Immune control of food intake: enteroendocrine cells are regulated by CD4+ T-lymphocytes during small intestinal inflammation.

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Abbreviations:
CCK, cholecystokinin  5-HT, 5-hydroxytryptamine
EEC, enteroendocrine cell  IL, interleukin
Th, T-helper  p.i., post infection
i.p., intraperitoneal  VCU: villus-crypt unit
Abstract

Background and aims: Gastrointestinal inflammation reduces food intake, but the biological mechanisms explaining suppressed feeding during inflammation are unknown. We have used a model of upper gut infection (*Trichinella spiralis* in the mouse) to study the effect of inflammation on food intake, and explored the role of a key enteroendocrine cell (EEC) in the regulation of feeding by the immune response.

Methods: Food intake of NIH mice infected with the intestinal nematode *Trichinella spiralis* was measured. Duodenal cholecystokinin (CCK) cells were counted. Plasma CCK was measured. Infected mice were treated with a specific CCK₁ receptor antagonist, and food intake reassessed. The influence of the immune response on food intake and CCK was mechanistically examined by treating mice with CD4 or mast cell neutralising antibodies. The role of the T helper 2 response was further explored in mice genetically deficient for IL-4, IL-13 or IL-4Rα.

Results: Food intake of infected mice was significantly reduced at the temporal peak of intestinal inflammation. Cholecystokinin (CCK)-expressing EEC were upregulated in infected mice, and plasma CCK levels were increased. A CCK₁ receptor antagonist restored the food intake of infected mice to a significant degree. Furthermore, suppression of food intake was completely abolished in the absence of CD4⁺ T-lymphocytes or IL-4Rα.

Conclusions: The data show for the first time that intestinal inflammation results in reduced food intake due to up-regulation of CCK. Moreover, following infection, food intake and CCK expressing cells are under the specific control of CD4⁺ T-cells, via the release of IL-4 and IL-13.
Inflammatory episodes in the intestine are strongly associated with reduced food intake \cite{1-3}, an important clinical issue with major nutritional consequences. Surprisingly, the physiological and cellular mechanisms that underpin this hypophagic response remain obscure.

In the short-term control of normal food intake, the proximal small intestine is the key sensory region of the gut. Neuroendocrine factors are released from epithelial enteroendocrine cells (EEC) in direct response to luminal nutrients\cite{4}, and activate vagal afferent neurons inputting to feeding control centres in the brain. EEC, along with enterocytes, goblet cells and Paneth cells, are derived from crypt stem cells. Differentiation into the EEC pathway is a highly regulated lineage commitment decision followed by a minority of cells (~1\% of total), and crucially dependent on the expression of several transcription factors \cite{5-7}. Cholecystokinin (CCK) is a key regulatory peptide secreted by a subset of predominantly duodenal EEC during eating to coordinate pancreatobiliary secretomotor responses, but which also induces satiety and limits ingested meal size. In large part these effects are achieved via a delay in gastric emptying \cite{8}.

A role for EEC in reducing food intake during intestinal disease episodes has not been investigated. In a proof of concept study, we have recently shown that plasma CCK levels are raised in symptomatic humans with a common small bowel infection, \textit{Giardiasis}\cite{9}. This returned to normal once the infection resolved. To explore this link mechanistically, we have now employed a mouse model of small intestinal infection, the nematode parasite \textit{Trichinella spiralis}, in order to investigate the role and function of CCK during small intestinal inflammation. This infection invokes a marked but transient inflammatory response, characterized by influx of T cells \cite{10}, mast cells \cite{11} and eosinophils \cite{12}. Intestinal parasite expulsion is mast cell and T cell dependent and in the absence of either cell type parasite expulsion is delayed \cite{10, 13, 14}. \textit{T.spiralis} evokes a Th2 dominated inflammatory response in which IL-4 and IL-13 are pivotal \cite{15, 16}. This infection is also usefully characterised by a delayed wave of secondary inflammation, when the parasite invades skeletal muscle. This represents a valuable internal control of extraintestinal inflammation induced by the same biological agent.

Using this model we have identified and characterised a novel aspect of intestinal immunoregulation, showing for the first time that Th2 cytokines mediate the control of food intake via altered EEC function during local inflammatory responses.

\textbf{METHODS}

\textbf{Mice and infection}

NIH and BALB/c mice were purchased from Harlan Olac (Bicester, UK). IL-13, IL-4 and \textit{IL4Rα} (receptor α subunit) deficient mice were generated as previously described \cite{17-19}. Mice were maintained under pathogen free conditions. Experiments obeyed the regulations of the United Kingdom Home Office Scientific Procedures Act (1986). Male mice (age 6-8 weeks) were infected with \textit{Trichinella spiralis} larvae by oral gavage of 300 larvae (day 0).

\textbf{Food intake, stomach and body weight}

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Chow (B and K, Hull, UK) was repeatedly weighed. The daily amount consumed per mouse was derived. The mice were weighed on day 0 and on day 9 p.i. In some, the stomach was removed whole and weighed. Small intestines were removed to recover and count parasites.

Immunohistochemistry

Duodena were removed and immediately fixed in 4% paraformaldehyde for 1 hour before transfer to 20% sucrose solution for a further 4 hours. Fixed tissues were frozen in liquid nitrogen-cooled isopentane and 5µm transverse sections cut, then attached to polylysine-coated glass slides. Sections were passed through graded 50% and 75% ethanol before blocking with 10% donkey serum (Jackson Immunoresearch, Westgrove, PA), then incubated with rabbit polyclonal anti-proCCK antibody L421 (kind gift from Dr Andrea Varro, University of Liverpool, UK) or anti 5-HT (Sigma) for 90 minutes at room temperature or overnight at 4°C. Following washing, sections were incubated with Alexafluor 594 goat anti-rabbit antibody (Molecular Probes, Oregon, USA) for 60 minutes at room temperature and mounted (Vectashield, Vector Labs, Peterborough, UK). Slides were examined on a Zeiss Axioplan 2 microscope and positive cells counted. Results are expressed as fluorescent (CCK-positive) cells per 15 villus crypt units (VCU).

CCK radioimmunoassay

Plasma CCK was determined using a commercially available kit (Euro-Diagnostica, Malmo, Sweden).

CCK₁ antagonism

Mice were injected once, intraperitoneally at 18:00 (to coincide with the predominantly nocturnal feeding pattern), on day 9 with either sterile distilled water or the CCK₁ receptor antagonist loxiglumide, 20mg.kg⁻¹ (Rotta Research Laboratorium, Monza, Italy). In a small pilot, loxiglumide was shown not to affect overnight food intake in non-parasitized mice. The amount of food consumed overnight was calculated. A subset of animals was later retreated with loxiglumide or a control injection on day 22 p.i., long after the enteritis had resolved.

Antibody neutralization of candidate cellular mediators

NIH mice were treated with an anti-CD4 antibody derived from rat hybridoma YTS 191. Infected mice received 0.5mg anti-CD4 i.p on days –1, 1, 3, 5 and 7 p.i. Control infected mice were simultaneously administered 0.5mg non-specific rat IgG (Sigma UK). Similar experiments were performed using an anti-c-kit antibody, which prevents the pronounced mastocytosis observed following infection with T.spiralis. Results are expressed as mean ± SE. Student's t test or Mann U Whitney tests were used to assess statistical significance as appropriate. A value of p<0.05 was deemed significant.

RESULTS

Small intestinal inflammation induces hypophagia

NIH mice were infected with T.spiralis and food intake was measured over the subsequent 30 days (fig 1). Infected mice became hypophagic by day 7 p.i., eating least

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at day 9 p.i., then returned to normal. Day 9 p.i. corresponds to the peak of intestinal inflammation\textsuperscript{10, 13, 20}. These events are associated with effective expulsion of worms from the intestine by day 9 p.i. (0.8±0.5 worms/mouse at day 9, compared to 82.5±9.4 at day 6, n=4). Body weight was also markedly reduced at day 9 post-infection, infected mice losing 1.8±0.1g from day 0. In contrast, uninfected mice gained 1.5±0.1g over the matched time period. Total gastric weights were lower in infected animals (naïve 0.71±0.1 g/mouse; infected 0.48±0.05g/mouse, p<0.05). This supports a direct inhibitory effect on feeding, rather than a simple limiting of food intake secondary to a delay in gastric emptying.

At day 25 p.i a second fall in food intake was observed (fig 1), despite resolution of the epithelial inflammation\textsuperscript{10, 13, 20}. This is when \textit{T.spiralis} larvae encyst in skeletal muscle, representing an extra-intestinal inflammatory response to the same biological agent\textsuperscript{21}.

**CCK cell numbers and secretion are increased during intestinal inflammation**

Numbers of duodenal CCK cells increased. (fig 2A), again peaking at day 9 p.i. By day 20, CCK cell numbers were back at naïve or pre-infected levels. Plasma CCK immunoreactivity was also elevated at this time point (naïve 1.1±0.3 pM vs infected 4±0.4 pM). This was not confined to CCK cells: serotonin-staining enterochromaffin cells were similarly increased in number (fig 2B).

**Inhibition of CCK\textsubscript{1} receptors improves food intake of infected mice**

A single treatment with the antagonist loxiglumide produced a significant, although not complete, restoration of food intake in parasitized animals (fig 3), which was recorded over a 15 hour period overnight from day 9 p.i. Gastric weights were also increased on the morning following loxiglumide (0.32±0.1g/mouse, loxiglumide vs 0.25±0.01g/mouse, control), suggesting a dominant effect of improving food intake via satiety and appetite mechanisms, rather than a purely gastroprokinetic effect of loxiglumide.

Interestingly, but in contrast, inhibition of CCK\textsubscript{1} receptors had no effect on feeding at day 22 p.i., the time point corresponding with the beginning of the second wave of hypophagia when \textit{T.spiralis} larvae encyst extra-intestinally in skeletal muscle (naïve 3.4±0g/mouse/15 hours; infected, i.p saline 2.4±0.030g/mouse/15 hours; infected, loxiglumide 1.6±0.10g/mouse/15 hours). This indicates that CCK directly contributes to intestinal inflammation-induced hypophagia, but that it does not mediate the hypophagia observed at this later, extra-intestinal inflammatory stage.

**Feeding and CCK cells are under the control of CD4\textsuperscript{+} T cells but not mast cells during inflammation**

CD4\textsuperscript{+} T cells play a decisive role in the resolution of intestinal infections. In the absence of functional CD4\textsuperscript{+} T cells, resolution of \textit{T.spiralis} infection was significantly delayed (fig 4a). The antibody had no intrinsic effect on food intake in a pilot study in uninfected mice (data not shown). Infected mice treated with non-specific IgG were hypophagic as before (fig 4b). The hypophagic effect was completely abolished in infected mice treated with CD4 neutralising antibodies. Gastric weights were also increased by antiCD4 (0.60±0.07g/mouse, vs 0.48±0.05g/mouse with control IgG)

In parallel, administration of CD4 neutralising antibodies significantly inhibited the increase in CCK-expressing cells in the duodenum of infected animals (naïve mice, 3.8±1.0 cells/15 villus:crypt units, rising in infected mice treated with IgG to 6.6±1.5 but...
not in infected mice treated with αCD4, 4.3±0.5; fig 4c), and attenuated the rise in plasma CCK (1.5±0.5pM at day 9, compared to 3.3±0.1 following control IgG).

Efficient resolution of *T. spiralis* infection is also dependent on mast cells (also under the control of CD4+ T cells), which can curiously degranulate in response to CCK. Anti-c-kit antibodies abrogate mucosal mastocytosis, but leave the T cell response intact. Food intake reduced after anti-c-kit treatment as in previous groups of infected animals (to 2.76±0.21g/mouse/day, anti-c-kit vs 2.34±1.04 g/mouse/day, control IgG). The anti-c-kit treatment was effective since worm burdens on day 8 were appropriately increased by anti-c-kit antibody, 147.3±15.0, compared with 66.0±5.4 after control IgG (p=0.006).

**Inflammation-induced T cell cytokines regulate food intake**

*T. spiralis* induces strong Th2 type cytokine responses in the intestine. Given that CD4+ T cells mediated food intake and CCK*+* cell hyperplasia in parasitized mice, we sought to investigate the influence of IL-4 and IL-13 on these parameters. Mice genetically deficient for IL-4 or IL-13 or IL-4Rα were infected, and food intake again measured during the course of infection. There was no significant difference in food intake between infected wild type mice or IL4 or IL13 deficient mice (fig 5a). In the absence of IL4Rα however, infection-induced hypophagia was fully abolished. IL4 and IL13 deficient mice lost less body weight over the course of infection compared to wild type animals, and this effect was again completely abolished in IL4Rα deficient mice (fig 5b). These results demonstrate that either IL4 or IL13 can influence nutritional parameters during inflammation, only in the functional absence of both cytokines together is this signal abolished. IL4 and IL13 exhibit some mutual redundancy, but both converge to signal via the IL4-Rα subunit.

**DISCUSSION**

Given the adverse nutritional consequences of gut inflammation widely observed in clinical practice, elucidation of specific molecular pathways causally linking inflammation to reduced nutrient intake would be of fundamental importance, particularly if accessible to existing therapeutic agents. Much recent physiological research has focussed on the role of EEC factors in the short term regulation of food intake in health, with evidence available from both human and animal models. The brain-gut axis peptide CCK has received the most attention; its physiological role in the control of food intake is widely accepted. CCK is most highly expressed in the duodenum, which bears the most nutrient chemo-sensitive epithelium gut. CCK is believed to influence meal size in part via a delay in gastric emptying rate, and in part via direct signals to brain feeding centres.

However, the possibility that increased CCK release is mechanistically implicated in inflammatory gut diseases associated with reduced food intake has not been explored.

In a preliminary study we recently demonstrated that humans with upper gut infections such as *Giardia lamblia* have elevated circulating plasma CCK concentrations during infection, and that this returns to normal afterwards. This raised the possibility that hyperCCKaemia may causally account for some of the observed symptoms, such as anorexia. In keeping with this, it is well established that intravenous infusion of high dose CCK to healthy humans induces similar symptoms.
Mice infected with the nematode parasite, *T. spiralis*, present a powerful model in which to dissect the molecular mechanisms operating in small bowel inflammation, including those underpinning reduced food intake. This model has 3 key advantages. First, the inflammation is transient and reproducible, and most marked in the key region of interest, the upper small intestinal mucosa. Second, the regulatory cell types and cytokines elicited in response to this parasite are very well characterised, and amenable to intervention including immunoneutralization and transgenics. Third, the biphasic nature presents an internal control, with a delayed extra-intestinal inflammatory phase.

As previously documented, infection caused initial proximal enteritis which peaks around day 9-10, when the parasites have just been expelled from the gut. Intestinal inflammation then resolves, the epithelium is functionally normalised by day 21, even by highly sensitive parameters such as epithelial permeability. However, some neuromuscular dysfunction persists after the epithelial changes have resolved, but this continues well beyond the transient hypophagia we have observed in this study. Given the degree of intestinal inflammation observed in these mice, it was unsurprising to initially demonstrate that food intake fell significantly, in a time course paralleling the well documented inflammatory response described above. However this basic observation had not been reported previously. Our study confirms that the *T. spiralis* parasitized mouse presents a tractable model to identify the biological effectors responsible.

Increased CCK cell numbers were evident from day 6 p.i, despite villus atrophy. This increase is absolute since the data are normalised to a numeric sample of crypt-villus functional units. The CCK cells clearly remain functional, since the plasma CCK concentration is elevated 4-fold. This can be directly linked to reduced food intake since this is partially abrogated by the specific CCK antagonist loxiglumide. The failure of loxiglumide to reverse the second phase of hypophagia, when skeletal muscle inflammation is active, is also of importance in confirming a specific mechanism of hypophagia in gut-centred inflammation. This demonstrates that the CCK-immune interaction is in large part mediated locally, in the intestinal epithelium, rather than representing a purely systemic effect, for example via circulating hypercytokinaemia. Loxiglumide is in phase III clinical trials for functional human gastrointestinal disorders, but has not yet been evaluated in the context of inflammatory disease or of nutritional status.

Since crypts are the stable niche for the stem cells which populate all 4 cell lineages in daughter villi, the data suggest a shift in lineage differentiation commitment. Given that both goblet cells and Paneth cells are increased in this model, the data suggest a shift in lineage differentiation commitment: from absorptive to secretory cell types. The transcription factor Math 1 has been reported as essential to commitment to the 3 secretory cell types rather than enterocytes and its role in mediating stem cell responses to inflammation now requires study. Subsequent endocrine cell differentiation is controlled by the Notch signalling pathway, neurogenin 3, pax 4 and pax 6. The factors governing final differentiation to a CCK cell are not yet known. The lag of 6 days before the CCK cell count increases is in keeping with the time course of epithelial cell turnover, which has been estimated at 3-4 days by kinetic studies. This rapid cell turnover probably also underpins the transient effect on EEC, unlike the neuromuscular changes which persist for months in these subepithelial cells of far greater longevity. The lag also suggests that it is necessary for the inflammatory response to be activated before cell lineage commitment is altered, rather than representing a direct effect of the parasite. It will be interesting to ascertain whether CCK cells have become...
hypersecretory basally, hyperfunctional only in response to nutrient exposure, or whether the increased plasma level purely mirrors increased cell numbers. However, pure primary EEC preparations to answer these questions cannot be obtained at present.

The role of the immune system we have now characterised is novel. A single previous report showed that orally administered glucocorticoids reduced hypophagia in nematode infected rats\textsuperscript{29}. However, corticosteroids are intrinsically highly orexigenic and thus do not allow discrimination between this property and their immunosuppressive effects. We adopted a more specific approach, investigating the role of CD4\textsuperscript{+} T cells on infection-induced hypophagia and CCK expression by treating infected animals with CD4 neutralising antibodies. In the absence of functional CD4\textsuperscript{+} T cells inflammation was much reduced and resolution of \textit{T.spiralis} infection was significantly delayed. Infected mice treated with non-specific IgG were hypophagic but, strikingly, this was completely abolished in infected mice treated with CD4 neutralising antibodies. Indeed, eating proceeded normally despite a very large worm burden. CD4 neutralising antibody had no independent effect on food intake in uninfected mice.

We have demonstrated that CD4\textsuperscript{+}T-lymphocytes are as pivotal to the enteroendocrine and hypophagic response as they are to worm clearance. This functional coupling via a single effector cell type suggests that the EEC-CD4 cell pathway represents an innate immunoregulatory role, rather than an aberrant response. The IL-4R\textsubscript{α} has a pivotal role here, and is activated by both IL-4 and IL-13: hence the redundancy observed when either cytokine is absent alone\textsuperscript{23}. The current model is at present most relevant to events coupled to a predominantly Th2 type immune response. However, it is likely that other inflammatory mediators are coupled to EEC biology. For example, T cell receptor alpha chain knockout mice have reduced EEC numbers in the colon\textsuperscript{30}, suggesting that this interaction may be constitutively important in health for epithelial cell differentiation, or in the response to endogenous bacterial flora. Gnotobiotic animals have been shown to have reduced EEC numbers, which increase when re-colonised with flora\textsuperscript{31}.

It is also clear that this effect is not restricted to CCK cells: enterochromaffin cells were increased in number in parallel. Secretion of 5-HT by human enterochromaffin cells is linked to the induction of nausea and vomiting in a variety of situations, particularly in chemotherapy and post-operative patients\textsuperscript{32}. Mice are not able to vomit, but the presence of nausea contributing to the reduction in food intake is highly plausible. This aversive sensation may in part be mediated via enterochromaffin 5-HT hypersecretion. 5-HT is also implicated in diarrhoea in some infections and in functional gastrointestinal disorders\textsuperscript{32,33}. Normal faecal pellets are still observed in our model, but with additional looser and mucoid material present in the cage, all observed for a few days at the time of peak inflammation. This may indeed be partly due to 5-HT hypersecretion, but difficult to unravel in the presence of an inflammatory enteropathy, villus atrophy with probable malabsorption, enhanced smooth muscle contractility and increased mucus secretion due to goblet cell hyperplasia. However, mast cells infiltrate the gut in this model, and murine mast cells are also an abundant source of 5-HT. In addition, the amine operates at multiple receptor subtypes, so it is not tractable to pharmacologically unravel these confounding factors. It will be interesting to discover whether all EEC lineages are affected in parallel, as seems likely given the general secretory switch to include increased goblet and Paneth cells\textsuperscript{27}, or only a restricted subset.
An immunoregulatory role of EEC may have short-term advantages, such as reduced energy expenditure and avoidance of ingestion of contaminated feed sources. CCK may have additional defensive roles, such as increased mucosal immunoglobulin secretion and enhanced mucosal blood flow. If trial therapeutic interventions with CCK antagonists were undertaken for anorexia triggered by gut inflammation, the possibility of possible adverse outcomes must therefore be considered. However this must be balanced against possible additional beneficial effects of CCK antagonists, particularly in the presence of infection. For example, gut propulsion is accelerated by CCK antagonism, and could contribute to a faster elimination of parasites or other pathogens. There is evidence that other EEC products, such as GLP-2, are restitutive to damaged epithelia, and studies are planned to assess other EEC lineages in this model.

However, beyond initial, short-term adaptive responses, ongoing inflammation may prove counterproductive to the health of the host organism. It would be detrimental to nutritional status if food intake remained suppressed. This may be of considerable importance when considering the prevalence of intestinal nematode infection in man and, indeed, in domestic stock where food intake and weight gain have economic implications in addition to health considerations. Conversely, subverting the molecular basis of this immunoregulatory pathway would present a novel therapeutic target for purposefully increasing satiety, thereby impairing food intake and inducing weight loss.

In summary, we have discovered and characterized for the first time an important aspect of enteric immunoregulation. We have shown that the reduction of food intake during small intestinal inflammation is linked to an increase in CCK expressing EEC, and under the control of IL-4 and IL-13 secreting CD4+ T cells.

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Figure legends

Figure 1. Effects of infection on feeding and weight. Food intake was measured following *T. spiralis* infection. Points represent the mean of 4 mice/group ± s.e from at least 3 independent experiments.

Figure 2. Effects of infection on EEC. Duodenal CCK cells significantly increased in number during the course of infection, as did duodenal 5-HT cells (B). Data are mean of 4 mice/group ± s.e, from at least 3 independent experiments.

Figure 3. Effect of a CCK antagonist on feeding, during and following small intestinal inflammation. 5 groups of 3-4 infected mice were treated *i.p.* with either sterile water or the CCK$_1$ receptor antagonist loxiglumide. Feeding was measured 15 hours later. Control mice, but not loxiglumide-treated mice at d9 p.i consumed significantly less than naïve animals.

Figure 4. The role of CD4 T-cells in controlling food intake during small intestinal inflammation. Naïve and infected mice received repeated doses of neutralizing CD4 antibody or rat IgG (control) *i.p.* Two independent experiments were undertaken, with 4 mice/group. Mice treated with αCD4 failed to expel their parasites by d9 p.i (A), yet their food intake remained comparable to naïve mice (B). Infected mice treated with control IgG had significantly more CCK$^+$ cells/15VCU than naïve or αCD4-treated infected mice (C).

Figure 5. The role of Th2 cytokines in controlling food intake during small intestinal inflammation. Wild type (wt), IL4-/-, IL13-/- and IL4Rα-/- mice were infected with *T. spiralis*. At day 9 p.i. food intake of infected IL4-/-, IL13-/- and wt mice was again reduced compared to IL4-Rα-/- mice, which consumed the same as naïve mice (A). Weight loss was reduced in infected IL4-/- and IL13-/- wt mice and abolished in infected IL4Rα-/- mice on day 9 p.i (B). Histograms represent mean of 4 mice/group ± s.e, from 2 independent experiments.
Fig 1

- Black line: Naive
- Dotted line: Infected

* p<0.01

Food Intake g/day vs Day post-infection
Fig 2A

CCK⁺ cells/15VCU

* p<0.05

Naive  Day 6  Day 9  Day 13  Day 20
Fig 2B

5-HT<sup>+</sup> cells/15 cvu

Naive  Day 6  Day 9  Day 13  Day 20

*
Fig 3

*p<0.05 vs naive day 9

food intake g/day/mouse

- day 9 naive
- day 9 control
- day 9 loxiglumide
**Fig 4A**

*Fig 4A shows the worm burden on day 9 for rats treated with rat IgG and αCD4. The bar graph indicates a significant difference (*p<0.002*) between the two groups.*
Fig 4B

- Infected + αCD4
- Infected + control
- Naive + αCD4
- Naive + control

* p<0.05
Fig 4C

CCK+ cells/15vcu

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Fig 5A

change in food intake g/day

WT WT IL-4 -/- IL-4R -/- IL-13 -/-

Naive Infected
Fig 5B

change in body weight g

WT  WT  IL-4 -/-  IL-4R -/-  IL-13 -/-
Naive  Infected

Infected
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