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EFFECTS OF INTESTINAL WORMS ON SKIN IMMUNITY AND CONTROL OF CO-INFECTION

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Cover image: Adult *H. polygyrus* worms. Photo by Nuno Sousa and edited by me.

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Effects of intestinal worms on skin immunity and control of co-infection

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Till mina och alla andra Vinhaggor där ute

ABSTRACT

Intestinal helminth infections remain a major health concern in developing areas of the world. Consequences of infection range from gastrointestinal discomfort to systemic manifestations. It has been suggested that individuals infected with intestinal helminths are more susceptible to other infections and mount weaker immunity to vaccination. Regions heavily burdened by intestinal helminths geographically coincide with areas plagued by infections with mycobacteria and the protozoan parasite *Leishmania spp*, causing tuberculosis (TB) and leishmaniasis, respectively. Further, it has been reported that people carrying intestinal worms mount weaker immune responses to the intradermally administered tuberculosis vaccine *Mycobacterium bovis* Bacillus Calmette Guérin (BCG). Intestinal helminth infections induce type 2 responses that aid in the expulsion of the worms, but also regulatory responses that facilitate chronicity of the worm infection. Both type 2 and regulatory responses are known to counteract type 1 immune responses required for protection against the intracellular pathogens mycobacteria and *Leishmania*, suggesting that worm infection may dampen protection to these infections. Yet, little is known about the systemic implications of intestinal worm infection, and the aim of the work in this thesis was to investigate the effects of intestinal helminth infection on skin immunity and control of co-infection.

To this end, mice were infected with the strictly intestinal nematode *Heligmosomoides polygyrus*, and at various time points after infection subjected to secondary infection, immunization, or were culled. At the end of each experiment, composition and function of immune cells in skin, skin-draining lymph nodes (LNs), liver, spleen and other tissues involved in responses to secondary infection, were analysed.

In Paper I, we found that mice infected with *H. polygyrus* were more susceptible to systemic infection with BCG and skin infection with *Leishmania major*. Increased susceptibility to BCG was accompanied by weaker IFN-γ production and fewer mycobacteria-specific transgenic p25 cells in spleen, and less *inos* expression and granuloma formation in livers. Delayed type hypersensitivity responses (DTH) induced in ears to BCG and *L. major*-derived antigens were dampened. Dendritic cell (DC) migration from footpad skin to the draining LN was reduced in worm-infected mice, as well as in mice where the footpad skin had been pre-conditioned with either *H. polygyrus* excretory-secretory (HES) products or recombinant human transforming growth factor β (TGF-β). *In vitro*, BCG-induced IFN-γ production by mycobacteria-specific T cells was reduced by HES, soluble worm antigens, or by TGF-β. This led us to hypothesize that *H. polygurus*-induced reduction of immunity to the T helper cell type 1(T_H1)-controlled organisms mycobacterium BCG and *L. major* was mediated by enhanced TGF-β production in worm-infected mice.

In Paper II, we saw that (similar to the situation with *H. polygyrus* – BCG co-infected animals) worm-infected mice were more susceptible to systemic infection with the T_H1-controlled pathogen *Leishmania donovani.* Reduced protection was accompanied by lower *inos* levels and granuloma formation in livers and higher *il10* levels in spleens.

In Paper III, we sought for the explanation to the weaker skin immunity seen in worm-infected mice (in Paper I). We found that mice infected with *H. polygyrus* had substantially smaller skin-draining LNs compared to worm-free animals. Both T cell and B cells were fewer, whereas no significant difference was observed in myeloid and stromal cell populations. As mentioned, numbers of DCs migrating from BCG-injected skin as well as p25 cells were less in skin-draining LNs of worm-infected mice. Notably however, numbers were directly proportional to the total number of cells in that particular LN. This led us to hypothesize that cells enter or are retained in an LN dependent on the original size of that node. As oppose to the atrophic skin draining LNs, the gut-draining mesenteric LNs were instead (as expected) dramatically increased in size. The lymphocyte pool cannot expand without limitation, and we suggested that worm-induced expansion of one LN occurred at the expense of other LN. Removal of worms restored the sizes of the non-draining nodes. However, this took time, since (according to out hypothesis) the atrophy of skin draining LNs and hyperplasia of mesenteric LN in itself decreased or increased infiltration or retention of cells into the respective nodes, maintaining this new "homeostasis".

In the last paper, Paper IV, we proceeded by investigating immune cells in the skin itself after *H. polygyrus* infection. We found that mice infected with *H. polygyrus* had fewer CD4+ cells producing IFN-γ in ear skin injected with whole cell lysate (WCL) from *Mycobacterium tuberculosis* in response to mycobacteria-specific *ex-vivo* re-stimulation, compared to worm-free mice. IFN-γ production was also lower in the contralateral, untouched ear. Interestingly however, the total number of CD4+ cells were higher in ear skin of worm-infected mice. CD4+ T cell numbers were also higher when comparing *H. polygyrus*-infected and non-infected animals without any skin stimulation, indicating that the intestinal infection, in itself, caused accumulation of CD4+ T cells in the skin. We found that the accumulated CD4+ T cells responded to *H. polygyrus* antigen by producing T_H2 associated cytokines and that they remained in the skin for several weeks after removal of worms from the intestine. In accordance, skin-homing chemokine receptors were up-regulated on CD4+ T cells in the mesenteric LNs and blood. We hypothesized that the increased number of T_H2 cells in the skin, in concert with the atrophy of skin draining LNs, were responsible for the lower protection to T_H1-controlled organisms in the skin.

In conclusion, mice chronically infected with the strictly intestinal nematode *H. polygyrus* were more susceptible to systemic and skin infection by T_H1-controlled organisms compared to worm-free mice. We suggest that less *inos* and granuloma formation contributed to lower protection to systemic infection and that a combination of atrophic skin-draining LNs and increased numbers of T_H2 cells in the skin caused weaker skin immunity. Taken together, this indicates that deworming may increase protection against secondary infection and increase beneficial effects of BCG vaccination.

FOR ALL NON-INFECTION IMMUNOLOGISTS / FÖR ALLA ICKE-INFEKTIONSIMMUNOLOGER

ENGLISH

In the first draft of this text, I began by painting a picture of what my PhD defence party would have been like if I had graduated 100 years earlier. We would wear fringed dresses and cloche hats, do the Charleston, carry beaded flapper bags – and many of us would also carry a bundle of parasitic worms in our intestines. Several would have tuberculosis bacteria in their lungs, and the constant threat of malaria would still be upon us. Some that saw me register as a PhD student would not be there for the graduation, as they would have succumbed to the Spanish flu, a virus believed to have killed more people than the world war raging in Europe at the same time. The purpose of this introduction was to give people a glimpse of what a society strongly affected by infectious diseases was like; to describe a time when infections had a major impact on our daily lives and were a constant threat to our health and survival. And Stockholm in the roaring twenties was such a society.

In the six months since that first draft was penned, another pandemic has showed us that also our modern society is highly vulnerable to infectious diseases, that they are not just a threat of the past. But unlike most written on infections right now, this thesis is not about Covid-19. Instead it is about those intestinal worms and other infections we would have had at that party in Stockholm 100 years ago. The infectious diseases we have basically eradicated in the West today – but which are still present in developing areas of the world, and thereby for most people living on this planet today.

Many here in Sweden may have encountered small and harmless pinworms during kindergarten or primary school, but other than that, we are nowadays basically free from worm infections. In low-income countries the situation is very different. It is estimated that one fourth of the human population is infected with intestinal worms, highly concentrated to children in developing areas of the world. The most common species alone, “the large roundworm”, currently infects almost one billion humans. The large roundworm can reach 0.6 cm in thickness and 30 cm in length and can (like many other worm species) cause intestinal symptoms, but also have outspread consequences for the infected person. For example, intestinal worms may cause problems with iron deficiency, stunted growth and reduced school performance. Most importantly for this thesis, they can also influence the immune system in a way that could change an individual’s ability to respond properly to vaccination or to protect themselves against other infections.

Tuberculosis is rare in Sweden today, but still kills around 1.5 million persons every year in developing countries, and one third of the human population is latently infected with the bacteria causing tuberculosis. Just as scientists today struggle to find a vaccine against Covid-19, at the time of my fictional defence party, Albert Calmette and his assistant Camille

Guérin were at the Pasteur Institute in Paris finalizing their development of a vaccine against tuberculosis. They named their invention after themselves: Bacillus Calmette Guérin – or BCG, and 100 years later BCG remains the only vaccine we have against tuberculosis. BCG is the most used vaccine in the history of humankind and is still the first injection most newborn babies around the world experience. In Sweden we removed BCG from the national vaccine program in the 1970's, but most Swedes born before then still carry the whitish scar on their upper arm as a memory of a time when tuberculosis was a major cause of death here as well. BCG is one of few vaccines still used today that actually contains live, although weakened, bacteria. However, it is debated whether or not BCG even protects against tuberculosis. When scientists are investigating the matter, some reports show that as many as 80% of individuals receiving the vaccine are protected, whereas others show that it has no protective effect whatsoever. There are many hypotheses as to the reason behind these diverging results, and the outcomes of the studies partially seem to depend on where in the world the study is performed. As it happens, the areas where BCG does not seem to work are the same areas where intestinal worms are still common. In addition, researchers have seen that humans infected with intestinal worms actually do not get as good result from a BCG vaccination as a worm-free person.

The reasons behind this observation are not fully known. A common hypothesis involves the fact that our immune system is divided into two major parts directed by two types of T cells, called T_H1 and T_H2. These two parts each consists of a bunch of cells and molecules specialized to combat a specific type of infection. T_H1 fights bacteria and parasites that want to live inside of our cells. The tuberculosis bacteria, as well as the BCG vaccine bacteria, belong to this category. The T_H2 section instead takes care of infectious organisms too big to fit inside a cell, such as intestinal worms. Interestingly, T_H1 and T_H2 do not only fight different infections, they also fight each other, and a good balance in between them is required for the immune system to function properly. In addition, a third fraction called T_{REG} dampens (regulates) both T_H1 and T_H2, and T_{REG} is also induced during intestinal worm infection.

The aim of my PhD work was to investigate if the immune reactions induced by intestinal worm infections (T_H2 and T_{REG}) could change the whole immune system of the host so that they would be less protected against other infections or be more difficult to vaccinate. We have focused on infections where the body need T_H1 reactions for protection or for initiating a vaccine response, and those infections that affect a separate part of the body than the gut where the worm is located. The BCG vaccination is delivered in the skin, and because of that we were also especially interested in investigating what happens in the skin after an intestinal worm infection. As mentioned, we were already aware that humans infected with worms could get weaker immune reactions to the BCG vaccine, but we wanted to understand the mechanism behind this observation. When investigating mechanisms behind a phenomenon that includes the whole body, animal experiments must be used, since many tissues are not possible to collect from humans for analysis. Although controversial, using animals for

medical research is still our main tool to understand how our bodies work. Animal experiments have contributed enormously to the development of drugs and vaccines that have saved millions of human lives. Mice are the most commonly used animal since they are small, easy to handle, and reproduce fast, yet, because they are mammals, their molecules, cells and tissues are quite similar to ours.

In our lab, we infect mice with an intestinal parasitic worm called *Heligmosomoides polygyrus* to imitate intestinal worm infection in humans. *H. polygyrus* is common in mice living in the wild, and the mice do not get noticeably sick from the infection. We infect the mice orally with the larval stage of the worm, and the larvae then migrate to the intestine of the mice where they develop into adult worms.

In **Paper I**, we confirmed that mice – like humans – had weaker immune responses to the tuberculosis vaccine BCG when they were infected with worms. We also saw that worm-infected mice were less protected against another infection by a small parasite that infects the skin, called *Leishmania major.* Infection with *L. major* causes lesions in the skin that may last for several months, and sometimes even years. *Leishmania* parasites are common in low-income areas of the world where they are spread by a tiny fly called the sandfly. Like the tuberculosis bacteria, *Leishmania* parasites live inside of cells, hence, the body uses T_H1 immunity to fight *Leishmania* infections.

There are several different types of *Leishmania* parasites, they all live inside of cells, but in different parts of the body. **Paper II** is about how intestinal worms affect immune responses to another type of *Leishmania*, causing so called visceral leishmaniasis and which attacks our inner organs. Visceral leishmaniasis (or Kala-azar, the "Black disease" in Hindi) is a deadly disease that kills 300,000 people every year in tropical regions, many of them in the north eastern parts of India where I travelled to a few times during my PhD. Visceral leishmaniasis is one of the most lethal infections existing in the world with almost 100% death rate without treatment, beating Ebola, HIV and malaria. Still the disease is unknown to most people living in the Western world, and is (along with intestinal worms) categorized by the WHO as a "Neglected tropical disease", as it receives much less research funding and attention considering the death and suffering *Leishmania* causes. Visceral leishmaniasis can be caused by the *Leishmania* species *Leishmania donovani.* In Paper II we saw that mice that we had infected with intestinal worms were less protected against *L. donovani* with more *Leishmania* parasites in their liver and spleens compared to the mice without worms. As we expected, mice infected with worms had weaker T_H1 responses in the infected organs.

In **Paper III**, we aimed to understand the mechanism behind why mice infected with worms had weaker immune reactions to BCG. Surprisingly, we found a completely novel mechanism behind this, not including the T_H1 and T_H2 responses described earlier. When a person or mouse gets the BCG vaccine injected into their skin, the lymph nodes close to the skin are vital in initiating an immune reaction to the bacteria, a reaction that will prepare the

body for a future tuberculosis infection. Surprisingly, we found that, in mice infected with intestinal worms these lymph nodes were smaller with fewer T cells, compared to lymph nodes of worm-free mice. Hence, we found a new mechanism that could explain why people infected with worms are more prone to having tuberculosis.

We then wanted to see what was going on in the skin itself in mice infected with worms. In **Paper IV**, we found that mice infected with *H. polygyrus* had more memory T_H2 cells in the skin, and these T_H2 cells had likely travelled from the intestine to the skin. It is commonly believed that memory immune cells mostly appear at the body site where the infection is located, so that the next time the person gets infected with the same microbe, the memory cells are in the right position, ready to protect. Hence, the reason why our results were remarkable is that the memory cells appeared in a completely different location than where the worm was situated. In addition to the smaller lymph nodes seen in paper III, we believe that these T_H2 cells in the skin may also contribute to the weaker vaccine responses to BCG seen in worm-infected mice.

The risk of us Swedes again having to deal with intestinal worms and tuberculosis is minute, and since we do not have any sandflies, we probably do not have to worry about leishmaniasis either. Yet, the coronavirus has reminded us that infectious diseases are not just something to only think about when travelling to tropical and developing regions of the world. During this spring everyone has suddenly learned about T cells and what a long time it takes for the body to make antibodies. What maybe has been surprising to many is how little scientists know about immune responses to infections, as highly esteemed professors with seemingly the same education can have completely different views on how to deal with the situation. This is to a large extent because the immune responses to infections are extremely complex and multifaceted, and even though an immense amount of ground-breaking research is constantly being conducted all over the world, are we still far away from fully understanding and being able to predict how our immune system will react to a new infection. Hopefully, the work included in this thesis will bring us one (albeit small) step closer to that goal.

SVENSKA

I mitt första utkast till den här texten, inledde jag med att måla upp en bild av hur min disputationsfest skulle ha varit om jag tog examen för 100 år sedan. Vi skulle ha på oss fransiga klänningar och klockhattar, dansa Charleston och bära pärlprydda handväskor – och många av oss skulle även bära ett nystan av parasitiska maskar i våra tarmar. Flera skulle ha tuberkulosbakterier i sina lungor och hotet från malaria skulle vara konstant närvarande. Vissa som såg mig registreras som doktorand skulle inte ens vara där vid min examen, då de hade avlidit i spanska sjukan, ett virus som tros ha dödat fler personer än världskriget som samtidigt härjade i Europa. Tanken med introduktionen var att ge en bild av hur ett samhälle starkt påverkat av infektionssjukdomar tedde sig; att beskriva en tid då infektioner hade stort

inflytande på våra dagliga liv och var ett konstant hot mot vår hälsa och överlevnad. Och Stockholm på det glada 20-talet var ett sådant samhälle.

Ett halvår efter att det första utkastet skrevs, har nu ännu en pandemi visat oss att även det moderna samhället är sårbart inför infektionssjukdomar, att de inte bara är ett hot från det förflutna. Men till skillnad från det mesta som skrivs om infektioner just nu så handlar den här avhandlingen inte om covid-19. I stället handlar den om de inälvsmaskar och andra infektioner som vi skulle ha haft på festen i Stockholm för 100 år sedan. De där infektionssjukdomarna vi i västvärlden sedan länge blivit av med – men som fortfarande är närvarande i mindre utvecklade delar av världen, och därmed också för de flesta människor som lever på den här planeten i dag.

Många här i Sverige kan ha stött på små och ofarliga springmaskar under dagis eller grundskolan, men utöver det så är vi i dag i princip helt fria från maskinfektioner i Sverige. I låginkomstländer är situationen mycket annorlunda. Det uppskattas att en fjärdedel av den mänskliga befolkningen är smittad med inälvsmask, och infektionerna är till mycket stor del koncentrerade till barn i utvecklingsländer. Den vanligaste arten, spolmask, står ensam för nästan en miljard infektioner. Spolmasken kan nå 0,6 cm i tjocklek och 30 cm i längd och kan (liksom många andra maskarter) orsaka tarmsymptom, men också ha mer utbredda konsekvenser för den smittade personen. Till exempel kan inälvsmaskar orsaka problem med järnbrist, dålig tillväxt och sämre prestation i skolan. Dessutom, och viktigast för denna avhandling: kan de påverka en persons immunsystem på ett sätt som kan förändra individens förmåga att reagera som man ska på vaccination eller förmågan att skydda sig mot andra infektioner.

Tuberkulos är sällsynt i Sverige idag, men dödar fortfarande cirka 1,5 miljoner personer varje år i utvecklingsländer och en tredjedel av den mänskliga befolkningen är latent smittad med de bakterier som orsakar tuberkulos. Precis som forskare idag kämpar för att hitta ett vaccin mot covid-19 men vid tiden för min fiktiva disputationsfest, jobbade Albert Calmette och hans assistent Camille Guérin vid Pasteur-institutet i Paris för att färdigställa utvecklingen av ett vaccin mot tuberkulos. De uppkallade sin skapelse efter sig själva: Bacillus Calmette Guérin – eller BCG, och nu, 100 år senare, är BCG fortfarande det enda vaccinet vi har mot tuberkulos. BCG är det mest använda vaccinet i människans historia och är fortfarande den första injektionen som de flesta nyfödda barn världen över upplever. I Sverige tog vi bort BCG från det nationella vaccinationsprogrammet på 1970-talet, men de flesta svenskar födda före det bär fortfarande det vitaktiga ärret på överarmen som ett minne av en tid då tuberkulos var en vanlig dödsorsak även här. BCG är ett av få vacciner som används idag som faktiskt innehåller levande, dock försvagade, bakterier. Förvånande nog efter all tid som gått, diskuteras det fortfarande ifall BCG överhuvudtaget skyddar mot tuberkulos. När forskare undersöker saken visar vissa rapporter att så många som 80% av individerna som får vaccinet är skyddade, medan andra visar att det inte har någon skyddande effekt alls. Det finns många hypoteser till orsaken bakom dessa motsägande resultat, och utfallen av studierna verkar

delvis bero på var i världen studien utförs. Som av en slump är många av de områden där BCG inte verkar fungera samma som de områden där inälvsmaskar fortfarande är vanliga. Dessutom har forskare sett att människor som är smittade med inälvsmask faktiskt inte verkar få lika bra resultat av en BCG-vaccination som en maskfri person.

Anledningarna bakom dessa observationer är inte helt klargjorda. En vanlig hypotes bygger på att vårt immunsystem är uppdelat i två huvuddelar, organiserade av varsin typ av T-cell: T_H1 och T_H2. Dessa två delar består vardera av ett gäng celler och molekyler som är specialiserade på att bekämpa en specifik typ av infektion. T_H1 bekämpar bakterier och parasiter som vill leva inuti våra celler. Tuberkulosbakterierna såväl som BCG-vaccinbakterierna tillhör denna kategori. T_H2-delen hanterar istället infektiösa organismer som är för stora för att få plats inne i en cell, såsom inälvsmaskar. Intressant nog bekämpar T_H1 och T_H2 inte bara skilda infektioner, de bekämpar också varandra, och det krävs en bra balans mellan dem för att immunsystemet ska fungera som det ska. Dessutom dämpar (reglerar) en tredje fraktion som kallas T_{REG} både T_H1 och T_H2, och T_{REG} framkallas också av inälvsmaskinfektion.

Syftet med mitt doktorsarbete var att undersöka ifall immunreaktionerna framkallade av inälvsmask (T_H2 och T_{REG}) kan förändra värdens hela immunsystem så att de skulle vara mindre skyddad mot andra infektioner eller vara svårare att vaccinera. Vi har fokuserat på infektioner där kroppen behöver T_H1-reaktioner för skydd, och på de infektioner som påverkar en separat del av kroppen än tarmen (där masken finns). BCG-vaccination ges i huden, och därför var vi särskilt intresserade av att undersöka vad som händer i huden vid inälvsmaskinfektion. Som nämnts visste vi redan att människor infekterade med maskar kunde få svagare immunreaktioner vid BCG-vaccination, men vi ville förstå mekanismen bakom detta för att kunna göra något åt det. När man undersöker mekanismer bakom ett fenomen som involverar hela kroppen måste man använda djurförsök, eftersom många vävnader inte är möjliga att samla in från människor för att kunna analysera. Även om det av många anses kontroversiellt är användning av djur för medicinsk forskning fortfarande vårt huvudsakliga verktyg för att förstå hur våra kroppar fungerar. Djurförsök har bidragit enormt mycket till utvecklingen av läkemedel och vaccin som har räddat miljontals människoliv. Möss är det mest använda djuret då de är små, lätta att hantera, och reproducerar snabbt. Men eftersom de är däggdjur har de flesta molekyler, celler och vävnader ganska lika våra.

I vårt labb infekterar vi möss med en inälvsparasitisk mask som heter *Heligmosomoides polygyrus*, för att imitera inälvsmaskinfektion hos människor. *H. polygyrus* är vanligt hos möss som lever i naturen, och mössen blir inte märkbart sjuka av infektionen. Vi infekterar mössen oralt med larvstadiet av masken, och larverna migrerar sedan till tarmen hos mössen där de utvecklas till vuxna maskar.

I **Artikel I** bekräftade vi att möss - liksom människor - hade svagare immunsvar mot tuberkulosvaccinet BCG när de var infekterade med mask. Vi såg också att maskinfekterade möss var mindre skyddade mot en annan infektion med en liten parasit som infekterar huden,

som heter *Leishmania major*. Infektion med *L. major* orsakar sår på huden som kan finnas kvar i flera månader, ibland till och med år. *Leishmania*-parasiter är vanliga i de fattigaste områden av världen där de sprids av en liten fluga som kallas sandfluga. Liksom tuberkulosbakterierna lever *Leishmania*-parasiter inuti cellerna, därför använder kroppen T_H1-immunitet för att bekämpa *Leishmania*-infektioner.

Det finns flera olika typer av *Leishmania*-parasiter: de lever alla inuti cellerna, men i olika delar av kroppen. **Artikel II** handlar om hur inälvsmaskar påverkar immunsvaret mot en annan typ av *Leishmania* som orsakar så kallad visceral leishmaniasis och som istället angriper våra inre organ. Visceral leishmaniasis (eller Kala-azar, den "svarta sjukdomen" på hindi) dödar 50 000 – 90 000 människor varje år i de fattigaste delarna av världen, många av dem i nordöstra delen av Indien vartill jag har rest några gånger under min doktorandtid. Visceral leishmaniasis är en av de mest dödliga infektionerna som finns i världen med nästan 100% fatalitet utan behandling, vilket slår ebola, HIV och malaria. Ändå vet de flesta människor som lever i västvärlden inte ens om att den finns. Visceral leishmaniasis är (tillsammans med inälvsmask) kategoriserad av WHO som en "försummad tropisk sjukdom", eftersom den får väldigt lite forskningspengar och uppmärksamhet proportionellt till hur mycket död och lidande den orsakar. Visceral leishmaniasis kan orsakas av *Leishmania*-arten *Leishmania donovani*. I artikel IV såg vi att möss som vi hade infekterat med *H. polygyrus* inte var lika bra på att skydda sig mot *Leishmania donovani* och hade fler *Leishmania*-parasiter i sin lever och mjälte jämfört med möss som inte hade mask. Och som vi anade hade möss som vi infekterat med mask svagare T_H1-svar i de infekterade organen.

I **Artikel III** försökte vi förstå mekanismen bakom varför möss infekterade med maskar hade svagare immunreaktioner mot BCG. Vi hittade en helt ny mekanism bakom detta, oberoende av de T_H1- och T_H2-celler som beskrivits tidigare. När en person eller mus får BCG-vaccinet injicerat i huden är lymfkörtlarna nära huden viktiga för att starta en immunreaktion mot bakterierna och därmed för att förbereda kroppen på en framtida tuberkulosinfektion. Överraskande nog såg vi att hos möss infekterade med inälvsmaskar var dessa lymfkörtlar betydligt mindre med färre T-celler jämfört med lymfkörtlar hos maskfria möss. Därmed hittade vi en ny mekanism som kan förklara varför människor smittade med maskar är mer benägna att få tuberkulos.

Vi ville sedan se vad som händer i själva huden hos möss infekterade med inälvsmask. I **Artikel IV** fann vi att möss infekterade med *H. polygyrus* hade fler minnesceller av T_H2-typ i huden, och att dessa T_H2-celler troligen hade färdats från tarmen till huden. Det antas att minnes-T-celler oftast uppkommer på samma ställen i kroppen som där infektionen är belägen, så att nästa gång personen smittas av samma mikrob, finns minnescellerna redan på rätt plats. Orsaken till att våra resultat var anmärkningsvärda att alltså att minnes-T-cellerna dök upp på en helt annan plats än där masken fanns. Utöver de mindre lymfkörtlarna från artikel II tror vi att dessa T_H2-celler i huden också kan bidra till det svagare vaccinsvaret mot BCG vi ser hos maskinfekterade möss.

Risken för att vi svenskar återigen skulle behöva hantera inälvsmaskar eller tuberkulos är väldigt liten, och eftersom vi inte har några sandflugor behöver vi förmodligen inte heller oroa oss för leishmaniasis. Däremot har coronaviruset påmint oss om att infektionssjukdomar inte bara är något man behöver tänka på när man reser till tropiska och mindre utvecklade delar av världen. Under våren har alla i Sverige plötsligt lärt sig vad T-celler är och hur lång tid det tar för kroppen att göra antikroppar. Men det som kanske har varit mest förvånande för många är hur lite forskare faktiskt vet om immunförsvar mot infektioner, då högt uppsatta professorer med till synes samma kunskap har haft helt olika åsikter om hur vi bäst ska hantera situationen. Detta beror mycket på att vårt immunsystem är extremt komplext och mångfacetterat, och även om en enorm mängd banbrytande forskning görs hela tiden över hela världen, är vi fortfarande långt bort från att förstå allt och att kunna förutsäga hur vårt immunsystem kommer att reagera på en ny infektion. Förhoppningsvis har arbetet bakom denna avhandling tagit oss ett (om än litet) steg närmare det målet.

LIST OF SCIENTIFIC PAPERS

I **Chronic gastrointestinal nematode infection mutes immune responses to mycobacterial infection distal to the gut.**
Obieglo K*, Feng X*, Bollampalli VP*, Dellacasa-Lindberg I, Classon C, Osterblad M, Helmby H, Hewitson JP, Maizels RM, Gigliotti Rothfuchs A, Nylen S. **Equal contribution.*
J Immunol (2016) 196: 2262-71

II **Intestinal nematode infection exacerbates experimental visceral leishmaniasis.**
Classon C, Feng X, Eidsmo L, Nylen S.
Parasite Immunol (2019). 41: e12618

III **Atrophy of skin-draining lymph nodes predisposes for impaired immune responses to secondary infection in mice with chronic intestinal nematode infection.**
Feng X, Classon C, Teran G, Yang Y, Li L, Chan S, Ribacke U, Rothfuchs AG, Coquet JM, Nylen S.
PLoS Pathog (2018) 14: e1007008

IV **Intestinal nematode infection transforms CD4+ T cell composition of skin.**
Classon C, Li M, Tibbitt C, Ma J, Stark J, Hokka-Zakrisson C, Feng X, Cardoso R, Villablanca E, Rothfuchs A, Eidsmo L, Coquet J, Nylén S.
Manuscript.

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Resident T cells in resolved psoriasis steer tissue responses that stratify clinical outcome.
Gallais Serezal I, Classon C, Cheuk S, Barrientos-Somarribas M, Wadman E, Martini E, Chang D, Xu Landen N, Ehrstrom M, Nylen S, Eidsmo L.
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STAT3 expression by myeloid cells is detrimental for the T- cell-mediated control of infection with *Mycobacterium tuberculosis*.
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Meta-taxonomic analysis of prokaryotic and eukaryotic gut flora in stool samples from visceral leishmaniasis cases and endemic controls in Bihar State India.
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PLoS Negl Trop Dis (2019) 13: e0007444

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LIST OF ABBREVIATIONS

AAM	Alternatively activated macrophages
ADCC	Antibody-mediated cellular cytotoxicity
APC	Antigen presenting cell
BCG	Bacillus Calmette-Guérin
CCR	CC chemokine receptor
CD	Cluster of differentiation
CL	Cutaneous leishmaniasis
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DETC	Dendritic epidermal T cell
DTH	Delayed type hypersensitivity
ES	Excretory secretory
Foxp3	Forkhead box P3
GI	Gastrointestinal
HEV	High endothelial vessels
HpARI	*H. polygyrus* alarmin release inhibitor
HpTGM	*H. polygyrus* TGF-β mimic
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IFN	Inferferon
IGRA	Interferon gamma release assay
IL	Interleukin
ILC	Innate lymphoid cell
iNOS	Inducible nitric oxide synthase
LC	Langerhans cell
LP	Lamina propria
LPS	Lipopolysaccharides
MCL	Mucocutaneous leishmaniasis
MHC	Major histocompatibility complex

NLO	Non-lymphoid organs
NO	Nitric oxide
nT_{REGs}	Natural regulatory T cells
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PKDL	Post kala-azar dermal leishmaniasis
PLO	Primary lymphoid organ
PP	Peyer's patch
PPD	Purified protein derivate
PRR	Pattern recognition receptor
pT_{REGs}	Peripheral regulatory T cell

1 INTRODUCTION

A vast number of humans and animals around the world are infected with one or several species of parasitic worms, also known as helminths. Intestinal helminth infection may cause both local gastrointestinal (GI) symptoms and have systemic consequences for the host. Some of the latter involves disturbances in the balance of T helper (T_H) cell subsets – vital for a well-functioning immune system. T_H cells orchestrate the immune response in the direction appropriate for the nature of the infection, and protective immunity to helminths is mediated by T_H cell type 2 (T_H2)-driven immune reactions. In addition, regulatory responses which protects against immunopathology, but also facilitate establishment of chronic infection, are induced. There is still a substantial lack of knowledge regarding the effects intestinal worms have on our immune system (1, 2). This thesis focuses on the distal consequences by intestinal helminth infection, and more specifically, effects of intestinal worms on skin immunity and control of co-infection.

1.1 T HELPER CELLS

When pathogens first infect a host, they need to be detected, engulfed and presented by antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages, to initiate an adaptive immune response. Next, the APCs give instructions of their findings to the conductors of an adaptive immune response – the T_H cells. T_H cells orchestrate other immune cells via cytokines capable of triggering activation, inhibition, proliferation, and differentiation of the receiving cell (3). Below, the generation and function of lymphocytes, with focus on T_H cells, are outlined.

1.1.1 The lymphatic system

The lymphatic system consists of primary (also called central) lymphoid organs (PLOs), secondary (also called peripheral) lymphoid organs (SLOs), and the lymphatic vessels. PLOs include the bone marrow from where all lymphocytes originate, and the thymus, responsible for T cell maturation. In the SLOs, adaptive immune responses to new antigens are initiated. SLOs include lymph nodes (LNs) and spleen, but also tissue specific structures such as tonsils, adenoids and the Peyer's patches (PPs). The lymphatic vessels transport lymph in between lymphoid organs, blood, and non-lymphoid organs (NLOs) such as lung, fat, liver, kidneys, intestine and skin (3).

1.1.2 Generation of T helper cells

The T in T cell is short for "thymus-dependent", as progenitors of T cells (thymocytes) migrate from the bone marrow to the thymus for maturation. B cells (short for "bursa of fabricius" where B cells where first found to mature in birds) mature completely in the bone marrow in mammals. The thymus is situated above the heart and consists of two lobes, each with an outer cortex and a central medulla. Cortex and medulla are separated by the cortico-medullary region where the thymocytes enter. The newly arrived thymocytes start their

development into mature T cells in the cortex as double negative cells, expressing neither cluster of differentiation (CD) 4 nor CD8 T cell receptor (TCR) co-receptor molecules. Maturation is initiated by the rearrangement of a TCR by variable, diversity, and joining (V, D and J) recombination, which together with random nucleotide insertions and deletions provide enormous variability to the T cell repertoire. During the TCR rearrangement, the cells will start to express both CD4 and CD8, thereby becoming double positive T cells. Double positive cells will first enter positive selection, where the cells that have succeeded in creating a functional TCR molecule capable of binding self-major histocompatibility complex-(MHC)-peptide complexes are selected for survival. Depending on if the TCR binds to an MHC class II or an MHC class I molecule at this stage, the T cell will become either a CD4 or CD8 single positive T cell, respectively. Single positive cells will migrate to the medulla where negative selection takes place. In the negative selection, cells with high affinity for self-MHC-peptide complexes are eliminated in order to remove possibly autoreactive cells. Specialized stromal cells in the thymus express tissue restricted antigens from peripheral organs to prevent autoimmune reactions to these tissues as well. Cells with intermediate affinity to self-MHC-peptide complexes may instead develop into regulatory T cells (T_{REGs}, discussed in section 1.2.3). The process of negative selection results in so-called central tolerance. The great majority of T cells (around 95-97%) will fail either positive or negative selection and never leave the thymus, but instead be targeted for apoptosis. T cells that pass will leave the thymus as mature, single positive, naïve cells. Naïve T cells circulate between blood and SLOs in search for its cognate antigen presented by an APC (3-6).

During aging, the thymus degenerates and the output of new cells decreases, a process known as thymic involution. In older humans, naïve T cells are mainly replenished by proliferation, whereas in mice the great majority still stems from thymic output. Yet, thymic involution occurs in both humans and mice (7).

1.1.3 Initiation of a T helper cell response in the lymph node

Adaptive immune responses are initiated in SLOs, of which T_H cell activation in LNs is most central for this thesis. LNs are bean-shaped pale-yellow organs situated where afferent lymph vessels converge, whose function is to create a meeting hub for naïve T cells and activated DCs. LNs consist of a peripheral cortex and a central medulla, all encapsulated by the subcapsular sinuses (SCS). The cortex is divided into an outer cortex mainly comprising B cells and an inner area (paracortex) primarily consisting of T cells. The spleen has a similar organization, but filters antigens in blood instead of from NLO (3).

Most immature DCs reside in NLOs, where they sample their environment in search for potentially harmful microbes. Pathogens are recognized by expression of pathogen associated molecular patterns (PAMPs) – i.e. molecules that are prevalent on the microbe and often essential for its fitness. Detection of PAMPs is done by various pattern recognition receptors (PRRs), abundant on DCs and other APCs. The most renowned PRRs are probably the toll-like receptors (TLRs) and the nod-like receptors (NLRs), recognizing for example

lipopolysaccharides (LPS; also called endotoxin) on gram-negative bacteria, lipoteichoic acid on gram-positive bacteria, and viral nucleic acids. Binding of a PAMP to the PPR can result in phagocytosis of the PAMP-expressing microbe as well as production of cytokines, chemokines and antimicrobial substances. DCs process the phagocytosed pathogens into peptide fragments for presentation on MHC molecules, and the APC thereby becomes activated. Activation causes the DC to downregulate tissue retention markers and instead upregulate markers that will allow it entry into the LN, such as CCR7. In addition, the DC will upregulate activation markers such as CD80 and CD86 (also called B7.1 and B7.2, respectively) required to stimulate naïve T cells. Active DCs travel in the lymph to a draining LN, which they enter through afferent vessels into the SCSs and continue to the paracortex.

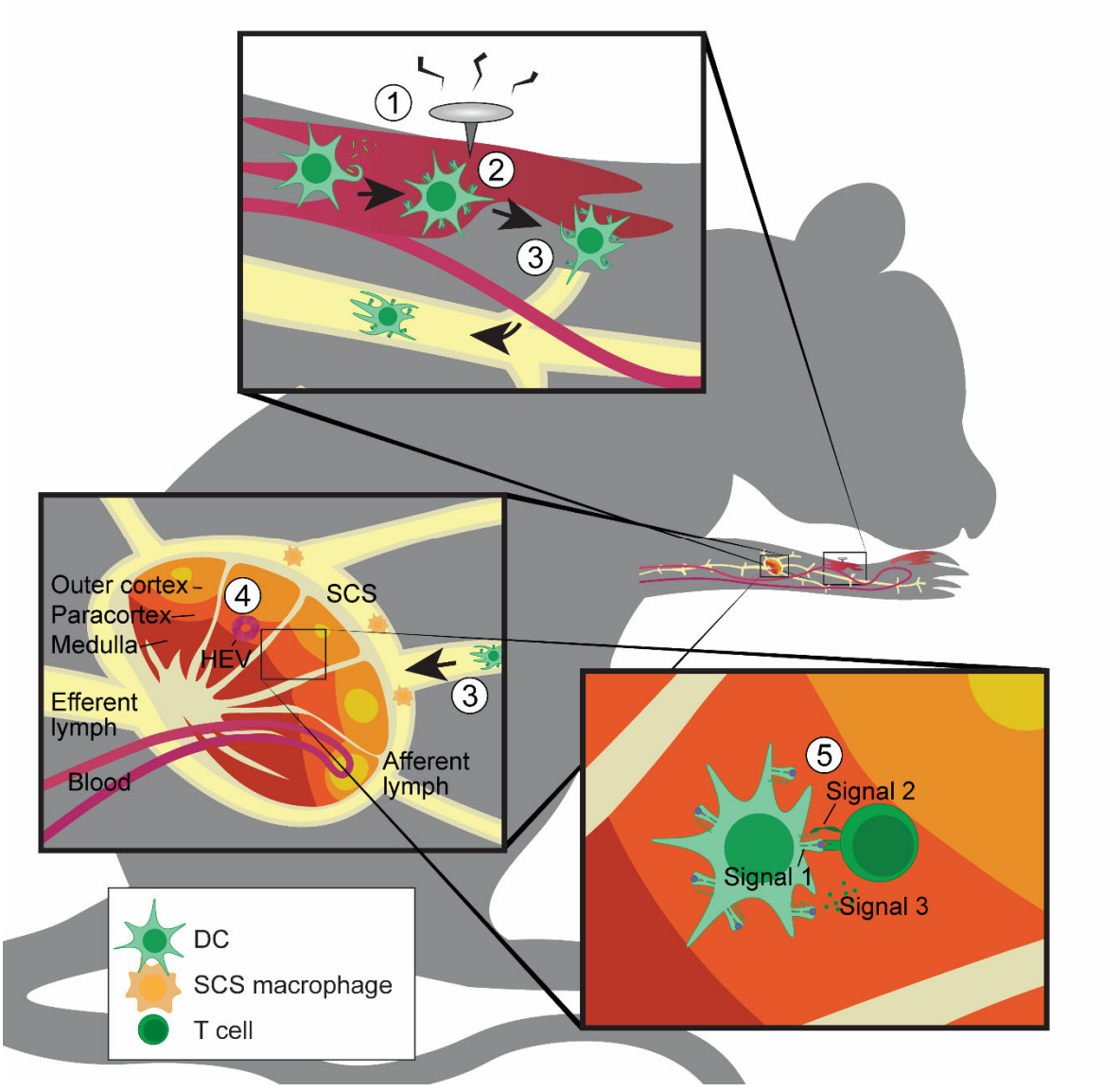


Figure 1: Antigen capture and T cell priming. After an infection, DCs engulf pathogens and process them into peptides (1), upregulate MHC-II-peptide complexes and co-stimulatory markers (2), and travel to the draining LN through afferent vessels (3). Naïve T cells enter the LN through the HEVs (4). In the paracortical area of the LN, DCs present antigens to T cells, which scans the DCs with their TCR. In the case of a match, the DC will prime the T cell (5). The MHC-II-peptide complex will then bind the TCR (signal 1) and co-stimulatory markers on the DC will bind to co-stimulatory receptors on the T cell (signal 2) which will induce cytokine production from the T cell (signal 3).

Antigens can also enter the LN free floating in the lymph and be engulfed by local DCs, macrophages, or B cells (which may induce a B cell response). Naïve lymphocytes circulating in blood instead enter the LN via the high endothelial venules (HEVs). HEV entry also leads to the paracortex, where the naïve lymphocytes meet with the activated DCs. Here the DCs present peptides on MHC-II and MHC-I to incoming CD4+ and CD8+ T cells, respectively. The entering T cells scan the peptide-MHC complexes with their TCRs. When a match is found, the T cell will become primed to activation by the DC (3).

Priming of naïve T_H cells by DCs occurs in three steps. Binding of the TCR-CD4 complex on the naïve T cell with the peptide-MHC II complex on the APC provides the signal one. Next, the co-stimulatory molecules (the activation markers) CD80 and CD86 on the DC bind to co-stimulatory receptors on the T cell, such as CD28, conveying signal two. If the DC is not properly activated and lacks co-stimulatory molecules, the T cell will become anergic (non-responsive) or tolerogenic. In addition to the activating co-stimulatory receptors, T cells also express inhibitory receptors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), that balances the signal. Further, the DC will produce cytokines of various kinds depending on which PRR it was activated via, and these cytokines are sometimes referred to as signal three. Depending on where in the body the pathogen was found and in which draining LN priming occurs, the T cell will upregulate specific chemokine receptors, promoting distinct tissue homing patterns. When primed, the T cell will proliferate dramatically by clonal expansion, differentiate into the appropriate T_H effector cell subset (discussed below) and return to the infected site to exert its effector functions (3).

1.1.4 T helper cell subsets

The functions of T_H cells are to enforce, balance and direct the immune response onto a path appropriate to control the invading pathogen and simultaneously limit tissue damage. To achieve this, T_H cells differentiate into various subsets producing distinct cytokine repertoires aiding the host to combat separate sorts of pathogens (3).

The first indication that the mammalian immune system is orchestrated by subsets of T_H cells was published in the 1980's, when Mitchell *et al* found that genetically different mice developed distinct immune responses to the intracellular protozoan parasite *Leishmania major* (8). C57BL/6 mice displayed a resistant phenotype with local skin manifestations whereas BALB/C mice were more susceptible and developed disseminated disease with rampant parasite propagation (8). That this could be attributed to different T_H cell populations with separate functionality was later demonstrated by Mosmann *et al* who named them T_H1 and T_H2 (9). T_H1 cells were seen to produce interferon (IFN)-γ and T_H2 cells to produce interleukin (IL)-4 (9). T_H1 cells and IFN-γ promoted cellular immunity that was seen to be protective against intracellular bacteria and parasites such as *L. major*, whereas T_H2 cells and IL-4 were detrimental for *L. major* control T_H2 cells and IL-4 were instead shown to mediate protective immunity to helminths (discussed in chapter 1.2.2) (10, 11). T_H1 and T_H2 cells act antagonistically on each other, as IFN-γ dampens IL-4 production and vice versa. This

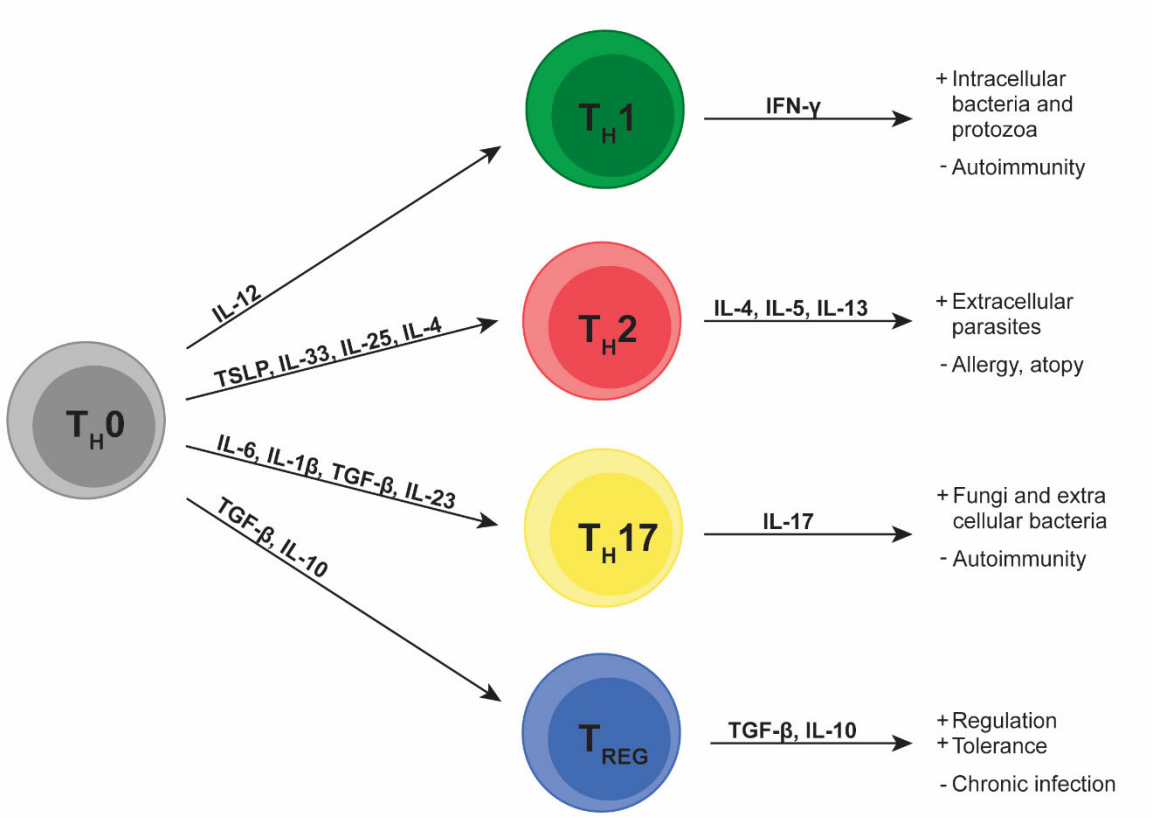


Figure 2: Differentiation into T_H cell subsets. Plus (+) indicates effector responses of the cells and minus (-) indicates common unwanted consequences.

dichotomy stood ground until early 2000 when the universe of T_H cell subtypes started to expand. The anti-inflammatory T_{REG} cells were discovered first (discussed in chapter 1.2.3) followed by the pro-inflammatory T_H17 cells producing IL-17. T_H17 cells are protective against extracellular bacteria and fungi and has been seen to promote several autoimmune conditions including psoriasis and rheumatoid arthritis (RA) (12, 13). Both T_H1 and T_H2 cells dampen T_H17 responses. IL-17A has however been seen to be important for the induction of T_H2 responses during helminth infection but to later dampen it, demonstrating the complexity of T_H cell regulation (14). Next T_H cell type to be discovered were the follicular T_H cells (T_{FH}) which were shown to orchestrate B cell responses in germinal centres. Two cell types are still under debate whether they should be considered their own subsets or included under T_H2 and T_H17 respectively, namely T_H9 and T_H22 cells. T_H9 cells promote mast cell recruitment and activation and are as T_H2 cells involved in helminth defence, whereas T_H22 cells promotes epithelial integrity (14, 15).

1.1.5 Immunological memory and vaccination

An adaptive immune response does not only protect the individual during the infection but leaves a trace of better preparedness for infection by the same pathogen, referred to as immunological memory. As previously described, when T cells are primed in the LN, effector T_H cells with appropriate functions expand to fight the ongoing infection. In addition, memory cells that will outlive the effector response develop. Memory T_H cells are typically divided into three types dependent on what tissues they patrol: central memory cells (T_{CM}s),

effector memory cells (T_{EMs}), and the most recent discovery – tissue resident memory cells (T_{RMs}). All memory (and effector) cells express the activation marker CD44 by which they can be distinguished from naïve cells. T_{CMs} circulate through blood and SLOs, and express markers such as CD62L and CC chemokine receptor (CCR) 7, allowing them to (like naïve cells) enter SLOs through the HEVs. T_{EMs} have lost expression of these markers, and instead circulate between blood and NLOs. T_{RMs} are believed to mainly remain in the NLOs, and if (and in that case how often) T_{RMs} recirculate is debated. It seems that CD8+ T_{RMs} are more stationary in the tissue whereas CD4+ T_{RMs} are more motile and prone to recirculate (16). T_{RMs} express the retention marker CD69 and some of them also CD103, the latter however to a varying degree. Like naïve cells, memory T_H cells continuously sample MHC II-peptide complexes throughout the body. When a memory cell encounters its antigen, co-stimulation ("signal two") is not required for activation, and any of the APCs, anywhere in the body, can activate a memory cell. In addition, this time around the response will be faster and stronger than the first time (16, 17).

Immunological memory provides the possibility for vaccination, where for example an attenuated version or a component of a pathogen is given in a controlled manner in order to initiate an adaptive immune response. If the individual later is infected, the body will have developed memory cells able to give a rapid response to the infection. It is currently estimated that vaccination prevents around two to three million deaths every year around the world (16-18).

1.1.6 Dynamics of lymphocyte recirculation

Lymph refers to the semi-transparent liquid in the lymphatic vessels and contains cellular debris, immune cells (mainly lymphocytes), fatty acids, and microbes, all to a varying degree depending on body site. Lymph emerge when interstitial fluid is drained from the NLOs into blind ended lymphatic capillaries. These merge into larger (afferent) vessels which in turn converge at the LN draining the tissue. Lymph leaves the LN by efferent vessels which merge into the thoracic duct and is then emptied into the blood. Plasma is filtered out from the blood into the NLOs by capillary filtration, and some remain in the tissue as interstitial fluid which can again be collected as lymph. Lymph circulates due to pressure emerging from muscles when the body moves and can only travel in one direction due to intraluminal valves situated throughout the vessels, hindering backflow (19).

At a given moment, most lymphocytes reside in the SLOs, many in tissues, and only around two percent in the peripheral blood. In a human, the total lymphocyte pool consists of around 10^{12} lymphocytes. During homeostatic conditions, the number remains relatively constant by a balance of thymic output, peripheral proliferation and cell death. The cytokine IL-7, produced by stromal cells in lymphoid organs, is vital for T cell survival and for maintaining lymphocyte homeostasis (20). A naïve T_H cell has been estimated to live around six to ten years in humans, but only around six to ten weeks in mice (7). The number of cells per TCR clone is highly heterogenous but is estimated to range between one in 10^6 to one in 10^5, and

the magnitude of the response has been seen to depend upon the initial number of clonal cells (4, 21-23).

Naïve lymphocytes enter LNs via the HEVs. HEVs act as gatekeepers for cell entry from the blood into an LN, maintaining the number of cells in the LN relatively constant during homeostatic conditions (24). When an LN is activated, more cells are allowed to pass the HEVs into the node, and in addition, fewer cells leave, a phenomenon known as "LN shutdown" which increases the size of the node. Levels of sphingosine-1-phosphate receptor (S1PR1) expression regulates LN egress (19). The ligand for S1PR1, sphingosine 1-phosphate (S1P), is found at high levels in blood and lymph, but low levels in lymphoid organs. Hence, upregulation of the receptor causes cells to follow the gradient and leave the LN (19). Naive T_H cells are highly motile within an LN with a mean velocity of around 10–12 μm/min whereas DCs travel slower (2–6 μm/min) (22, 25). Each DC meet around 500-5000 T cells per hour, and each contact last only around a few minutes if the displayed antigen is not a match with the TCR (22). Studies on lymphocyte travel time through LNs of mice indicates that CD4+ T cells travels the fastest, followed by CD8+ T cells, and that B cells moves the slowest through the node (22, 25-27). In mice, a naïve T_H cell has been estimated to spend an average of 12.2 and 9.6 hours traveling through a peripheral and a mesenteric LN, respectively (27).

1.2 INTESTINAL HELMINTHS

The word helminth stems from the ancient Greek word *hélminthos*, meaning "intestinal worm". Historically, all vertebrate animals, including humans, have been infected with intestinal worms for, probably, as long as their species have excited. Today, the burden of disease in humans is almost exclusively limited to developing countries and regions, where poor sanitary conditions contribute to dissemination of infection. In the 1930th, researchers set up the first experimental animal models of helminthic disease. These early studies laid grounds for further research, including studies on how our immune system is affected by presence of intestinal worms (28-30).

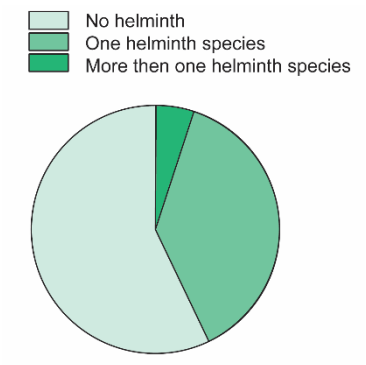


Figure 3: Helminth prevalence in an Indian population. Samples were collected in Bihar, India (2017) by Cajsa Classon.

1.2.1 Intestinal helminths in humans

Intestinal helminths are widespread in most vertebrates, including humans. The number of people infected is not known, but the World Health Organization (WHO) estimates it to be more than 1.5 billion individuals, corresponding to 24% of the human population. The great majority of cases are found in developing countries and the disease burden of helminthiasis is severely neglected. Children in rural areas of many low-income countries are often infected by multiple helminth species simultaneously throughout a large part of their childhood (31, 32). Intestinal helminths inhabit the GI tract of their host in the

adult stage of the life cycle, where they produce immense numbers of eggs that are excreted with the host's faeces. Parameters such as poor accessibility to sanitary facilities, high population densities, and warm climate, increase the abundance of intestinal worms within a population (31, 32).

Intestinal helminths infecting humans range from a few millimetres to several meters in size. They can be divided into three major groups based on morphology and means of nutrient acquisition and reproduction: nematodes (roundworms), cestodes (tapeworms) and trematodes (flukes). Nematodes are cylindrical in shape and range from a few millimetres up to 30 centimetres in length. They have a proper GI tract and most species have distinct male and female individuals. Cestodes are the lengthiest, and (although rare) individual worms have been shown to measure up to 30 meters. They attach to the intestinal mucosa with a head structure, called scolex. The body consists of a chain of flattened hermaphroditic segments (proglottids) that contains eggs and are released with the host's faeces. Cestodes have no proper GI tract but absorbs nutrients from their host's intestinal lumen through the tegument. The third group, flukes, have a GI tract and a solid body instead of proglottids. Many are hermaphroditic, except for the blood-flukes that have separate male and female individuals. Flukes are often quite small, ranging from a few millimetres up to around eight centimetres, and have a leaf-like shape. Around 300 helminth species have been found to infect humans but a small number of nematode species are responsible for the great majority of infections, namely *Ascaris lumbricoides*, *Trichuris trichura* and the two hookworm species *Necator americanus* and *Ancylostoma doudenale* (31-34).

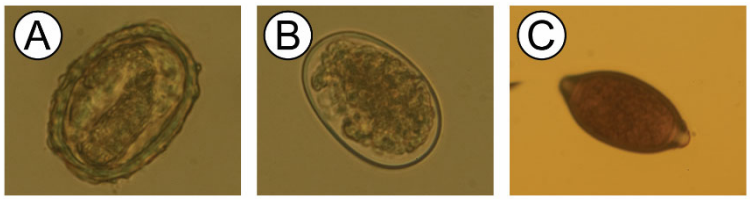


Figure 4: Eggs from *Ascaris sp* (A), hookworm (B), and *T. trichura* (C). Samples collected in Bihar, India (2017) by Cajsa Classon. Photo by Nuno Sousa.

Many intestinal helminth infections are asymptomatic, but symptoms may range from mild local effects including intestinal discomfort, constipation or diarrhoea, to systemic implications such as deficits in nutritional status, reduced weight, stunted growth, cognitional complications, reduced school performance, and most important for this thesis: reduced vaccine efficacy and immunity to secondary infections. In addition, hookworms can cause iron-deficiency anaemia by attaching to the intestinal mucosa and feed on host blood. Intestinal worms infect by various routes including contaminated food or water (e.g. *Ascaris spp.* and *T. trichura*) and penetration of skin (e.g. hookworms). The skin infection route may cause itchiness and redness (ground itch) at the site of penetration. Some species, such as *Ascaris spp.* and hookworms, have extraintestinal larval migration routes bypassing the lungs, and may thereby cause lung manifestations such as wheezing or shortness of breath (2).

1.2.2 Protective immunity to intestinal helminths

During an intestinal helminth infection, the host initiates type 2 responses, orchestrated by T_H2 cells, to expel the worms. Type 2 reactions in the intestines result in increased peristaltic movements, accelerated turnover of gut epithelial cells, and hyperplasia of goblet cells causing elevated mucus production – effects that are collectively referred to as the "weep-and-sweep" response. The weep-and-sweep response is mediated primarily by the signature T_H2 cytokines IL-4, IL-5 and IL-13, and by inflammatory mediators, histamines, and prostaglandins released by for example mast cells. In addition, type 2 responses result in eosinophilia and increased class-shifting to the IgE and IgG1 isotypes of antibodies. Most IgE immediately attach to the high affinity IgE receptor FcεRI on mast cells and basophils, and very little is free in serum. Eosinophils contain granules which content can damage the cuticle of the helminth. Type 2 responses are also elicited to allergens and during asthmatic reactions, and can have severe consequences if initiated in the skin or airways (3, 33).

How a type 2 response is initiated is still not fully elucidated, but a number of mechanisms have been suggested, that are likely to be overlapping. IL-4 is crucial for type 2 immunity, yet, DCs (known as the fundamental bridge between the innate and adaptive immune response) does not produce IL-4 (35). DCs can however induce T_H2 cells via Notch ligand and OX40 signalling (35). Basophils on the other hand can function as APCs to promote T_H2 differentiation and are significant producers of IL-4 (36). Epithelial cells damaged by the parasites produce alarmins such as thymic stromal lymphopoietin (TSLP), IL-25 and IL-33, known to induce type 2 immunity. Recent studies have identified tuft cells, a rare type of epithelial cell found at mucosal barriers, as an early source of type 2 cytokines (especially IL-25), in the context or intestinal helminth infection (37). Innate lymphoid cells (ILCs) also serve as an early source of type 2 cytokines and amplifies the response (37). The transcription factors GATA3, signal transducer and activator of transcription (STAT) 6, and STAT5 promotes T_H2 differentiation and stimulates production of IL-4, IL-5 and IL-13. IL-4 strengthens the T_H2 profile and promote B cells to produce IgE. IL-5 mainly recruits and activates eosinophils, whereas IL-13 induces smooth muscle cell contraction (causing peristaltic movements of the intestine), increases mucus production by goblet cells, and promotes enterocyte shedding (3, 33).

1.2.3 Regulatory responses during intestinal helminth infection

Despite induction of type 2 responses aiming to expel intestinal helminths, worms often manage to stay in the gut and the infection becomes chronic. Chronicity of helminth infection is facilitated by strong induction of regulatory responses. Regulatory signalling acts broadly anti-inflammatory and dampens all other immune reactions (including type 2 responses) in order to limit immunopathology. Consequently, regulatory responses may help the worms to remain in the intestine (2). $T_{REG}s$ are central for regulatory signalling. There are two main subtypes of $T_{REG}s$: natural $T_{REG}s$ (n$T_{REG}s$, also called thymus-derived $T_{REG}s$) which arise in the thymus, and induced $T_{REG}s$ (i$T_{REG}s$, also called peripheral $T_{REG}s$). i$T_{REG}s$ develop in the

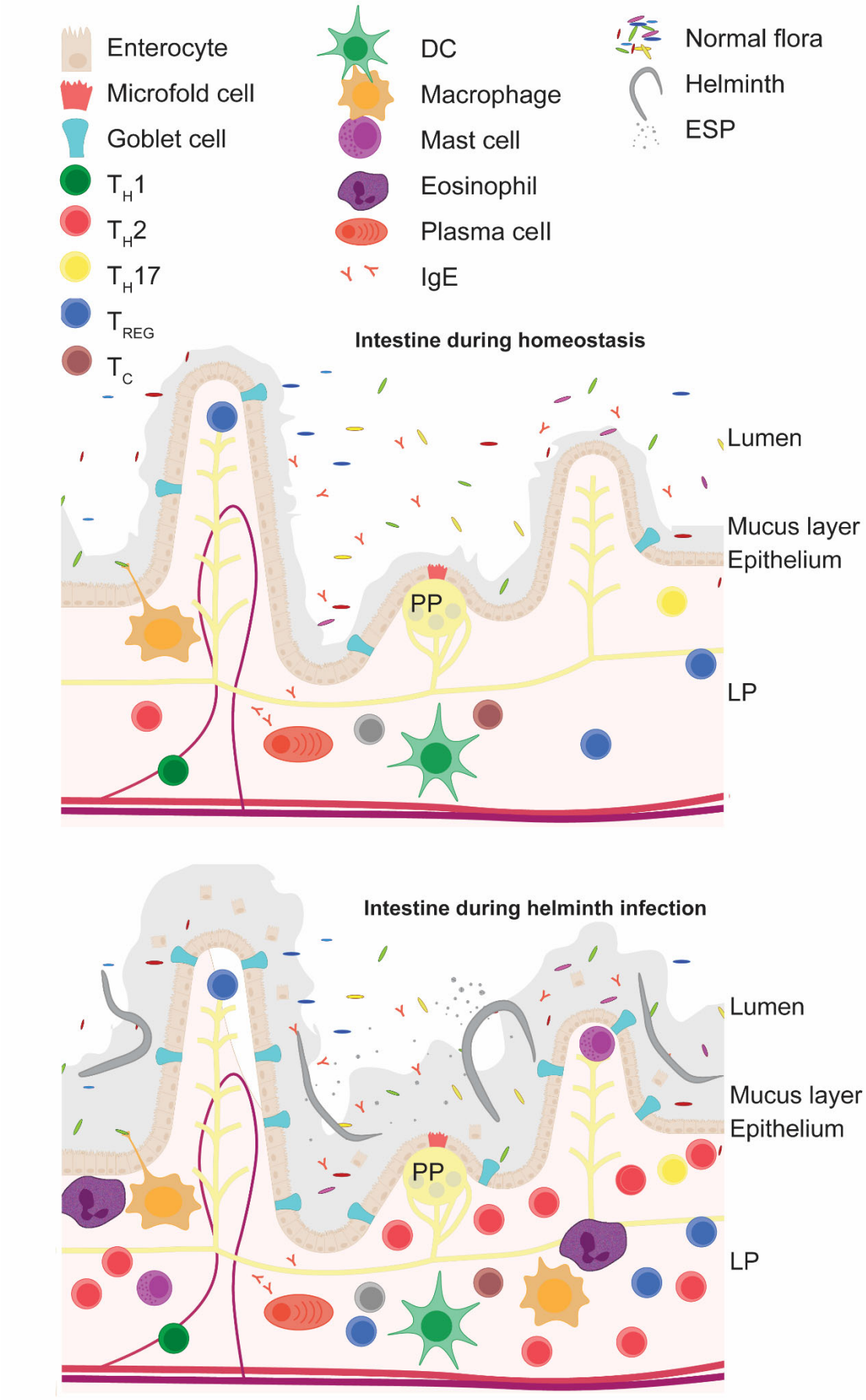


Figure 5: Immune response during intestinal helminth infection. During an intestinal worm infection, type 2 signalling triggers goblet cell hyperplasia (which causes thickening of the mucus layer) and enterocyte shedding. T_H2 cells and T_{REGs} expand and mast cell and eosinophils accumulate.

periphery if the cell encounters its cognate antigen during inadequate co-stimulation. T_{REGs} exert their suppressive function by a variety of mechanisms, including cell-cell contact, metabolic interference, and cytokines. T_{REGs} are both induced by and produce the regulatory cytokines transforming growth factor β (TGF-β) and IL-10 and are defined by expression of the transcription factor forkhead box P3 (Foxp3). TGF-β is excreted in an inactive form that is stored in the extracellular matrix until activated enzymatically. Genetic absence of any of the components of the TGF-β pathway is lethal during the first weeks of life due to rampant inflammatory disease. During intestinal helminth infection, robust TGF-β production is induced by the host, and in addition, many helminths produce TGF-β homologues that acts on host cells to reinforce the response (further discussed in chapter 1.2.6) (38, 39). IL-10 was first categorized as a T_H2 cytokine and does indeed mediate key T_H2 functions such as activation of mast cells. Nowadays however, IL-10 is mainly considered a regulatory cytokine critical for dampening inflammation and for wound healing. IL-10 is produced by Foxp3 expressing T_{REGs} but also by a Foxp3 negative population called type 1 regulatory (Tr1) cells, known for their high IL-10 production. Tr1 cells mediate immunosuppression by many of the same mechanisms as Foxp3+ T_{REGs} and are known to dampen protective immunity during helminth infection (6, 33, 39-41).

1.2.4 Macrophage subtype differentiation during worm infection

Another immunological feature promoted by helminth infection is the development of an anti-inflammatory type of macrophages, M2 macrophages (also called alternatively activated macrophages [AAMs]), as opposed to the classically activated pro-inflammatory M1 phenotype. M2 macrophages are induced by the type 2 cytokines IL-4 and IL-13 and are important for protective anti-helminthic immunity as well as healing of wounds caused by migrating helminth larvae. M2 and M1 macrophages differ in their ways to process arginine. M2 macrophages express the enzyme arginase-1 that synthesizes urea and ornithine from arginine. M1 macrophages are instead induced by IFN-γ and express the enzyme inducible nitric oxide (NO) synthase (iNOS) that generates citrulline and NO out of arginine, the latter which is vital for type 1 immunity in mice. As arginase-1 and iNOS compete for the same substrate and are activated by counter-inhibiting cytokines, M1 and M2 (like T_H1 and T_H2) act antagonistically against each other (42-44).

1.2.5 *Heligmosomoides polygyrus*

The work included in this thesis is centred around responses induced by the commonly used model organism *Heligmosomoides polygyrus bakeri*, previously known as *Nematospiroides dubius*, and onwards referred to as *H. polygyrus*. *H. polygyrus* is a strictly intestinal nematode and a natural parasite of mice. Out of the human-infecting helminths, *H. polygyrus* is taxonomically closest to hookworm, and the eggs are almost morphologically identical to those of hookworms. However, *H. polygyrus* lacks migratory route and does not feed on host blood. *H. polygyrus* infects orally as L3 stage larvae and enters the submucosa within the first 24 hours after infection. The larvae moult twice (via L4 and L5 stages) in the intestinal wall,

before exiting into the lumen as adult worms around eight to ten days after infection. As adults, *H. polygyrus* inhabit the small intestine, where they are curled up around the villi and feed on the host's intestinal tissue. After mating, the worms produce eggs which are excreted with the host's faeces. Around six to eight days after release, the eggs hatch, and via L1 and L2 larval stages develop into new infective L3 stage larvae (2, 33, 45, 46).

As most intestinal helminths, *H. polygyrus* induces type 2 responses in tissues close to the gut, such as mesenteric LNs, PPs, and lamina propria (LP). ILC2s are vital for induction of T_H2 cells and for protective immunity against *H. polygyrus* (47). The type 2 cytokines IL-4, IL-13 and IL-9 are crucial for protection, whereas the roles of IL-5 and the accompanying eosinophils are not as clear (11, 48). Mast cells are involved in worm expulsion (45, 49) and B cells, M2 macrophages and basophils are important for protection against re-infection (43, 50). The type 2 response peaks around two weeks after infection after which regulatory responses take over and dominate during the chronic stage (2, 33, 45, 47). During the tissue stage of infection, granulomas consisting of neutrophils, macrophages, DCs, and eosinophils form around the larvae in the submucosa. After reinfection, T_H2 cells and M2 macrophages accounts for a large fraction of cells in the granulomas. Similar to natural helminth infection, *H. polygyrus* establishes chronic infection in many laboratory mouse strains, including the IFN-γ prone C57Bl/6. In more IL-4 skewed strains such as BALB/c, the infection is typically cleared within a few weeks (2, 33, 45).

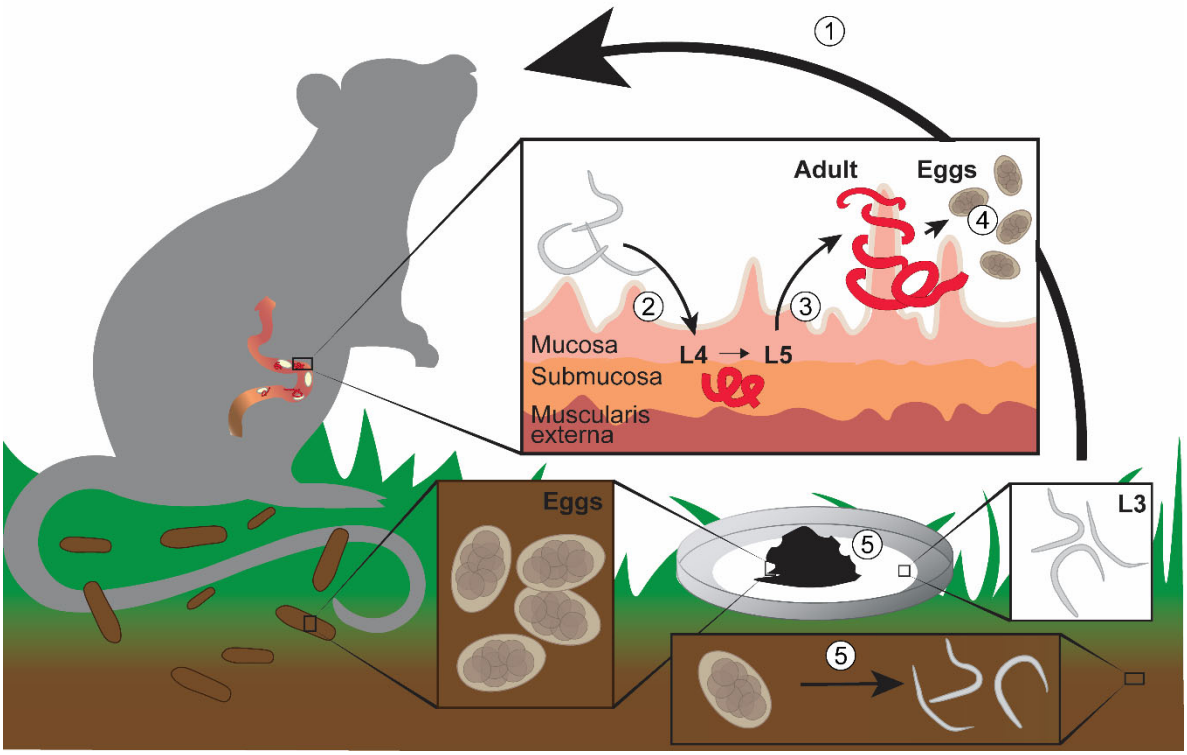


Figure 6: Life cycle of *H. polygyrus*. Mice are infected by ingestion of L3 stage larvae (1) that migrate to the small intestine where they enter the submucosa (2). Here they moult twice and exit into the lumen as adult worms eight to ten days after infection (3). Adult worms produce eggs that are shed in the host's faeces (4). Eggs will hatch in the environment (or in a moist Petri dish in the lab) and develop into new L3 stage larvae (5).

1.2.6 Excretory-secretory products

Helminths produce a plethora of molecules known as excretory-secretory (ES) products. These include both by-products of the worms' metabolism and lifecycle, as well as actively secreted molecules that can promote helminth establishment, survival and/or have immunomodulatory effects. Many acts to enhance regulatory responses, interfere with type 2 immunity, or dampen deleterious inflammation. This is done by for example modulating DC polarization, reducing cell T proliferation, preventing cell migration, and inhibiting mast cell activation. ES products can also for example act as proteases and degrade host tissue or inhibit blood coagulation to facilitate blood feeding. Many ES products have been seen to reduce both systemic and organ specific inflammatory conditions and one molecule often confers several effects. Commonly, the different larval stages and adult worms produce a distinct setup of ES products, tailored to their needs. ES products consist of various types of molecules, including proteins, glycoproteins and lipids. The majority are polypeptides ranging from around ten to several hundreds of kilodaltons, and many are glycosylated. Production of ES products is evolutionary conserved, as they can be found in all helminth phyla (51-53).

The first discovered ES product, ES-62, is still one of the most extensively studied ones and was found in the rodent filarial nematode *Acanthocheilonema viteae*. ES-62 is an example of a molecule with an array of mechanisms: it protects against neutrophil and eosinophil infiltration in the lung, induces IL-10 and blocks IL-17 production by T cells, and inhibits degranulation of mast cells (52, 54). Several ES products act on the TGF-β pathway and some of these have arisen by convergent evolution, indicating their significance for the fitness of the worms (53). One striking example is the so called TGF-β mimic produced by *H. polygyrus* (HpTGM), which functions as a TGF-β receptor ligand with similar effects as (but no structural homology to) mammalian TGF-β (38, 52, 55). HpTGM can induce Foxp3-expressing regulatory T cells *de novo* (38, 52, 55). Interestingly, HpTGM can also act on human T cells, even though *H. polygyrus* does not infect humans (53). Another ES product of *H. polygyrus* known as *H. polygyrus* alarmin release inhibitor (HpARI) blocks the IL-33 pathway both by suppressing its release from the nuclei of damaged epithelial cells and by binding directly to IL-33 (53, 56).

1.2.7 Systemic dissemination of type 2 and regulatory immunity

Many helminths have extra-intestinal larval migration routes passing though several tissues such as skin, blood, liver, and lungs. It is thus not surprising that a response is elicited in all organs encountering the migrating larvae. However, type 2 and regulatory cells and associated effector molecules have been observed at sites distal to the intestine also after infection with non-migrating helminths. It is established that humans and mice with intestinal worms have elevated levels of type 2 cytokines and eosinophils in the blood (57). The degree of T_H2 cell and T_{REGs} dissemination to NLOs has however only started to become investigated. In BALB/c mice infected with *H. polygyrus,* IL-4 producing T_H2 cells detected

by a fluorescent reporter did not only expand in the vicinity of the gut, but had disseminated systemically two weeks after infection (58, 59). Most T_H2 cells were found in bronchoalveolar lavage fluid, the peritoneal cavity and in the liver, and were still apparent (although diminished) four weeks after deworming (58). Similarly, others have shown that functionally protective tissue resident T_H2 cells can be found in the peritoneal cavity eight weeks after deworming from *H. polygyrus* (60). Increased levels of Foxp3+ T_{REGs} have been reported in axillary LNs, mediastinal LNs and spleen four weeks after *H. polygyrus* infection (61). Recently, regulatory myeloid-derived suppressor cells were seen to have disseminated to peritoneal fluid, blood and bone marrow of *H. polygyrus*-infected mice (62).

1.3 HELMINTH CO-INFECTIONS

Humans plagued by intestinal worms are to a large extent the same populations which are highly exposed to other infections. Due to the wide-spread effects of intestinal helminth infection on the host immune system, intestinal worms can be presumed to have implications on immunity to secondary infections and on vaccine responses. Effects have been investigated in humans and mice, but the results are generally diverging and varies with helminth species, secondary pathogen, secondary infection site, timing of infections, human population studied, or mouse strain employed. Further, a number of mechanisms for the observed effects have been proposed, including dissemination of type 2 responses, regulatory responses, or ES products, and alterations in the microbiota or in metabolic pathways (32, 63, 64).

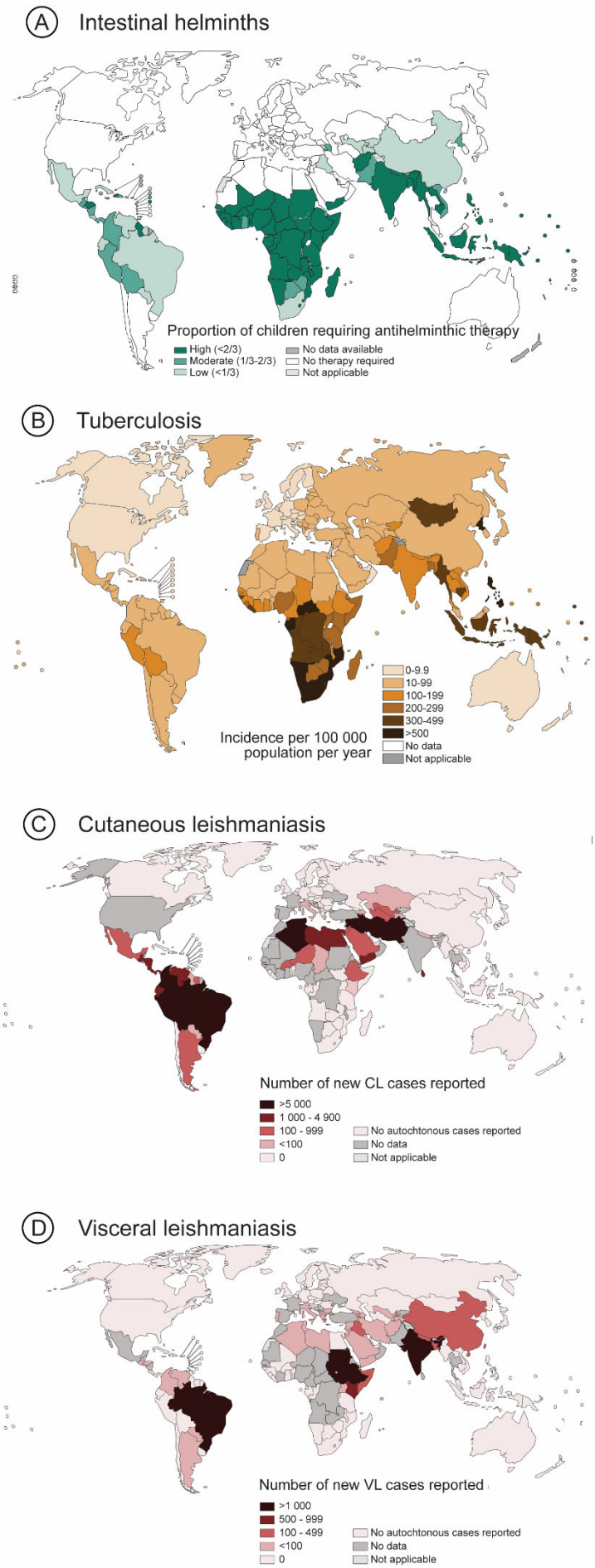
Due to the inhibitory effects of type 2 cytokines on T_H1 cells, infections requiring type 1 immunity for protection can be expected to be especially affected by presence of intestinal worms. Mycobacteria, e.g. *Mycobacterium tuberculosis* causing tuberculosis (TB), and the protozoan parasites of the *Leishmania spp*. (causing various forms of leishmaniasis) are such pathogens. *M. tuberculosis* and *Leishmania spp.* are completely unrelated organisms and their disease pathogenesis distinct, yet, both live intracellularly with macrophages as main residence which causes the host to employ in many respects similar immune reactions for protection. T_H1 responses are induced by IL-12 from APCs which will in turn promote production of the signature cytokine IFN-γ by T_H cells. In mice, IFN-γ induce NO production in the infected cell, which will contribute to killing of the pathogen (65). In humans on the other hand, reactive oxygen species and antimicrobial peptides are more important for protection. The necessity of CD4+ T cells in defence against mycobacteria and *Leishmania spp.* is underscored by overrepresentation of human immunodeficiency virus (HIV) positive individuals among patients progressing to active disease and that succumb to the infections, as HIV specifically eliminates CD4+ cells (66, 67).

1.3.1 Intestinal helminths and mycobacteria

TB has plagued humans for thousands of years and remains a leading cause of death by infectious diseases worldwide. TB is a disease of the poor and mainly affects developing areas of the world. Around ten million individuals progressed to active clinical disease from latent infection and around 1.5 million patients died from the disease in 2018. It is estimated that almost one third of the worlds' population is latently infected with *M. tuberculosis*. However, only a minor part of the infected individuals (five to ten percent) develop active disease during their lifetime – a phenomenon highly influenced by the immunological status of the patient (66).

In 1882, Robert Koch identified *M. tuberculosis* as the disease-causing agent of TB, and *M. tuberculosis* is indeed responsible for the great majority of TB cases. Other mycobacteria, such as *Mycobacterium bovis* may also case TB, but mainly in immunocompromised individuals. The only available vaccine against TB, Bacillus Calmette-Guérin (BCG), is a live attenuated form of *M. bovis* named after the developers Albert Calmette and Camille Guérin. Calmette and Guérin passaged *M. bovis* for attenuation at the Pasteur Institute in Paris, France, in the early 20th century, and used BCG as a vaccine against TB for the first time in 1921. Since then, BCG is the most ever used vaccine in the history of mankind, but the estimated efficiency is highly variable between studies, ranging from zero to 80%. Protection has been shown highest in children, especially for protecting infants against meningeal and miliary TB, and lowest against pulmonary TB in adults. Several reasons have been suggested for the variable efficiency of the vaccine, including differences between BCG strains, geographic location of the study, host genetics and immune status, method of immunization, presence of environmental mycobacteria, and as discussed below, presence of co-infections, such as intestinal helminths (66, 68-73).

BCG is administered as an intradermal injection into the skin. Active, latent or cleared TB infection, successful vaccination, or exposure to environmental mycobacteria can be detected by a so-called tuberculin skin test (TST; also known as Mantoux test), sometimes used to diagnose TB. A TST is performed by injecting purified protein derivate (PPD, a mixture of mycobacterial proteins) into the skin, which induces a delayed type hypersensitivity (DTH) reaction in exposed individuals. The diameter of the induration is measured 48 or 72 hours later and gives an indication of the robustness of the individual's mycobacteria-specific cellular immune response (74). Areas heavily affected by helminths geographically overlap with areas with high TB burden and low BCG efficiency (76). It has been estimated that around 20-35% of TB patients are co-infected with, or have been exposed to, intestinal helminths (77). Proper control of mycobacterial infection is linked to T_H1 cytokines and reactivation of TB disease has been associated with a shift towards T_H2 cytokines in mice (78). Accordingly, patients with active TB have been reported to have a mixed T_H1/T_H2 profile (79). Despite the theoretical support of that the T_H2 response to intestinal worms would disturb development of proper T_H1 immunity to TB, data from co-infection studies have been diverging. In some studies, higher prevalence of intestinal nematode infection has



been found in TB patients compared to healthy controls (58, 80-82), whereas other have shown no such association (83, 84). Intestinal nematode infection had no impact on progression from latent to active TB in HIV-infected TB patients in Uganda (85) and neither in a large longitudinal cohort study in India (86). However, HIV infected individuals are a complicated study population for the purpose due to low levels of the critical CD4+ T cells (85). In the Indiastudy, stool examinations were only performed at baseline and not during follow up when the patient had developed TB (75). Clinically, patients with intestinal worms have been seen to have more severe radiological pulmonary disease with larger lung areas affected at the end of TB treatment (76). Regarding cytokine expression, peripheral blood mononuclear cells (PBMCs) from helminth-infected TB cases have been seen to produce less IFN-γ and more IL-10 and IL-5 in response to PPD (57, 76-78). However, other studies have surprisingly shown increased IFN-γ production in patients with intestinal worms (57). Some studies report higher frequencies of Foxp3+ T_{REGs} cells in blood (78, 79) and other no difference (80). BCG-induced lymphocyte proliferation *in vitro* has been shown to be reduced in cells

Figure 7: Areas with high prevalence of intestinal worms are geographically overlapping with areas highly affected by TB and leishmaniasis. Proportions of children (1-14) years old in the country requiring treatment for intestinal helminthiasis (WHO, 2014, A). Estimated incidence rate of TB, (WHO, 2018, B), CL (WHO, 2018, C) and VL (WHO, 2018, D), around the world.

isolated from worm-infected individuals (80). Furthermore, intestinal helminth infection has been associated with decreased reactivity in diagnostic tests, such as TST and the interferon gamma release assay (IGRA) (57, 71, 77), indicative of weaker mycobacteria-specific immune responses in these individuals. Consistently, presence of helminths has been suggested to affect performance and limit interpretability of the IGRA assays in children (81). In a study in South Africa, an inverse correlation between *Ascaris* IgE status and a positive TST test was found (82), however, a study in Venezuela showed positive correlation between TST positivity and helminth infection (83). In a study of DNA methylation patterns of CD4+ T cells in blood after helminthic disease, it was found that active *Ascaris* and *S. mansoni* infection alters methylation in a direction presumably negative for anti-TB immunity (84). The *Ascaris*-induced methylation was restored in people that had cleared the infection, whereas the *S. mansoni*-induced patterns remained and lasted six months after deworming (84). Importantly, deworming from intestinal helminth has been shown to significantly improve T cell proliferation and IFN-γ production and to restore IL-10, eosinophil and Foxp3+ cell levels in blood (78, 85). Dewormed individuals have also in some studies been seen to mount stronger PPD-specific immunity and TST reactivity after BCG vaccination compared to those still carrying worms (86-88), whereas other studies have shown no such effect (89). Clinical TB scores have been seen not to improve within the first two months after deworming (85).

Similarly, some studies in animals indicate reduced TB protection in helminth-infected mice, whereas other show no effect (90). Pre-existing (five days) infection with *N. brasiliensis* have been seen to ameliorate aerosol *M. tuberculosis* infection in BALB/c mice, partly by inducing AAMs via the IL-4 pathway (91). Intraperitoneal *N. brasiliensis* infection of C57Bl/6 mice four days prior to intranasal *M. bovis* BCG caused lower mycobacteria-specific IFN-γ production in mediastinal LNs but no difference in BCG load (92). In BALB/c mice infected with *T. muris* followed by *M. bovis* BCG, CFUs and histopathology of the lung were similar between the two groups but specific cytokine responses to each organism were reduced in the co-infected mice (93). However, another study instead showed that *N. braziliensis*-infected BALB/c mice were more protected with lower bacterial load in lungs and increased IFN-γ production (94). Protection was mediated by macrophages and increased neutrophil recruitment to the lung (94). Further, other studies have shown no effect of pre-existing *H. polygyrus* or *Toxocara canis* infection on *M. tuberculosis* infection, despite dissemination of Foxp3+ regulatory T cells to the lungs and mediastinal LNs (61, 95).

1.3.2 Intestinal helminths and *Leishmania spp.*

Leishmaniasis remains one of the world's most neglected diseases, affecting the poorest of the poor, mainly in low-income countries. Leishmaniasis is caused by flagellated protozoan parasites of the genus *Leishmania*. It is a vector-borne disease, causing risk of infection to increase with for example inadequate housing, open sewers, and outside sleeping, and is transmitted by blood-sucking female *Phlebotomine* sandflies. Similar to helminths,

leishmaniasis is severely understudied. There are three main forms of leishmaniasis with dramatically distinct clinical manifestations. Cutaneous leishmaniasis (CL) causes skin-restricted lesions, whereas in mucocutaneous leishmaniasis (MCL), the lesions in addition or instead affect the mucus membranes. In visceral leishmaniasis (VL), the parasites infect visceral organs (e.g. spleen, liver and bone marrow) causing a highly lethal condition. The type of disease that develops depends largely on which of the around 20 *Leishmania* parasite species that has infected the patient (65, 67).

CL causes the mildest symptoms but is the most common type of leishmaniasis with around 600 000 to one million new cases each year (67). Lesions primarily develop at the site of the sandfly bite but parasites may disseminate and secondary lesions appear, often along the lymphatic vessels. Exposed skin which is more easily attainable for the sandfly, such as that of the face and hands, is mostly affected. Lesions often self-heal within a few months or up to two years, depending on infecting species. The highly visible lesions in concert with the association to poverty causes the disease to be stigmatizing, and concerns of scarification after healing are common. Healed infection causes life-long immunity. Parasites mainly reside in macrophages of the skin and skin-draining LNs (96). In some cases, certain species of *Leishmania* parasites, belonging to the brasiliensis complex, disseminate to mucous membranes such as mouth, nose and throat cavities, causing a more serious and difficult-to-treat form of the disease, mucocutaneous leishmaniasis (MCL). Mucous membranes beocomes destructed causing disabling and stigmatization. Almost 90% of MCL cases occurs in Brazil, Peru, and Bolivia (67).

The most severe form of leishmaniasis is the visceral type, also called Kala-Azar (Hindi for “black fever”), which affects around 50 000 to 90 000 individuals yearly and is close to 100% lethal without treatment (31). VL is associated with low-grade or intermittent fever, hepatosplenomegaly, weight loss and anaemia. The great majority (94%) of VL cases occur in only seven countries, namely Brazil, India, Ethiopia, Kenya, Somalia, South Sudan and Sudan. After the sandfly bite, parasites disseminate systemically and propagate in liver, spleen, bone marrow and LN. In general, therapy is expensive and toxic with significant side effects, but has a high rate of success. Patients who suffered from VL caused by *L. donovani* may develop a sequel of skin manifestations termed post kala-azar dermal leishmaniasis (PKDL). PKDL patients lack the clinical signs of VL, and inversely, the skin of VL patients look histologically normal. Parasites are however found in the skin of VL patients, where they are believed to be evade and survive treatment (97). Similar to the situation with TB, only around five to ten percent of infected individuals develops VL. What makes a patient progress to active disease is not clear, but is known to be affected by the immune status of the patient (67, 98-100)

Very few studies have been published on the association between intestinal helminths and *Leishmania* infection in humans. In a study in in Brazil, intestinal helminths were associated with poor response to *Leishmania* therapy in cutaneous forms of leishmaniasis caused by *L.*

braziliensis (101). This was accompanied by an immune response shifted toward T_H2 and higher eosinophil counts (101). The reduced therapy response was repeated in another study, when also higher prevalence of mucosal lesions was noted in worm-infected patients (102). However, a third study in the same region showed no difference in success of *L. braziliensis* treatment between worm-infected patients receiving anti-helminthic treatment immediately or 60 days after initiation of the *Leishmania* therapy (103). A possible explanation for the discrepancy may be that the immunological effects of the helminths remain long after removal of the worms, masking potential effects of deworming. In a study aiming at evaluate the association between intestinal helminths and VL in humans, no obvious association between the infections nor any difference in *L. donovani* load between worm-infected and worm-free individuals was noted ((104), own unpublished data). However, a larger sample size would be required to credibly exclude such an association (104).

In animals, the magnitude of footpad swelling after *L. major* infection have been shown not to be altered by concurrent or five days pre-existing *N. brasiliensis* infection (nor by IL-4 treatment) of C57Bl/6 mice (105). However, blocking IL-4 in BALB/c mice increased control of *L. major* infection (105). In C57Bl/6 mice infected with *Strongyloides ratti* in the footpad, followed by *L. major* infection in the same footpad six days later, the pre-existing *S. ratti* did not influence control of *L. major*, but even enhanced proinflammatory cytokine production in the draining LNs (106).

1.4 THE SKIN

Our skin is the major barrier between us and the outside world. The average human has a surface skin area of around 1.8 m^2 and the skin accounts for approximately 16% of the body weight. The skin provides one of our first lines of defence against the external environment, protecting us from mechanical, chemical, and microbial damage (107).

The skin is divided into two main compartments: the outer epidermis and the inner dermis. The epidermis mainly consists of keratinocytes, but also a few immune cells. The outermost layer of the epidermis, the cornified layer (*stratum corneum*), consists of dead keratinocytes called corneocytes that gives the skin its rigid structure. Corneocytes are terminally differentiated cells that have lost their nucleus and cytoplasmic organelles. Underneath lies the translucent layer (*stratum lucidum*, only found in palms and soles) followed by the granular layer (*stratum granulosum*), in both which the cells also lacks nuclei. The next layer is the first one consisting of living cells, the spinous layer (*stratum spinosum*), where the keratinocytes are connected with cell-to-cell junction proteins called desmosomes. The final layer (stratum basale), is one cell layer thick and contains keratinocyte stem cells. The basal layer contains melanocytes responsible for pigmentation of skin by producing melanin. The human epidermis consists of between five to ten layers of live cells, whereas mouse skin only has two to three layers. Turnover time of keratinocytes from basal layer is 40-65 days in human skin but only eight to ten days in mice. However, the most prominent difference between human and mouse skin is probably the hair follicle density, resulting in fury skin in

mice. The epidermis is not vascularised or enervated, as oppose to the underlying layer – the dermis. In between the epidermis and dermis lies a basement membrane to which both are attached. The dermis is mainly made up of extracellular matrix, fibroblasts, and structural cells, but also some immune cells. The dermis in turn is supported by underlying subcutaneous tissue, mainly consisting of fat, that separates it from the muscular layer (107-111).

1.4.1 The cutaneous immune system

Immune cells are relatively sparse in skin. Yet, due to its large surface, the skin contains a sizeable total number of immune cells. It has been estimated that human skin comprises approximately twice as many T cells as the circulation (112). The few immune cells found in the epidermis are located in the spinous layer, and mainly consists of αβ T cells (mostly CD8+), and Langerhans cells (LC). LCs comprise a unique type of DC only found in the skin, where they with long protrusions constantly survey and sample their surroundings. Further, a type of γδ T cells unique to the skin called dendritic epidermal T cells (DETCs) is common in mice but seem mostly absent in humans. Dermal immune cells include DCs, macrophages, αβ T cells (equally many CD4+ and CD8+ cells), γδ T cells, plasma cells, and ILCs (108, 112, 113)

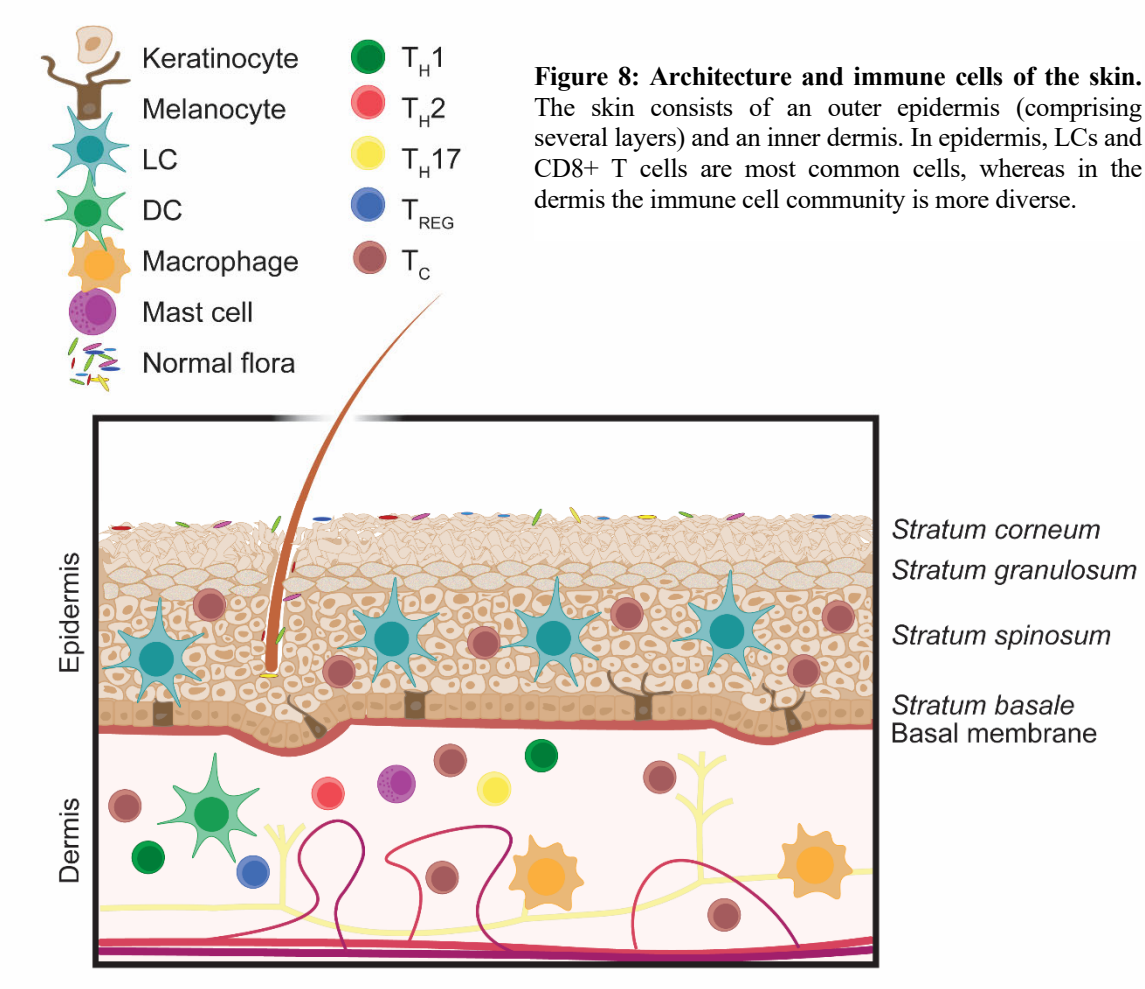


Figure 8: Architecture and immune cells of the skin. The skin consists of an outer epidermis (comprising several layers) and an inner dermis. In epidermis, LCs and CD8+ T cells are most common cells, whereas in the dermis the immune cell community is more diverse.

1.4.2 The gut-skin axis

A large part of this thesis addresses the skin and the gut, however, not as separate entities, but rather the link between the tissues and more specifically, what implications an intestinal infection may have on skin. The gut and skin are seemingly different and far apart, yet, the two tissues have a lot in common. Both are barrier tissues and provide a first line defence against pathogens, requiring resilient structures and robust immune protection. Yet, both also encounter an immense number of harmless substances such as food, commensal microbes and environmental material (114).

That there are associations between pathological conditions in the gut and the skin have for long been appreciated in the clinic. For example, constipation and increased intestinal permeability are associated with acne, *Helicobacter pylori* infection (a bacteria often linked to stomach ulcers) as well as small intestinal bacterial overgrowth (SIBO) are associated with rosacea, inflammatory bowel disease (IBD) with psoriasis, and coeliac disease with dermatitis and psoriasis (114, 115). Many studies indicate associations between diet and inflammatory status in the skin. Possibly the greatest interest has been shown in the probiotic field, which hopes to provide us with more resilient skin and shinier hair. For example, mice and humans given *Lactobacilli* as oral probiotics has been seen to acquire increased dermal thickness and barrier functions, which was associated with increased levels of anti-inflammatory cytokines systemically (115, 116). Oral *Lactobacilli* treatment has also been shown slightly protective against atopic dermatitis (114, 115, 117, 118). Further, oral antibiotics early in life of mice has been seen to increase susceptibility to imiquimod-induced psoriasis (119). A few mechanisms of crosstalk have been proposed, involving metabolic, neuroendocrine, and immunological pathways or the partly shared microbiota (114). Recently, mice infected with *H. polygyrus* were seen to be less susceptible to a model of skin contact hypersensitivity by an unknown mechanism independent of T_{REGs} (120). In general, not much research has been conducted if the crosstalk between intestine and skin and the so called “gut-skin” axis is a quite new concept (114).

2 AIMS

Intestinal helminth infection has systemic implications on the host's immune system and has been suggested to affect vaccine responses and immunity to other infections. In general, little is known about the consequences intestinal worms have on host immunity.

The overall objective of my PhD studies was to better understand the impact of intestinal helminths on immune responses distal to the gut, primarily effects on skin immunity and on control of co-infection.

More specifically, the aims were:

- To elucidate the effects of *H. polygyrus* on co-infection and skin immunization with T_H1-controlled pathogens (Paper I).

- To evaluate the impact of *H. polygyrus* infection on secondary infection with *L. donovani* (Paper II).

- To find the mechanism behind the reduced skin response to *M. bovis* BCG in worm-infected mice, seen in Paper I (Paper III).

- To investigate the effects of *H. polygyrus* on skin T cells (Paper IV).

3 ETHICAL CONSIDERATIONS

Intestinal helminth infections are severely understudied. Both helminthiasis and leishmaniasis are on the list of diseases which WHO classifies as “neglected tropical diseases”, meaning that the amount of resources spent on research and control of the diseases are substantially less than the morbidity and mortality they give rise to. TB is not classified as neglected, but despite persistent efforts to stop the TB pandemic, the disease still kills and disable vast numbers of people each year. All approaches to increasing the efficacy of the BCG vaccine should therefore be considered (31).

Approximately 350 000 animals were used for medical research in Sweden in 2016. Thirteen percent of these were used to study the immune system in one way or another (121). Alternative methods to animal research are constantly being developed and techniques such as organoids, safety evaluations for using human volunteers, computational modelling, and advanced cell culture systems win increasing amounts of grounds in research. Despite intense development of new techniques, there are still many phenomena impossible to study without using animals. The questions addressed in this thesis involve mechanistic investigations of systemic effects by a localized infection. There are so far no substitutes to whole animal models to understand the interactions between distal tissues, since the complete organism and the possibility to collect multiple tissues at the end of the experiment are required. Yet, there is a huge need for this type of knowledge. To better understand the effects of intestinal helminths on vaccine responses and control of co-infection would enable better targeted interventions that could improve quality of life for many.

4 BRIEF SUMMARY OF RESULTS

With the work included in this thesis, we have seen that *H. polygyrus* infection have widespread implication on its host's immune system, affecting lymphocyte distribution, skin immunity, and control of co-infection.

In **Paper I** we found that worm-infected mice mount weaker immunity to systemic infection by *M. bovis* BCG, reduced DTH responses to BCG and *Leishmania* and less DC migration from skin to draining LNs. In **Paper II**, we found that worm-infected mice are less protected against systemic infection with *L. donovani*. In **Paper III** we searched for the mechanism of reduced skin immunity of worm-infected mice found in Paper I and discovered that skin-draining LNs were drained of cells, leaving them atrophic. We found so signs of dissemination of type 2 or regulatory cells to skin-draining LNs that would explain the reduced priming efficiency in worm-infected mice. Mesenteric LNs were, as expected, extensively expanded in worm-infected animals. In **Paper IV**, *H. polygyrus*-specific type 2 CD4+ T cells were found to accumulate in the skin and remain after deworming. Skin homing chemokine receptors were upregulated in the mesenteric LNs and blood.

5 DISCUSSION

The four papers included in this thesis all address the effects of infection by the strictly intestinal nematode *H. polygyrus* on the host immune system. *H. polygyrus*, and intestinal worms in general, are extremely multifaceted in ways of affecting host immunity, and with each study we found new means of modulation. Below follows an integrative discussion that aims to place our new findings into a wider perspective. First, choice of mice, models and methods, will be discussed.

1.1. Mice

All data generated for this thesis are based on experiments in mice with C57Bl/6 background, a strain known to be skewed towards T_H1 in their immunological response (45). Intestinal helminth infections in humans and animals often becomes chronic, and the T_H1 bias in C57Bl/6 mice similarity causes *H. polygyrus* to linger and become chronic. BALB/c mice on the other hand, which have stronger T_H2 response, usually clear *H. polygyrus* infection during the effector phase of the T_H2 response and does not establish chronic infection. In humans, the distribution of helminths is uneven in a population, as a minority of individuals often carry a large proportion of the worm burden and are traditionally referred to as "wormy" people (122, 123). The reason has been suggested to be a variety of the T_H1/T_H2 gene ratio in the population, where the wormy people are, similarly to C57Bl/6 mice, T_H1 prone, and the non-wormy people T_H2 prone, similar to, BALB/c mice. Some of our findings we also tested in BALB/c mice. For example, in coherence with the requirement for chronicity for T_H2 cell accumulation in skin, CD4+ T cells did not accumulate in skin of BALB/c mice (own unpublished observations). However, skin-draining LNs were atrophic also in *H. polygyrus*-infected BALB/c mice (own unpublished observations).

Intestinal helminth infections are most frequent in school aged children, even if some species, mostly hookworms, are also common in adults (32). In an attempt to mimic this, we infected mice at a young age (four to five weeks of age) in most experiments, followed by secondary infection when the worm infection was considered chronic. A mouse at four weeks of age has passed the weaning stage and are just about to become sexually mature, hence can be compared to a school aged human child (124).

In Paper I, II and III, both female and male mice were used. Male mice were in many instances preferred, since they tend to keep the infection for longer ((45) and unpublished observations). In Paper II however, when the skin itself was analysed, the fact that male mice tend to fight more left no option but to choose female mice, as the risk of having to exclude animals due to skin scratches was too large with male mice. This have caused some difficulties in comparisons between the studies, as the female mice have ended up with a lower worm load or in some cases cleared the infection before the time of analysis. However, mice were always age and sex matched within a single experiment.

We have during the time of these studies moved our laboratory facilities, changed animal house and switched from inhouse breeding of C57Bl/6 mice into purchasing from vendors. Despite being the same strain of mice, this have likely caused differences in genetic makeup and the microbiota of the mice, which may also have caused differences between studies.

1.2. Models

We have used *H. polygyrus* as a model for intestinal worm infection, *Mycobacterium bovis* BCG, *L. donovani* and *L. major* as models for mycobacterial and leishmanial disease respectively, and DSS-induced colitis as model for non-infectious intestinal inflammation.

H. polygyrus in a strictly intestinal worm, without any systemic migratory route. In humans, strictly intestinal helminth species are less common then migrating ones (32). Yet, the migrating species spends the majority of time in the intestine and only a brief period in other organs (years versus days). Since organs distal from the intestine have been studied, it is likely that a migrating or systemic helminth model would have in some experiments yielded more pronounced results (e.g. co-infection with systemic T_H1 organisms and skin lymphocytes), whereas other results might not have at all been discovered (e.g. atrophy of skin-draining LNs). Helminth models where infection is performed subcutaneously such as *N. brasiliensis* or the *Strongyloides* species, would have caused difficulties in sorting out which reactions were effects of migratory larvae and which were due to actual dissemination of responses. *T. muris* is strictly intestinal but only causes acute infection in most mouse strains, and many of our key findings were not significant until the chronic stage of *H. polygyrus* infection, including DTH responses to mycobacteria, atrophy if skin-draining LNs, and T_H2 cell accumulation in skin. Other investigators have studied the effects of systemic helminths on mycobacteria and *Leishmania* infection, with varying outcome depending on model of choice. Some studies on mycobacteria indicate an detrimental effect by the worms (125-127) whereas other show no difference (64, 128, 129). On *Leishmania*, some studies show that helminths have negative effects for protection against *Leishmania* (127, 130-133). Surprisingly however, other studies show beneficial effects by the worms. Protection to *L. donovani* due to cross-reactivity has been seen in hamsters infected with the filarial nematode *Brugia malayi* (134, 135), underscoring the complexity of this type of studies.

In most experiments, we infected mice with a quite high dose (200-300 larvae) by a bolus injection. Both dose and means of injection is standard in the field which facilitates comparison of results to studies of other researchers (45). In nature, one could assume that helminth infection rarely occurs as a single high dose, but rather by low but continuous exposure. Such “trickle infection” of *H. polygyrus* has been employed by others with similar results as a single bolus infection, where T_H2 prone mouse strains got rid of the infection whereas T_H1 prone strains accumulated worm burden (136). No further examinations on the dissemination of HES have been performed during our studies. One could argue that this would have been of importance due to the nature of our questions, and future projects will if feasible entail such experiments.

We used the attenuated vaccine strain *M. bovis* BCG to study mycobacterial infection and vaccine responses in order not having to consider the confounding safety precautions associated with more virulent mycobacteria. In the experiments attempting to understand actual BCG vaccine immune response, this was also scientifically more appropriate. In Paper I, BCG was injected systemically, which is not the normal route of vaccination nor infection. Yet, these experiments gave general insight into the effects of intestinal helminths on infection with systemic T_H1 organisms. Similar results were obtained from the *L. donovani* co-infection model, which provided more credibility to both studies. Two types of *Leishmania* parasites have been employed during our studies, one skin tropic (*L. major*) and one visceralizing (*L. donovani*) to investigate the impact of *H. polygyrus* on skin and systemic infection, respectively. Other *Leishmania* species of similar tropism might have given other results, yet, the chosen parasites are two of the dominating *Leishmania* species world-wide (137).

The policies and practices of BCG vaccination differs between counties. In most developed countries, such as Sweden, BCG is no longer a part of the general vaccination program but is instead only given to risk groups. In many other countries however, BCG is still the first vaccine a child receives after birth, and in some, booster vaccinations are given around school age (74). This means that few individuals in endemic regions will actually be exposed to helminths before receiving the BCG vaccine, and one can argue that the opposite experiments, to immunize with BCG prior to the helminth infection would provide a more realistic scenario. Such experiments have been performed by us and others, with some results indicating decreased helminth control and others showed no difference ((92, 93) own unpublished data). Yet, the experimental setup used for our studies have provided valuable information that can be extrapolated to implications on efficacy of other vaccinations.

Incidence of VL is highest between five to 20 years of age (138), and CL is also more common in younger individuals (139). The overlapping age span of helminths and *Leishmania* infections increases the likelihood of the same individual being infected with intestinal helminths while being exposed to *Leishmania* and emphasizes the relevance of worm and *Leishmania* co-infection studies. The inverted setup have also in the case of *Leishmania* been studies by others, where superimposed *Leishmania* infection have in some instances caused reduced control of the worms and in other studies not altered the outcome (106, 127, 131, 140, 141).

1.3. Hypothesis evolution

Before the work in this thesis was initiated, it was well established that helminths induce type 2 and regulatory responses whereas mycobacteria and *Leishmania* requires T_H1 responses for protection, as well as that T_H2 and regulatory responses dampens T_H1 responses (57, 142). That this would be the mechanisms of which *H. polygyrus* dampened responses to mycobacteria and *Leishmania spp.* was naturally our primary hypothesis.

Both Paper I and Paper II proposed that the reduced protection against systemic BCG and *L. donovani* infection, respectively, was due to decreased *inos* expression and granuloma formation in livers. In Paper I, we proposed that increased TGF-βR signalling contributed to weaker control of infection, diminished DTH responses to mycobacteria and *Leishmania*, downmodulation of DC migration from skin to draining LN, and other immune dampening effects observed in worm-infected mice. This was based on that *H. polygyrus* products did (similar to TGF-β) decrease BCG-induced DC migration and downregulated BCG-induced IFN-γ production *in vitro* in a TGF-βR-dependent manner. In addition, worm-infected mice had increased frequency of LAP-expressing cells (a substitute measure for TGF-β) in spleen. In Paper III, aiming to further elucidate the mechanisms of *H. polygyrus*-mediated dampening of skin responses to BCG, we found that the atrophy of skin draining LNs observed in worm-infected mice better explained worm-mediated immunosuppression, as no dissemination of regulatory nor type 2 responses was observed in the skin-draining LNs. During the course of our study (in 2017), King *et al* published a paper supporting our theory (143). The authors saw that *H. polygyrus*-infected mice had muted responses to pneumococci-vaccine and influenza infection, delivered in the footpad and intranasally (respectively), and had atrophic draining LN at both sites (143). In Paper IV, we found an accumulation of *H. polygyrus*-specific T_H2 cells in the skin of helminth-infected mice that was suggested to also contribute to reduced IFN-γ production in response to mycobacterial WCL skin injection. Reports published by other investigators during the course of this work also suggest extraintestinal T_H2 memory cell deposition after *H. polygyrus* infection, supporting our findings (45, 53, 60). In light of these new discoveries, it is possible that the TGF-β mediated mechanism seen in Paper I is of lesser importance for worm-mediated immunosuppression of skin responses than first suggested. As an alternative, it is likely that reduced skin immunity is due to a combination of local increase in T_H2 cell abundance and reduced priming in the atrophic skin draining LNs, found in Paper IV and III, respectively.

1.4. Mass drug administration or helminth therapy?

The WHO encourages deworming by mass drug administration (MDA) to school aged children once or twice yearly (for areas of >20% and >50% prevalence, respectively) without diagnosing infection, in order to reduce the helminth burden in the population (31). The benefits of these de-worming programs are however debated, as the high incidence of re-infection may render them useless and that they may contribute to development of anthelminthic resistance, a widespread problem in veterinary medicine (31, 144-147). Yet, due to the morbidity caused by intestinal worms and especially for those with high load infection, the beneficial consequences of MDA are by many considered to outweigh the negative effects (148, 149).

There are two sides to every coin, fascinatingly also to the impact of intestinal worms on our immune system. With the economic development and increased availability of hygiene facilities in Western countries, worm infections have largely disappeared, and suddenly, a notable fraction of humans are worm-free. Not long ago it was norm rather than exception for

all humans to be infected with at least one intestinal helminth species. The mammalian immune system has been shaped in presence of intestinal worms which has resulted in an intricate relationship between host and parasite. Thus, it would not be surprising if the abrupt elimination of parasitic worms has had impact on the immune system of the modern human. Eradicating intestinal parasites has been suggested to play a part in the increase of inflammatory, asthmatic, allergic and autoimmune diseases we see in the Western world today (63, 150, 151). This notion was first postulated in the 19th century when it was noticed that people living in urban areas had higher prevalence of hay-fever compared to rural populations, and was later coined "The Hygiene Hypothesis" by David P. Stratchan in 1989 (152). Lately, this have raised the idea of reintroducing helminths into our bodies hoping that the worm-induced regulatory responses would dampen immuno-pathologies. Even though impressive results have been seen in experimental animal models, they have not been successfully replicated in human trials (153, 154). There are also obvious concerns with using live helminths for therapy, including safety issues for the host (especially for immunosuppressed individuals), risk of spread to other individuals, and that many patients might not feel comfortable with such a treatment. Instead, the potent immunoregulatory effects seen by isolated ES products have raised the idea to exploit these mechanisms for therapeutic use (51). Administration of ES products in animal models have been shown to inhibit intestinal inflammation in models of colitis, EA, T1D and collagen-induced arthritis (51, 153, 155).

1.5. Translatability and clinical importance

While animal models provide an excellent tool to demonstrate a proof of principle, the transability to humans by this type research should be interpreted with caution. *H. polygyrus* does not infect humans and none of the most important human intestinal helminths infects mice. Mice do not develop similar disease to *L. donovani* infections as humans and intravenous injection of BCG is an artificial rout of infection. The lesions from *L. major* and BCG skin injections however have clinical similarities to those of humans. Despite being a commonly used model of IBD and irritable bowel syndrome (IBS), DSS-induced colitis does not capture all aspects of either disease.

However, the immune responses evoked by helminth, *Leishmania ssp.*, and mycobacterial infections share large similarities between mammals and the value of these models to understand immunity to infection have been enormous. Mice and human share many features of lymphocyte migration, T_H cell orchestration, counter-inhibition between T_H cell subsets, and protective responses during infection. Hence, when the primarily goal as in our case is not to study the pathogenesis of a particular organism or disease, but rather to get an understanding of the immunological responses to that agent, mice provide a tool of unmatched value for new insights. Similarly, the aim with the DSS was to induce intestinal inflammation rather than to study IBS or IBD disease pathogenesis.

Paper I, III and IV support previously published studies, but also provide new suggestions that can explain why BCG vaccinations are less efficient in certain parts of the world. Considering the weak protection BCG provides against pulmonary TB in adults, the massive amounts of people infected, and the continual high numbers of death due to TB, every contribution to more robust mycobacterial immunity should be considered. Whether worm-infected humans actually have smaller skin-draining lymph nodes and more T_H2 cells in skin remains to be elucidated. If so, it is possible that also other skin infections would be aggravated in worm-infected individuals. In addition, if what our data and others suggest is correct, worm-infected people would have weaker DTH responses to BCG. This does not only have consequences on vaccine efficacy, but possibly even more importantly, worm-infected individuals with suspected TB may be less likely to be properly diagnosed by TST. Failure to find TST positive individuals may have implications both for the individual by potentially delaying treatment, but also for clinical studies that may underestimate the prevalence of TB in a population.

Co-infection studies between intestinal worms and leishmaniasis in humans showed no association between the diseases (104). That our studies in mice (Paper II) and in humans showed different results can have many reasons. For example, mice are not diseased by the infection, lacking fever and rampant parasite propagation. It is possible that VLs disease in human cause an unpleasant environment for the worms. In addition, in mice, we solely study the immunological features of co-infection, excluding epidemiological and vector-associated parameters. Hence, the result from Paper II may not be directly translatable to human VL. Yet, considering the findings on systemic *M. bovis* BCG in Paper I, Paper II provides additional support for that intestinal worms may affect systemic immunity to T_H1 controlled organisms

6 CONCLUDING REMARKS

Intestinal helminth infection has profound effect on the host's immune system. Our results support the notion that removal of worms may improve vaccine efficacy and control of co-infection. The rate of reinfection after treatment of worms is extremely high (144). Most likely, improved sanitary conditions would instead or in combination with treatment better reduce the burden on intestinal helminthiasis in a society. However, there are also positive effects of intestinal helminths, and ideally, we could learn how to utilize these without the negative consequences of infection. Hence, to better understand the effects intestinal worms have on our immune system will not only help us to get rid of an ancient disease, but also provide cues for innovative future solutions.

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