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# Snapshot of spatio-temporal cytokine responses to single and co-infections with helminths and bacteria

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ABSTRACT

Cytokines play a key role in maintaining communication between organs and in so doing modulate the interaction between concurrent infections. The extent of these effects depends on the properties of the organ infected and the intensity and type of infections. To determine systemic bystander effects among organs, IFN-γ, IL-4 and IL-10 gene expression was quantified at 7 days post-challenge in directly infected and uninfected organs during single and co-infections with the respiratory bacterium *Bordetella bronchiseptica* and the gastrointestinal helminths *Graphidium strigosum* and *Trichostrongylus retortaeformis*. Results showed that cytokine expression in a specific organ was influenced by the type of infection occurring in another organ, and this bystander effect was more apparent in some organs than others. Within the same organ the relative cytokine expression was consistent across infections, although some cytokines were more affected by bystander effects than others. For the infected gastrointestinal tract, a stronger cytokine response was observed in the tissue that harbored the majority of helminths (i.e. duodenum and fundus). Overall, co-infections altered the intensity but to a lesser extent the relative cytokine profile against the focal infection, indicating clear bystander effects and low organ compartmentalization. However, organs appear to actively modulate cytokine expression to avoid potential immuno-pathological consequences.



## 1. Introduction

Pathogens that colonize the same or different organs can interact *via* cytokine-mediated pathways. Cytokines can modulate immune responses both at the local site of infection and systemically through bystander effects, and by doing so play a key role in facilitating communication between organs, constituent tissues and ultimately influencing the establishment and survival of pathogens that infect these organs [1,2]. Pathogen specific cytokine responses lead to different cytokine signals, however, similar groups of pathogens often show similar profiles such as the clearly identified mutual inhibition between IFN-γ and IL-4 in the control of bacterial/viral and helminth infections, respectively, [3]. For instance, the liver fluke *Fasciola hepatica* caused up-regulation of IL-4 expression and down-regulation of IFN-γ against *Bordetella pertussis*, resulting in delayed bacterial clearance from the lower respiratory tract [4]. However, this may be far from universal. In the *Trichuris muris–Schistosoma mansoni* mouse model, where *T. muris* is restricted to the intestine and *S. mansoni* migrates through different organs during the parasitic life cycle, *T. muris* was associated with increased IL-10 and suppression of protective IFN-γ and IL-4 in the lungs, but not the liver [5]. This facilitated increased survival and migration of *S. mansoni* larvae from the lungs to the liver, where they developed into adults and caused augmented pathology.

More recently, it has been suggested that systemic cytokine effects are influenced by inherent differences in the structure, function and immune conditioning of the infected organ, rather than being simply driven by pathogen specific responses [6–10]. Likewise, groups of organs and tissues share similarities in the responses and contribute to analogous bystander effects by being more intimately connected than others [8,10]. Collectively, this implies that in addition to shaping the local cytokine signature, specific organ–pathogen interactions can modulate the immune profile of distal organs that may or may not be infected. Immune

*Abbreviations:* AR-1, autoregressive function of order 1; B, *B. bronchiseptica* single infection; BG, *B. bronchiseptica*+*G. strigosum* dual-infection; BT, *B. bronchiseptica*+*T. retortaeformis* dual-infection; BTG, *B. bronchiseptica*+*T. retortaeformis*+*G. strigosum* triple infection; DPI, days post-infection; GLM, generalized linear models; IFN-γ, Interferon-gamma; IL-10, Interleukin-10; IL-4, Interleukin-4; LME-REML, linear mixed effect models with restricted maximum likelihood; SI, small intestine; T, *T. retortaeformis* single infection; TG, *T. retortaeformis*+*G. strigosum* dual helminth co-infection

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cells synthesizing cytokine mRNA but not protein against the intestinal helminth *Heligmosomoides polygrus* or the lung-resident Influenza A virus have been found in uninfected and seemingly unrelated non-lymphoid organs (i.e. lungs, peritoneal cavity and liver), indicating the potential bystander impacts of localized infections but also stronger connectivity among some tissues compared to others [11,12].

Understanding the complexities of cytokine expression and their bystander stimuli during co-infections requires the quantification of these molecules against the constituent pathogens at the local site of infection and how they differ from single infected hosts. Here, we performed single and co-infections with the respiratory bacterium *Bordetella bronchiseptica* and the gastrointestinal helminths *Graphidium strigosum* and *Trichostrongylus retortaeformis*, pathogens that colonize distinct organs and thus prevent any confounding effect caused by their direct interaction [13,14]. These infections are commonly found co-inhabiting the European rabbit (*Oryctolagus cuniculus*) with animals getting initially exposed around 1–2 months of age based on the pathogen [13,14]. Our approach was to perform infections with all the possible combinations of these three pathogens and take a snapshot of cytokine gene expression at one sampling point [7 days post-infection (DPI)] across infected (lungs, stomach and small intestine) and uninfected organs (spleen, mesenteric lymph node and when available, uninfected small intestine and stomach) to identify changes in cytokine expression across different organs and type of infections. We focused on three cytokines widely identified to be instrumental in bacteria–helminth infections: IFN-γ, IL-4 and IL-10 [15,16].

*B. bronchiseptica* is a gram-negative bacterium that colonizes the respiratory tract of a wide range of mammalian hosts *via* oral–nasal infection [17]. We recently showed that rabbits single infected with this bacterium mounted IFN-γ mediated antibody and neutrophil responses that led to phagocytosis and bacterial clearance from the lungs, whereas IL-10 acted antagonistically by delaying clearance [18,19]. This pattern was consistent in single and co-infections with *T. retortaeformis*, with no apparent delay in the dynamics of infection induced by the helminth [19]. In the nasal cavity however, colonies persisted with high intensities throughout the trial in both single and co-infected hosts [18,19], in line with mouse models [20,21]. *G. strigosum* and *T. retortaeformis* are extracellular gastrointestinal helminths with direct life-cycle and infections that occur by ingestion of free living third stage larvae (L3). *T. retortaeformis* inhabits the small intestine (SI) where it tends to concentrate in the duodenum and to a lesser extent in the ileum [22,23], whereas *G. strigosum* colonizes the stomach with a preference for the fundus compared to the antrum [22,24]. We previously showed that rabbits single infected with *T. retortaeformis* mounted a mixed IFN-γ/IL-4 mucosal immune response where IFN-γ appeared to be associated to tissue damage and microflora infiltration during larval establishment, while IL-4 was directed at controlling parasite abundance [22]. Hosts were unable to clear *T. retortaeformis* when single infected but did so successfully when co-infected with *B. bronchiseptica* [19,22]. Single infections of rabbits with *G. strigosum* showed a strong mucosal IL-4 but low IL-10 response with parasite persistence throughout the infection [22].

Based on these observations, we predicted strong bystander effects of *B. bronchiseptica* on cytokine expression against the helminths and this would have been more apparent for *T. retortaeformis*, which induces a mixed type1/type2 response, than *G. strigosum*, which elicits a strong type 2 reaction [18,19,22]. We also predicted the helminths to affect IFN-γ but not IL-10 against the bacterium. Cytokines were measured at the sites of infection, as indicative of a local response, and in the lymph nodes, spleen as well as uninfected stomach and small intestine (depending on the type of infection) to describe systemic effects. Our results are discussed in relation to patterns of bacteria–helminth immune-mediated interactions across infection types and the role of cytokines in maintaining local immune homeostasis.

## 2. Materials and methods

### 2.1. Study design

The general experimental design and procedures were performed as outlined in Pathak et al. [18] and Murphy et al. [22]. All listed animal protocols were pre-approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University (USA) and the Home Office of the University of Glasgow (UK). Briefly, 60-day old, male New Zealand White rabbits were challenged with either a primary single infection (*B. bronchiseptica* or *T. retortaeformis*), a simultaneous primary infection with two (*B. bronchiseptica*+*T. retortaeformis*, *B. bronchiseptica*+*G. strigosum*, *T. retortaeformis*+*G. strigosum*) or three pathogens (*B. bronchiseptica*+*T. retortaeformis*+*G. strigosum*). A total of 6 experiments were performed. Rabbits were intranasally inoculated with 20,000 CFUs of *B. bronchiseptica* RB50 in 1 ml of sterile PBS solution (gift of Dr. Eric Harvill, The Pennsylvania State University) and/or infected with helminths by oral gavage of 3 ml mineral water solution containing 5500 L3 *T. retortaeformis* and/or 670 L3 *G. strigosum* (larvae were collected from pure cultures maintained at PSU). Control individuals were sham-inoculated with sterile PBS or mineral water. For each experiment we used 4 infected and 2 control animals. Individuals were euthanized at 7 DPI with 1 ml of Euthasol (Mid-West Scientific, PA, USA). Lungs, small intestine, stomach, spleen and mesenteric lymph nodes were collected and processed immediately for quantification of cytokine gene expression and where applicable, pathogen abundance [18,22]. No stomach or spleen tissues were sampled at 7 days post challenge in single infection against *G. strigosum* or *B. bronchiseptica*, respectively; mesenteric lymph nodes were collected only during infections of the small intestine. In the dual bacteria–helminth infections we also collected the uninfected gastrointestinal organs, for example, in the *B. bronchiseptica*–*G. strigosum* infection the uninfected duodenum was sampled and cytokines estimated. Our previous work showed that 7 DPI is when we observe the strongest cytokine response during the course of these infections, thus we focused on this time point for our analysis [18,19,22,25–27] (unpublished data). In the current study we chose not to re-stimulate any organ but quantified cytokine gene expression in organs preserved *in situ*.

The lungs were blended for 10 s and 3 g of tissue is diluted into 30 ml of RNAlater (Ambion, TX, USA) for storage at −80 °C. The remaining lung tissue was transferred to ice-cold PBS and bacterial counts obtained by plating 10-fold serial dilutions of the homogenate on Bordet–Gengou blood agar plates supplemented with streptomycin (VWR Intl., West Chester, PA) [18]. The small intestine was divided into four equal sections, SI-1 to SI-4, from the duodenum to the ileum, respectively. Each section was further divided into four equal segments. Segments 1 and 3 from the SI-1 and SI-4 sections were transferred to PBS (pH 7.4) for *T. retortaeformis* counts [22]. Five pieces of tissue (0.5 cm × 0.5 cm) were collected from segments 2 and 4, transferred to 5 ml RNAlater and stored at −80 °C. The stomach was divided into two parts, fundus and antrum (i.e. top and bottom). Each section was then divided longitudinally and the right sections with the food contents stored in PBS for *G. strigosum* counts. Five pieces of tissue were collected from the top and bottom of the left sections and stored in RNAlater at −80 °C for subsequent cytokine gene expression analyses [22]. The spleen and mesenteric lymph nodes were cut

into 0.5 cm × 0.5 cm sized pieces, transferred to 5 ml of RNAlater and stored at −80 °C. It should be noted that the titration of the whole lungs precluded the quantification of cytokine expression in regions of high and low infection intensity, which was scattered within the organ and not consistent among individuals, in contrast to the gastrointestinal sections. Indeed, histopathological analyses confirmed the patchy inflammation in the lungs (unpublished data).

### 2.2. RNA extraction and quantification of cytokine gene expression

Except for the lung where the entire tissue was processed, 2 pieces of small intestine and stomach (approx. 0.5 $cm^2$ each) from each RNAlater preserved tissue were homogenized with a rotor-stator homogenizer (Brinkmann Instruments, Long Island, NY) and RNA extracted with 5 ml of TRIzol reagent according to manufacturer's instructions (Life Technologies, Carlsbad, CA). RNA estimation, integrity tests, DNase digestions, reverse transcriptions and quantitative PCR with IFN-γ, IL-4, IL-10 and Hypoxanthine phosphoribosyl transferase (HPRT, house-keeping gene) specific primers and probes were performed as described by Pathak et al. [18]. Expression of each gene was quantified in duplicate for each individual.

### 2.3. Data analysis

Cytokine data were transformed following the comparative $2^{-\Delta\Delta Ct}$ approach [28]. Specifically, for each organ, the normalized $C_t$ value of every replicate from an infected individual was normalized over the average of the controls across the infections for that organ as $Xijk^*_{1/2} = Xijk_{1/2} - Xj$, where $Xijk_{1/2}$ is the cytokine value from individual *i*, organ *j* experiment *k* and replicate 1 or 2, and *Kj* is the average of the same cytokine from the controls for organ *j* and across all the infections where organ *j* has been measured. For every individual the average between the normalized replicates was calculated and used in the subsequent analysis. To highlight differences in cytokine expression within an organ between infection types, a pair-wise Tukey Honest test was performed. Pair-wise relationships between cytokines from the same organ and between intensity of infection and cytokine expression were quantified using generalized linear models (GLM) or linear mixed effect models

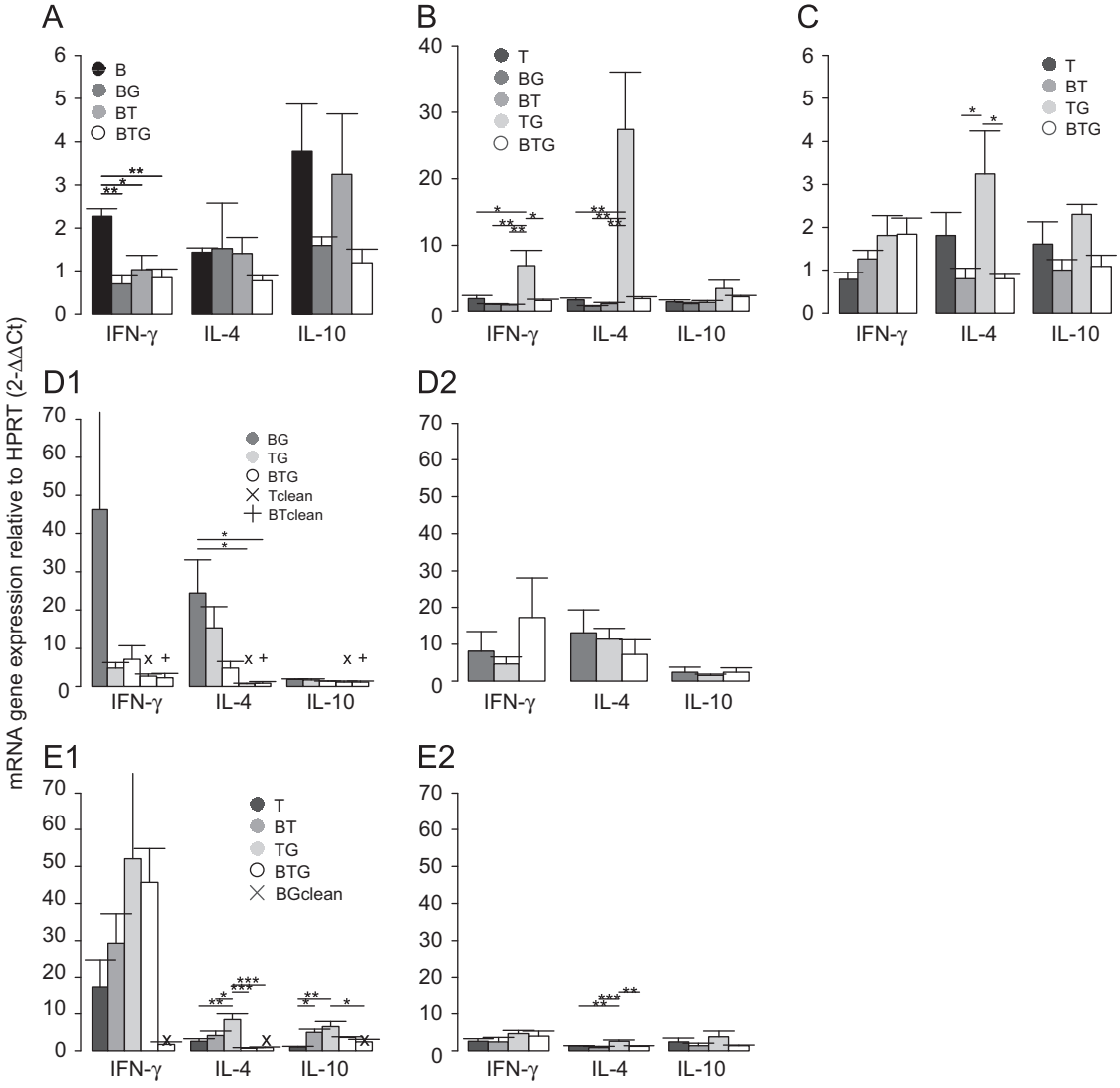


**Fig. 1.** Average cytokine gene expression by organ and type of infection. Data are represented as fold-change in gene expression relative to sham inoculated control animals sampled at 7 DPI. Lungs (A), stomach fundus (D1) and antrum (D2), small intestine duodenum (E1) and ileum (E2), spleen (B) and mesenteric lymph nodes (C). Reported are also the average cytokine responses in uninfected stomach fundus from the single *T. retortaeformis* (Tclean) and *B. bronchiseptica*+*T. retortaeformis* (BTclean) infection (D1), uninfected duodenum from the *B. bronchiseptica*+*G. strigosum* co-infection (BG clean, E1). Single infection: B=*B. bronchiseptica*, T=*T. retortaeformis*. Co-infection: BT=*B. bronchiseptica*+*T. retortaeformis*, BG=*B. bronchiseptica*+*G. strigosum*, TG=*T. retortaeformis*+*G. strigosum*, BTG=*B. bronchiseptica*+*T. retortaeformis* +*G. strigosum*. Significant pair-wise Tukey Honest test are reported; $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.001$.

(LME-REML with restricted maximum likelihood) if different parts of the same organ were used (e.g. SI-1 and SI-4 or top and bottom stomach). In this latter case and to take into account the non-independent sampling of different parts of the same organ from the same individual, the individual ID was included as a random effect and/or autoregressive function of order 1 (AR-1).

## 3. Results

### 3.1. Cytokine gene expression within organs between infections

To examine if co-infection with a second pathogen affected cytokine expression in other organs, pairwise Tukey Honest test was performed for each cytokine between types of infection in infected and uninfected organ (Fig. 1).

In the lungs, IFN-γ against *B. bronchiseptica* was significantly suppressed when hosts were co-infected with the helminths (both dual and triple co-infections) (Fig. 1A). Similarly, strong IL-10 expression was observed in the single infection, however, a large variability was found between infections. IL-4 showed no variation among infections and levels were close to baseline ($2^{-\Delta\Delta Ct}=1$).

In the spleen, the levels of cytokine expression were relatively low and generally similar between infections with the significant exception of the relatively high IFN-γ and IL-4 values in the dual helminth infection (Fig. 1B).

In the mesenteric lymph nodes, cytokines were usually close to baseline values with the exception of IL-4 in the dual helminth infection (Fig. 1C).

In the stomach, the expression of cytokines against *G. strigosum* was similar amongst infections (Fig. 1D). The only exception was in the fundus where consistently higher IL-4 was found in the *B. bronchiseptica–G. strigosum* co-infection compared to the uninfected stomach from the *T. retortaeformis* single and *B. bronchiseptica–T. retortaeformis* co-infection (Fig. 1D1). No significant differences in cytokine expression were found between fundus and antrum from the same infection type (Fig. 1D1 and D2).

In the small intestine, the cytokine response against *T. retortaeformis* was consistently different between infections in the duodenum (Fig. 1E1) and to a lesser extent in the ileum (Fig. 1E2). In general, IL-4 and IL-10 showed significantly higher expression in the dual helminth infection and lower in the triple or single infection. The remaining infections showed intermediate values for both cytokines. IFN-γ was highly expressed in the duodenum, although no significant differences were found across infections (Fig. 1E1). Within the ileum, only IL-4 differed between infections and it was mainly driven by the high expression in the *T. retortaeformis–G. strigosum* co-infection (Fig. 1E2). The average expression of cytokines in the duodenum was consistently higher compared to the ileum but significant values were only observed for IFN-γ and IL-4 in the dual helminth infection (Tukey test: $P<0.05$ and $P<0.001$, respectively). The uninfected duodenum from the *B. bronchiseptica–G. strigosum* co-infection exhibited baseline values (Fig. 1E1).

These findings suggest that bystander effects by a second pathogen are more apparent in some organs and appear to affect the expression of some cytokines more than others.

### 3.2. Relationship between cytokines by organ across infections

To determine whether the relationship between cytokines was consistent among types of infection, pair-wise regressions were performed between IFN-γ, IL-4 and IL-10 gene expression from the same organ and combining the infections together. The uninfected small intestine (from the *B. bronchiseptica*+*G. strigosum* co-infection) and stomach (from *B. bronchiseptica*+*T. retortaeformis* and single *T. retortaeformis* infections) were also included in the analysis. Significant positive associations between cytokines were found for the spleen, the small intestine and partially the stomach, but not for

**Table 1**
Pair-wise relationships between cytokines within each organ and across types of infections. Analyses are reported for the whole small intestine as well as duodenum (SI1) and ileum (SI4), and the whole stomach as well as fundus and antrum. Generalized linear models (GLM) or mixed linear models (LME-REML) were applied; only the relationships with a significant slope (S) and/or intercept (I) are reported. A significant slope indicates a significant relationship between the two cytokines examined, while a significant intercept indicate that the initial conditions of this relationship (i.e. difference in cytokine expression between infections) are significant. All the relationships reported showed a positive trend. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

| | | Lungs | | Spleen | | Stomach | | S. intestine | | MLN | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IL-4 | IL-10 | IL-4 | IL-10 | IL-4 | IL-10 | IL-4 | IL-10 | IL-4 | IL-10 |
| Lungs | IFN-γ | | I* | | | | | | | | |
| | IL-4 | | I* | | | | | | | | |
| Spleen | IFN-γ | | | I*** | S** | | | | | | |
| | | | | S*** | | | | | | | |
| | IL-4 | | | | I* | | | | | | |
| | | | | | S* | | | | | | |
| Stomach | IFN-γ | | | | | I** | | | | | |
| | | | | | | S* | I*** | | | | |
| | | | | | | Fundus: S*, I* | Fundus: I* | | | | |
| | | | | | | Antrum: S**, I*** | Antrum: I** | | | | |
| | IL-4 | | | | | | S*, I*** | | | | |
| Small intestine | IFN-γ | | | | | | | I* | I*** | | |
| | | | | | | | | S*** | S*** | | |
| | | | | | | | | SI1: S** | SI1: S*, I** | | |
| | | | | | | | | | SI4: I* | | |
| | IL-4 | | | | | | | | I** | | |
| | | | | | | | | | S*** | | |
| | | | | | | | | | SI1: S**, I** | | |
| | | | | | | | | | SI4: S*** | | |
| MLN | IFN-γ | | | | | | | | | | |
| | IL-4 | | | | | | | | | | |

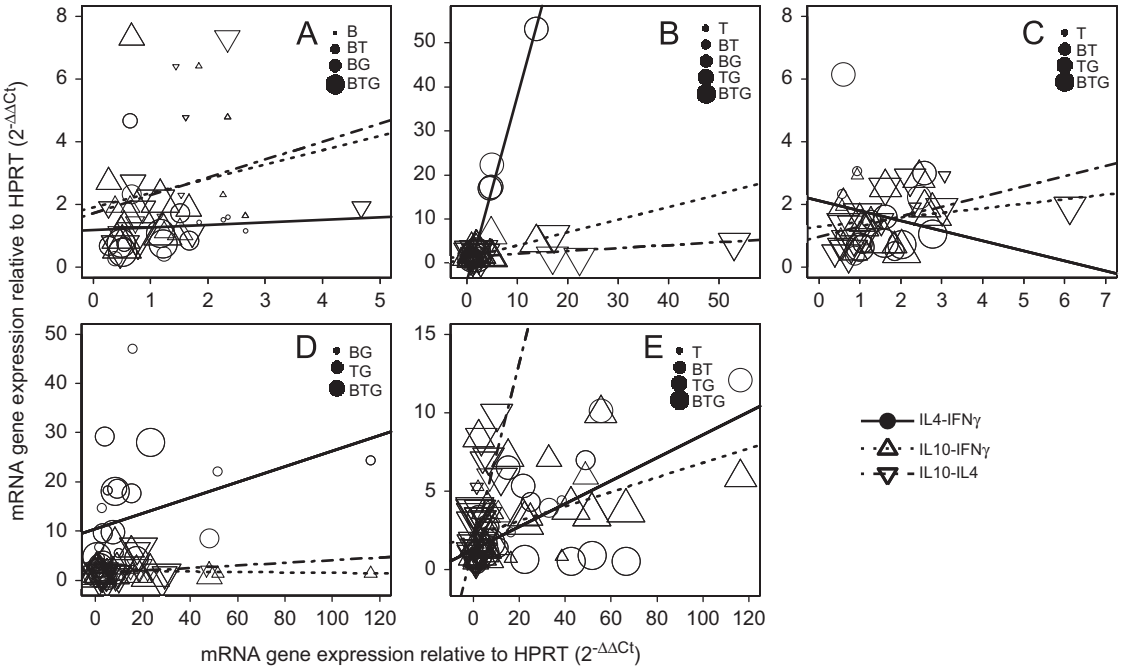


**Fig. 2.** Pair-wise relationships (GLM or LME) between cytokine expression at 7 DPI for every organ and across types of infections. Organs are as follow: lungs (A), spleen (B), mesenteric lymph nodes (C), stomach (D), small intestine (E). Single infection: B=*B. bronchiseptica*, T=*T. retortaeformis*. Co-infection: BT=*B. bronchiseptica*+*T. retortaeformis*, BG=*B. bronchiseptica*+*G. strigosum*, TG=*T. retortaeformis*+*G. strigosum*, BTG=*B. bronchiseptica*+*T. retortaeformis*+*G. strigosum*. The following relationships in cytokine expression across infections within organs are presented: open circle and single unbroken line=IL-4 vs. IFN-γ, upright triangle and dotted line=IL-10 vs. IFN-γ, inverted triangle and dashed line=IL-10 vs. IL-4. The significance of the slope and intercept from the pair-wise relationships between the cytokines and their meaning are reported in Table 1. The size of filled dots in the legend matches the infection type and the corresponding open symbols in the plot. Data are represented as fold-change in gene expression relative to sham inoculated control animals sampled at 7 DPI.

the lungs or the mesenteric lymph nodes (Table 1, Fig. 2). These positive relationships were observed despite differences in the level of expression among infections; indeed, the significant intercepts from the regression analysis suggested that there is variability in local cytokine expression among infection types (Table 1).

The significant association between cytokines in the spleen was mainly influenced by the *T. retortaeformis–G. strigosum* co-infection as the remaining infections showed close to baseline cytokine expression. The pattern in the small intestine was driven by the strong relationships in the duodenum, although a significant association between IL-10 and IL-4 was also observed in the ileum (Table 1). A significant positive IL-4–IFN-γ relationship was found in the total stomach as well as the fundus and antrum but was mainly caused by a couple of individuals with strong IFN-γ expression (Table 1).

### 3.3. Cytokine expression and intensity of infection

The relationship between cytokine expression and intensity of infection was assessed for lungs, small intestine and stomach (Fig. 3). No relationship was found between cytokines and bacteria in the lungs except a positive association between IL-4 and bacterial numbers in the dual *B. bronchiseptica–G. strigosum* infection (coeff ± SE: $0.889 \pm 0.216$ $P=0.003$) (Fig. 3A). Similarly, no significant relationships were found for *G. strigosum* except a positive relationship between IFN-γ and the helminth in the fundus ($7.230 \pm 2.378 \times 0.038$) (Fig. 3B). In the small intestine, *T. retortaeformis* intensity was positively associated with IFN-γ ($0.579 \pm 0.070$ $P<0.0001$), IL-10 ($0.124 \pm 0.054$ $P=0.037$) and IL-4 (only when interacting with the dual helminth infection: $0.236 \pm 0.104$ $P=0.043$) (Fig. 3C). The analysis repeated independently for the duodenum and the ileum showed a significant negative relationship between helminth abundance and IL-4 ($-0.966 \pm 0.253$ $P=0.002$) and IL-10 ($-0.533 \pm 0.248$ $P=0.0498$) in the duodenum; no significant associations were observed in the ileum.

## 4. Discussion

A snapshot at day seven post challenge of cytokine gene expression in a number of relevant organs and across a combination of infections with a respiratory bacterium and two gastrointestinal helminths revealed two main patterns. First, the level of local IFN-γ, IL-4 and IL-10 expression in a specific organ was affected by the type and number of co-infections occurring in other organs; this bystander effect was more apparent in some organs than others and not for every cytokine measured. Specifically, IFN-γ against *B. bronchiseptica* in the lungs was suppressed by helminth co-infections, the expression of the three cytokines against *G. strigosum* in the stomach were also suppressed in hosts concurrently infected with *B. bronchiseptica,* while the cytokine response against *T. retortaeformis* in the small intestine was generally augmented in the presence of co-infections. Second, within the same organ, relative cytokine expression was consistent across different infections and exhibited a general positive trend. In other words, a second pathogen altered cytokine expression levels against the first pathogen in another organ, but not the positive relationship between cytokines. These findings suggest that there is relatively low tissue compartmentalization among the organs examined. However, while some organs are more sensitive to bystander effects than others, they appear to adjust the cytokine pool to levels that avoid immuno-pathology. Indeed, the positive correlation between cytokines among infections from the same organ is indicative of cross-regulation between these cytokines. Interestingly, baseline cytokine expression in the uninfected stomach and small intestine suggests that there are relatively weak bystander signals in these organs in the absence of active infections.

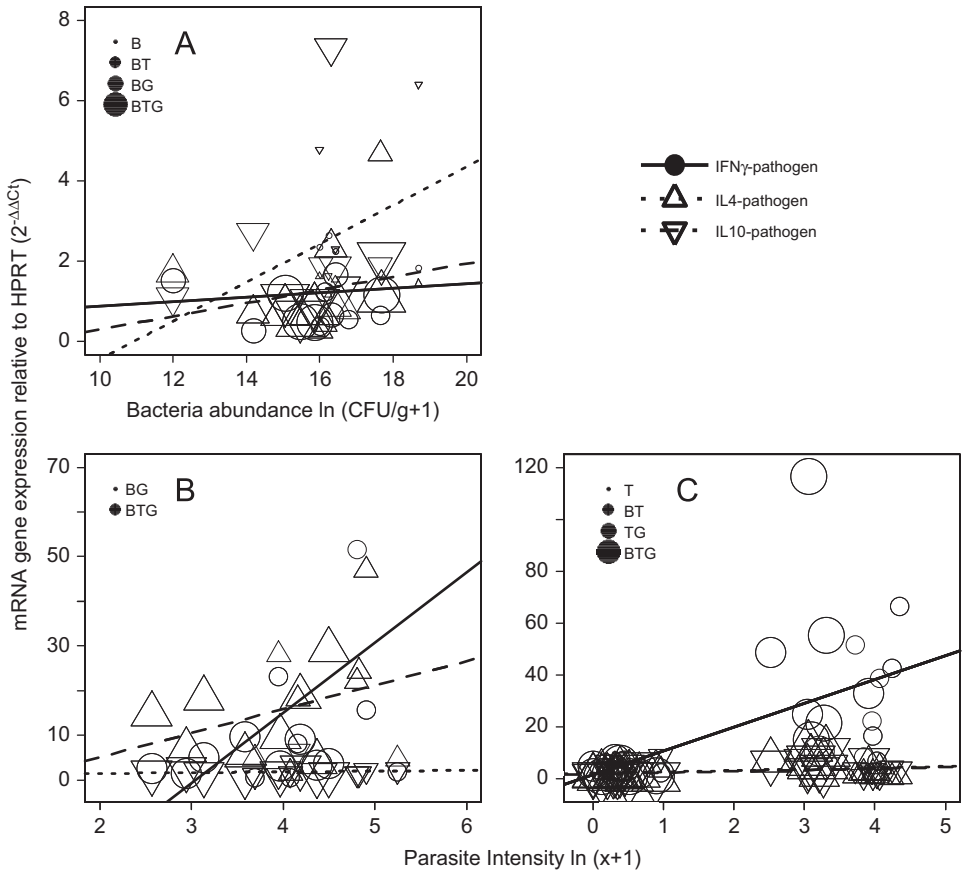


**Fig. 3.** Relationship between cytokine expression and intensity of infection for *B. bronchiseptica* colony forming units (CFU) in the lungs (A), *G. strigosum* in the stomach (B) and *T. retortaeformis* in the small intestine (C). The size of filled dots in the legend matches the size of the corresponding open symbols in the plot. The following cytokine–intensity relationships across infections within organs are reported: open circle and single unbroken line = IFN-γ vs. pathogen, upright triangle and dotted line = IL-4 vs. pathogen, inverted triangle and dashed line = IL-10 vs. pathogen.

Mutual regulation between IFN-γ, IL-4 and IL-10 has been well-characterized for *T. muris* and *S. mansoni* in mice where it is critical for the balance between parasite survival and host protection [29–31]. A similar cytokine regulation has also been described for *B. bronchiseptica* where the absence of IL-10 expression hastened pathogen clearance *via* increased production of IFN-γ [25]. Yet, it is important to highlight that pathogens themselves can induce cytokine expression, thus challenging the host to maintain the cytokine balance at local level but also contributing to bystander effects. For example, *B. bronchiseptica* has been suggested to induce up-regulation of IL-10 through the type III secretion system (TTSS) modulation of T cell subtypes and inhibit a protective IFN-γ response in the lower respiratory tract of mice [25]. As such, the positive relationship between IFN-γ and IL-10 in the lungs could be partly caused by the host mediated IFN-γ production as a response to bacteria mediated IL-10 expression. Indeed, our recent modeling of the *B. bronchiseptica–T. retortaeformis* infection also showed that higher IL-10 expression in the duodenum throughout the dual compared to the single helminth infection was due to bystander effects induced by the *B. bronchiseptica* TTSS [19].

The lack of significant relationships between cytokines in some organs should not be interpreted as the absence of interaction, but rather as an environment (i.e. tissue) biased towards a specific cytokine or groups of cytokines at a particular time point. Indeed, the low IFN-γ/IL-4 relationship in the lungs of co-infected individuals was probably the result of the concurrent helminth infections in the gastrointestinal tract, which down regulated IFN-γ but did not up-regulate IL-4 in a proportional manner [19]. Similarly, IL-10 expression in the stomach and spleen was consistently low across infections. However, this appeared to be counterbalanced by a strong and positive IFN-γ–IL-4 relationship that probably prevented a bias in cytokine over-expression. Previous studies have suggested that the IFN-γ/IL-4 inhibitory balance may in fact involve continual co-regulation of expression in bacteria–helminth infections such as that proposed for *Onchocerca ochengi* and *Mycobacterium bovis* in cattle [32]. Likewise, mice infected with the intestinal helminth *H. polygyrus* showed low IFN-γ expression to the stomach bacterium *Helicobacter felis* but up-regulation of IL-4 and IL-10 expression with a corresponding reduction in gastric atrophy relative to single infected animals [33].

Contrary to our expectation cytokine levels in the mesenteric lymph node did not closely follow the pattern observed in the small intestine. The lymph nodes showed a negative IFN-γ–IL-4 relationship and large variability in IL-4 and IL-10 expression compared to the small intestine. Similarly, low draining cytokines to and from organs was also observed for the spleen. Differences in the properties and functions of these organs may have caused this discrepancy. Similar patterns have also been found in other gastrointestinal helminth–host systems for example, *Teladorsagia circumcincta*–sheep and *Trichostrongylus tenuis*–red grouse, where low expression of several immune response genes, including cytokines, was observed in the local draining lymph nodes relative to the site of infection [34,35].

Cytokines showed a tendency to be associated with the level of infection, although this was apparent only for *T. retortaeformis* in the small intestine and to a lesser extent, *G. strigosum* in the stomach. Within the same organ, distal tissues showed a positive

relationship between helminth intensity and cytokine expression. For example, *T. retortaeformis* colonizes the entire small intestine but concentrates in the duodenum where it stimulated a stronger cytokine response than in the ileum. A similar trend was observed for *G. strigosum* between the fundic and antrum parts of the stomach, although this distinction was less obvious probably because of the more compact and smaller size of the organ. The lack of a significant cytokine–bacteria relationship in the lungs was probably the consequence of the confounding bystander effects of the two different concurrent helminth infections. Collectively, these findings show that changes in the cytokine profile between tissues within organs can contribute to increase the variability in the immune response and thus alter individual heterogeneity to infections.

It is important to stress that the observed patterns represent a snap-shot at 7 days post challenge in the infection process and it is also when IFN-γ, IL-4 and IL-10 responses against our pathogens were at the highest [18,19,22,25–27] (unpublished data). We previously showed that relative cytokine expression changes with the course of the trial in the focal infected organs, however, the general bystander effects and the modulatory properties of the organs examined appear to be conserved throughout the infection [18,19,22]. Our current study offers additional insights into the spatial patterns of cytokine expression during co-infections, how they change from single infections and how organs balance the local and systemic responses.

## 5. Conclusions

This study showed that cytokine gene expression against *B. bronchiseptica* in the lungs and *T. retortaeformis* and *G. strigosum* in the gastrointestinal tract modulated, and were modulated by each other through systemic bystander effects. The intensity of these signals/responses was driven by the type and characteristics of the infecting agents as well as the properties of organs. We focused on three cytokines identified as strategic in controlling bacterial and helminth infections; however, other cytokines may have been involved in regulating the expression of our focal cytokines, or more generally, the dynamics of these infections [15,19,26]. More studies need to be done on the spatial and temporal immuno-dynamics of co-infection. We simply cannot predict how the immune system reacts to co-infections based on our knowledge of single infections [19]. Indeed, one of the challenges facing disease biologists is to disentangle how pathogens in different organs communicate through the immune response and how organs filter these messages.

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The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

## Disclosure statement

The authors declare no potential conflicts of interest.

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