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Short Communication

Respiratory infection with a bacterial pathogen attenuates CNS autoimmunity through IL-10 induction

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ABSTRACT

Infection with viral or bacterial pathogens has been linked with the development of multiple sclerosis (MS), while infection with helminth parasites has been associated protection against MS and other autoimmune diseases. Here we have used a murine model of MS, experimental autoimmune encephalomyelitis (EAE), to examine the effect of infection with the respiratory pathogen *Bordetella pertussis* infection on development of CNS inflammation. The data demonstrate that infection of mice with *B. pertussis* significantly attenuates the clinical course of EAE induced by active immunization or cell transfer. This was reflected in a significant reduction in VLA-4 and LFA-1 expression on T cells and infiltration of IL-17⁺, IFN- γ ⁺ and IFN- γ ⁺IL-17⁺ CD4 T cells into the CNS. Infection with *B. pertussis* induced IL-10 production from dendritic cells in vitro and enhanced the frequency of IL-10-producing CD25⁺Foxp3⁺ CD4⁺ T cells in vivo. Furthermore, the suppressive effects of *B. pertussis* infection on EAE were lost in IL-10^{-/-} mice. Our findings demonstrate that a bacterial infection of the respiratory tract can attenuate EAE by promoting production of the anti-inflammatory cytokine IL-10 that may suppress licensing of autoaggressive T cells in the lungs, thereby preventing their migration into the CNS.

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1. Introduction

Epidemiological studies have reported an association between infections with certain bacterial or viral pathogens and the development of autoimmune diseases, including multiple sclerosis (MS) (Mills, 2011). There is evidence that exacerbations of symptoms, and loss of neurological function in patients suffering from relapsing remitting MS often occur around the time of a clinical infections, most commonly upper respiratory infections (Turchinovich and Hayday, 2011). Furthermore, it has been suggested that systemic infections may trigger an increase in relapses in relapsing-remitting MS patients through bystander activation and sensitisation of auto-reactive myelin-specific T cells (Correale et al., 2006). *Chlamydia pneumoniae* infection is also associated with enhanced MRI spinal lesions in MS (Sotgiu et al., 2001). Furthermore, the detection of Epstein Barr virus latent protein and herpes simplex virus in the brains of patients with MS has

provided circumstantial evidence that pathogens may trigger or exacerbate the onset or progression of MS (Serafini et al., 2007).

Conversely, the prevalence of human infections, especially with helminth parasites, has been linked with a reduced incidence and severity of allergic and autoimmune diseases, including MS. This has been explained by the 'hygiene hypothesis', whereby regulatory T (Treg) cells induced by helminths and possibly other pathogens can suppress Th2 and Th17 responses that mediate allergy and autoimmunity respectively (McSorley and Maizels, 2012). Indeed it has been demonstrated that concurrent infection with helminths induced regression of new lesions and promoted remission in MS patients (Correale and Farez, 2007). Studies in a murine model of MS, experimental autoimmune encephalomyelitis (EAE), have shown that infection with the helminth parasite *Fasciola hepatica* attenuated symptoms of EAE through TGF- β -mediated suppression of Th1 and Th17 responses (Walsh et al., 2009). The bacteria *Staphylococcus aureus* is also capable of suppressing EAE through an extracellular adherence protein, which has anti-inflammatory effects by binding to ICAM-1 and preventing the infiltration of myelin specific T cells across the blood brain barrier into the CNS (Waubant et al., 2001).

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Bordetella pertussis causes whooping cough which, despite high vaccine coverage, is a re-emerging infectious disease that affects a high proportion of adults as well as children (Cherry, 2014). Infection with *B. pertussis* is persistent even in an immunocompetent host, with the bacteria taking weeks or months to be cleared through a combination of innate and adaptive immune responses, the latter involving IFN- γ -secreting Th1 cells and IL-17-producing Th17 cells (Higgs et al., 2012). However, these adaptive immune responses that eventually eliminate the pathogen are slow to develop and this reflects the various immune subversion strategies employed by *B. pertussis*, including induction of innate IL-10 and recruitment of Treg cells to the lungs during the acute stage of disease (McGuirk et al., 2002).

In this study we examined the influence of respiratory infection with *B. pertussis* on the development of EAE. The data reveal that infection with *B. pertussis* significantly attenuated the clinical symptoms of EAE. The protective effect of *B. pertussis* infection was associated with enhanced IL-10 production and impaired infiltration of encephalogenic T cells into the CNS, which was reversed in IL-10^{-/-} mice. Our findings demonstrate that bacterial infection may not only be involved in precipitating or exacerbating autoimmunity, but like helminth infections, may also have a protective role through bystander regulation of pathogenic immune responses that mediated autoimmune diseases.

2. Materials and methods

2.1. Mice

Breeding pairs of IL-10-defective (IL-10^{-/-}) mice on a C57BL/6 background were purchased from Jackson laboratories. C57BL/6 and IL-10^{-/-} were bred in house under pathogen free conditions and were maintained according to European Union regulations. All experiments were performed under license (BI00/2412) from The Health Products Regulatory Authority of Ireland and with approval from the Trinity College Dublin BioResources Ethics Committee.

2.2. Induction and assessment of EAE

Mice were immunized s.c. with 100 μ g MOG_{35–55} peptide (GenScript) emulsified in complete Freund's adjuvant containing 4 mg/ml (0.4 mg/mouse) of heat-killed *Mycobacterium tuberculosis* (Chondrex). Mice were injected i.p. with 500 ng pertussis toxin (PT; Kaketsuken) on days 0 and 2. Alternatively, EAE was induced by adoptive transfer of MOG-specific cells from C57BL/6 mice immunized with MOG and CFA following stimulation of lymph node and spleen cells with MOG (100 μ g/ml) and IL-23 (10 ng/ml) + IL-1 β (10 ng/ml) for 72 h (15 $\times$ 10⁶ cells injected i.p.). PT was not administered in the transfer model. Disease severity was assessed by weight change and clinical scores as follows: no clinical signs, 0; limp tail, 1; ataxic gait, 2; hind limb weakness, 3; hind limb paralysis, 4; tetra paralysis/moribund, 5.

2.3. *B. pertussis* respiratory infection

Respiratory infection of mice was induced by aerosol challenge as described (McGuirk et al., 2002). Briefly, *B. pertussis* Tohoma 1 was grown on Bordet-Gengou agar plates for 4 days, and bacteria were transferred to Stainer–Scholte liquid medium for 24 h at 37 °C. Bacteria were resuspended at 1 $\times$ 10⁹ colony forming units (CFU)/ml in physiological saline containing 1% casein, and aerosol challenge was administered over a period of 15 min using a nebulizer. Infection was confirmed by performing CFU counts on the

lungs of mice 3 h and 21 days post-infection as described (McGuirk et al., 2002).

2.4. Flow cytometry analysis on CNS and spleen cells

Mice were sacrificed by an anaesthetic overdose of pentobarbital sodium (Euthetal). The mice were perfused intracardially through the left ventricle with 20 ml of ice cold PBS. The brain and spinal cord were isolated. Tissue was lysed using the Qiagen tissue lyser at 30 rps for 5 min total. Cells were then washed in PBS and resuspended in 5 ml of 40% Percoll. The homogenate was overlaid onto 5 ml of 70% Percoll. The Percoll gradients were centrifuged at 1300g for 20 min. Mononuclear cells were removed from the interface of the Percoll gradients and washed twice with medium. Cells were stained with CD11b, F4/80 and Ly-6G, and FACS analysis was performed to determine the frequency of macrophages and neutrophils. Alternatively, spleen cells or mononuclear cells from brain or were stimulated for 5 h with PMA (10 ng/ml) and ionomycin (1 mg/ml) in the presence of brefeldin A (5 mg/ml). Cells were washed and stained for surface CD3 and CD4 (eBioscience). Cells were then fixed and permeabilized (Fix and Perm cell permeabilization kit; Caltag Laboratories) and stained for intracellular IL-17A, IFN- γ or IL-10 (eBioscience). Flow cytometric analysis was performed using a LSR Fortessa (BD Biosciences), and analyzed with FloJo software.

2.5. Antigen-specific cytokine production

Spleen (2 $\times$ 10⁶ cells/ml) were stimulated with MOG_{35–55} peptide (2–50 μ g/ml) or medium only. Supernatants were recovered after 72 h, and IL-17 and IFN- concentrations were determined by ELISA (R&D Systems).

2.6. Dendritic cells

Mouse bone-marrow derived immature DCs were generated as described (Marks et al., 2009). Bone marrow-derived immature DC (10⁶/ml) were cultured at 37 °C for 24 h with heat killed (HK) or live *B. pertussis* (10⁵–10⁸/ml). Supernatants were removed after 24 h, and the concentrations of IL-10 were quantified by ELISA (R&D Systems).

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism statistical analysis software. Group differences were analyzed by unpaired Student t test or two-way ANOVA with multiple comparisons, followed by Bonferroni posttest comparisons, for three or more groups. Differences between groups for clinical scores in EAE were analyzed by two-way ANOVA with repeated measures. The *p* values $\leq$ 0.05 were considered significant.

3. Results

3.1. Respiratory infection with *B. pertussis* attenuates EAE and infiltration of Th1 and Th17 cells into the CNS

We examined the immunomodulatory effects of *B. pertussis* infection on the development of EAE and found that respiratory infection of mice with *B. pertussis* significantly attenuated the clinical symptoms of EAE and associated weight loss (Fig. 1A). The mean clinical score of mice infected with *B. pertussis* was 1.75 compared with a mean clinical score of 3.97 for uninfected mice with EAE. The onset of symptoms was also delayed in the mice infected with *B. pertussis*. The suppressive effect was most pronounced

Fig. 1. Respiratory infection with *B. pertussis* suppresses attenuates EAE, associated with suppression of pathogenic T cell migrating into the CNS. (A) EAE was induced in C57BL/6 mice that were un-infected or infected with *B. pertussis* on day-1. Clinical scores and body weights were recorded daily. (B) Repeat of experiment shown in A with clinical scores out to day 35. (C) EAE clinical scores in mice convalescing from *B. pertussis* (36 days post challenge) or in control naïve mice. (D and E) Mononuclear cells were isolated from the brains on day 23 of mice with EAE or EAE and infected on day-1 with *B. pertussis* (Bp). (D) Cells were restimulated for 5 h with PMA + ionomycin and brefeldin A, and stained for surface CD3, CD4, and for intracellular IL-17 or IFN-γ and analysed by flow cytometry; results are mean absolute numbers of cells for 6 mice per group, with representative dot plots. (E) Cells from brain stained with for surface CD11b, CD19, Ly6G and F4/80 to identify neutrophils and macrophages. (F) Mice were sacrificed on day 13 of EAE and spleen and lymph node cells were re-stimulated with MOG peptide (0, 5, 20 μg/ml). After 72 h supernatants were removed and analyzed for IL-17 by ELISA. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 versus *B. pertussis* infection only, **P* < 0.01, ***P* < 0.001 versus medium by ANOVA and Bonferroni post hoc analysis. (G and H) EAE was induced by passive transfer (15×10^6 cells i.p. per mouse) of lymph node and spleen cells (from MOG immunized mice; cells re-stimulation in vitro with MOG, IL-23 and IL-1β) into naïve C57BL/6 recipient mice or mice infected with *B. pertussis* 1 day prior to transfer. (G) EAE clinical scores. (H) Mononuclear cells were isolated from the brains on day 14 post transfer and re-stimulated for 5 h with PMA + ionomycin and brefeldin A, and stained with anti-CD3, anti-CD4 and for intracellular IL-17 or IFN-γ and analysed by flow cytometry. Results are mean absolute numbers of cells (*n* = 6 mice per group) with representative dot plots (gated on CD4⁺ T cells).

during the period of acute infection; when mice were monitored for EAE at extended interval after *B. pertussis* challenge, when the bacteria numbers in the respiratory had declined (McGuirk et al.,

2002), symptoms of EAE did appear, but the time of onset was significantly delayed compared with un-infected mice (Fig. 1B). Furthermore, the course of EAE was not altered when it was

induced in convalescent mice 36 days after challenge with *B. pertussis* around the time of bacterial clearance from the lungs (Fig. 1C).

Intracellular cytokine staining of mononuclear cells from brain and spinal cord of mice on day 23 showed significantly reduced numbers of IL-17⁺, IFN- γ ⁺ and IL-17⁺IFN- γ ⁺ CD4⁺ T cells in the brain (Fig. 1D) and spinal cord (data not shown) of *B. pertussis* infected mice with EAE when compared with uninfected mice with EAE. Furthermore, there was a non-significant reduction in the numbers of infiltrating neutrophils and macrophages into the brain of infected mice with EAE (Fig. 1E). We examined the possibility that *B. pertussis* modulated peripheral MOG-specific T cells responses. Spleen and lymph node cells from mice with EAE, with or without *B. pertussis* infection, produced significantly more IL-17 when stimulated with MOG compared with medium only (Fig. 1F). Furthermore, spleen and lymph node cells from mice with EAE, with or without *B. pertussis* infection, produced significantly greater IL-17 (Fig. 1F) and IFN- γ (data now shown) when compared with *B. pertussis* infected mice without EAE. These findings suggest that *B. pertussis* infection does not inhibit induction of pathogenic T cell but may inhibit their migration to the CNS. In order to confirm and extend these findings, we used the T cell transfer model where MOG-specific Th17-polarized cells were transferred to recipients that were then infected with *B. pertussis*. In this model with did not use PT to induce EAE, allowing us to rule out any role for *B. pertussis* infection in modulating the potentiating effects of PT on EAE. *B. pertussis* infection significantly impaired the ability of spleen and lymph nodes cells from mice immunized with MOG and CFA to transfer EAE to recipient mice (Fig. 1G). Recipient mice that were infected with *B. pertussis* had significantly fewer IL-17-secreting CD4⁺ T cells migrating into the brains (Fig. 1H). Collectively these findings suggest that rather than suppressing induction of autoantigen-specific Th1 and Th17 cells, *B. pertussis* infection attenuates EAE and that this is associated with a reduction of pathogenic T cell migration into the CNS.

3.2. *B. pertussis* attenuates EAE through IL-10 production

One explanation for the modulatory effect of *B. pertussis* infection on the induction of EAE was that the bacteria enhanced immunosuppressive cytokine production by cells by innate immune cells and/or T cells. We have previously reported that *B. pertussis* induces TLR4-dependant production of IL-10 as well as the pro-inflammatory cytokines IL-1 β , IL-6, TNF, IL-12 and IL-23 (Higgins et al., 2006). Here, we found that DCs produced IL-10 at high concentrations when stimulated with live *B. pertussis* and at lower concentrations in response to killed *B. pertussis* (Fig. 2A). We have also reported a significant increase in the numbers of IL-10-producing Foxp3⁺CD25⁺ CD4⁺ T cells in the lungs during infection with *B. pertussis*, which peaked at day 14 (Coleman et al., 2012). Consistent with this, we found that there was a significant increase in the number of IL-10-producing CD4⁺ T cells from the spleens of mice 14 days post infection with *B. pertussis* compared with naive controls (Fig. 2B). Furthermore, we demonstrate a significant increase in IL-10-producing T cells in the lungs of mice infected with *B. pertussis* where EAE was induced by passive (Fig. 2C) or active immunization (Fig. 2D and E) and approximately 30% of these IL-10-producing CD4⁺ T cells were CD25⁺Foxp3⁺ (Fig. 2F). These findings demonstrate that *B. pertussis* induces IL-10 producing T cells in the lungs and this constrains Th1 and Th17 responses specific for *B. pertussis*, but may also exert bystander suppression of the function or migration of Th1 and Th17 cells that are pathogenic in EAE.

To determine if *B. pertussis* induced IL-10 mediated the modulatory effect of infection on EAE, we examined the effect of *B. pertussis* infection on the course of EAE in IL-10^{-/-} and WT mice.

Consistent with the data in Fig. 1A, infection of WT mice with *B. pertussis* significantly attenuated EAE, but this effect was completely reversed in IL-10^{-/-} mice (Fig. 2G). The reduced infiltrating IL-17- and IFN- γ -producing CD4⁺ T cells observed in the brains of *B. pertussis* infected WT mice (Fig. 1D) was less pronounced in IL-10^{-/-} mice (data now shown). These findings demonstrate that *B. pertussis* attenuates EAE through IL-10 induction. When taken together with the observation that infection also suppresses EAE induced by T cell transfer, which was associated with suppressed migration to the CNS, suggest that IL-10 induced by *B. pertussis* infection in the lungs may inhibit migratory activity of encephalitogenic T cells. In order to provide evidence for this conclusion, we examined the effect of IL-10 on MOG-specific Th17 cells in vitro and found that IL-10 inhibited expression of VLA-4 and LFA-1, integrins known to be involved in migration of T cells (Fig. 2G). Furthermore, infection of recipient mice with *B. pertussis* suppressed VLA-4 and LFA-1 expression on CD4 T cells in the lungs when assessed 4 days after transfer of MOG-specific T cells (using the protocol to induce passive EAE) (Fig. 2H). Collectively these findings demonstrate that IL-10 induced by the respiratory pathogen *B. pertussis* suppresses integrin expression on T cells in the lungs and this may explain the attenuating effect of the infection on migration of pathogenic Th1 and Th17 cells and the induction of CNS autoimmunity.

4. Discussion

The significant new findings of this study are that infection of mice with a common human bacterial pathogen can significantly reduce CNS inflammation and attenuate clinical symptoms of EAE, a mouse model of MS. There was a significant reduction in disease onset, disease scores and weight loss following induction of EAE in mice infected with *B. pertussis*. This was also reflected in a reduced number of infiltrating neutrophils, macrophages, Th1, Th17 cells in the brains and spinal cords of *B. pertussis* infected mice with EAE. However, the reduced infiltration of IL-17 and IFN- γ producing T cells into the CNS in *B. pertussis* infected mice was not reflected by a corresponding reduction in peripheral MOG-specific Th1 and Th17 in infected mice, suggesting that the bacterial infection may prevent the migration of encephalitogenic T cells into the CNS and thereby limiting CNS pathology associated with EAE.

Autoimmune diseases, such as MS, develop as a result of breakdown in immune tolerance resulting in the development of adaptive immune responses against self antigens. The precipitating factors are believed to include pathogen-associated molecular patterns (PAMPs) released during viral or bacterial infections, danger-associated molecular patterns (DAMPs) released from dead or dying cells during sterile inflammation or microbe-associated molecular patterns (MAMPs) released from inflammatory commensal bacteria (Mills, 2011). These molecules bind to pathogen recognition receptors on innate immune cells leading to DC maturation and production of IL-1 β , IL-6, IL-12 and IL-23 that direct the induction of pathogenic Th1 and Th17 cells. Indeed development of MS and other autoimmune diseases has been linked with certain viral and bacterial infections (Mills, 2011). However there is also evidence that infection with helminth or 'friendly' microbiota can reduce the symptoms of autoimmunity through induction of anti-inflammatory cytokines, including IL-10 and TGF- β , that promote induction of FOXP3⁺ Treg or IL-10-secreting Tr1-type cells (Finlay et al., 2014). Our study has extended these findings through the demonstration that the bacteria *B. pertussis*, which promotes IL-10 production by DC and induces *B. pertussis*-specific Treg cells, exerts bystander suppression on pathogenic Th1 and Th17 responses that mediate CNS inflammation and the development of EAE. It has been demonstrated that autoreactive T cells can be

Fig. 2. The protective effect of *B. pertussis* on EAE is mediated by IL-10, which inhibits integrin expression on T cells. (A) Bone marrow-derived murine DCs were stimulated with increasing concentrations of live or heat killed *B. pertussis* ($1\text{--}100 \times 10^6$ CFU/ml or equivalent) for 24 h. Supernatants were removed and IL-10 quantified by ELISA. (B) Spleen were isolated from *B. pertussis* infected (day 14) or naive mice and cells were stimulated for 5 h with brefeldin A and stained with anti-CD3, anti-CD4, and for intracellular IL-10 and analysed by flow cytometry. Results are mean absolute numbers of cells from 4 mice with representative dot plots $^{**}P < 0.01$ versus naive by unpaired student *t*-test. (C–E) Mononuclear cells were isolated from the lungs of infected mice with EAE induced by passive (C) or active (D and E) immunization. Cells were stimulated for 5 h with PMA + ionomycin and brefeldin A, and stained with anti-CD3, anti-CD4 anti-CD25 and for intracellular IL-10 and intranuclear Foxp3 and analysed by flow cytometry. Results are mean absolute numbers of cells (C and D) with representative dot plots for Foxp3 versus CD25 on IL-10 $^{+}$ CD4 $^{+}$ T cells (E). (F) C57BL/6 WT or IL-10 $^{-/-}$ mice were aerosol infected with *B. pertussis* on day-1. EAE was induced in infected or uninfected mice on day 0 and clinical scores were assessed daily. $^{***}P < 0.001$ by two one-way ANOVA and Bonferroni post hoc analysis. (G) MOG-specific Th17 cells were expanded from mice immunized with MOG and CFA by stimulation in vitro with MOG, IL-23 and IL-1 β in the presence or absence of IL-10. After 72 h, cells were stained with anti-CD3, CD4, VLA-4 (CD49d) and LFA-1 (CD11a) and analyzed by flow cytometry. (H) Lymph node and spleen cells from MOG immunized mice and stimulated in vitro with MOG, IL-23 and IL-1 β were transferred into naive C57BL/6 recipient mice or mice infected with *B. pertussis* 1 day prior to transfer. 4 days post transfer, mononuclear cells were isolated from the lungs of infected mice and stained with anti-CD3, CD4, CD49d and CD11a. Results are mean absolute numbers of cells with representative FACS plots.

reprogrammed in the lungs enabling these T cells to migrate to the CNS during EAE (Odoardi et al., 2012). We demonstrated that EAE induced by transfer of MOG-specific T cells was, like active EAE, inhibited during infection with *B. pertussis*, and this was associated with reduced migration of the transferred T cells into the CNS. Together with the observation that the MOG-specific T cell responses induced in the lymph nodes and spleen by active immunization with MOG and CFA was not suppressed in mice infected

with *B. pertussis*, suggests that the suppressive mechanism involved inhibition of T cell migration or effector function.

Our studies using IL-10 $^{-/-}$ mice demonstrated that the suppressive effects of *B. pertussis* infection on the development of EAE were mediated by IL-10; *B. pertussis* infection failed to suppress clinical symptoms of EAE in IL-10 $^{-/-}$ mice. Furthermore, the suppression of Th1 and Th17 migration into the CNS following infection was mostly reversed in IL-10 $^{-/-}$ mice. Furthermore, we demonstrated

that expression of the integrins, VLA4 and LFA-1, which are known to be involved in cell migration into the CNS, was suppressed on MOG-specific Th17 cells in the lungs during *B. pertussis* infection or when cultured in vitro with IL-10. These findings are consistent with reports that overexpression of IL-10 protects against EAE disease, whereas deletion of IL-10 exacerbated disease (Bettelli et al., 1998; Cua et al., 2001). In MS patients, it has been reported that serum concentrations of IL-10 are increased during disease remission (Waubant et al., 2001). Furthermore, the efficacies of IFN- β and glatiramer acetate as therapies in MS are partly attributed to their induction of IL-10 production (Ersoy et al., 2005; Putheti et al., 2003). IL-10 is a potent inhibitor of the inflammatory process (Fiorentino et al., 1991a) and inhibits Th1 and Th17 responses either by directly suppressing T cell cytokine production or by inhibiting APC function (Fiorentino et al., 1991b). However, our study reveals that bacteria-induced IL-10 may suppress the licensing of autoaggressive T in the lungs, thus preventing them from migrating into the CNS to mediate autoimmune inflammation. The findings add further weight to the hygiene hypothesis, but suggest that suppression of autoimmunity by infectious agents is not confined to parasites, but can also be mediated by a common bacterial pathogen of man.

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