on the expression of the *B. bronchiseptica* TTSS. The decreased surface expression of CD40 and decreased secretion of IL-12, a potent Th1-polarizing cytokine, were dependent on the expression of a nontype III secreted factor, ACT. We also tested other mutant *B. bronchiseptica* that are defective in the expression of various nontype III secreted virulence factors, including FHA, fimbriae, and pertactin, and all of these mutants showed a similar phenotype to the wild-type bacteria in their interactions with BMDCs (data not shown).

The failure of TTSS mutant bacteria to stimulate the increased surface expression of MHCII and CD86 was unexpected. The presence of LPS and other immunostimulatory molecules displayed by live Gram-negative bacteria should activate BMDCs to up-regulate MHCII and CD86 independent of a functional TTSS. Nevertheless, functional type III secretion was required for the up-regulated surface expression of these molecules. These results also suggest that other unidentified bacterial factors may be involved in the suppression of DC activation. These factors may be specific for the inhibition of surface stimulatory and costimulatory molecules because bacteria deficient in type III secretion were able to stimulate increased IL-6 secretion by BMDC.

Although our results demonstrating the ACT-dependent down-regulation of CD40 and IL-12 expression confirm those recently published by Ross et al. (19), they contradict the ACT-dependent up-regulation of MHCII, CD86, and CD80. This apparent discrepancy may reveal experimental differences inherent to comparing studies conducted with purified protein and studies with live bacteria expressing the same protein. Alternatively, these results may indicate fundamental differences between *B. pertussis* and *B. bronchiseptica* infection. The acute nature of *B. pertussis* infections and the lifelong persistence of *B. bronchiseptica* infection may reflect underlying differences in DC activation and maturation driven by each *Bordetella* species.

Our work shows that live *B. bronchiseptica* can drive DCs into a semimature phenotype characterized by high surface expression of certain stimulatory and costimulatory molecules concurrent with reduced secretion of a T cell-polarizing cytokine. A similar DC phenotype has been linked to the induction of tolerogenic immune responses (31, 32). DCs pulsed with high quantities of *B. pertussis* FHA (20), TNF-α (33), or apoptotic cells (34) are driven into a semimature regulatory DC phenotype capable of directing the generation of regulatory T cells that ultimately results in reduced Th1 response. Additionally, ACT-mediated inhibition of CD40 surface expression could negatively affect IL-12 secretion during DC-T cell interactions because optimal secretion of IL-12 relies on CD40–CD40L interactions (35–37). The secretion of IL-6 by tolerogenic DCs has not been reported. However, IL-6 production by pulmonary DCs has been shown to impede Th1 immune responses (38). Because BMDCs that are infected with wild-type *B. bronchiseptica* show the phenotypic similarities to known regulatory DCs, we are currently determining the functionality of wild-type and mutant *B. bronchiseptica*-infected DCs to drive possible tolerogenic T cell responses. Guerard et al. (17) reported that *B. bronchiseptica*-reactive splenocytes produced low levels of IL-10 in addition to IFN-γ and IL-2 after infection. The presence of IL-10 may indicate the presence of IL-10-secreting regulatory T cells as a result of wild-type *B. bronchiseptica* infection. We are currently determining whether the secretion of IL-10 by restimulated splenocytes is due to the presence of *Bordetella*-specific regulatory T cells generated by a mechanism dependent on the interaction of bacterial virulence factors with APCs.

Our study also suggests that the bacteria-driven changes in DC phenotype may result from differential activation of DC MAPK signaling by *Bordetella* virulence factors. Because MAPK signaling plays a key role in DC maturation (39, 40), these pathways represent ideal targets for bacterial modulation. Our findings that type III secretion activates the ERK 1/2 MAPK signaling pathway while ACT inhibits the p38 MAPK pathway suggest that the combination of ERK 1/2 activation and p38 inhibition may alter the maturation and functionality of DC that can result in bacterial persistence. A recent report has shown that activation of the Fas cell death signaling pathway leads to phenotypic and functional maturation of murine BMDCs. In that study, specific inhibition of ERK 1/2 led to the reduced expression of maturation markers, including MHCII and CD86 (41). Therefore, it is possible that type III secreted factor(s) may activate a Fas-dependent signal transduction pathway resulting in the activation of ERK that in turn leads to the up-regulation of stimulatory and costimulatory molecules. Such a mechanism could explain why in vivo cell death is a prominent feature of BMDCs infected with wild-type *B. bronchiseptica*. Cell death may be averted in vivo by prosurvival signals from the surrounding environment because increased DC migration to local lymph nodes is evident following infection by *B. bronchiseptica* (17). Alternatively, in vivo cell death could release cellular components into the extracellular milieu, resulting in an
15 altered phenotype and functionality of neighboring DCs (42). In addition to the activation of ERK 1/2, the inhibition of p38 signaling could be responsible for partially deactivating DCs. Because increased p38 signaling has been linked to an increased ability of DCs to activate naïve T cells (26), any inhibition of p38 signaling by \textit{B. bronchiseptica} could lead to decreased stimulation of \textit{Bordetella}-specific T cells.

It has become increasingly clear that DC maturation status plays a key role in determining the effector functions of naïve T cells. By affecting DC signaling pathways that control DC maturation, \textit{B. bronchiseptica} could alter DC functions, leading to increased bacterial persistence within the host. Previous studies have shown the importance of the \textit{B. bronchiseptica} TTSS (e.g., type III mutants are cleared more readily from the trachea and elicit higher anti-\textit{Bordetella} Ab titers in infected mice) and ACT in the down-regulation of host immune functions (9, 22), and our present studies provide some insights into the possible molecular mechanisms used by these virulence factors. We are currently testing the functionality of \textit{B. bronchiseptica}-matured DCs to direct possible tolerogenic T cell responses and determining whether the IL-10 detected by previous investigations is secreted by regulatory T cells. Pathogen-driven development of a tolerogenic adaptive immune response could represent a novel mechanism by which a microorganism is able to avoid host immunity and establish a persistent infection.

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