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# *Helicobacter hepaticus* infection in BALB/c mice abolishes subunit-vaccine-induced protection against *M. tuberculosis*

Isabelle C. Arnold[a], Claire Hutchings[b], Ivanela Kondova[c], Ariann Hey[b], Fiona Powrie[a], Peter Beverley[b,1], Elma Tchilian[b,*]

[a] *Kennedy Institute of Rheumatology, Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences (NDORMS), University of Oxford, Roosevelt Drive, Oxford, UK*
[b] *The Peter Medawar Building for Pathogen Research, University of Oxford, South Parks Road, Oxford, UK*
[c] *Biomedical Primate Research Centre, Rijswijk, The Netherlands*

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ABSTRACT

BCG, the only licensed vaccine against tuberculosis (TB), provides geographically variable protection, an effect ascribed to exposure to environmental mycobacteria (EM). Here we show that altering the intestinal microbiota of mice by early-life infection with the commensal bacterium *Helicobacter hepaticus* (*Hh*) increases their susceptibility to challenge with *Mycobacterium tuberculosis* (*Mtb*). Furthermore *Hh*-infected mice immunised parenterally with the recombinant subunit vaccine, human adenovirus type 5 expressing the immunodominant antigen 85A of *Mtb* (Ad85A), display a reduced lung immune response and protection against *Mtb* challenge is also reduced. Expression of interleukin 10 (IL10) messenger RNA is increased in the colon of *Hh* infected mice. Treatment of *Hh*-infected Ad85A-immunised mice with anti-IL10 receptor antibody, following challenge with *Mtb*, restores the protective effect of the vaccine. These data show for the first time that alteration of the intestinal microbiota by addition of a single commensal organism can profoundly influence protection induced by a TB subunit vaccine via an IL10-dependent mechanism, a result with implications for the deployment of such vaccines in the field.



## 1. Introduction

Tuberculosis (TB) is an important cause of morbidity and mortality worldwide with nearly 9 million new infections annually and 1.4 million deaths. The difficulty of reducing this disease burden is compounded by the spread of drug resistant organisms and the greatly increased susceptibility of HIV patients to TB [1].

In the face of these problems, vaccination is an attractive strategy but the protective efficacy of the only licensed vaccine, BCG, varies geographically and wanes with time. Furthermore, boosting with BCG is generally ineffective [2]. This has led to attempts either to replace BCG with a more effective vaccine or to improve its efficacy with booster vaccines.

A common hypothesis to account for the variation in BCG effectiveness in different locations is that this is due to environmental effects, including exposure to environmental mycobacteria (EM), which might may prevent growth of BCG, mask BCG-induced protection or induce regulatory T cells, as demonstrated experimentally in mice [3]. Clearly if subunit booster vaccines are to be used to improve the efficacy of BCG, it will be important to know whether their efficacy is similarly subject to environmental influences. In fact, when EM are administered orally and a subunit vaccine parenterally, protection against *Mtb* challenge is reduced [4]. However, in these experiments large quantities of EM were continuously administered in the drinking water, so that the question remained whether less artificial changes in the microbiota of the intestine might also influence protective immune responses to a subunit TB vaccine.

Multiple studies have highlighted the immunomodulatory effect of enteric microorganism exposure on lung immune responses. Experimental helminth infection in mice has been shown to impair innate pulmonary host defence against *Mtb* through the IL4 receptor signalling pathway [5] and helminth infection has been associated with increased TB incidence and reduced BCG vaccine efficacy in affected populations [6]. In contrast,

*Abbreviations:* Hh, *Helicobacter hepaticus*; Mtb, *Mycobcterium tuberculosis*; CFU, colony forming units.

* Corresponding author. Present address: The Pirbright Institute, Ash Road, Pirbright, Surrey, UK. Tel.: +44 0 483231410.
*E-mail address:* elma.tchilian@pirbright.ac.uk (E. Tchilian).
[1] Present address: Respiratory Infection and Airway Disease Infection Section, National Heart & Lung Institute (NHLI), Imperial College London, London, UK.

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epidemiological evidence suggests that infection with the gastric bacterium *Helicobacter pylori* is associated with protection against TB [7], while neonatal infection of mice with *H. pylori* prevents the induction of allergic lung disease later in life [8].

The related gram-negative bacterium, *Helicobacter hepaticus* (*Hh*), is a non-invasive organism commonly found in the murine lower intestinal tract in mouse colonies [9]. *Hh* does not cause histological lesions in immunocompetent mice but IL10 insufficient mice develop typhlocolitis [10]. Inflammation develops via induction of Th17 cells in the intestinal mucosa that rapidly extinguish IL17A production and become IFNγ producing Th1 cells [10–12].

In these experiments, we investigate the effect of *Hh* on immune response and protection induced by the recombinant subunit vaccine, human adenovirus type 5 expressing the immunodominant Antigen 85A of *Mtb* (Ad85A) [13].

## 2. Materials and methods

### 2.1. Mice

All experiments were performed with BALB/c mice bred in house. Sentinel mice were screened every 3 months by Harlan Orlac (Blackthorn, UK) to exclude the presence of *Helicobacter* species and breeders were replaced after every experimental *Hh* infection. The experiments were approved by the animal use ethical committee of Oxford University and complied with UK Home Office guidelines.

### 2.2. Hh infection and quantitation

*Hh* NCI-Frederick isolate 1A (strain 51449) was grown as described previously [14]. Seven day old BALB/c mice from *Helicobacter*-free breeders were fed on two consecutive days with *Hh* 1A ($\sim 2.5 \times 10^7$ CFU) by oral gavage. *Hh* colonisation was analyzed in caecal contents collected upon sacrifice. DNA was isolated using the DNA Stool kit (QIAGEN) and SYBR Green real-time PCR with *Hh*-specific primers against the *cdtB* gene (Fwd: CCG CAA ATT GCA GCA ATA CTT; Rev: TCG TCC AAA ATG CAC AGG TG) was performed in triplicate using the CFX96 detection system (Bio-Rad Laboratories). Results represent arbitrary units normalised to uninfected control samples.

### 2.3. Ad85A immunisation

Four to five weeks after *Hh* administration, infected and matched control mice were immunised with $2 \times 10^9$ virus particles of Ad85A equally divided between the two quadriceps muscles.

### 2.4. Infection with Mtb and determination of mycobacterial load

Five to ten mice were anesthetised with isoflurane and infected i.n. with *Mtb* (Erdman strain) in 40 μl PBS. The number of organisms deposited was determined 24 h after challenge (~200 CFU). Mice were sacrificed 5 weeks post-challenge. Lungs were homogenised and 10-fold serial dilutions of tissue homogenates were plated on Middlebrook 7H11 agar plates (E&O Laboratories Ltd, Bonnybridge, UK) to determine mycobacterial load. Colony-forming units (CFU) were enumerated after 3–4 weeks of incubation at 37 °C in 5% $CO_2$.

### 2.5. Treatment with antibody to the IL-10 receptor

Naive or *Hh* infected mice were immunised with Ad85A i.m. or left unimmunised. Following challenge with *Mtb*, mice were injected intraperitoneally on day 0, 7 and 17 with 1 mg of anti-IL-10R antibody (clone 1B1.2) or PBS as control.

### 2.6. Histopathological assessment

Five weeks after *Mtb* challenge, lungs from infected mice were removed and fixed in buffered 4% formalin. 4–5 μm paraffin-embedded sections were stained with haematoxylin and eosin, and histopathology of the lungs was assessed semi-quantitatively in a blinded fashion by a trained pathologist.

### 2.7. Isolation of lymphocytes from lungs, spleen, gut and MLN

Lungs were perfused with PBS, cut into pieces and digested with 0.7 mg/ml collagenase type I (Sigma) and 30 μg/ml DNase I (Sigma) for 45 min at 37 °C. Digested fragments were crushed through a cell strainer using a syringe plunger, washed with PBS, layered over Lympholyte (Cederlane, Ontario, Canada) and centrifuged at $1000 \times g$ for 25 min. Interface cells were collected and washed. Spleens and MLNs cell suspensions were prepared by mashing the tissue through a cell strainer using a syringe plunger. For spleens, red blood cells were removed with lysis buffer (Qiagen, Crawley, UK) and the cells were washed with PBS. For colonic lamina propria cell isolation, colons were opened longitudinally, washed in PBS 0.1% BSA and cut into pieces. Pieces were washed twice in HBSS supplemented with 4% FBS, 100 U/ml penicillin/streptomycin and 5 mM EDTA at 37 °C with shaking to remove epithelial cells. Tissue was then digested at 37 °C in a shaking incubator with 1 mg/ml type VIII collagenase (Sigma-Aldrich) and 0.5 mg/ml DNase I in RPMI-1640 medium supplemented with 4% FBS and 100 U/ml penicillin/streptomycin. The isolated cells were layered on a 40/80% Percoll gradient and the interface was collected and washed.

### 2.8. Flow cytometry

For surface staining, cells were washed and incubated with CD16/CD32 monoclonal antibody to block Fc binding. Cells were stained with a fixable viability dye and a combination of the following antibodies: CD45 (30-F11), CD3 (17A2), CD4 (RM4-5), CD8 (53-6.7), CD44 (IM7), CD62L (MEL-14) and CD25 (PC61.5) surface markers. For Tregs staining, cells were fixed and permeabilised with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions and stained with Foxp3 (FJK-16s). For intracellular cytokine staining in response to 85A stimulation, cells from Ad85A immunised animals were stimulated for 6 h with a pool of 66 15-mer peptides overlapping by 10 amino acids, covering the 85A protein sequence (Peptide Protein Research Ltd, Fareham, UK), in Hepes buffered RPMI with 10% heat-inactivated FCS, L-glutamine, penicillin and streptomycin. Each peptide was 2 at μg/ml. After 2 h at 37 °C, Golgi Plug (BD Biosciences, Oxford, UK) was added according to the manufacturer's instructions. Cells were stained for surface markers then for intracellular IFNγ (XMG1.2) and TNFα (MP6-XT22) (eBioscience, Hatfield, UK) using the BD Cytofix/Cytoperm kit according to the manufacturer's instructions. All cells were run on a LSRII (BD Biosciences) and analysed using FlowJo software (Tree Star Inc, Ashland, Oregon, USA).

### 2.9. Quantitation of gene expression using TaqMan qPCR

RNA was isolated from snap-frozen lung and colonic tissue using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions, including an on-column DNase I digestion step. cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) for the candidate genes was performed using TaqMan gene expression assays (Life Technologies). cDNA samples were analysed in duplicate using the CFX96 detection system (Bio-Rad Laboratories) and values were normalised to *Hprt* according to the

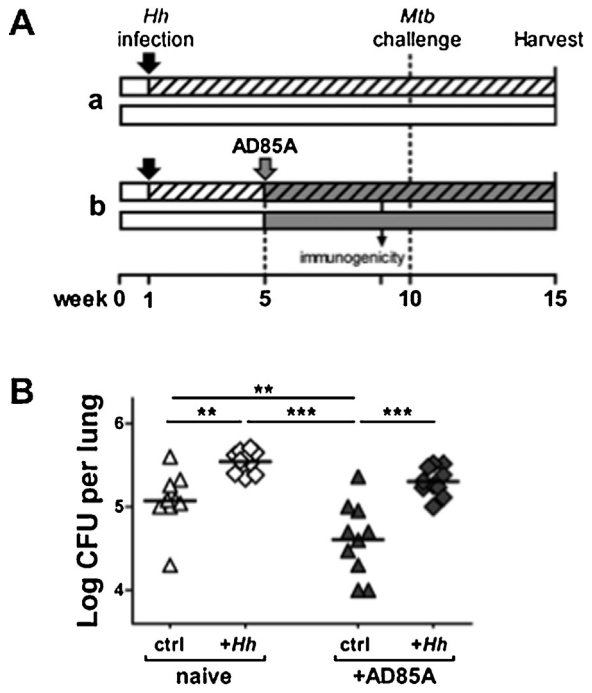


**Fig. 1.** *Protection against Mtb challenge.* BALB/c mice were infected with *Hh* and 5 weeks later immunised with $2 \times 10^9$ v.p. Ad85A i.m. Controls were uninfected naïve or uninfected Ad85A immunised animals. Mice were challenged with *Mtb* 5 weeks after immunisation and lung *Mtb* CFU were determined 5 weeks after challenge. (A) Schematic depiction of the treatment regimen. (B) Lung *Mtb* CFU. Symbols show individual mice and horizontal lines indicate the mean. *** $p < 0.001$, ** $p < 0.01$ between the indicated groups as determined by one-way ANOVA with Tukey's post-test. Data are representative of two independent experiments with similar results.

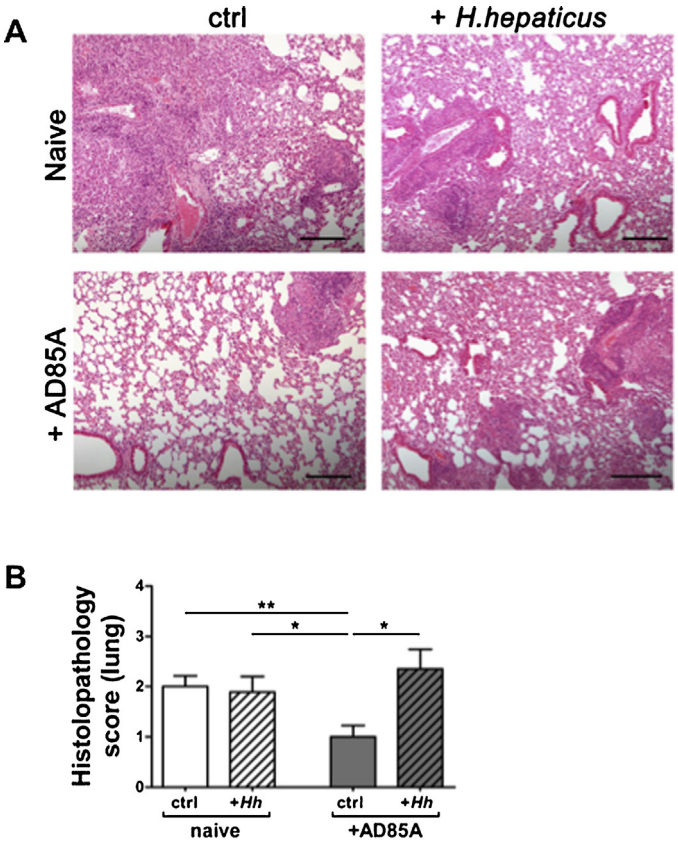


**Fig. 2.** *Histopathology.* (A) Representative histopathology of the lungs of mice 5 weeks after *Mtb* challenge. Naïve mice are compared to the lungs of mice immunised with Ad85A or mice infected with *Hh* or infected with *Hh* and immunisd with Ad85A. Haematoxylin/eosin-stained paraffin-embedded lung sections. Bar is 100 μm. (B) Histograms show mean and standard error of semi-quantitative scoring of the extent of pathology in groups of 10 mice as 10–15%, 15–25%, 25–50%, 50–70% and >70%. ** $p < 0.01$, * $p < 0.05$ between the indicated groups by Mann Whitney *U* test.

Δ-Ct method. TaqMan Gene Expression Assays were performed for mouse *Hprt* (Mm01545399_m1), *Il10* (Mm00439614_ml), *Tbx21* (Mm00450960_m1), *Gata3* (Mm00484683_m1), *Rorc* (Mm01261022_m1) and *Foxp3* (Mm00475162_m1).

### 2.10. Statistical analysis

Data were analysed using one-way ANOVA followed by Tukey's multiple comparison tests. Immune responses and histopathological scores were assessed by Mann–Whitney *U* with Bonferroni's post-test.

## 3. Results

### 3.1. Hh infection increases Mtb bacterial load and abolishes the protective effect of Ad85A immunisation

In order to understand how early life exposure to a bacterial trigger could influence the response to *Mtb* infection and vaccine efficacy, we assessed the impact of neonatal infection with *Hh* on *Mtb* challenge in mice. One week old BALB/C mice were infected with *Hh* orally and challenged intranasally at ten weeks of age with *Mtb* along with uninfected littermates. Mice were assessed for mycobacterial load in the lungs 5 weeks post-challenge (Fig. 1A). We observed that *Hh* infected animals had an average 0.6 log increase of *Mtb* CFU in the lungs compared to their uninfected littermates (Fig. 1B).

Similarly, groups of *Hh* infected or uninfected mice were immunised with Ad85A at weeks 6 of age and challenged 5 weeks later with *Mtb* (Fig. 1A). As previously reported, Ad85A immunisation efficiently reduced the *Mtb* colonisation level in the lungs of uninfected mice by ~0.5 $\log_{10}$ [4]. However, early-life infection with *Hh* strikingly abolished the protective effect of Ad85A (Fig. 1B).

Histological analysis shows that naive mice have multifocal coalescing granulomatous lesions composed of macrophages and lymphocytes with small aggregates of neutrophils and scattered multinucleate cells, making up 50–70% of the sections in most animals (Fig. 2A). In contrast, Ad85A immunised mice show significantly smaller areas of interstitial inflammation with a low number of lymphohistiocytic aggregates (Fig. 2B). Lungs from *Hh*-infected animals show multifocal lesions with mild interstitial infiltrates and foci of peribronchial lymphocytes, often with focal extravasated red blood cells (haemorrhage), mild oedema and congestion. Interestingly, the pathological appearance in *Hh*-infected animals immunised with Ad85A is similar to their non-immunised counterparts, but in several animals the inflammation is severe, with intra-alveolar inflammatory infiltrates in addition to areas of haemorrhage and oedema. Thus, the histopathological appearances reflect the abolition of the protective effect of Ad85A by *Hh* infection.

### 3.2. Effect of Hh infection on antigen specific responses and lung cell composition

To characterise in more detail the effect of *Hh* infection on Ad85A immunisation, we measured 85A-specific responses in the lungs and spleen 4–5 weeks after immunisation, at the peak of the response to Ad85A. Intramuscular administration of Ad85A has

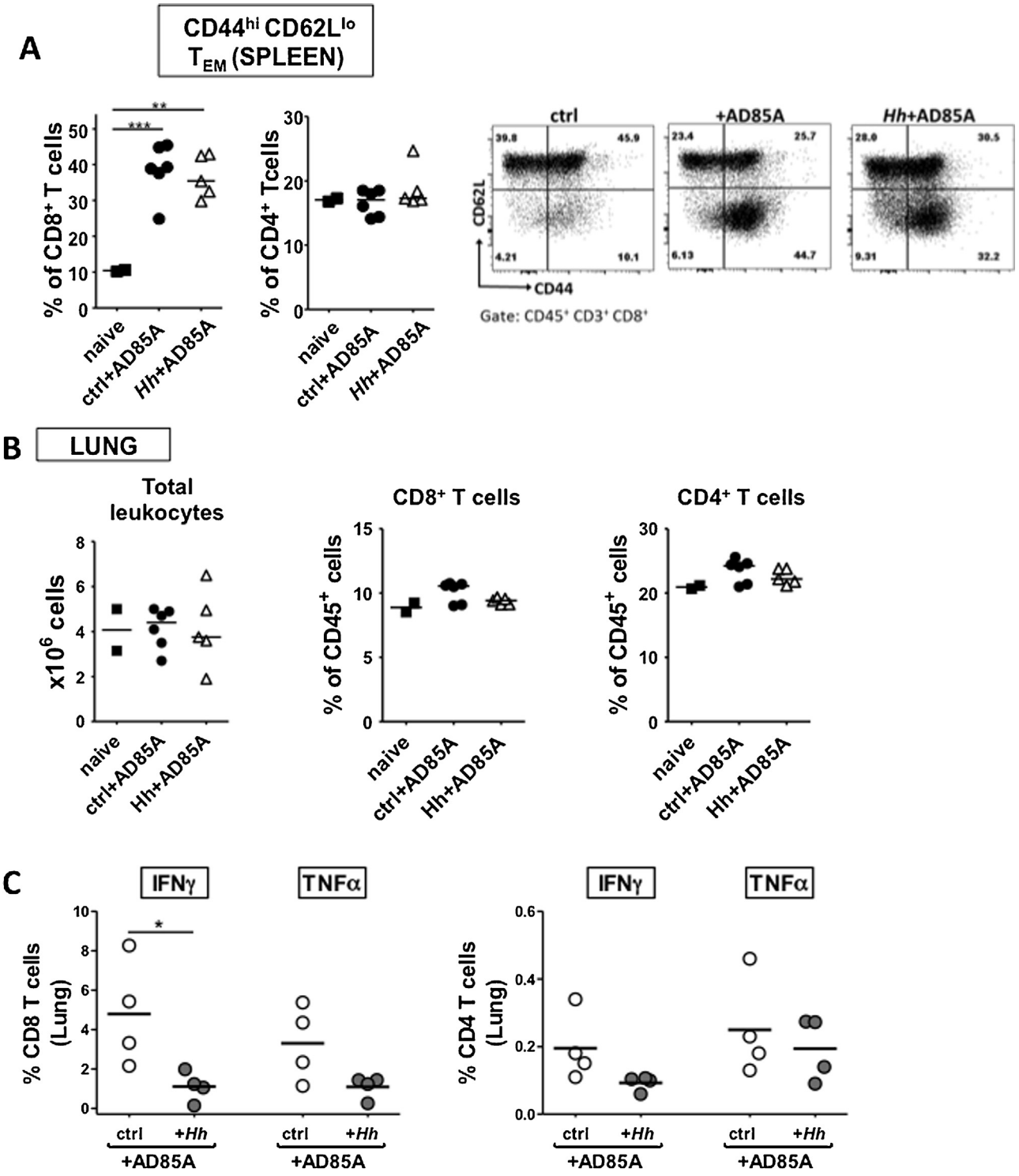


**Fig. 3.** *Antigen 85A specific T cells responses.* BALB/c mice were infected with *Hh* and 5 weeks later immunised with Ad85A. Controls were uninfected naïve or uninfected Ad85A-immunised animals. Lung and splenic lymphocytes were isolated 4 weeks post immunisation. (A) Frequencies of $CD44^{hi}$ $CD62L^{lo}$ effector memory T cells among splenic $CD8^+$ and $CD4^+$ T cells and representative flow-cytometry staining gated on live $CD45^+$ $CD3^+$ $CD8^+$ T cells. (B) Absolute numbers of lung leukocytes and frequencies of lung $CD8^+$ and $CD4^+$ T cells among total $CD45^+$ cells. (C) Frequency of lung of lung $CD8^+$ and $CD4^+$ T cells expressing IFNγ and TNFα determined by flow-cytometry following 6 h in vitro stimulation with 85A peptides. Data points represent individual mice and horizontal bars represent medians. * $p < 0.05$ as determined by one-way ANOVA with Tukey's post-test.

previously been reported to induce a strong CD8 but weaker CD4T cell response [13,15] and both *Hh*-infected and uninfected control mice show a similar increase in the frequency of $CD8^+$ effector memory T cells in the spleen following immunisation (Fig. 3A). In the lungs, the total number of leukocytes is similar in control and *Hh*-infected mice immunised with Ad85A and the frequencies of CD4 and CD8 cells are unchanged (Fig. 3B). However, while in uninfected mice immunised with Ad85A, 4–5% of CD8 and ~0.2% of CD4 lung T cells produce IFNγ and TNF upon antigenic restimulation with 85A peptides, 85A-specific CD8T cell IFNγ responses are significantly reduced in immunised mice that have been previously infected with *Hh* (Fig. 3C).

Taken together, these results indicate that *Hh* infection of neonatal BALB/c mice increases the lung mycobacterial load after *Mtb* challenge. Furthermore, *Hh* infection reduces antigen specific lung immune responses after Ad85A i.m. immunisation and abolishes protection against *Mtb* challenge.

### 3.3. *IL10 is a key determinant of the Hh-mediated suppression of Ad85A vaccine-induced protection against Mtb challenge*

IL10 plays a key role in preventing pathological intestinal inflammation induced by *Hh* [10–12] and IL10$^{-/-}$ mice infected with *Hh* develop severe inflammation in the caecum and colon. In contrast, *Hh*-infected wild-type mice mount an antigen-specific regulatory T cell response to the bacterium that prevents bacteria-induced colitis in an IL10-dependent manner [16]. We therefore examined the expression of *Il10* mRNA in the colon and lungs of naive and *Hh*-infected mice. We first confirmed that *Hh*-infected animals are similarly colonised by *Hh* eight weeks after their initial infection (Fig. 4A). Both unimmunised and Ad85A-immunised mice infected with *Hh* display significantly up-regulated *IL10* mRNA expression levels in the colon but not the lungs (Fig. 4B), with a trend towards reduced T-bet and GATA3 mRNA expression, transcription factors associated with Th1 and Th2 cell differentiation, respectively (Fig. 4C). However, *Hh* infection does not affect *Foxp3* tissue expression level (Fig. 4D) or the frequency of Foxp3$^{+}$CD4$^{+}$ regulatory T cells in the colon, lung, spleen and mesenteric lymph nodes (Fig. 4E) in Ad85A-immunised mice.

Because IL10 has been shown to play an important role in regulating immunity to *Mtb* infection [17], we investigated whether this pathway was involved in the *Hh*-mediated abrogation of parenteral Ad85A-induced protection. We therefore assessed the effect of IL10R signalling blockade during *Mtb* challenge in control, uninfected immunised or *Hh*-infected immunised mice, using a treatment regime shown capable of inducing colitis in normal mice infected with *Hh* [16]. Anti-IL10R treatment of unimmunised naive mice does not alter lung *Mtb* CFU numbers, nor does it affect protection in uninfected mice immunised with Ad85A (Fig. 4F). However, the protective effect of Ad85A is restored in *Hh*-infected mice treated with anti-IL10R, strongly suggesting that IL10 is a crucial determinant of the *Hh*-mediated suppression of Ad85A vaccine protection against *Mtb* challenge.

## 4. Discussion

BCG has long been known to vary in efficacy in different geographical locations, an effect often ascribed to exposure to EM [18]. As many candidate TB vaccines are now entering clinical trials [19], it will be important to determine whether these will also show variable efficacy in different locations and whether this variability is linked to EM exposure only or reflects larger differences in the gut microbiota composition across populations. Understanding the mechanisms underlying variable vaccine efficacy may allow the development of strategies to overcome it.

Experimentally, parenteral administration of EM to mice does not affect protection induced by subunit vaccines (ESAT-6 or Ag85B-ESAT-6 proteins), also administered parenterally [3]. In contrast, concurrent oral administration of EM or BCG abrogates protection induced by the parenterally administered subunit vaccine, Ad85A [4]. That oral but not parenteral administration of EM affects protection is in agreement with recent observations indicating the importance of the intestinal microbiota in both, development of the immune system and immune regulation.

In germ free mice most populations of lymphocytes are greatly reduced in number, while infection with host specific microbiota leads to re-population of the immune system [20]. However, this is not true of all lymphocyte populations; for example, CXCR6$^{+}$ iNKT cells are specifically increased in the colon and lung of germ free mice, due to higher expression of CXCL16 at mucosal surfaces [21]. The link between intestinal and lung immunity is supported by many other studies and manipulation of the microbiota by administration of a single bacterial species can greatly alter immune responses. For example, monocolonisation of germ-free animals with *Bacteroides fragilis* increases the induction of IL10-producing Tregs [22]. Furthermore, there is an inverse relationship between numbers of Foxp3$^{+}$ Tregs and Th17 cells in the intestinal lamina propria [23] and differentiation of Th17 cells, which are important in protection against bacterial and fungal infections at mucosal surfaces, is critically dependent on the presence of segmented filamentous bacteria in the intestine [24]. These data indicate that production of two cytokines, IL10 and IL17A that play important roles in immunity to TB [17,25] is regulated by components of the intestinal microbiota.

In other experiments, neonatal *H. pylori* infection of mice has been shown to prevent the induction of lung allergic responses through the reprograming of dendritic cells and induction of highly suppressive Tregs in an IL10-dependant manner [8,26]. IL10 also plays a key role in preventing the pathological colonic inflammation induced by infection with *Hh* [10–12], an effect attributed mainly to the induction of *Hh* antigen-specific regulatory T cells [16]. However, additional mechanisms may be involved, since *Hh* infection leads to its dominant presence among the local bacterial pool and has been associated with a decreased overall diversity of the intestinal micobiota [27].

Irrespective of whether the effect is directly attributable to *Hh* itself, here we show that neonatal infection of BALB/c mice with *Hh* up-regulates *IL10* mRNA expression within the colonic lamina propria and increases susceptibility to later *Mtb* challenge, as evidenced by higher lung *Mtb* CFU. *Hh*-infected mice immunised parenterally with the recombinant subunit vaccine Ad85A further display reduced protection against *Mtb* challenge compared to their non-infected counterparts, an effect that is dependent on IL10R signalling.

IL10 has been shown to modify protective immunity to several organisms including *Mtb*. In *Mtb* infection there are two main effects. Protection against infection is generally increased in IL10 insufficient mice and mycobacterial counts are lower. This is thought to be mainly due to the effects of IL-10 on myeloid cells, which become less responsive to IFNγ activation and therefore less able to kill mycobacteria, while antigen presentation to T cells and induction of Th17 responses are also inhibited. In contrast, IL10 may dampen down Th1-mediated protective responses later in infection and lessen immune mediated pathology. However these effects are both mouse and *Mtb* strain dependent as well as subject to environmental effects (reviewed in [17]).

In our experiments, *Hh* infection alone increases significantly *Mtb* lung CFU after challenge, a result in accord with the known effects of IL10 on *Mtb* infection. However, the abolition of parenteral Ad85A-induced protection is much more surprising (Fig. 1B). The exact mechanisms of this reduction of vaccine-induced protection remain to be determined, as there was no increase in lung *Il10* message. In an earlier study, protection induced by parenteral but not intranasal administration of Ad85A was abolished by oral administration of EM, suggesting that priming of Ad85A specific cells in peripheral lymph nodes or migration of cells into the lung, rather than expression of local immunity, may be affected. Recent experiments on the effects of intestinal flagellin-bearing bacteria on systemic immune responses suggest that the mechanism involves changes in myeloid cell function in lymph nodes [28]. Here we show here that following parenteral immunisation, numbers of antigen specific CD8$^{+}$ T cells (the principal population induced by Ad85A in BALB/c mice) in the lung are reduced in *Hh* infected mice.

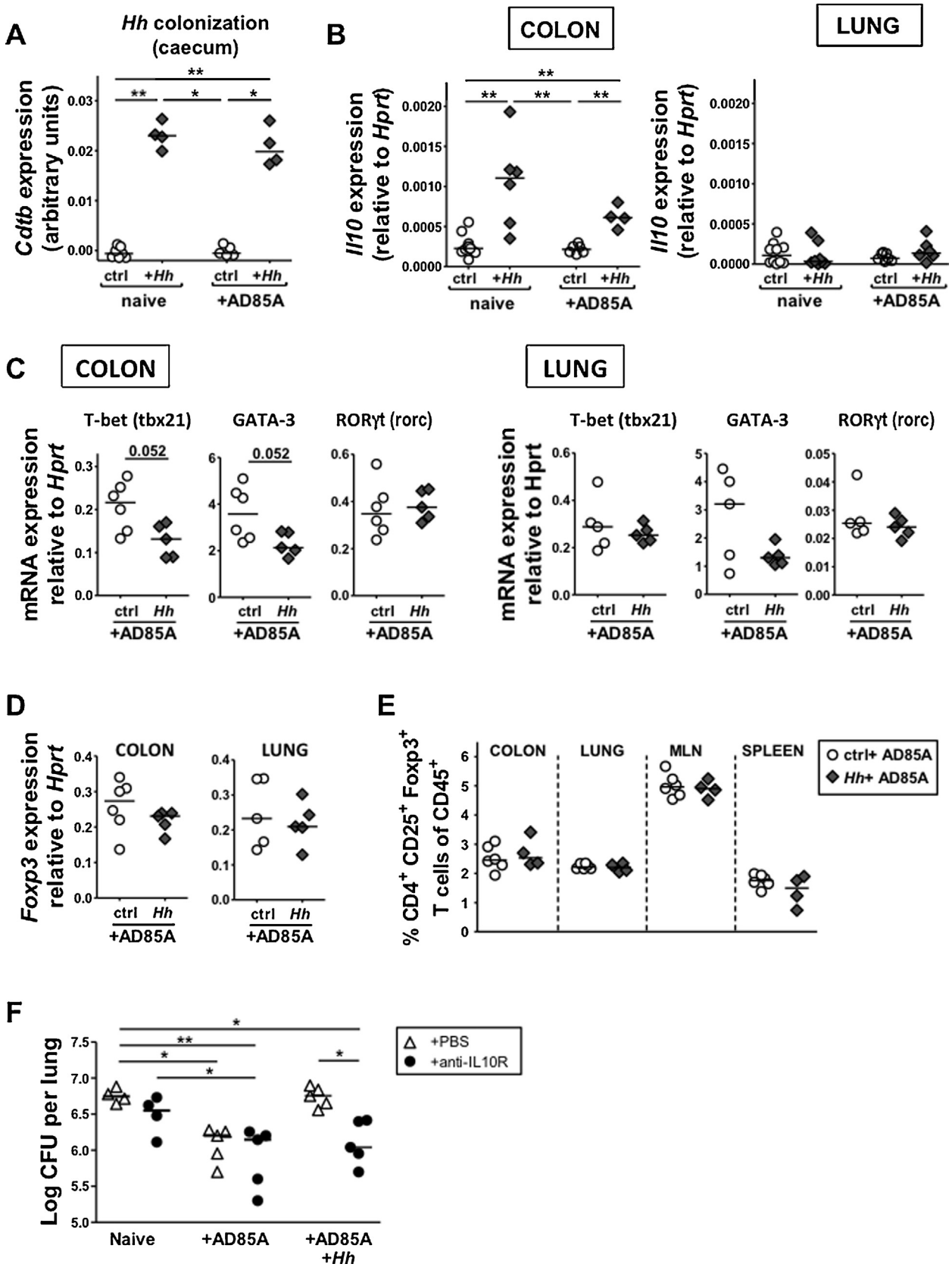


**Fig. 4.** *Protection against Mtb challenge depends on IL10R signalling.* Control or neonatally *Hh*-infected BALB/c mice were immunised with Ad85A and were compared to unimmunised controls. (A) *Hh* colonisation levels quantified by SYBR qPCR (arbitrary units). (B–D) *IL10*, *T-bet*, *GATA-3* and *RORγ*t mRNA expression of total colonic and lung tissue quantified by TaqMan qPCR and normalised to *Hprt*. (E) Frequencies of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T-cells among total live $CD45^+$ cells in the colon, lungs, mesenteric lymph nodes (MLNs) and spleen. Data are representative of or pooled from two independent experiments. (F) Naive or *Hh* infected mice were immunised with Ad85A or left unimmunised. Following challenge with *Mtb*, mice were treated with anti-IL10R mAb or PBS on day 0, 7 and 17. Lung CFU were evaluated 5 weeks post challenge. Symbols show individual mice and horizontal lines indicate the means. ** $p < 0.01$, * $p < 0.05$ between the indicated groups as determine by Mann–Whitney *U* test (A–E) or one-way ANOVA with Tukey's post-test (F).

However it remains to be determined whether this reduction is due to impaired priming in systemic lymph nodes or if altered migration is responsible.

Irrespective of the exact mechanisms involved, this is the first direct evidence that experimental *Hh* infection can profoundly influence the magnitude and protective efficacy of immune responses to Ad85A, a candidate TB vaccine, which has already entered clinical trials [29]. These data indicate that it may be expected that not only BCG but subunit vaccines against TB will suffer from geographical variation in efficacy. Furthermore these results add to the arguments for further exploring the use of the pulmonary route for administration of TB vaccines [30], since at least under some circumstances this can overcome the suppressive effect of alterations in intestinal flora [4].

## Conflict of interest statement

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.02.041.

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