Immune modulation by helminths and the impact on the development of type 2 diabetes

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SUMMARIZING DISCUSSION

Adapted from: Helminths, Hygiene Hypothesis and Type 2 Diabetes.

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Parasite Immunology (2017)

WHAT WAS ALREADY KNOWN ABOUT HELMINTHS, THEIR IMMUNOMODULATORY EFFECTS AND THE ASSOCIATION WITH TYPE 2 DIABETES?

Helminth parasites are the strongest natural inducers of type 2 immunity, and landmark studies have shown that conditions which induce type 2 immune responses improve metabolic disorders (1-6). Furthermore, in several epidemiological studies an inverse association between helminth infections and the prevalence of type 2 diabetes (T2D) or metabolic syndrome was found (7-11). However, the cross-sectional design of these studies have prevented conclusions on the causal relationship between helminth infections and T2D in humans.

Although the mechanisms underlying the potential association between helminth infections and T2D are still largely unknown, experimental infections with helminths or helminth-derived molecules in high fat diet-induced obese mice have indicated that changes in the immune cell composition, in particular in white adipose tissue, contribute to the insulin-sensitizing effects. While obese adipose tissue is characterized by pro-inflammatory type 1 cytokines, a type 2 cytokine environment is present in metabolic tissues under homeostatic, insulin sensitive conditions. Group 2 innate lymphoid cells (ILC2s) and $CD4^+$ T helper 2 (Th2) cells produce the type 2 cytokines interleukin (IL)-4, IL-5 and IL-13 in healthy adipose tissue and sustain a white adipose tissue (WAT) eosinophil and alternatively macrophage (AAM) axis that is largely driven by eosinophil-produced IL-4 (1, 12). Of note, whereas a recent study showed an association between soil-transmitted helminth (STH) infections and insulin sensitivity in humans, no indication for differences in systemic inflammation between subjects with and without STH infections was found (11).

The induction of a strong regulatory network involving regulatory T cells (Tregs) is another important hallmark of helminth infections which might explain the possible beneficial effects of helminth infections. Although filarial antigen-mediated improvement in glucose tolerance in obese mice was shown to occur independently of Tregs (5), the establishment of a regulatory network may contribute to the control of overt immune responses, restricting the chronic low-grade inflammation in adipose tissue that is key to the development of insulin resistance (13). Wammes et al. recently found that not Treg frequencies, but the expression of CTLA4, a molecule expressed by Tregs and involved in putting the brake on immune activation, significantly declined in anthelmintic-treated individuals, indicating that helminths have a modulatory effect on Tregs resulting in increased regulatory capacity (14).

In addition to modulation of the host immune response, helminth infections might confer protection against the development of T2D through caloric restriction, induced by changes in digestion and decreased absorption of nutrients (15, 16). It was previously shown that helminth infections were associated with a lower body mass index (BMI) and waist hip ratio (WHR). However, after adjustment for BMI, the negative association

between STH infections and HOMA-IR persisted, indicating that this association cannot be explained by effects of STH infections on BMI alone (11).

Taken together, previous work has identified the interplay between helminths, inflammation and metabolic homeostasis as an exciting new area that needs further dissection.

HOW DID OUR STUDIES ADVANCE THE FIELD?

Helminths and metabolic homeostasis

To investigate the causal relationship between helminth infections and the development of T2D, we assessed the effect of anthelmintic treatment on changes in insulin resistance (IR), in an area endemic for STH on Flores Island, Indonesia. To this end, a double-blind, household-cluster-randomized, placebo-controlled trial was conducted which is described in **chapter 2**. During one year, study participants (≥ 16 years of age) received four rounds of albendazole or matching placebo with three-month intervals, for three consecutive days. In **chapter 3** we describe the trial outcomes, showing that intensive anthelmintic treatment significantly reduced STH prevalence, as well as infection intensity. Moreover, type 2 immune responses, assessed by eosinophil counts and total IgE levels in peripheral blood, were significantly reduced in albendazole-treated subjects. Although treatment did not lead to an increase of whole-body IR, assessed by homeostatic model assessment of IR (HOMA-IR), at the community level, a significant increase in HOMA-IR was observed among helminth-infected subjects. The latter was accompanied by a significant increase in BMI, and pathway analysis showed that adjustment for BMI and eosinophil count attenuated the treatment effect on HOMA-IR among helminth-infected subjects. Altogether, this is the first cluster-randomized trial in humans demonstrating the causal relationship between helminth infections and whole-body IR in an area endemic for STH. However, it should be noted that the anthelmintic treatment-induced increase in IR might have a relatively small contribution to the multi-factorial pathogenesis of IR, and the assessment of other more established factors such as diet and physical activity will be needed to investigate this.

Experimental infections with helminths in obese mice have enabled us to investigate the potential mechanisms by which helminths can influence metabolic outcomes. Moreover, the identification of single, active helminth-derived molecules has gained increasing attention, as administration of these molecules eliminates the potential helminth-induced pathological condition, and excludes the possibility that the beneficial effects of helminth infections on metabolic homeostasis are simply a result of parasitism. **Chapter 4** builds on previous work showing that chronic treatment with a mixture of *Schistosoma mansoni* soluble egg antigens (SEA) improved whole-body metabolic homeostasis in high-fat diet (HFD)-induced obese mice (4). In this chapter, we investigated the effects of two plant-produced glycosylation variants of omega-1 (ω1), a glycoprotein present in SEA which was previously identified as the major immunomodulatory component in SEA (17, 18), on whole-body metabolic homeostasis. Both recombinant ω1 glycovariants decreased fat mass and

improved whole-body metabolic homeostasis in obese mice, an effect associated with increased adipose tissue Th2 cells, eosinophils and AAM. The use of mice deficient for STAT6, a transcription factor which is essential for IL-4/IL-13 receptor-mediated signaling, allowed us to assess whether type 2 immunity induced by either SEA or ω1 is required for its metabolic effects. Remarkably, although the metabolic effects of SEA were abolished (data not shown), those of ω1 were still observed, despite the abrogation of the Th2-mediated immune response in these Stat6-deficient mice. In addition, ω1 was found to inhibit food intake in both WT and Stat6-deficient mice, suggesting that the improvement of metabolic homeostasis in insulin-resistant obese mice by plant-produced recombinant ω1 glycovariants is independent of their Th2-inducing capacities, and may be explained by brain-mediated inhibition of food intake and/or immune-independent direct interaction of ω1 with metabolic cells. Further studies are required to investigate the anorexigenic effect of ω1, which might be a specific property of this molecule since it was not described previously when mice were chronically infected with *S. mansoni* or treated with SEA (4). Thus, although the schistosome egg antigen mixture improves metabolic homeostasis through type 2 immune responses, the single molecule ω1, which is capable of inducing strong type 2 responses, improves metabolic homeostasis, but not through its effect on type 2 immune responses.

In summary, our work on helminth infections and metabolic disorders, in both human and mice, has provided valuable insights into the effects of helminths and their molecules on metabolic homeostasis (**Figure 1**). The clinical trial revealed that in helminth-infected subjects, anthelmintic treatment significantly increases IR, highlighting the need for education and prevention strategies for noncommunicable diseases such as T2D, to go hand in hand with infectious disease control measures such as mass drug administration programs. Furthermore, repetitive injections of ω1 in a pathogen-free setting were found to have a beneficial effect on metabolic homeostasis in obese mice. However, as this effect appeared independent of the Th2-inducing capacity of ω1, it questions the role of type 2 immune cells in this particular experimental model.

Immune modulation by helminths

In the subsequent chapters, we further characterized the immunomodulatory effects of helminths as these might be associated with improved metabolic homeostasis. Eosinophilia is a well-known hallmark of helminths, and considering the presumed role of WAT eosinophils in maintaining whole-body metabolic homeostasis, we assessed the effect of STH infections on the activation status and responsiveness of eosinophils. In addition, neutrophil activation was analyzed as, despite a recent study showing enhanced neutrophil activation in subjects infected with helminths (19), little is known about the role of neutrophils during helminth infections. **Chapter 5** describes a field-applicable method that was developed to monitor changes in the expression of certain granulocyte surface molecules by flow cytometry, in order to assess granulocyte activation. As we were limited

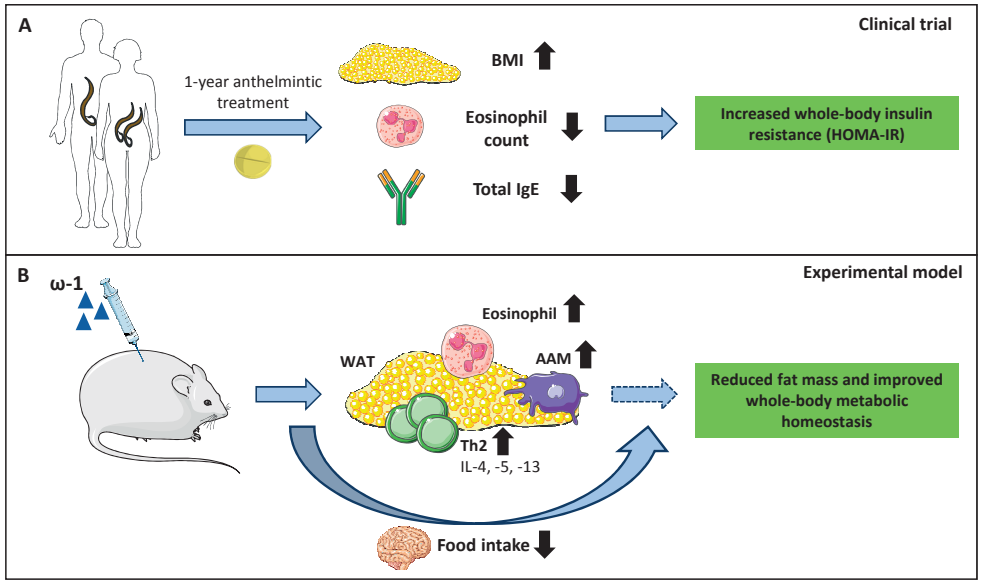

Figure 1. Summary of findings demonstrating the causal relationship between helminths or the helminth-derived molecule ω1 and whole-body metabolic homeostasis. This figure summarizes the results described in chapter 3 & 4. A) Intensive anthelmintic treatment in an STH-endemic area significantly reduced both STH infection prevalence and intensity, as well as its related type 2 immune responses (eosinophil counts and total IgE levels), while BMI increased. Deworming did not lead to an increase of whole-body IR at the community level, but it increased IR among those with a microscopy-detected STH infection. B) Treatment of obese mice with plant-produced recombinant ω1 glycovariants decreased fat mass and improved whole-body metabolic homeostasis, independent of its Th2-inducing capacity (indicated by the arrows with dashed lines), and may be explained by brain-mediated inhibition of food intake (indicated by the arrow with uninterrupted lines). BMI, body mass index; AAM, alternatively activated macrophages; WAT, white adipose tissue.

by the resources at the field study site with no direct access to a flow cytometer, we set up a method to analyze granulocyte activation markers in whole-blood samples that were lysed, fixed and cryopreserved. Although marker intensities varied when comparing fresh and fixed granulocytes, most likely due to intracellular staining and increased eosinophil autofluorescence as a consequence of fixation, it was shown that the responsiveness to stimuli could still be clearly measured after fixation.

We applied this method in our clinical trial (chapter 2) and collected samples for flow cytometric analysis from a subset of 300 subjects, before and after one year of albendazole treatment. **Chapter 6** describes that, although anthelmintic treatment effectively reduced the prevalence of helminth infections and circulating eosinophil numbers (similar to the results described in Chapter 3), helminths did not affect the activation status, nor responsiveness of eosinophils and neutrophils as assessed by the expression of activation markers (CD11b, CD35, CD69, CD66b and CD62L). Serum levels of eosinophil granule proteins represent another measure of eosinophil activation, as these cytotoxic proteins

are released upon eosinophil degranulation. Whereas the levels of major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) were not different between helminth-infected and uninfected subjects at baseline, treatment significantly reduced the levels of EDN in those infected at baseline. In contrast to previous studies (19-26), the findings in this chapter indicate that STH infections do not induce increased eosinophil activation, although they do increase eosinophil numbers. However, it should be noted that this applies to eosinophils circulating in the peripheral blood, and it might be different at the site of infection or in the case of tissue-residing eosinophils. This study is the first placebo-controlled trial studying the effect of helminth infections on granulocyte activation and derives strength from its large sample size. Nevertheless, future studies, preferentially analyzing fresh instead of fixed eosinophils, are needed to investigate this further.

Besides eosinophilia, the type 2 response induced by helminths is also characterized by high levels of IgE and elevated signature cytokines such as IL-5. In **chapter 7** we assessed the effect of anthelmintic treatment on these two different components - total plasma IgE levels and IL-5 responses to mitogen PHA - of the Th2-mediated response, by analyzing immunological data obtained from a previously conducted randomized placebo-controlled trial of three-monthly single-dose albendazole treatment in an area highly endemic for STH (27). Remarkably, we showed that two years of anthelmintic treatment might have differential effects on different arms of Th2-mediated responses. Whereas IgE levels significantly declined in albendazole-treated subjects, IL-5 production by peripheral blood cells in response to PHA was not significantly affected by the treatment and rather tended to increase. The latter could be described as reversed T-cell hyporesponsiveness, a phenomenon which has been associated with chronic helminth infections and is thought to be mediated by Tregs (28). However, in **Chapter 8** (see below), we observed a significant reduction in PMA+ionomycin stimulated cells producing IL-4/IL-5/IL-13 after albendazole treatment, which supports the decrease seen in total IgE, yet it contrasts with the increased IL-5 production in PHA-stimulated whole blood. A combination of higher responsiveness to PHA, a different composition of cells in whole blood producing IL-5, and the stimuli used could account for this anomaly. This raises the question whether different types of Th2 cells are involved in various arms of the type 2 response.

As the sample collection during our field study progressed, so did the technology to study immune cells, and in **chapter 8** we used a recently developed platform termed mass cytometry (cytometry by time-of-flight; CyTOF) to get a better understanding of immune modulation by helminths and to identify specific cells that might be important in this process. Mass cytometry is an advanced form of flow cytometry that allows single-cell analysis of more than 40 different cellular markers, without the need for spectral compensation (29). Together with advances in computational analysis approaches such as Hierarchical Stochastic Neighbor Embedding (HSNE) (30), implemented in Cytosplore (31, 32), mass cytometry has emerged as a powerful tool to dissect the cellular composition of the immune system at a single-cell level.

We performed unbiased immune profiling of Indonesians who were infected with STH, before and 1 year after 3-monthly anthelmintic treatment, as well as of healthy European volunteers who had not been exposed to helminths. Expanded populations of total Th2 cells and ILC2s, but not Tregs, were found in STH-infected Indonesians compared to Europeans. After deworming, the frequency of Th2 cells significantly decreased and this was marked by a decrease in $CD161^+$ Th2 cells, a subpopulation which might be identical to previously described pathogenic effector Th2 cells (33). Interestingly, whereas anthelmintic treatment did not affect the frequency of ILC2s, the proportion of ILC2s producing type 2 cytokines declined after treatment, indicating decreased functional activity. Whereas ILC2s represent the innate source of type 2 cytokines, little is known about their role in human helminth infections and this is partly due to their low frequencies in peripheral blood. Here, we demonstrated a helminth-induced expansion of ILC2s in blood and future studies are needed to investigate their relative contribution to the different effector arms of the type 2 immune response.

Although the frequency of total Tregs was similar in Europeans and Indonesians, the use of mass cytometry allowed us to investigate the heterogeneity within Tregs. A significantly expanded population of $CTLA4^+$ Tregs was identified in STH-infected Indonesians compared to Europeans, which decreased after deworming. CTLA4 is a molecule which is crucial for the suppressive function of Tregs (34) and interestingly, further analysis showed that this $CTLA4^+$ subpopulation contained clusters expressing HLA-DR, CD38 and/or ICOS, all markers which have been associated with distinct Treg capacities and modes of suppression (35-37). Taken together, this indicates that helminths induce a particular Treg phenotype which could be represented by cells with increased regulatory capacity.

The use of mass cytometry also enabled us to identify rare cell populations such as type-2 cytokine producing $CD8^+$ T cells (termed Tc2 cells) and γδ T cells, subsets which were expanded in STH-infected Indonesians but did not change after treatment. Moreover, IL-10 producing B cells (regulatory B cells; Bregs) were identified and $CD11c^+$ B cells were shown to be the main IL-10 producers among B cells in Indonesians, a subset which is almost absent in Europeans.

Collectively, these results provide us with a detailed insight into the specific cell populations that participate in the type 2 and regulatory networks, and show that treatment of helminths affects specific cell subsets in these networks. In addition, we demonstrated that the combined use of mass cytometry and HSNE allowed us to identify rare cell populations in blood, such as ILC2s, Tc2 cells and Bregs.

In summary, the data presented in chapter 6, 7 and 8 have shed light on the immunomodulatory effects of helminths involving different components of the immune response. Whereas chapter 6 focused on eosinophil activation, chapter 7 studied the effects of anthelmintic treatment on IgE levels and the production of IL-5 in response to PHA, followed by a detailed characterization of circulating Th2 cells, ILC2s and Tregs before and after deworming in chapter 8. Regarding the beneficial effects of helminth infections on metabolic homeostasis, our work showed that $CD161^+$ Th2 cells, $CTLA4^+$ Tregs and ILC2s are of particular interest and enhancement of these populations might reduce systemic inflammation, and hence should be further analyzed. Whereas the relative contribution of each cell type to the beneficial effects of helminth infections in humans remains unknown, the helminth-induced regulatory network involving Tregs is thought to play a key role. However, experimental studies describing the effects of helminth-derived molecules on Tregs residing in WAT have shown inconclusive results. Previous work showed that treatment with ω1 (6), as well as with filarial antigens (5), resulted in increased Treg abundance in WAT of obese mice, while we demonstrated no change in WAT Treg frequencies following repetitive injections with ω1 (chapter 4). Moreover, a single footpad injection of ω1 increased both the percentage of total Tregs and of $CTLA4^+$ Tregs in the draining lymph nodes of non-obese diabetic (NOD) mice, indicating the potential of helminth-derived molecules to upregulate the suppressive function of Tregs (38). Therefore, further studies are needed to elucidate the functional role of Tregs in WAT in the context of helminth infections and metabolic homeostasis.

DIRECTIONS FOR FUTURE RESEARCH

Effects of deworming on type 2 and regulatory immune responses: Part II

Recent advances in cellular immunology methodologies and computational analysis approaches have now enabled detailed profiling of the immune system. However, mass cytometry is currently still limited by its slow throughput compared to flow cytometry. When considering large scale clinical trials, the application of this technique therefore provides the point of departure for larger studies. By using flow cytometry, future studies can further explore the findings described in chapter 8 in a larger subset of the study population, including placebo-treated individuals, to assess whether the observed effects can be truly attributed to the removal of helminths and to investigate if changes in the immune profile are reflected in clinical outcomes (e.g. changes in HOMA-IR). In addition, cellular assays can be performed to further investigate the function of particular cell populations. For example, suppression assays in which Tregs and T effector cells are co-cultured in the presence of blocking antibodies, can be used to evaluate the suppressive function of Tregs expressing CTLA4 and/or HLA-DR and/or CD38 and/or ICOS that were found to be expanded in STH-infected Indonesians.

To assess the effect of deworming on the cytokine production of immune cells, cells need to be stimulated *ex vivo*. Whereas stimulation with PMA+ionomycin (used in chapter 8) is very strong, non-specific, and not even mediated via the T cell receptor complex, stimulation with helminth-antigens is more physiological and will provide insight into the function of antigen-specific T cells. For example, stimulation with schistosomal antigens (schistosome soluble egg (SEA) and adult worm antigens (AWA)) was previously used to investigate the adaptive immune responses in *Schistosoma haematobium*-infected schoolchildren (39, 40). However, up to now, it has been challenging to isolate STH-derived antigens which can induce detectable cytokine responses.

Controlled human infections

Although longitudinal field studies in areas endemic for helminths provide a unique opportunity to study the real-life biological settings of infection, the interpretation of the results is complicated due to the presence of other infections that could be affected by treatment, the chance of reinfection and the observation that protective effects of helminths might persist long after treatment. Meanwhile, controlled human infections (CHI) in which healthy volunteers are experimentally infected with helminths, have generated much interest as a complementary approach to study the host-pathogen interaction in a very specific and controlled manner (41). CHI models will allow the characterization of the dynamics of immune responses that develop to helminth infections in a highly controlled setting, and will enable the assessment of helminth-induced effects on metabolic homeostasis. Moreover, these models can be used to study the therapeutic potential of helminth-derived molecules.

Another advantage of the CHI model compared to a field setting where clinical resources are limited, is the possibility to obtain human adipose tissue biopsies from helminth-infected individuals. Although experimental infections with helminths or helminth-derived molecules have shown that the type 2 immune pathways in WAT have protective roles that support maintenance of metabolic homeostasis, little is known about the effects on the immune cell composition of human WAT. However, additional insights on tissue-resident cells might be more relevant for the control of metabolic homeostasis, compared to circulating immune cells which are most often studied in helminth-infected individuals. Previous work identified ILC2s in human adipose tissue and found that frequencies are lower in obese compared to non-obese individuals (42). The same was observed for Tregs, whose frequencies appear to decrease in obese individuals (13). It would be very interesting to study these immune cell frequencies in WAT of obese individuals in the setting of a controlled helminth infection, the hypothesis being that type 2 and regulatory immune cells will expand after infection, thereby promoting insulin sensitivity. Moreover, the use of WAT biopsies would allow the study of macrophages, cells that are absent in peripheral blood but have shown to play a key role in the association between helminths and metabolic homeostasis.

Although white adipose tissue is the most studied organ in terms of immune-metabolic interactions in the context of obesity and metabolic disorders, it will also be interesting to explore the impact of helminths and helminth-derived molecules on other metabolic tissues such as brown adipose tissue, pancreas, liver, brain, muscle and intestine. These (predominantly rodent) studies will provide a deeper understanding of how the immune and metabolic systems interact to support metabolic homeostasis. To this end, novel technologies such as imaging mass cytometry (43), which couples high-density analysis by mass cytometry to conventional histology, could be used to study cell interactions in the context of the tissue microenvironment.

Gut microbiome

Besides alteration of the immune response, changes in the composition of the gut microbiome might be another possible helminth-mediated mechanism associated with improved metabolic homeostasis. Although a recent study reported the impact of human gut microbiome on insulin sensitivity (44) and chronic *Trichuris muris* infection was shown to alter the host microbiota *in vivo* (45), there have been no studies specifically studying the interaction between helminth infections, gut microbiome and insulin resistance. Interestingly, in mice, helminth infections are associated with an increase of intestinal short-chain fatty acids (SCFA) (46), the end products of dietary carbohydrates fermentation, which have been shown to play an important role in the control of body weight and insulin sensitivity (47).

Despite the growing interest in the analysis of the composition of the human gut microbiome, sample collection, storage techniques and bacterial DNA extraction methods are key steps required for the accuracy of these studies (48). While immediate freezing of fecal samples at -20˚C or below is considered the gold standard for microbiome preservation, this approach is not feasible for many field studies in remote areas (49). Studies showed that long-term sample storage at room temperature or multiple freeze-thaw cycles alters microbial community stability, while preservation methods improve stability but could alter community structure (50). Therefore, the microbiome field urgently needs a sampling method which can be readily applied outside of a clinical or well-equipped environment and which produces an accurate representation of the microbiota composition (48). Recently developed commercial kits such as OMNIgene GUT (DNA Genotek), allowing storage of fecal samples at room temperature, are now available and these might be suitable for large-scale field studies since there is no need for cold-chain transportation.

CONCLUDING REMARKS

This thesis has contributed to further understanding of immune modulation exerted by soil-transmitted helminths and how this can affect the development of type 2 diabetes. Every experimental study design has its pros and cons, and whereas this work has been based on a large-scale field study conducted in a rural area of Indonesia and an animal model of HFD-induced obese mice, the implementation of the controlled human infection model is expected to provide complementary insights into the mechanisms underlying the beneficial effects of helminths on metabolic homeostasis. Moreover, by investigating the effects of helminth-derived molecules on the immune response, future studies may offer new insights towards the development of novel therapeutics for the treatment of metabolic disorders.

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