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Oral administration of helminth fluid modulates distinct tuft cell and immune-metabolic cues linked to reduced body fat

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Abstract
Intestinal tuft cells have been shown to induce type 2 immune responses during viable parasite infections, but whether oral supplementation with a parasitic exudate is able to promote type 2 immune responses that have been shown to positively regulate obesogenic metabolic processes is yet unresolved. High-fat fed mice were gavaged with pseudocoelomic fluid (PCF) derived from the helminth Ascaris suum or saline thrice a week during weeks 5–9, followed by examination of intestinal tuft cell activity, immune, and metabolic parameters. Helminth PCF upregulated expression of distinct genes in small intestinal tuft cells, including genes involved in regulation of RUNX1 and organic cation transporters. Helminth PCF also enhanced levels of innate lymphoid cells in the ileum, and eosinophils in epididymal white adipose tissue (eWAT). Network analyses revealed two distinct immunometabolic cues affected by oral helminth PCF in high-fat fed mice: one coupling the small intestinal tuft cell responses to the fat-to-lean mass ratio and a second coupling eosinophils in eWAT to general regulation of body fat mass. Our findings point to specific mechanisms by which oral supplementation with helminth PCF may translate into systems-wide effects linking to reduced body and fat mass gain in mice during high-fat feeding.

KEYWORDS
energy metabolism, helminth, immune responses, systems biology, tuft cells

1 INTRODUCTION

Dysregulated immune responses in metabolically active organs contribute to development of metabolic disorders including obesity. 1
Type 2 immune responses are gaining increased attention for their role in immune surveillance at tissue barrier sites such as the lung and gut, and as regulators of energy homeostasis in adipose tissues counteracting obesity development. 2 In obesity, type 2 responses are suppressed and associated with dysregulation of the IL-33-ILC2-IL-5/13-eosinophil-IL-4/13-M2 macrophage axis in adipose tissues resulting in decreased numbers of innate lymphoid cells type 2 (ILC2s), eosinophils, and M2 macrophages combined with decreased levels of IL-5, IL-4 and IL-13. 3–5 By contrast, type 1 associated immune cells and cytokines including M1 macrophages, pro-inflammatory ILC1s,
CD11c+ DCs, CD8+ T-cells, Th1 T cells and Th17 T cells are reported to be increased in adipose tissue in the obese state along with increased TNF-α and IFN-γ levels.\(^6,7\)

Viable helminths, including the prevalent human nematode *Ascaris lumbricoides* and its equivalent in pigs *A. suum*, are known for their ability to induce a type 2 immune response in the host during infection, involving infiltration of eosinophils, ILC2 and/or Th2 cells into the affected tissues, and enhanced expression of IL-13 to increase mucus production.\(^8-10\) More recently, the involvement of tuft cell-derived IL-25 in response to infecting helminths was reported.\(^11,12\) Helminth-induced tuft cell hyperplasia, which requires IL-13, also occurred in Rag\(^{−/−}\) mice\(^13\) showing that type 2 responses may be induced by viable helminths and tuft cells independently of adaptive immune response. The innate source of IL-13 was identified to be ILC2-based,\(^13\) thus exemplifying a tuft-cell-IL-25-ILC2-IL-13-axis in response to helminth infection.

In obese mice, intraperitoneal injection of IL-25\(^14-16\) and/or IL-4\(^17\) or Schistosoma mansoni soluble egg antigens\(^18\) was previously reported to increase eosinophil and M2 macrophage counts in white adipose tissue (WAT), associated with decreased weight gain, reduced lipid accumulation, and increased energy expenditure indicating that type 2 activation may improve systemic metabolism. Although not proven directly in previous studies, oral supplementation of type 2 immune inducing agents could be speculated to hold potential to improve obesity-associated disorders via stimulation of signalling cues that enhance type 2 immune responses in WAT.

The objective of this study was to examine if oral supplementation with pseudocoeolic fluid (PCF) from the helminth *A. suum* to mice on a high-fat diet would be able to induce type 2 immune responses in the small intestine or systemically, thereby influencing whole body metabolic regulation. PCF is an antigenic rich fluid derived from the perenteric body cavity of adult worms, and has been shown to recapitulate the effects of live *A. suum* infection in vitro and in vivo models, such as exerting strong immunosuppressive properties in monocyte-derived dendritic cells exposed to type 1 inflammatory conditions.\(^19,20\) Moreover, subcutaneous and systemic administration of helminth PCF have been shown to inhibit ongoing pro-inflammatory conditions and induce a regulatory immune phenotype.\(^21-24\) PCF contains a number of putatively bioactive molecules such as fatty acid- and haem-binding proteins which may be responsible for its strong immunomodulatory effects,\(^25,26\) and thus, may serve as a feasible alternative to live parasite colonisation for inducing anti-inflammatory and/or type-2 immune activity. Here, we explored immune-metabolic interplays involving small intestinal tuft cells, and immune compartments in the small intestinal lamina propria, epididymal WAT (eWAT), and inguinal WAT (iWAT) in mice provided a high-fat diet for 9 weeks, in which helminth PCF or saline was administered by oral gavage for the last 4 weeks of the intervention. Altogether, oral supplementation with helminth PCF was found to influence two different pathways, one in the small intestine and one in eWAT, both coupled to beneficial metabolic regulation.

### 2.1 Experimental design

Male C57BL/6J (Taconic Biosciences A/S, Lille Skensved, Denmark) mice 5 weeks of age at arrival (week 0) were housed with four mice per cage in a climate-controlled room (temperature 30°C and 55% humidity). Mice were subjected to 12 h light/dark cycle (6:00 AM to 6:00 PM) with ad libitum access to water and food, and assigned to the treatment groups: high-fat diet (HFD) + saline or HFD + PCF according to MRI scans at arrival (similar body weight distribution in each treatment group). During the first week of acclimatization to the new environment (week 0–1), all mice were fed normal chow (Altromin 1324, Brogaarden, Denmark), and starting from week 1, groups were changed to receive irradiated synthetic high-fat diet (EF D12492 (l), Ssniff, Germany), which they received for the remaining study period. All animals were handled similarly using standard procedures including weekly changes of bedding and cages, body weight measurements, bi-weekly food intake per cage recordings, and general cleaning procedures at the animal facility. From week 5 until week 9, all mice were orally gavaged every second day with 200 μL of either 50 mg/mL (weight of dry matter per volume of sterile normal saline) PCF extracted from the helminth *Ascaris suum* (Helminth PCF) as detailed\(^19\) or sterile saline. The study was repeated three times, and included four mice per group in each study. To measure lean and fat mass development during the experiment, MRI was performed on mice at week 0, 5, 8 and 9 using EchoMRI as per the manufacturer’s instructions. All mice were sacrificed at week 9 by cervical dislocation followed by dissection of the small intestine, liver, epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), interscapular brown adipose tissue (BAT) and determination of the masses of the four latter. Mice experiments were approved by the Danish Animal Experiment Inspectorate (Ref. No.: 2014-15-2934-01027) and performed in accordance with EU Directive 2010/63/EU for animal experiments.

### 2.2 Preparation of single cell suspensions for tuft cell isolation and immune cell profiling

Epithelial and lamina propria single-cell suspensions were prepared from the small intestine (20 cm of the small intestine measured from the terminal ileum and upwards) in two steps. In all single-cell preparations, DMEM, PBS (Mg\(^2+\) and Ca\(^{2+}\)-free, pH 7.4), foetal bovine serum (FBS, heat-inactivated) and FACS buffer (3 mM EDTA in PBS + 5% FBS) were used. The small intestines were flushed with ice-cold PBS, followed by removal of visible mesenteric fat and Peyers’ patches, then cut open and washed with PBS to remove remaining intestinal contents. Intestinal tissue was cut into 2 cm pieces and incubated on a rocking shaker (900 rpm) with occasional flicking at 37°C for two times 20 min in 1.5 mL 30 mM EDTA in PBS. Obtained epithelial cell suspensions were washed (500g, 5 min at 5°C) in 3 mL PBS and incubated on a rocking shaker (300 rpm) with occasional flicking at 37°C for two times 10 min in...
1.5 mL PBS containing 20 μg/mL Dispase DH and 20 μg/mL DNase I. Afterwards, epithelial cell suspensions were passed through 70 μm nylon filters while homogenizing the particulate matter, washed in 15 mL DMEM containing 5% FBS and suspended in FACS buffer. Cells were kept at ice until processing for tuft cell staining and isolation by FACS.

For lamina propria single-cell preparation, the left-over intestinal tissues after EDTA treatment were rinsed with 10 mL PBS containing 5% FBS, minced into 1–2 mm pieces with scissors, incubated on a rocking shaker (500 rpm) with occasional flicking at 37°C for two times 30 min in 1.5 mL digestion buffer (DMEM containing 5% FBS, 40 μg/mL Liberase DH and 50 μg/mL DNase I). Afterwards, 1 mL supernatant was transferred to a new tube, remaining cell suspension was repeatedly drawn through an 18-gauge needle to further mince the intestinal pieces, 1 mL fresh digestion buffer was added, and the cell suspension was incubated again under the above-mentioned conditions. Afterwards, the cell suspension was passed through a 70 μm nylon filter while homogenizing the particulate matter, washed in 20 mL DMEM containing 5% FBS and suspended in FACS buffer. Cells were kept on ice until staining for flow cytometry analysis.

For eWAT and iWAT single-cell preparations, respective tissues were harvested, finely chopped with scalpel blades and incubated at 37°C for 40 min in 1 mL DMEM containing 10% FBS and 20 mg/mL collagenase in a shaking incubator (150 rpm). 14 mL PBS containing 1% FBS was added to dilute the digestion reaction and afterwards the digest was passed through 70 μm nylon filters, washed (400g, 10 min at 5°C) in PBS, and subjected to red blood cell lysis using 1 mL of lysis buffer (154.95 mM ammonium chloride, 9.99 mM sodium hydrogen carbonate, 0.0995 mM disodium EDTA in PBS) for 5 min at room temperature. Finally, the cell suspensions were washed again in 14 mL PBS and suspended in FACS buffer. Cells were kept on ice until staining for flow cytometry analysis. All single-cell suspensions were counted on a Nucleocounter NC-200 (ChemoMetec) and plated into 96-well plates for flow cytometry-based staining and analysis.

2.3 Staining and flow cytometry analysis for immune cell profiling and tuft cell sorting

All cell suspensions were incubated and analysed on the day of study termination. Firstly, single-cell suspensions were incubated with Fc-block followed by staining with antibodies against surface markers and kept at 4°C throughout the staining procedure.

Single-cell epithelial preparations were stained with antibodies against CD45-eVolve 605, EpCAM-AF647 and SiglecF-FIPE for 30 min at 4°C and were stained in FACS buffer followed by 0.3 μM 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI, 1.2 μL/10E6 cells) addition for dead cell exclusion. Viable CD45-EpCAM+SiglecF+ tuft and CD45-EpCAM+SiglecF- non-tuft cell populations were sorted using an Aria III (BD Biosciences) with a four laser (405, 488, 562 and 633 nm) configuration directly into RNA lysis buffer from the Quick-RNA Microprep Kit and were stored at −20°C immediately after sorting and at −80°C afterwards. Flow cytometric counting beads (CountBright Absolute) were added to enumerate total live cell numbers.

Lamina propria, eWAT and iWAT single-cell preparations were divided into two fractions and both fractions were first stained with fixable violet live/dead stain as per manufacturer’s instructions and then incubated with Fc-block. Afterwards, one fraction was stained for surface markers (30 min at 4°C) with an antibody cocktail in FACS buffer containing 2 μM monensin for intracellular cytokine staining (Sigma-Aldrich, M5273 ) and another fraction without monensin. The monensin supplemented antibody cocktail included antibodies (Table S2) against CD45, CD11c, CD11b, F4/80, CD206, SiglecF, FceR1, CD117 and TfnA. The antibody cocktail used for staining without monensin addition included antibodies against NKP46, CD90.2, ST2, KLRG1, IL-17rb, CD45, CD4, TCRab, GATA3, Tbet, Rorgt, and lineage cocktail (B220, Ter-119, CD8a, CD49b, NK1.1, CD11b, CD11c, FceR1). For intracellular (TFN-a) and intranuclear (GATA-3, T-bet, ROYrt) staining, the Fixation/Permeabilization Solution Kit and FoxP3/Transcription Factor Staining Buffer Set (both BD Biosciences) were used according to manufacturer’s instructions, respectively. Samples were analysed on an LSR II (BD Biosciences) with a four laser (355, 405, 488 and 633 nm) configuration. All data were subsequently analysed using FlowJo software (V10.0.7, Tree Star). Gating strategies are shown in Figure S1 (tuft cells), Figure S2 (innate immune cells), Figure S3 (additional immune cells) and Figure S4 [Fluorescence Minus One (FMO) controls for intracellular staining].

2.4 RNA extraction, RT-qPCR and RNA sequencing of enriched small intestinal tuft cells

Total RNA from the viable-sorted CD45-EpCAM+SiglecF+ small intestinal tuft cells was isolated using the Quick-RNA Microprep Kit. RNA concentrations were measured using a Qubit 2.0 fluorometer and Qubit RNA HS Assay kit and quality checked using a Bioanalyzer according to the manufacturer’s protocol. Reverse transcription was performed with 5.6 μL of purified RNA and the resulting cDNA was amplified following the Smart-seq2 protocol with minor modifications using a UNO96G Gradient thermocycler (VWR) and primers and reagents (Table S1). cDNA was prepared as described in Arora et al. Real-time qPCR (RT-qPCR) of the amplified cDNA obtained from viable-sorted CD45-EpCAM+SiglecF+ small intestinal tuft cells was performed with TaqMan Fast Universal PCR Master Mix and a 7900HT Fast Real-time PCR system using the specified primers and probes (Table S1). The PCR reactions were run under the following conditions: 95°C for 20 s; 40 cycles of 95°C for 1 s and 60°C for 20 s. Transcripts were normalized to Dclk1 expression and relative expression was obtained using ΔCt.

For RNA sequencing, the sequencing libraries from amplified cDNA from sorted viable EpCAM+CD45+ Siglec-F+ small intestinal epithelial cells were generated using the Nextera XT DNA library preparation kit with multiplexing primers, according to manufacturer’s
protocol and as reported in Arora et al. Resultant cDNA libraries were purified using AMPure XP beads, quantified using Qubit HS dsDNA Assay Kit, checked for library fragment size distributions using a High-Sensitivity DNA chip. Afterwards, libraries having insert sizes of 200–700 bp were subjected to 150 bp paired-end sequencing on the Illumina NovaSeq 6000 sequencing platform (Novogene Corporation, China). Raw data, after de-multiplexing, were filtered using Cutadapt (v1.15) to remove sequencing adapters and low-quality reads. Mapping and alignment of the clean reads was performed using STAR (v2.6.0) and transcript quantification was performed using RSEM. Afterwards, the gene matrices were filtered to include genes that were present in at least six samples with at least five reads, resulting in 11,581 genes for further analysis. One sample was removed due to low effective library size (~188,000 reads). BSEQ-sc v1.0 and CIBERSORT were applied to single cell data from Haber et al. to perform cell type deconvolution, and to identify gene count contributions from tuft cells, we weighed the gene count distribution of genes expressed by multiple cell types by the cell type frequency estimated by BSEQ-sc. For genes with too few reads to confidently estimate the tuft cell proportion of gene counts, we retained 95% of the reads distributed symmetrically around the mode of the log10-transformed gene count distribution, and used the lower limit of these read distribution as an additional weighing parameter in the estimation of tuft cell gene count proportions. Genes with at least three reads in at least 20% of any non-tuft cell types and with at least three reads in less than 20% of tuft cells, and genes that were identified as non-tuft cell marker genes were also removed from the dataset. Genes were functionally annotated using Reactome, and differential expression of genes (DEG) was identified using SAM-seq v1.0, while fold-change estimates were obtained using edgeR v3.24. Differentially regulated pathways were identified by summing the read counts for all pathway-related genes followed by differential expression analysis as for DEG.

2.5  |  Tissue cytokine analysis

During dissections, sections of ileum (flushed and mesenteric fat free), eWAT and iWAT tissue samples were harvested and snap frozen at −80°C until further analysis. For cytokine analysis approximately 50 mg tissue was weighed, added into the extraction buffer at 100 mg tissue/mL extraction buffer (w/v) (1 protease-inhibitor cocktail tablet (Complete ULTRA) per 5 mL PBS (Mg²⁺ and Ca²⁺-free, pH 7.4)) and homogenized using a homogenizer (Biospec 1001) for 3 × 20 s until uniform tissue homogenization. Afterwards, the homogenized sample was centrifuged at 500g at 4°C for 10 min and supernatant was collected. Homogenate concentrations of IL-4, IL-5, IL-13, IL-25, IL-33 and TNF-α cytokines were detected using electrochemiluminescence measurements based on the Meso Scale Discovery platform (Meso Scale Discovery) according to the manufacturer’s instructions. Minimum detectable cytokine amount across all tissues (in pg/g tissue): IL-33: 117, IL-25: 0.05, IL-4: 0.27, IL-5: 0.01, IL-13: 0.12, TNF-α: 0.04.

2.6  |  Statistical analysis

All experiments were performed using randomly assigned mice. Statistical details for each analysis is provided in the figure legends. Statistical analyses were performed in R v3.5.2 (R Core Team, 2018). Statistical differences between the two treatment groups were tested by non-parametric Wilcoxon rank tests and data were subsequently adjusted for multiple comparisons using multiple testing by FDR (Benjamini–Hochberg) for each subfigure (either boxplots or heatmaps). Unless otherwise stated, Spearman’s rank correlation analysis was performed with q-values < 0.1 as statistically significant. Networks based on Spearman correlations were built using Cytoscape v3.3.0.

3  |  RESULTS

Oral intake of helminth PCF during obesity development induces distinct transcripts in small intestinal tuft cells that link the fat-to-lean mass ratio without contributing to changes in ileal type 2 immune responses

To examine if oral supplementation with helminth PCF affected metabolism in the context of high-fat feeding, we fed mice a high-fat diet for a total of 9 weeks, and concomitantly supplemented with helminth PCF (PCF) or saline by gavage every second day from week 5 to 9. The 4 weeks of oral supplementation with PCF to the high-fat fed mice resulted in no statistically significant differences in body metabolic parameters, but showed an overall trend of a lower weight gain in mice of the PCF group from week 5 to 9, which was due to reduced fat-to-lean mass ratio in PCF as compared to saline supplemented mice (Figure 1A, p(body weight gain) = .107; p (fat-to-lean mass) = .133). The fat-to-lean mass ratio presented expected highly significant positive relations to other metabolic parameters (Figure S5). Helminth infections have been shown to increase tuft cell-derived IL-25 production in the intestine, so one of the focus points of this study was to address if in vivo supplementation with PCF would influence small intestinal tuft cell numbers, their expression of IL25, as well as Tslp. In order to do so, we enriched small intestinal tuft cells (EpCAM+/Siglec-f+) by FACS. Please refer to Figure S1 for gating and sorting strategy for tuft cells. The 4 weeks intervention with PCF in high-fat fed mice did not result in significant changes in tuft cell numbers (p = .45), expression of IL25 (p = .93) or Tslp (p = .09) (Figure 1B). In order to get a global view on possible transcriptional regulation induced by PCF in the small intestinal tuft cells, we next performed RNA sequencing on RNA extracted from the FACS-enriched tuft cells. This revealed that transcripts of genes involved in RUNX1-regulated pathways were specifically enriched in high-fat fed mice receiving PCF (Figure 1C, Table S3). The identified RUNX1-regulated pathways have previously been associated with immunoregulatory events in T, B, and myeloid cells, but the genes involved in these pathways have not previously been found to be expressed in tuft cells. PCF also enhanced expression of genes involved in transport of several organic cations via different Slc22-family transporters, including Slc22a1, Slc22a4, Slc22a5, Slc22a15, Slc22a18 and Slc22a21. These Slc22-transporters are
reported to function as transporters of endogenous compounds (a1; choline, histamine, adrenaline), and drugs (a1; quinine, metformin), carnitine (a4, a5, a15, a21), chloroquine/quinidine (a18) and ergothioneine (a4), amongst others, indicating that oral intake of PCF may enhance the expression of transporters for a diverse set of compounds in tuft cells.
We next examined if oral PCF supplementation influenced the secretion of the type 2-related cytokines IL-4, IL-5, IL-13, IL-25 and IL-33 as well as the pro-inflammatory cytokine TNF-α in the small intestinal tissue. Besides tuft cells, IL-25 can be expressed by other cell types in the gut, but we found no significant changes in IL-25 at the protein level in the small intestinal tissue for PCF administered mice (Figure S6a), and moreover, tuft cell expressed Il25 mRNA and small intestinal IL-25 did not correlate (Figure S6b). Additionally, none of the cytokines IL-4, IL-5, IL-13, IL-33 and TNF-α were statistically significantly changed by oral PCF supplementation (Figure S6c). Due to the great individual responses within the mice, we next performed co-correlation analyses between the different parameters in order to identify if certain parameters were correspondingly altered across mice. When correlating the expression of genes involved in the RUNX1-regulated pathways to the tuft cell and small intestinal cytokine data, we, upon adjusting for multiple testing, identified significant correlations between numbers of tuft cells and eight of the 17 tuft cell expressed genes induced by PCF (Figure 1D), hence implying that oral supplementation with PCF directly affected expression of these distinct genes in tuft cells, but without affecting type 2 immune cytokines or pro-inflammatory TNF-α levels in the small intestine.

We next examined the relationship between the PCF-associated tuft cell markers, and fat and lean mass to examine if the variation in some of these parameters may be interlinked. After adjustment for multiple testing, we identified an inverse relation between fat-to-lean-mass ratio, the tuft cell fraction, and the four transcripts of multiple testing, we identified an inverse relation between fat-to-lean-mass ratio, the tuft cell fraction, and the four transcripts of}

\[ \text{Fat-to-lean-mass ratio} \times \text{Tuft cell fraction} \times \text{IL-25 mRNA} \times \text{IL-13 mRNA} \times \text{IL-33 mRNA} \times \text{TNF-α mRNA} \]

...and other transcripts (Figure S7a).

We next examined the effect of PCF administration on the percentages of small intestinal immune cells. Based on an extensive analysis of the major innate and adaptive immune cell types (Figures S2 and S3 for gating strategy), we found that the proportion of innate lymphoid cells in the small intestine was significantly increased in response to oral PCF supplementation \((p = .004, q(FDR) = 0.034, \text{Figure 2A})\). An ILC sub-group analysis revealed that especially the proportion of a non-ILC1/2/3 subset was significantly increased by oral PCF supplementation \((p = .001, q(FDR) = 0.007, \text{Figure 2B})\). At this point, the non-ILC1/2/3 represents an uncharacterized ILC subset, but we speculate that the non-ILC1/2/3 cells could represent a regulatory ILC phenotype, as the identified cells did not express the transcription factors necessary for development of ILC1 (T-bet), ILC2 (GATA3) or ILC3s (RORγt) (Figure S3). No other small intestinal immune cell subsets of the monocyte, macrophage, dendritic cell, Th cell or ILC lineages were significantly affected by oral PCF supplementation (Figure S7a).

The PCF-associated non-ILC1/2/3 proportion in the small intestinal lamina propria trended to correlate inversely to small intestinal TNF-α levels \((p = .040, q(FDR) = 0.167, \text{Figure 2C})\), but did not correlate to the earlier identified PCF-associated tuft cell markers (Figure 2D), nor to any of the body composition parameters (Figure 2E), hence suggesting that the change in the non-ILC1/2/3 subset induced by oral PCF might be non-tuft cell related and directed at the intestine where it may contribute to the slight reduction in TNF-α levels during HFD conditions.

Since the focus of this study was to examine the appearance of type 2 immune cues after oral PCF supplementation in a HFD setting, we also examined the phenotype of ILC2 cells based on their expression of the IL-33 receptor ST2 and the IL-25 receptor IL-17rb in concert with KLRG1 (Figure S3 for gating strategy). The distribution of the six derived ILC2 subtypes showed > 50% of ILC2s in the small intestine of this obesogenic mouse model to be KLRG1 + ST2-IL17rb-, while KLRG1 + ST2 + IL17rb- ILC2s made up around 25%, and KLRG1 + ST2-IL17rb + ILC2s represented around 10% (Figure S7b). However, there were no statistically significant differences between PCF and saline supplemented high-fat fed mice with respect to the ILC2 subsets (Figure S7c).

We next performed a network analysis, which was based only on the strongest significant correlations \((0.5 < SCC < 0.5)\) between the oral PCF-associated tuft cell markers, overall small intestinal immune cells and cytokines, and overall metabolic parameters (Figure 2F, direct PCF effects marked with a star). This systems biological approach showed some strong noteworthy associations of relevance for general type 2 immune and metabolic interactions. Independently of PCF, we identified a significant positive link between small intestinal IL-13 and IL-33, inversely correlated with liver mass and eWAT mass for both IL-13 and IL-33, as well as body weight, body weight gain and total fat mass for IL-13. IL-4 correlated positively with both IL-13, IL-33 and Th2 cell proportions, but not directly with any of the body composition parameters. Another subpart of this small intestinal and metabolic network was formed by significant associations between small intestinal immune cell subsets. The immune cell subsets did not correlate significantly to any of the measured cytokines nor to the PCF-associated tuft cell markers, but the subnetwork of intestinal immune cells was associated to the body composition parameters via a positive correlation between small intestinal proportions of CD11b-hi monocytes and body weight, liver mass, total fat mass, BAT mass, iWAT mass and the fat-to-lean-mass ratio. Non-Th1/Th2/Th17 cells (that may be regulatory T cells) also associated positively to BAT mass, total fat mass, and fat-to-lean-mass ratio. Non-Th1/Th2/Th17 cells (that may be regulatory T cells) also associated positively to BAT mass, total fat mass, and fat-to-lean-mass ratio, and negatively with tuft cell numbers. A third subpart of the network centred on the earlier shown PCF-associated relations in Figure 1E; with small intestinal tuft cell proportions correlating positively with many of the genes in the PCF-enhanced RUNX1-regulated pathways and organic cation transport and negatively with the fat-to-lean-mass ratio. The ileal non-ILC1/ILC2/ILC3 cellular subset that was enhanced by oral PCF correlated negatively to ileal-NK cell numbers only.

\[ \text{Oral helminth PCF supplementation increases eosinophil numbers in eWAT that associate to reduced body fat} \]

Besides investigations into the local effects in the small intestine, we also examined if oral supplementation with PCF to high-fat fed
Oral intake of helminth PCF increases an innate lymphoid cell subset in the small intestine of high-fat fed mice. (A) Immune cells in the ileal lamina propria were quantified by flow cytometry, and shown as percentage of total viable CD45+ cells. (B) ILC subsets, subgrouped based on the presence or absence of key transcription factor markers (ILC1: Tbet+, ILC2: GATA3+, ILC3: RORγt+, Tbet+ ILC3: RORγt−Tbet+, Non-ILC1/2/3: Tbet−GATA3−RORγt−), as percentage of total viable CD45+ cells. (C-E) Co-correlation heatmaps displaying SCC between the PCF-enhanced non-ILC1/2/3 subsets versus (C) concentration of ileal cytokines, (D) differentially expressed tuft cell pathway genes, and (E) metabolic parameters. (F) Network showing the interrelations between intestinal and metabolic responses to oral PCF supplementation in HFD mice. Correlations with SCC > 0.5 (red) or < −0.5 (blue) and correlation p-values < 0.05 are shown. Asterisks indicate factors found to be significantly regulated by oral PCF supplementation upon adjustment for multiple testing (q < 0.05). Data in (A) and (B) are gated as displayed in Figure S2 and S3. Boxes in (A) and (B) represents the first to third quartiles and the median, and whiskers show 1.5* of the interquartile range (of Q1 and Q3). The individual data points are shown as single dots. Statistical significance was tested for using Wilcoxon rank tests followed by FDR correction (*, q < 0.05, **, q < 0.01). Statistically significant correlations in (C), (D) and (E) were based on q-values. All immune cell subsets for correlation analyses were transformed using isometric log ratio transformation. All data are based on 12 mice per group derived from three repeated experiments.

mice affected immune cell composition and cytokine production in the two fat depots, eWAT and iWAT. In eWAT, oral PCF was found to significantly increase the number of eosinophils (Figure 3A, p = 0.010, q(FDR) = 0.083), while no significant changes were identified for other major or minor immune cell subsets (Figure S8a). There was no changes induced in the levels of the cytokines IL-5, IL-13, IL-33, and TNF-α in eWAT by oral PCF supplementation (IL-4 and IL-25 were not detectable, Figure 3B). Eosinophils in eWAT correlated inversely with fat mass (p = 0.004, q(FDR) = 0.018), fat-to-lean-mass ratio (p = 0.004, q(FDR) = 0.018), eWAT mass (p = 0.012, q(FDR) = 0.036), and iWAT mass (p = 0.043, q(FDR) = 0.097) (Figure 3C), but did not correlate with eWAT cytokines (Figure 3D) and other eWAT immune cell subsets (Figure 3E). These findings suggest that oral PCF supplementation holds the potential to regulate processes outside the intestine of relevance for obesity development via enhancing the proportion of eWAT eosinophils that link to whole-body metabolic parameters.

In iWAT, we found no changes in the composition of major and minor immune cell subsets upon oral PCF supplementation (Figures 3F and S8b), but PCF trended to enhance IL-13 (p = 0.045, q(FDR) = 0.153) and IL-5 (p = 0.06) production (Figure 3G). IL-13 levels in iWAT correlated inversely with body weight gain (p = 0.008, q(FDR) = 0.0745) (Figure 3H), but not with iWAT cytokines (Figure 3I) or other immune cell types in iWAT (Figure 3J). Hence, oral PCF supplementation significantly enhanced eosinophils, which represent a type 2 immune cell type, in eWAT, that correlated inversely with several whole-body metabolic parameters, while levels of the type 2 cytokine IL-13 that correlated inversely to body weight gain trended to be significantly increased in iWAT, thus demonstrating differential regulation in the two adipose tissues of oral PCF supplementation.

Oral helminth PCF supplementation influences metabolic regulation during obesity development via two distinct tissue-specific cues

In order to provide a systems-wide overview of the PCF-induced effects across the three tissues: small intestine, eWAT and iWAT, we established an integrative co-correlation network based on the strongest significant associations (−0.5 < SCC < 0.5, between tuft cell numbers and transcripts, cytokines and immune cell subsets in the small intestine, eWAT and iWAT, and the measured whole-body metabolic...
parameters, Figure 4). The network analysis revealed that the parameters that were directly increased by oral PCF supplementation (indicated by stars), grouped separately within the network, thus representing a small intestinal tuft cell-based cue (described in Figure 2F), and an eWAT-eosinophil-based cue, where eosinophils linked inversely to eWAT mass and body fat mass. Both cues interlinked via their inverse association to fat-to-lean mass ratio. Altogether, this study demonstrates that oral PCF supplementation during diet-induced obesity development in mice may facilitate activation of two largely independent circuits that both affect anti-obesogenic features; one cue activating distinct genes in small intestinal tuft cells, and one imprinting changes via enhancing the levels of eosinophils in eWAT.

4 | DISCUSSION

Helminth infections are acknowledged to induce profound activation of type 2 immune responses in the intestine, which are needed to eradicate the infecting helminths. Recently this process was reported to involve intestinal tuft cell relays via enhanced IL25 transcriptional activity coupled to increased IL-13 expression and tuft cell hyperplasia. In the present study, we aimed to examine if tuft cells could be activated to promote type 2 immune responses by oral supplementation with helminth products derived from PCF of the helminth A. suum in high-fat fed mice, and if so, to examine if effects could also translate into local and systemic type 2 immune responses that link to whole-body metabolic regulation.

We found that oral supplementation with helminth PCF in high-fat fed mice enhanced the innate type 2 immune cells, eosinophils, in eWAT, but not in the intestine. Instead, in the small intestine we identified distinct increases in expression of genes involved in RUNX1-regulation and organic cation transport in small intestinal tuft cells that linked inversely to the fat-to-lean mass ratio. The proportions of eosinophils in eWAT linked inversely to several fat mass parameters. Oral PCF supplementation also enhanced the proportion of a non-characterized ileal non-ILC1/2/3 subset that correlated
negatively to ileal-NK cell numbers only. Overall this points to two main immunometabolic cues that are involved in reducing body fat acquisition upon oral supplementation with helminth products during diet-induced obesity development in mice: (i) distinct activation of non-type 2 intestinal tuft cell pathways and (ii) enhanced eWAT eosinophils.

**FIGURE 3** Oral intake of helminth PCF increases eosinophil infiltration in eWAT of high-fat fed mice. (A) Immune cell populations (shown as percentage of CD45+ cells) in eWAT. (B) Cytokine concentrations in eWAT. (C–E) Co-correlation heatmap displaying SCC between eWAT eosinophils and metabolic parameters (C), concentrations of eWAT cytokines (D), and immune cell populations as percentage of CD45+ cells in eWAT (E). (F) Immune cell populations as percentage of CD45+ cells in iWAT. (G) Cytokine concentrations in iWAT. Boxes in (A, B, F, G) represent the first to third quartiles and the median, and whiskers show 1.5* of the interquartile range (of Q1 and Q3). The individual data points are shown as single dots. All immune cell subsets for correlation analyses were transformed using isometric log ratio transformation. Statistical difference was tested using Wilcoxon rank tests followed by FDR correction. Statistically significant correlations in (C–E) are based on q-values [*], q < 0.1; *, q < 0.05; **, q < 0.01. All data are based on 12 mice per group derived from three repeated experiments. ND, non-determined.
In regard to previous reports on induction of IL-25 in intestinal tuft cells by helminth infections, it should be noted that we found none of the PCF-affected pathways in the small intestine to involve tuft cell-derived IL-25 production, which could suggest that IL-25 induction in tuft cells might require tissue damaging interactions or products produced by infecting helminths.

At this stage very little is known concerning transcriptional regulation in small intestinal tuft cells upon food intake. It is therefore not surprising that it has not previously been reported that genes belonging to the RUNX1-regulated pathways in small intestinal tuft cells are modulated by oral helminth products and tuft cell proportions vs immune cell subsets, tissue cytokines, and tuft regulated genes were used as input for the network. All data are based on 12 mice per group derived from three repeated experiments.

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Whether similar mechanisms operate in the gut environment needs further investigation, but given the inverse correlation between Ccnd3 expression and fat-to-lean-mass ratio in our data and the fact that the cyclin D3 protein encoded by CCND3 is able to promote Runx1 activity, we speculate that this may be a potential mechanism. Expression of Pml has also previously been shown to link to fat metabolism in that Pml−/− mice on a high-fat diet displayed increased fat gain as a consequence of increased PPARG- and CEBPA-mediated adipogenesis. Altogether, these findings imply that oral supplementation of helminth PCF might hold the potential to downregulate and/or inhibit obesogenic trajectories via activation

**FIGURE 4** Gut-adipose tissue-metabolic crosstalk with emphasis on nodes affected by oral helminth PCF supplementation. Correlation network of intestinal and adipose tissue responses in relation to metabolic parameters in mice fed a high-fat diet for 9 weeks and supplemented with either PCF or saline three times per week from week 5 to 9. Parameters that were statistically significantly enhanced by oral helminth PCF supplementation (upon adjustment for multiple testing) are marked by an asterisk. Only significant correlations with SSC > 0.5 (red) or < −0.5 (blue) and p-values < .05 between the metabolic parameters and tuft cell proportions vs immune cell subsets, tissue cytokines, and tuft regulated genes were used as input for the network. All data are based on 12 mice per group derived from three repeated experiments.
of specific molecular relays either in small intestinal tuft cells, or promoted via their activation. PCF-induced expression of the gene Slc22a21, encoding an organic cation transporter, in small intestinal tuft cells further supports this hypothesis. Slc22a21 promotes uptake of carnitine from the gut lumen and carnitine itself has been positively associated with the alleviation of high-fat diet-induced fat mass gain and improved glucose tolerance. While the transporter encoded by Slc22a18 has previously been reported to promote lipid accumulation and adipogenesis in vitro (3T3-L1 cells) and in vivo (NCrj rats) when expressed in adipocytes, we found here that small intestinal tuft cell-specific Slc22a18 expression levels correlated inversely with fat-to-lean-mass ratio. These observations point towards a species- and tissue-specific function of Slc22a18 in the context of intake of a high-fat diet. Our finding of a link between certain small intestinal tuft cell genes and systemic energy metabolism, independently of intestinal type 2 immune involvement, indicates that small intestinal tuft cells may hold a specialized function in metabolic regulation, which is unrelated to their recently described type 2 immunoregulatory role. Based on publically available data, Runx1 appears to be highly expressed in intestinal tuft cells, while expression in other epithelial cell types is negligible, hence stressing the importance of intestinal tuft cells in Runx1-mediated gut-metabolic interplays.

An inverse association between fat mass and eWAT levels of eosinophils has previously been reported by others, but to our knowledge this is the first time to show induction of type 2 immune responses in adipose tissues after oral supplementation of helminth products. A few studies have reported on helminth-derived products being effective in inducing different diseases in experimental animal models, including arthritis, type 1 and type 2 diabetes (reviewed in ). In the case of the latter, which is of most relevance for the present study, S. mansoni infection or i.p. injection of S. mansoni eggs was shown to induce diet-induced insulin resistance and reduce weight gain and fat mass, while mediating recruitment of alternatively activated macrophages and eosinophils into eWAT. Also, infection with N. brasiliensis in mice on a high-fat diet resulted in reduced weight gain, but whether this to some part is mediated by the infection has not yet been resolved. The present study is thus adding a new layer of information to the current body of knowledge into metabolic effects of helminth products, especially in relation to our identification of a role for induction of genes involved in RUNX1 activity and certain organic ion transporter in small intestinal tuft cells, and to the involvement of adipose specific type 2 immune cues.

**CONCLUSION**

Our findings that oral supplementation with helminth PCF induces changes in expression of distinct genes within small intestinal tuft cells as well as increased levels of eosinophils in eWAT during high-fat dieting in mice, point to a role for helminth products in indirectly promoting anti-obesogenic effects during diet-induced obesity development as the enhanced eosinophils in eWAT is indicative of a more healthy, lean-like adipose phenotype. These findings enhance our understanding of the functional aspects of the small intestine and type 2 immune responses of adipose tissues in relation to diet-induced metabolic dysregulation and suggest that dietary intake of suitable type 2 immune response-inducing compounds could be beneficial to counter-regulate diet-induced obesogenic cues.

**AUTHOR CONTRIBUTIONS**

Pankaj Arora, Daniel Andersen, Karsten Kristiansen, Susanne Brix conceived and designed the study. Andrew Richard Williams provided access to PCF. Si Brask Sonne, Karsten Kristiansen and Susanne Brix supervised the animal experiment. Pankaj Arora, Daniel Andersen, and Niels Banhos Danneskiold-Samsøe performed the experiments. Data were analysed by Daniel Andersen (data integration, flow cytometry gating), Janne Marie Moll (RNA-seq analysis), Pankaj Arora (flow cytometry gating). Susanne Brix, Daniel Andersen and Pankaj Arora wrote the paper, Karsten Kristiansen, Janne Marie Moll, Si Brask Sonne, Niels Banhos Danneskiold-Samsøe, and Andrew Richard Williams revised the paper. All authors approved the manuscript.

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**CONFLICT OF INTEREST STATEMENT**

The authors declare no conflicts of interest.

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**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**REFERENCES**


**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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