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Repurposing diabetes drugs for the treatment of intestinal helminth infection

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Research Masters Thesis

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I, Megan Dooley, confirm that the work presented in this thesis is my own and has not been submitted in substantially the same form for the award of a higher degree elsewhere. Where information has been derived from other sources, I confirm this has been indicated in the thesis.

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1. Abstract

Intestinal helminth infection affects over 1.5 billion people in the most deprived communities. These helminth infections cause significant morbidity, especially in children, with symptoms including malnutrition, stunted growth, and cognitive impairment. Resistance to current anthelminthic drugs is increasing, particularly in *Trichuris trichiura* "whipworm" infection, leading to the urgent need for new treatments. Recently, a group of immune cells at the intestinal barrier, intraepithelial lymphocytes (IELs), have been found to possess receptors for the epithelial produced peptide hormone, glucagon-like peptide-1 (GLP-1). However, how these GLP-1 receptor (GLP-1r)+ IELs respond during infection is completely unknown. We used the mouse model of whipworm *Trichuris muris* to investigate whether already clinically approved GLP-1r agonist treatment could influence mice chronically infected with the parasite. We utilised GLP-1r reporter, receptor null (GLP-1rKO) and immunocompromised RAG-/- mice as well as flow cytometric cytokine and epithelial pulse chase turnover experiments. We found that GLP-1r+ IELs expand at the intestinal barrier in response to chronic *Trichuris* infection and *ex vivo* treatment of GLP-1r+ IELs with GLP-1r agonists favoured a Th2/Th1 cytokine balance. *In vivo* treatment of already chronically infected mice with GLP-1r agonists significantly increased epithelial turnover, a known mechanism of intestinal parasite expulsion. Excitingly, this was coupled with rapid expulsion of the parasite, which we did not observe when treating infected GLP-1rKO or RAG-/- mice. These data suggest that GLP-1 is modulating the local intestinal immune response through GLP-1r+ IELs to induce increased epithelial turnover and expulsion of the parasite, offering an exciting potential therapeutic option for Trichuriasis. Furthermore, use of GLP-1r agonists, already approved for use in diabetics, would allow a quick and simple repurposing for treatment of these helminth infections greatly improving the quality of life of many children in developing countries.

2. Literature Review

2.1. Soil Transmitted Helminths

Over 1.5 billion people in the most deprived countries are infected with soil transmitted helminth (STH) infection (Palmeirim et al., 2018). STHs are a group of infections including roundworm *Ascaris lumbricoides,* hookworms *Necator americanus* and *Ancylostoma duodenale,* and whipworm *Trichuris trichiura.* The World Health Organisation considers these infections to be neglected tropical diseases, causing significant morbidity, and severely impacting the quality of life of infected individuals (Hotez et al., 2014; Ngwese et al., 2020). STH infections were responsible for the loss of 5.18 million disability-adjusted life-years (DALYs) worldwide in 2010 and negatively impact the economic growth of infected communities (Hay et al., 2017; Hotez et al., 2014). STH infection is common in low-middle income countries with poor sanitation and hygiene but can also occur in high-income countries with vulnerable populations (Hotez et al., 2014). Individuals with greater worm burden have higher morbidity while light infections can be asymptomatic (Ngwese et al., 2020).

Children of school age are the most vulnerable to infection (Fig. 2.1), and pregnant women are also at higher risk (Adegnika et al., 2007; Bethony et al., 2006). Infected children suffer a range of symptoms including stunted growth, cognitive impairment, anaemia, malnutrition, and intestinal blockage (Bethony et al., 2006; Ngwese et al., 2020). Safe drugs are donated free to endemic countries, however only half of the at-risk children received treatment in 2016. Furthermore, the end goal of mass drug administration is to control morbidity through reduction of infection intensity, rather than curing the infection (Jourdan et al., 2018).

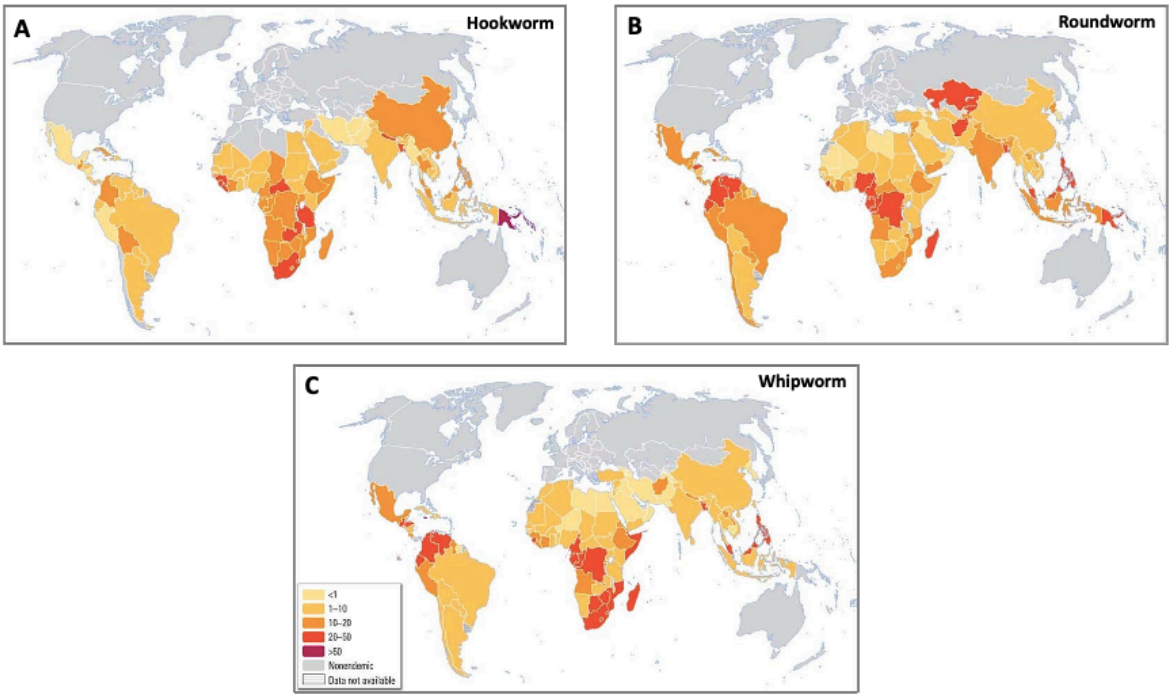

Figure 2.1: Global distribution of Soil Transmitted Helminth infection, by species, in pre-school and school age children, 2015. (A) Prevalence of hookworm infection. (B) Prevalence of roundworm infection. (C) Prevalence of whipworm infection. The distribution of soil transmitted helminth infection reflects social and environmental factors. There is greater transmission of hookworm infections in rural areas while prevalence of roundworm and whipworm infection is greater in per urban environments. Figure adapted from (Bundy et al., 2017).

2.2. Current Treatments

The World Health Organisation aims to control STH infection via preventative chemotherapy with drugs including albendazole, mebendazole, levamisole, and pyrantel pamoate (Keiser and Utzinger, 2008; Stephenson et al., 2000). Preventative chemotherapy aims to reduce morbidity by lowering prevalence of moderate-to-heavy infection in preschool and school age children, women of reproductive age, pregnant women in the second or third trimester, and adult groups with high risk of exposure to infection (Freeman et al., 2019; World Health, 2012). Albendazole and mebendazole are the only two drugs commonly distributed in preventative chemotherapy, usually as a single oral dose (400mg albendazole or 500mg mebendazole) taken once or twice a year (Freeman et al., 2019; Organization, 2017a; Organization, 2017b; Schulz et al., 2018; World Health Organization, 2017). The NHS state that for effective treatment of soil transmitted helminth infection, mebendazole should be taken twice daily for 3 days (NHS., 2019).

Although they are effective against other STH infections, mass drug administration programmes are substantially less effective against *Trichuris trichiura* (whipworm) infection and reinfection with *T. trichiura* after treatment is very common (Hotez, 2017). Meta-analysis of randomised controlled trials of the benzimidazoles (albendazole and mebendazole) found them to have unsatisfactory results in treating *T. trichiura,* with cure rates of 28% and 36%, respectively (Keiser and Utzinger, 2010). More recently, a further randomised trial showed even lower cure rates for albendazole (2.6%) and mebendazole (11.8%) (Speich et al., 2014). Egg reduction rates for albendazole (49.9%) and mebendazole (66%) are greater than placebo, although are still not at acceptable success rates (Keiser and Utzinger, 2010). The desired efficacy is an egg reduction rate of greater than 90% (Olliaro et al., 2011). A significant decrease in both cure rate and egg reduction rate are seen for albendazole and mebendazole after the year 2000 due to developing resistance of the parasite to these drugs. Levamisole and pyrantel pamoate are less commonly used due to their lower efficacy, need for weight-dependant dosing, and lack of access to the drugs (Marchiondo, 2016). A recent study found levamisole and pyrantel pamoate to be no more effective than placebo (Moser et al., 2017).

Over 1 billion people with STH infection have been treated with albendazole, putting high drug pressure on the parasites and leading to drug resistance (Moser et al., 2017). With *T. trichiura* now becoming resistant to these commonly used drugs, the cure rates and egg reduction rates will only continue to decrease (Adegnika et al., 2015). There is further controversy in opinion on whether these preventative chemotherapy programmes are effective for *T. trichiura* infection due to reinfection being so common after treatment, and adults generally not being treated allowing them to remain as reservoirs of reinfection for treated children (Asbjornsdottir et al., 2017; Jourdan et al., 2018). The uniqueness of *T. trichiura* makes it a difficult infection to treat, especially with its increasing resistance to current therapies. Therefore, there is an urgent need for new therapeutic options for this infection in order to improve the quality of life of many children in developing countries.

2.3. Trichuris trichiura

Human trichuriasis is common in low-income countries, especially in regions with warm and moist conditions. The combination of poor sanitation and a lack of education in these areas leads to an environment favouring infection and transmission of the parasite (Else et al., 2020). Previously infected individuals are more likely to become infected again after treatment than an individual who has never been infected (Wright et al., 2018). Up to 80% of *T. trichiura* infections are recorded in children, who suffer with the greatest worm burden and therefore most significant symptoms (McDowell and Rafati, 2014). Even mild infection in children can lead to stunted growth while heavy worm burden can lead to *Trichuris* dysentery syndrome (TDS) and colitis. TDS is a severe illness accompanied by symptoms including chronic dysentery, rectal prolapse, anaemia, poor growth, and clubbing of the fingers (Bundy and Cooper, 1989; Stephenson et al., 2000). Infected children may also present with failure to thrive and cognitive impairment (Bundy and Cooper, 1989; Ezeamama et al., 2005; Simeon et al., 1995).

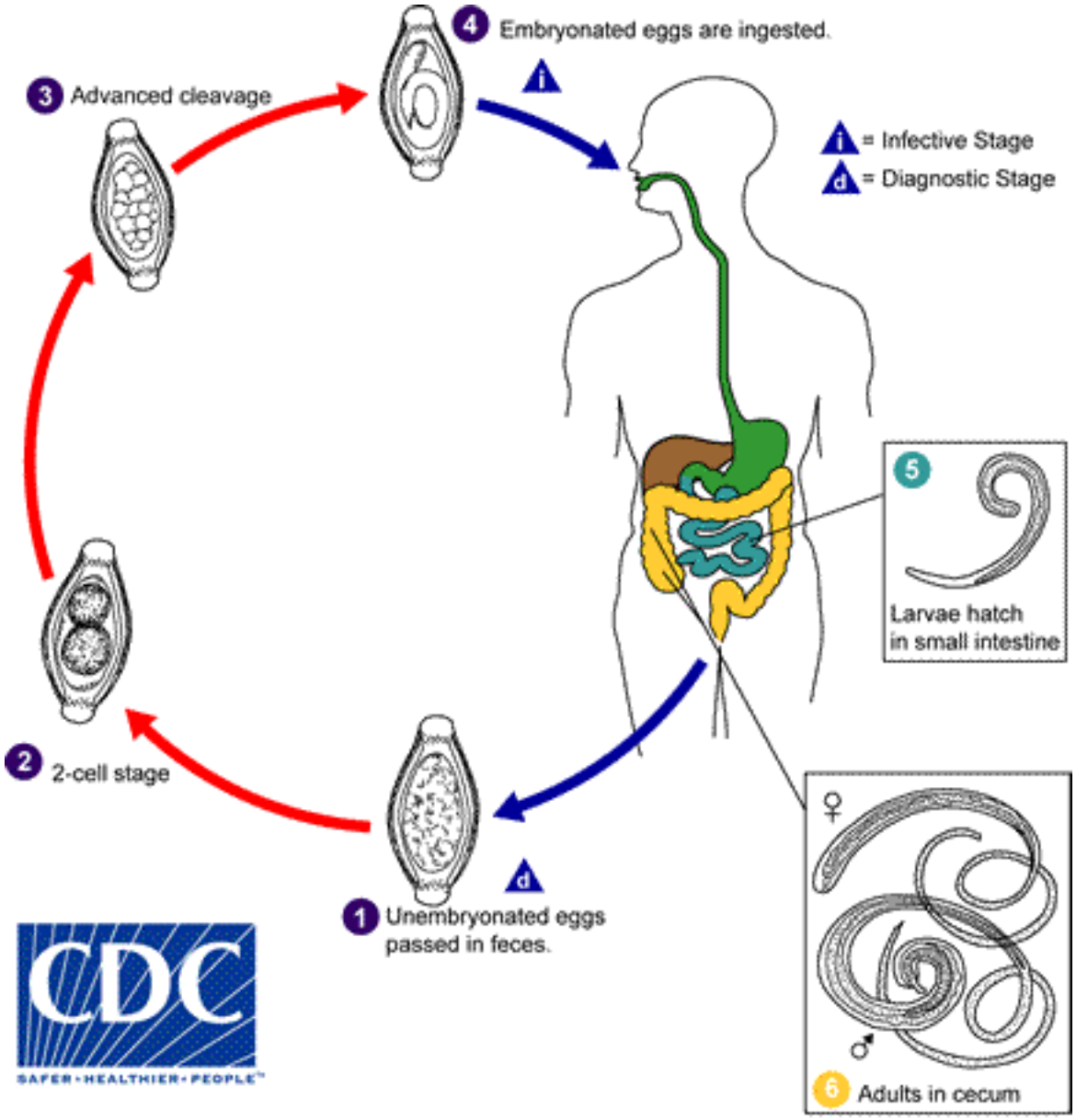

Figure 2.2: Trichuris trichiura life cycle. Unembryonated eggs are passed in faeces and become embryonated through a 2-cell stage (2) and an advanced cleavage (3). Embryonated eggs become infective within 15-30 days. Eggs are ingested via contaminated hands or food and then hatch in the small intestine of the host to release larvae which mature through 4 moult stages before establishing as adult worms within the colon. Adult worms live in the host cecum and ascending colon with the thin anterior of the worms embedded in the mucosa. Females shed eggs into the intestinal lumen and these eggs are passed in the faeces. Image adapted from (Centres for Disease Control and Prevention, 2013).

Transmission of *T. trichiura* (Fig. 2.2) requires embryonation of eggs in the environment, which can occur in temperatures between 0-37°C (Else et al., 2020). Infection occurs upon ingestion of embryonated eggs which hatch in the host intestine through contact with

bacterial type 1 fimbriae which bind to proteins at the poles of *T. trichiura* eggs (Hayes et al., 2010). Hatching of embryonated eggs releases L1 larvae into the intestinal lumen and these larvae penetrate epithelial cells at the base of intestinal crypts to form syncytial tunnels (Cliffe and Grencis, 2004). L1 larvae grow and moult through the four larval stages and subsequent adult stages within these syncytial tunnels. The posterior end of the L3, L4, and adult worms protrudes into the gut lumen while the anterior end containing the stichosome remains embedded within the syncytial tunnel (Else et al., 2020). Adult worms survive in this partially intracellular niche of the host caecum and proximal large intestine for up to 8 years, with fertilized females releasing 2,000 to 8,000 eggs per day (Else et al., 2020; Pike, 1969). These eggs are then released in the faeces into the environment where they become embryonated and the lifecycle can begin again (Else et al., 2020).

Key to understanding *T. trichiura* has been the use of the murine parasite, *Trichuris muris.* These mouse models of trichuriasis have allowed us to increase our understanding of the role of the host immune response in promoting either susceptibility, through a Th1 response, or resistance, through a Th2 response, to infection. The involvement of cell populations, including innate lymphoid cells, dendritic cells, and regulatory T-cells, have been studied using the *T. muris* model. Using mouse models, we have also developed an understanding of mechanisms of worm expulsion and possible targets for treatments (Klementowicz et al., 2012).

2.4. Trichuris muris

T. muris exists as a natural chronic infection of wild rodents and has very similar antigenic cross-reactivity, morphology, and niche as human parasite, *Trichuris trichiura* (Else et al., 2020). These characteristics allow *T. muris* to act as a well-established experimental model for research into human Trichuriasis, including in development of new therapeutics and to study host immune response (Hurst and Else, 2013; Roach et al., 1988). This model is well established and the immune response to *T. muris* in mice is well characterised (Else and Grencis, 1991). The model also allows us to control variables such as infection level, host genetics, and nutrition (Bancroft and Grencis, 2021).

Depending on host strain, host susceptibility, and parasite dose, *T. muris* can either establish a chronic infection in the host or quickly be expelled (Cliffe and Grencis, 2004). The majority of inbred laboratory mouse strains will expel a high dose (200 eggs) infection while immunodeficient mice will allow high dose infection to become chronic (Antignano et al., 2011; Klementowicz et al., 2012). Some mice that are normally resistant to a high dose infection, such as C57BL/6 mice, can be made to be susceptible by delivery of a low-dose infection of 30 eggs (Hurst and Else, 2013). Trickle infection (repeated low-dose exposure) can be used to model a natural infection (Glover et al., 2019).

Embryonated *T. muris* eggs contain a fully developed larva which will hatch in the caecum and invade the mucosa of the host caecum and large intestine (Klementowicz et al., 2012; Vejzagic et al., 2015). Worms reach patency around 33 days post infection and survive for prolonged periods, with some adult worms beginning to die from senescence from 100 days onwards (Bancroft and Grencis, 2021).

2.6. Worm Expulsion

Adult worms are extensively embedded between intestinal epithelial cells and are physically a challenge to remove (Tilney et al., 2005). Invasion of the worms into the colonic mucosa induces an immune cascade and a Th2 cytokine response, leading to profound changes in tissue physiology, the 'weep and sweep' response, to expel the parasites (Darlan et al., 2021). This response involves goblet cell hyperplasia and smooth muscle hypercontractility (Fig. 1.3) induced by type 2 innate lymphoid cells (ILC2s) and Th2 cytokines, including IL-13, IL-33, and IL-25 (von Moltke et al., 2016). A T-helper2 (Th2) response, characterised by the expression of type 2 cytokines, including IL-4, IL-5, IL-9, and IL-13, is essential for control of *Trichuris* infection. A Th1 response leads to susceptibility to infection, allowing worms to establish and the infection to become chronic (Cliffe et al., 2005).

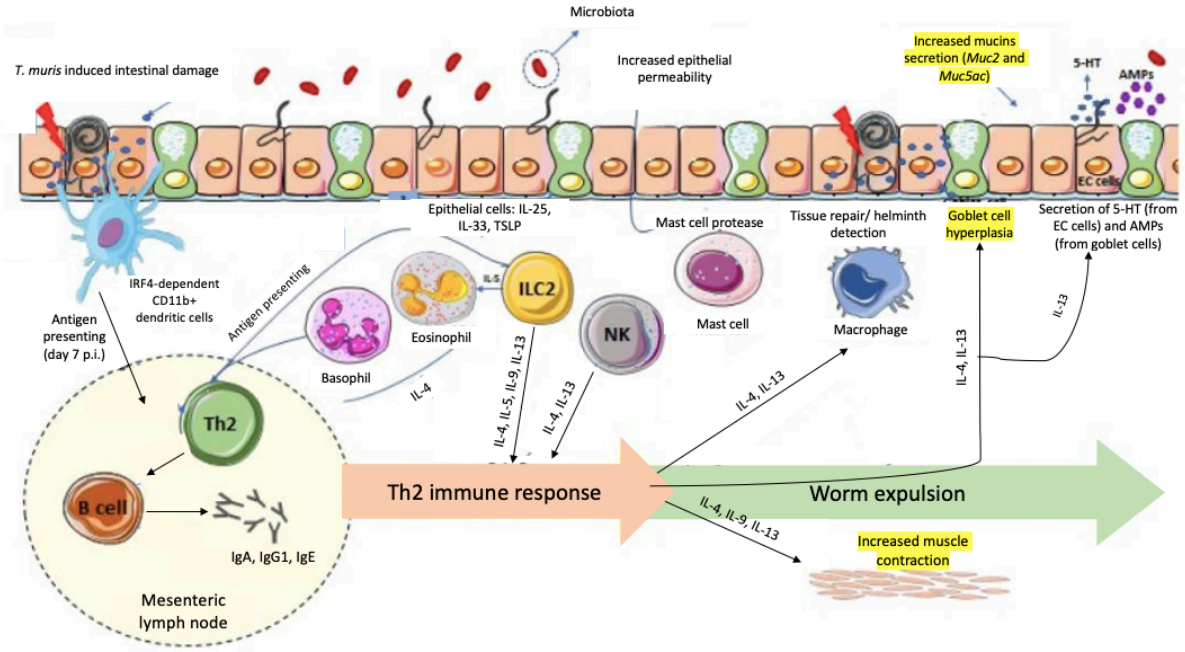

Figure 2.3: Representative schematic of the host immune response to infection with *T. muris*. *T. muris* larvae breach the intestinal epithelium, causing damage and releasing antigen, initiating the immune response. Intestinal epithelial cells produce alarmins (IL-25, IL-33, and TSLP) to recruit immune cells. Dendritic cells present *T. muris* antigen to adaptive immune cells in the mesenteric lymph node at day 7 post infection, activating a Th2 response. A Th2 response, associated with resistance to infection, increases epithelial permeability, induces goblet cell hyperplasia and increased mucin production, and increased smooth muscle contraction, leading to expulsion of the worms. Figure adapted from (Yousefi et al., 2021).

The roles of a Th1 or Th2 response in susceptibility or resistance to infection are now supplemented by the roles of innate lymphoid cells, basophils, dendritic cells, and regulatory T cells, which are all proposed to play a role in the response to *Trichuris* (Klementowicz et al., 2012). Th2-driven antiparasitic immunity is associated with eosinophilia, caecal mastocytosis, and elevated parasite-specific IgG1 and IgE antibodies. However, studies focusing on the outcome of infection in the absence of each effector individually have proved expulsion of *T. muris* to not critically depend on eosinophils, mast cells, or antibody-binding mechanisms (Betts and Else, 1999).

The immunological control of eosinophilia is completely dependent upon IL-5 (Coffman et al., 1989; Sher et al., 1990), and thus Betts and Else (1999) ablated IL-5 to uncover any role of eosinophilia during *T. muris* infection. Mice treated with anti-IL-5 antibody had eosinophil numbers lower than those of naïve mice but were still able to expel all worms. This

experiment proves that eosinophils have no essential role in resistance to *T. muris* infection. Furthermore, no changes in parasite specific IgG1 and IgG2a were detected in the anti-IL5 group compared to infected controls. Serum IgG1 (indicative of a Th2 response) were higher than IgG2a (indicative of a Th1 response) in both groups, as routinely found in resistant mouse strains (Betts and Else, 1999; Else et al., 1994).

Helminth-induced mastocytosis is controlled by a number of cytokines, including IL-3, IL-4, IL-9, and IL-10, but ablation of any one of these only suppresses mastocytosis rather than completely inhibit it (Madden et al., 1991). However, stem cell factor plays a critical role in mast cell differentiation and thus, abrogation of mast cell differentiation was achieved by blocking stem cell factor receptor (*c-kit*). Mast cell numbers and worm burden were determined at several timepoints throughout infection. At day 18 p.i., full ablation of intestinal mastocytosis was achieved with mast cell numbers reduced to below naïve levels in mice treated with anti-*c-kit.* It was found that abrogation of mastocytosis does not impede worm expulsion (Betts and Else, 1999). This experiment proved complete ablation of mastocytosis to have no effect on the ability of mice to expel *T. muris*.

The role of antibody-dependent cell-mediated cytotoxic (ADCC) mechanisms in resistance to *T. muris* were investigated using FcγR-/- mice. FcγR-/- mice are unable to express FcγRI, FcγRIII, or FcεRI and therefore are deficient in any effector function requiring high affinity binding of IgG or IgE (Takai et al., 1994). The FcγR-/- mice respond to infection very similarly to wildtype C57BL/6 mice, with worm burdens reduced by 21 and eliminated by day 36. Analysis of the cytokine profiles of these mice found FcγR-/- to produce higher levels of Th2 cytokines IL-4, IL-5, and IL-19 than wildtype C57BL/6 mice. FcγR-/- mice also produced less Th1 cytokine IFNγ than the wildtype C57BL/6 mice. IgG1 is associated with resistance to *T. muris* infection while IgG2a is associated with susceptibility. Infected C57BL/6 and FcγR-/- mice were found to have very similar IgG1 profiles, indicating resistance to infection. Serum IgG2a titre was very low in both C57BL/6 and FcγR-/- mice (Betts and Else, 1999).

There is some evidence that dendritic cells have an important role in the development of type 2 immunity in response to *T. muris* infection. Circadian studies found the circadian

clock of dendritic cells to affect, in part, the outcome of *T. muris* infection. Hopwood et al. (2018) utilized C57BL/6 mice kept on a 12-hour light/dark cycle. Mice were given a high dose (200 eggs) *T. muris* infection either in the morning (zeitgeber time 0, the point at which lights were turned on) or night (zeitgeber time 12, the point at which lights were turned off). Mice infected in the morning were more resistant to infection than those infected at night. Mice infected at night had a significantly greater worm burden at day 21 post infection than mice infected in the morning. However, both groups had largely cleared the infection by day 28 post infection (Hopwood et al., 2018).

2.6.1. Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are a part of the innate immune system most abundant at barrier surfaces where they have a role in response to pathogens (Messing et al., 2020). ILCs are divided into three subsets (ILC1, ILC2, and ILC3) based on surface markers, cytokine profiles, and function. These subsets closely resemble those of T-helper cells (Th1, Th2, and Th17, respectively) of the adaptive immune system. However, ILCs lack T-cell receptors and antigen specificity (Spits et al., 2013). ILC2s produce IL-5 and IL-13 in response to helminth infection (Bando and Colonna, 2016; Price et al., 2010).

Studies indicated that innate immunity may play a role in host protection during intestinal nematode infection, including *N. brasiliensis* and *H. polygyrus,* through tuft-cell induced type 2 innate lymphoid cell (ILC2) production of IL-13 (Gerbe et al., 2016; Oliphant et al., 2014; Pelly et al., 2016; von Moltke et al., 2016). However, Glover et al., (2019) found that ILC2s do not have an important role in resistance to *T. muris* infection. ILCs from the intestine and mesenteric lymph node were analysed via flow cytometry. Total intestinal ILC numbers decreased throughout infection compared to naïve animals, with a decrease in the proportion of ILC2s and ILC3s relative to ILC1s. The ILC populations of the mesenteric lymph node were unaffected during infection. A small but significant increase in tuft cell numbers in the caecum was observed, however there was a significant decrease in ILC2s at this time point (Glover et al., 2019). ICOS-T mice can be specifically depleted of ILC2s using DTx treatment. Glover et al. (2019) confirmed their results using these ICOS-T mice with both trickle and high-dose infections. Worm burdens of mice depleted of ILC2s were not significantly different to that of control mice for those with trickle infection or those that

received a high dose. Therefore, resistance to *T. muris* infection, unlike other intestinal helminths, is not reliant on ILC2s (Glover et al., 2019).

2.6.2. Tuft Cells

Tuft cells are a rare epithelial cell type in the steady-state epithelium (Gerbe et al., 2011). These cells were found to have a role in initiating a Th2 response to parasites through a cytokine-mediated mechanism and, with goblet cells, have important roles in protecting the colonic epithelium from damage (Gerbe et al., 2016; Van der Sluis et al., 2006).

Previous studies found a significant increase in tuft cells during *N. brasiliensis* and *H. polygyrus* infection, playing an important role in expansion of ILC2s through secretion of IL-25 (Gerbe et al., 2016; Howitt et al., 2016a). After *T. muris* trickle infection, a small but significant increase in tuft cells was observed, identified by expression of Dclk1, a tuft cell marker (Glover et al., 2019). IL-33 is an alarmin cytokine that is known to be upregulated in response to helminth infection (Artis and Grencis, 2008; Humphreys et al., 2008; Waddell et al., 2019) and can also expand ILC2s and hence tuft cells. Tuft cells constitutively express IL-25, another alarmin cytokine, which activates ILC2s during helminth infection to produce IL-13. IL-13 further drives proliferation of tuft cells, forming a circuit between innate lymphoid cells and tuft cells during helminth infection (Howitt et al., 2016b; von Moltke et al., 2016).

2.6.3. Mucin Production

The mucosal barrier is the primary defence against intestinal pathogens (Hurst and Else, 2013). The barrier consists of two major components: the intrinsic barrier of continuous epithelial cell lining and the extrinsic barrier which is a combination of secretions from the goblet cells within the epithelium (Linden et al., 2008). Changes to the mucus barrier have been observed in response to *T. muris* infection. Goblet cell hyperplasia occurs in animals resistant to *T. muris,* whereas hyperplasia of enterocytes is observed during a chronic infection (Artis et al., 1999; Hasnain et al., 2010). Goblet cell hyperplasia is under control of Th2 cytokines, including IL-4, IL-5, and IL-13, although some studies suggest that IL-4/IL-13 independent goblet cell hyperplasia can occur (Gerbe et al., 2016; Marillier et al., 2008).

Muc2, the main component of mucus at the intestinal barrier is increased in mice resistance to *T. muris* infection. This increase is seen only in the caecum, the niche that the parasite occupies. This increase in Muc2 was not observed in susceptible mice, thus supporting the hypothesis that Muc2 contributes to host protection during infection with *T. muris.* Muc2-deficient mice were found to have significant delay in worm expulsion, although did eventually expel all worms. This delay appears to not be caused by alterations in the adaptive immune system, as this was unaltered between wildtype and Muc2-deficient mice. Hasnain et al. (2010) then proved Muc5ac to be a significant component of mucus in response to *T. muris* infection, increasing shortly before worm expulsion. This was first observed in Muc2-deficient mice but, interestingly, was also found to also be significantly up-regulated in wildtype mice at days 14 and 21 post infection. Interestingly, the de novo expression of *Muc5ac* was observed only in mice strains resistant to *T. muris* infection and not in susceptible animals. No changes were observed in the cell surface mucins, *Muc1, Muc4,* and *Muc17*, which are thought to play a role in mucosal protection (Hasnain et al., 2010). Muc5ac-deficient mice, on a resistant C57BL/6 background, were found to be susceptible to *T. muris* infection, with no significant decrease in worm burden even after 45 days post infection (Hasnain et al., 2011a).

2.6.4. Intestinal Muscle Contraction

Hypercontractility of smooth muscle is a known mechanism of intestinal helminth expulsion, including *T. muris.* IL-9 has been found to play a key role in colonic muscle hypercontractility and subsequent worm expulsion during intestinal helminth infection, including *T. muris*. Administration of IL-9 to non-infected C57BL/6 mice was found to significantly increase jejunal muscle contractility and in C57BL/6 mice infected with *Trichinella spiralis,* significantly accelerated worm expulsion (Khan et al., 2003). Khan et al. (2003) vaccinated C57BL/6 and BALB/c mice against IL-9 to neutralise IL-9 activity and then infected them with *T. muris* (100 eggs). This neutralisation significantly attenuated colonic muscle hypercontractility and inhibited worm expulsion. The lower degree of muscle contractility was found to correlate with the presence of higher worm burden in the caecum (Khan et al., 2003). IL-9 has been previously shown to be important in expulsion of *T. muris.* One study found IL-9 transgenic mice, which constitutively overexpress IL-9, infected with a high dose (300 eggs) *T. muris* infection to expel worms more quickly than wildtype mice, expelling all

worms by day 13 post infection (Faulkner et al., 1998). Richard et al. (2000) vaccinated C57BL/6 mice, which are normally resistant to a high dose *T. muris* infection, with IL-9-OVA and infected with 200 *T. muris* eggs. They found vaccinated mice to be unable to expel the worms by day 34 post infection, while seven of the eight control mice had completely cleared the infection (Richard et al., 2000).

2.6.5. Epithelial Turnover

Increased epithelial turnover is a key mechanism in expulsion of *T. muris*, physically moving the parasite out of its optimal niche (Cliffe et al., 2005; Oudhoff et al., 2016b). As epithelial cells move from the bottom of the crypt (the proliferation zone) towards the top (the shedding zone), the embedded parasite is moved with the cells towards the lumen, where both the cells and parasite are shed (Klementowicz et al., 2012). Cliffe et al. (2005) used a bromodeoxyuridine (BrdU) pulse-chase experiment to look at the rate of epithelial turnover in mice resistant to *T. muris* infection (BALB/c) and mice susceptible to infection (AKR). They found cell turnover to be elevated in both strains after infection. However, in resistant BALB/c mice, epithelial turnover was seen to be double that of susceptible AKR mice at day 14 post infection. Turnover in the susceptible mice was elevated at day 21 post infection, to levels comparable to BALB/c mice at day 14 post infection. Determination of worm burden found that increased turnover at day 21 is no longer sufficient to dislodge *T. muris.* Between days 14 and 21 post infection, the worms will have undergone a further moult stage and will have quadrupled in length. At this point, the worms reside much higher in the crypt and therefore are no longer in the compartment at which the fastest movement of cells is occurring (Cliffe et al., 2005).

Cliffe et al. (2005) then investigated the roles of Th2 cytokines IL-4 and IL-13 in epithelial turnover to expel *T. muris.* They utilized BALB/c IL4-/- mice, as females of this strain can expel worms through an IL-13 dependent mechanism, while males suffer chronic infection (Bancroft et al., 2000). Animals were infected and worm burden and rate of epithelial turnover were assessed. At day 28 post infection, when all wildtype controls had expelled their worms, parasite expulsion was underway in female IL4-/- mice but male IL4-/- mice were still infected. Female IL4-/- mice were found to have significantly faster epithelial

turnover than the males, suggesting a prominent role of IL-13, but not IL-4, in this turnover (Cliffe et al., 2005).

Chronic *T. muris* infection is dependent on IFNγ and IFNγ-induced protein 10 (IP-10) has been found to reduce the rate of epithelial turnover (Else et al., 1994; Sasaki et al., 2002). At day 21 post infection with *T. muris*, IP-10 was detected in epithelial cells of the large intestine of susceptible AKR mice, coinciding with expression of IFNγ. Both IFNγ and IP-10 were undetectable in resistant BALB/c mice. IL-13 was also found to be expressed in the gut of AKR mice on day 21 and day 35, explaining the increased epithelial turnover observed at these timepoints. These data suggest that IP-10 has a dominant effect even in the presence of IL-13. *In vivo* neutralization of IP-10 proved this, as infected AKR mice were able to then expel their worms and showed significantly increased epithelial turnover compared with untreated mice. Furthermore, neutralization of IP-10 in severe combined immunodeficient (SCID) mice was found to cause a significant reduction in worm burden compared to controls. These data suggest that increased epithelial turnover alone is sufficient to induce worm expulsion, and may even be able to expel adult worms if the elevation in epithelial turnover is enough (Cliffe et al., 2005).

2.7. Intraepithelial Lymphocytes

The intestinal epithelium has an important role acting as a barrier against the huge number of pathogens it constantly encounters, including intestinal helminth infections, and therefore hosts one of the largest immune systems in the body (Peterson and Artis, 2014). Intraepithelial lymphocytes (IELs) are a specialised population of intestinal immune cells located between the basolateral surfaces of intestinal epithelial cells, in direct proximity to antigens in the intestinal lumen, with roles in modulation of innate immunity and control of gut barrier functionality, epithelial turnover, and host response to enteric pathogens (Qiu and Yang, 2013). IELs are predominantly CD8+ T cells which are immunologically distinct from peripheral T cells (Kunisawa et al., 2007). IELs possess some, but not all, properties of activated T cells and are therefore constantly partially activated (Montufar-Solis et al., 2007).

IELs are divided into 2 subsets based on their unique features. Type-a IELs arise from conventional T cells, so are major histocompatibility complex (MHC) class II- and class I-restricted, and express TCR$\alpha\beta$ with CD4 or CD8α. These cells are specific for non-self-antigen due to positive and negative selection in the thymus. Type-b IELs either express TCR$\alpha\beta$ or TCR$\gamma\delta$, and are either CD8$\alpha\alpha^+$ or CD8$\alpha\alpha^-$ (Qiu and Yang, 2013). The majority of IELs are TCR$\gamma\delta$+ and are fast acting cells able to be activated without antigen presenting cells (Park et al., 2010). IELs produce cytokines which modulate the mucosal barrier of the intestine and important cellular functions to trigger downstream signalling pathways with roles in barrier homeostasis and injury (Qiu and Yang, 2013). Intestinal $\gamma\delta$ T-cells have important roles in regulation of epithelial barrier function, epithelial cell turnover, and protection in response to pathogens (Inagaki-Ohara et al., 2004; Inagaki-Ohara et al., 2011; Roberts et al., 1996).

Intestinal IELs are found in the exact place *Trichuris* occupies. TCR$\gamma\delta$ T-cells can mediate immunity and can be a source of Th2 cytokines, raising the possibility for these cells to have a role in the immune response to helminth infection (Ferrick et al., 1995; Inagaki-Ohara et al., 2011). Inagaki-Ohara et al. (2011) used TCRδ-/- mice to investigate the role of TCR$\gamma\delta$ T-cells in response to *Nippostrongylus brasiliensis* infection. Wildtype and TCRδ-/- mice were challenged with 500 third-stage *N. brasiliensis* larvae and course of infection was compared. All wildtype mice survived the infection whereas 70% of the TCRδ-/- mice succumbed to infection within 16 days. At day 10 post infection, wildtype mice had cleared all worms from their intestine whereas TCRδ-/- mice retained a high worm burden (Inagaki-Ohara et al., 2011).

Interestingly, IELs have recently been found to possess the glucagon-like peptide-1 (GLP-1) receptor (GLP-1R), indicating that peptide hormones could be influencing the intestinal immune response to helminth infection. Activation of the GLP-1r on intestinal IELs with the receptor agonist Exendin-4 (Ex-4) was found to suppress expression of inflammatory cytokines, including interferon-γ (IFNγ) and tumour necrosis factor-α (TNFα), *in vitro*. C57BL/6 mice with dextran sodium sulfate (DSS) induced colitis were treated with Ex-4 and

it was found that Ex-4 treated mice had significantly increased levels of *Il13* mRNA compared to PBS control mice.

Furthermore, the severity of intestinal injury is increased in *Glp1r-/-* mice. Yusta et al. (2015) compared the severity of DSS induced colitis in *Glp1r-/-* mice and *Glp1r+/+* mice. The *Glp1r-/-* mice lost significantly more weight and exhibited significantly greater disease activity scores and epithelial damage in the colon (Yusta et al., 2015). Given that GLP-1 signalling has been proven to be involved in host immune responses to intestinal injury or infection, this GLP-1-IEL-GLP-1R axis appears to be a very interesting drug target for treatment of helminth infection, including *T. trichiura*.

2.8. GLP-1

GLP-1 is synthesised in response to nutrient detection and secreted from enteroendocrine L-cells after posttranslational processing of proglucagon by prohormone convertase 1/3 (Campbell and Drucker, 2013; Lafferty et al., 2021). Enteroendocrine cells (eecs) are specialised trans-epithelial signal transduction conduits dispersed throughout the gut that comprise 1% of the intestinal epithelium, collectively forming the largest endocrine system in the human body (Worthington et al., 2013). Eecs secrete a number of peptide hormones in response to luminal stimuli and these hormones have roles in metabolism and act as local neurotransmitters (Gribble and Reimann, 2016; Yusta et al., 2015). Studies have focused primarily on the role of eecs in post-prandial assimilation of nutrients via alterations in gastrointestinal secretion, gut motility, insulin release, and satiety (Begg and Woods, 2013). However, more recent research indicates that these cells have chemosensory mechanisms to detect stimuli beyond nutrient intake, and hence have important roles beyond those in appetite and digestion. Eec signalling can be protective to the intestine as peptide hormones have been proven to modulate barrier function and chemosensory eecs are able to mediate mucosal immunity (Worthington et al., 2018).

Helminth infections have been found to alter eec function, likely due to the close association of helminths with the epithelium. This was first observed in livestock (Bosi et al., 2005; Dynes et al., 1998; Forbes et al., 2009; Scott et al., 1998; Yang et al., 1990) before

experimental murine models were used to investigate these alterations further. CCK+ cell hyperplasia and increased CCK levels have been observed during *Trichinella spiralis* infection in mouse models, coupled with hypophagia during enteritis. Mice lacking CCK displayed no period of hypophagia, identifying CCK as the mediator of hypophagia in *Trichinella spiralis* infection (McDermott et al., 2006; Worthington et al., 2013). These findings do not apply to all helminth infections, as CCK levels are reduced in *Nippostrongylus brasiliensis* infection in rat models (Ovington et al., 1985). Gastrin levels are reduced in the serum of *T. spiralis* infected rats (Castro et al., 1976). Enterochromaffin cells, another type of EEC, produce 5-HT in the colon. During *T. muris* infection, resistant BALB/c mice were found to possess significantly higher numbers of enterochromaffin cells than susceptible AKR mice. BALB/c mice also had significantly increased levels of 5-HT in the colon than AKR mice. This study found significant correlation between worm burden and the number of enterochromaffin cells and 5-HT levels (Motomura et al., 2008). Taken together, these studies indicate a role for EECs and peptide hormones in helminth infection.

Peptide hormones control metabolism through regulation of energy intake, digestion, absorption, and storage and disposal of digested nutrients (Drucker, 2007). GLP-1 is an incretin peptide hormone with a range of functions including glucose induced insulin secretion through stimulation of proinsulin gene expression and biosynthesis (Drucker et al., 1987). Other functions of GLP-1 include inhibition of glucagon release, delay of gastric emptying, and reduction of glucose production (Campbell and Drucker, 2013). GLP-1 is an integral component of the incretin axis, with transient blocking of GLP-1 receptor signalling reduces insulin levels, increases glucagon levels, and impairs glucose tolerance (Campbell and Drucker, 2013). GLP-1 binds receptors in the pancreas to stimulate glucose-dependent insulin secretion from β-cells, enhancing insulin biosynthesis and inhibiting glucagon secretion (Ahrén, 2003). GLP-1 also reduces pancreatic β-cell apoptosis and promotes β-cell proliferation, and as such, is used as a treatment for type 2 diabetes mellitus (Farilla et al., 2002; NICE., 2021a). GLP-1 may also have direct effects on the hypothalamus to decrease appetite, promoting earlier satiety and therefore weight loss. This weight loss then reduces insulin resistance and facilitates normoglycaemia (Ali et al., 2011). Aside from its main role in the pancreas, GLP-1 receptor is widely distributed throughout the body, found on cells in the brain, heart, kidneys, liver, heart, fat, and gastrointestinal tract (Cheang and Moyle,

2018; Holst, 2007). GLP-1 has a wide range of different functions throughout the body, as seen in figure 2.4.

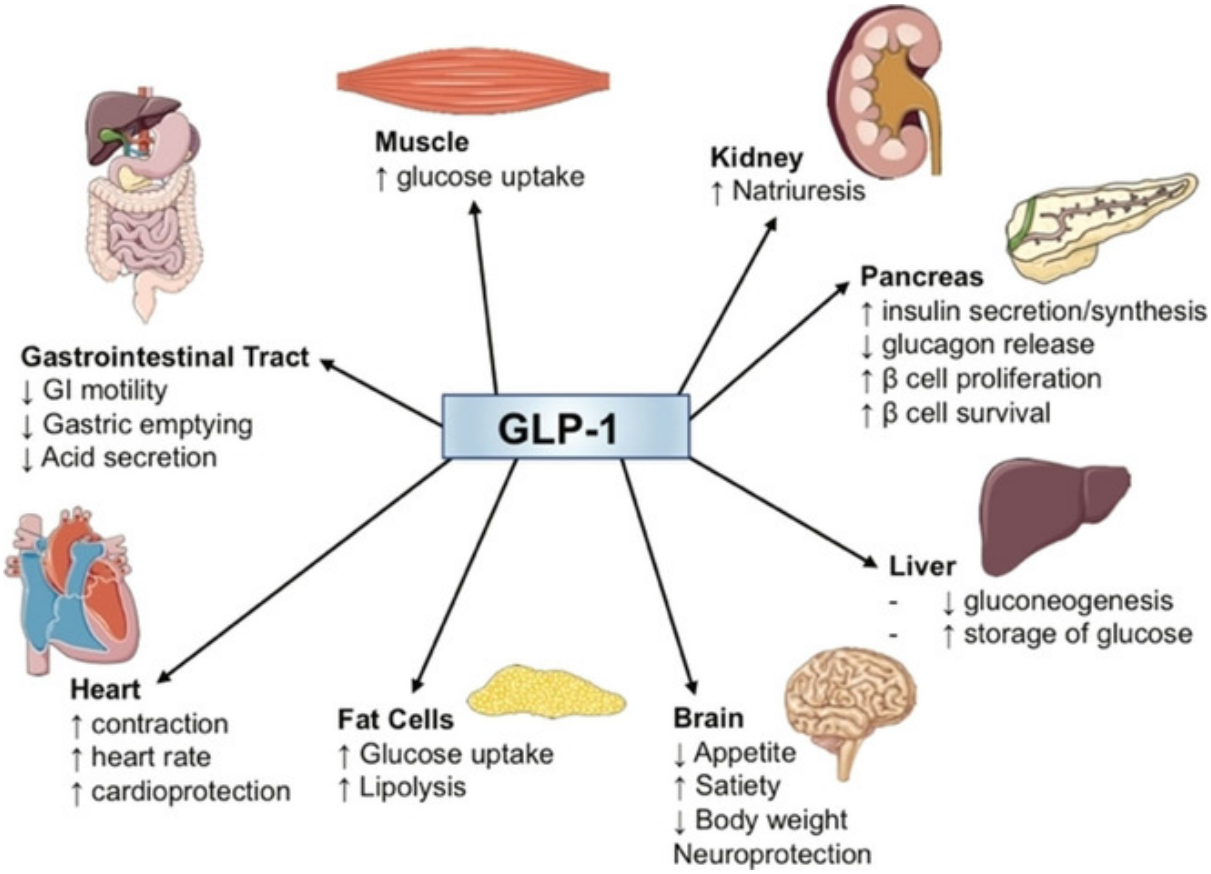

Figure 2.4: Biological functions of GLP-1. The GLP-1r is found on many cells in the body and has a range of functions. The main functions of GLP-1 are in the pancreas where it stimulates insulin secretion and inhibits glucagon release in a glucose dependent manner. Figure adapted from (Cheang and Moyle, 2018).

In response to stimulation by nutrients, enteroendocrine L-cells of the intestine produce proglucagon which liberates multiple proglucagon-derived peptide hormones, including glucagon-like peptides 1 and 2 (GLP-1 and GLP-2) (Bewick, 2012; Lafferty et al., 2021). GLP-1 and GLP-2 have been linked to intestinal growth in both the small intestine and colon (Drucker et al., 1996; Koehler et al., 2015). Drucker et al. (1996) administered synthetic GLP-1 or GLP-2 to female CD1 mice twice daily for 10 days. They found that doses as low as 6.25 μg of GLP-2 twice daily increased the weight of the small intestine by up to 2-fold. Mucosal thickness and villus height were found to be increased, while crypt depth and muscle thickness were unaffected. Time course experiments proved the growth-promoting

properties of GLP-2 to be evident after only 4 days of treatment with the peptide, with no evidence of cell proliferation in other organs examined, including the spleen, kidneys, lung, heart, brain, or liver. Cell proliferation rates were assessed using proliferating cell nuclear antigen (PCNA) as an indicator of proliferation. The number of PCNA positive cells were significantly increased in GLP-2 treated mice, indicating that GLP-2 has a role as a major determinant of epithelial proliferation in the small intestine (Drucker et al., 1996). Koehler et al. (2015) treated C57BL/6 mice with GLP-1r agonist, exendin-4 (Ex-4), daily for 7 days. Ex-4 treatment significantly increased the weight of the colon but did not enhance cell proliferation. They then treated with Ex-4 twice daily for 1 month. After a month, the number of crypts in the colon was significantly increased, but crypt depth was unaffected. These results suggest that sustained GLP-1r activation increases crypt number, rather than crypt cell proliferation, to promote colon growth. These effects are mediated through the GLP-1r as they are not seen when *Glp1r-/-* mice are treated with GLP-1r agonists (Koehler et al., 2015).

Levels of GLP-1 have been found to increase rapidly in response to intestinal injury or mucosal inflammation with studies showing that *Glp1r-/-* mice have dysregulated gene expression, abnormal microbial species in faeces, and enhanced sensitivity to intestinal injury. *Glp1r+/+ and Glp1r-/-* female mice were maintained on drinking water containing 3% w/v dextran sodium sulfate (DSS) or regular drinking water for 7 days. Severity of induced colitis was compared, and it was found that *Glp1r-/-* mice lost significantly more weight and had significantly shorter colon lengths. Furthermore, the *Glp1r-/-* mice exhibited significantly increased disease activity scores and epithelial damage, reflected by a significantly greater colon damage score. These results suggest that activation of GLP-1 receptor signalling seems to establish a generalised cytoprotective response in murine intestine. Acute treatment with GLP-1 receptor agonist, Exendin-4, has been found to markedly increase expression of genes that play a role in pathogen clearance, including those encoding Th2 cytokines, IL-5 and IL-13 (Yusta et al., 2015).

2.9. GLP-1 Receptor Agonists

The usefulness of endogenous GLP-1 as a therapy for diabetes is limited by its rapid degradation by the enzyme dipeptidyl peptidase-4 (DPP-4), which is broadly expressed on cell surfaces and present in circulation, resulting in a half-life of only 2 minutes (Cheang and Moyle, 2018; Gilbert and Pratley, 2020). Cleavage by DPP-4 inactivates GLP-1 and generates a species that acts as an antagonist to the GLP-1 receptor (de Graaf et al., 2016). The practical limitations of native GLP-1 as a therapy have been overcome through the development of GLP-1 receptor agonists (GLP-1rA) that are resistant to the DPP-4 enzyme (Gilbert and Pratley, 2020). GLP-1RAs mimic the action of native GLP-1, binding the GLP-1 receptor with specificity and can be short- or long-acting based on their pharmacokinetic and pharmacodynamic properties (Drucker, 2018; Gilbert and Pratley, 2020) (Fig. 2.5). Some GLP-1r agonists are approved for use in humans for treatment of type 2 diabetes mellitus. Currently, there are five GLP-1r agonists approved for use in the UK, all of which are self-administered via subcutaneous injection (National Institute for Health and Care Excellence, 2021). These therapies are particularly attractive due to their glucose-dependent incretin effects, propensity for weight loss, and potential cardiovascular benefits (Smilowitz et al., 2014).

GLP-1r agonists are often approved as add-on therapies to exogenous insulin therapy to reduce insulin dose requirement and to boost therapeutic effects (DeFronzo et al., 2008). GLP-1 receptor agonists are generally well tolerated and can be given to non-diabetic people, as risk of hypoglycaemia is low due to their glucose-dependent mechanism of action (Cheang and Moyle, 2018; Gilbert and Pratley, 2020). These drugs induce insulin secretion in hyperglycaemic patients, but not those with normal glucose levels, thus reducing the risk of hypoglycaemia (Elkinson and Keating, 2013).

Figure 2.5: Amino acid structures of GLP-1 receptor agonists in comparison to biologically active native GLP-1. Exendin-4 is a naturally occurring GLP-1 receptor agonist, now commercially available for use in humans in

treatment of type 2 diabetes. Lixisenatide is a synthetic analogue of Exendin-4, also approved for treatment of type 2 diabetes mellitus in humans. Amino acids highlighted in grey are those that differ from native GLP-1 and those in black are unique to lixisenatide. Figure adapted from (Werner et al., 2010).

2.9.1. Exendin-4

Exendin-4 (Ex-4) is a GLP-1 receptor agonist found naturally in the saliva of the Gila monster lizard *(Heloderma suspectum)* which has a remarkably similar mode of action to that of native GLP-1 (Yap and Misuan, 2019). GLP-1 and Ex-4 share only 50% sequence similarity, however Ex-4 is still a full agonist for the GLP-1 receptor, causing a dose-dependent, glucose-induced secretion of insulin (Goke et al., 1993). Ex-4 has been commercialised into its synthetic form, exenatide (under the brand name Byetta® or Bydureon®) (Yap and Misuan, 2019). The UK approved exenatide for use in treatment of human type 2 diabetes mellitus in 2006, therefore we have a solid understanding of its side effects (Kyriacou and Ahmed, 2010).

2.9.2. Lixisenatide

Lixisenatide (brand name, Lxyumia) is a synthetic analogue of Exendin-4 and GLP-1 receptor agonist that is approved for the treatment of type 2 diabetes (Elkinson and Keating, 2013; Newsome, 2017). The structure of lixisenatide is based on Ex-4, with modification via deletion of a proline residue, and addition of six C-terminal lysine residues, and exhibits fourfold higher binding affinity for the GLP-1 receptor than native GLP-1 (Christensen et al., 2011; Horowitz et al., 2013). The amino acid modifications to lixisenatide confer resistance to cleavage by DPP-4, extending the half-life to 3 hours, in comparison to the very short half-life of 2 minutes of native GLP-1 (Aroda, 2018). Still, lixisenatide is considered to be a short-acting GLP-1 receptor agonist in comparison to longer-acting GLP-1 receptor agonists such as liraglutide and once-weekly exenatide (Anderson and Trujillo, 2016; Christensen et al., 2011). Due to its short acting nature, there is only transient exposure to lixisenatide once injected (Nauck et al., 2017).

The GetGoal-X study found lixisenatide recipients to be significantly less likely than exenatide recipients to experience symptomatic hypoglycaemia (Rosenstock et al., 2011).

Furthermore, lixisenatide produces only mild and transient side effects including nausea, vomiting, diarrhoea, and headaches. Other adverse reactions to lixisenatide included dizziness, back pain, flu-like symptoms, drowsiness, and stomach ache (Elkinson and Keating, 2013).

2.10. Aims

T. trichiura infection is a neglected tropical disease affecting over 1 billion people worldwide, and the parasites are gaining resistance to the current treatments in use. Symptoms can be severe and cause great morbidity, especially in children, and therefore there is urgent need for new therapeutics. Based on the literature, GLP-1r+ IELs seem to be a good drug target for *T. trichiura* infection. Using the well-defined model of *T. muris* in C57BL/6 mice, this project aims to investigate whether GLP-1r agonist treatment is effective against Trichuriasis and the mechanism by which GLP-1rA-induced worm expulsion would occur. Use of GLP-1r agonists, already approved for diabetes, would allow a quick and simple repurposing for treatment of *T. trichiura,* greatly improving the quality of life of many children in developing countries.

3. Methods

3.1. Animals

C57BL/6 mice, C57BL/6 RAG-/- mice (a kind gift from Dr K Okkenhaug; Babraham Institute, Cambridge, England), GLP-1rKO (Scrocchi et al., 1996), and GLP-1rCre reporter (Richards et al., 2014) (housed at Lancaster University) were kept in individually ventilated cages on a 12-hour light/dark cycle at 22 $\pm$ 1 °C and 65% humidity. All procedures were carried out on 6–12-week-old littermates after 1 week of acclimatisation and in accordance with the Home Office Science Act (1986). All experiments carried out at Lancaster University were carried out under the project licence PP4157153. All experiments conformed to the Lancaster University Animal Welfare and Ethical Review Body (AWERB) and ARRIVE guidelines. All animals were humanely killed by cervical dislocation followed by terminal exsanguination.

3.1.1. Genotyping

GLP-1rKO and GLP-1rCre mice were genotyped using a pinch of ear tissue. Tissue was digested in digestion mix (1mM EDTA, 50mM Tris - hydrochloric acid, 20mM sodium chloride, 1% tween, 10mg/ml proteinase K). All samples were then left on a heat block at 55°C overnight. The next day, samples were vortexed until the tissue completely disintegrated before being left on the heat block at 95°C for 10 minutes. Samples were then moved immediately onto ice for 10 minutes. Samples were amplified in master mix (7.125μL qH2O, 1μL primer, 3μL MgCl2 (25mM, Promega), 0.75μL dNTPs (10mM, Promega), 0.125μL GoTaq G2 Flexi DNA polymerase (Promega), 5μL GoTaq® Flexi buffer (Promega), and 4μL betaine (5M, MP Biomedicals)) using the programs shown in tables 1 and 2. Samples were run at 75V for approximately 80 minutes on a 2% agarose gel containing 7.5μL safe view nucleic acid stain (Fig. 3.1).

Table 3.1: GLP-1rKO PCR cycles

	94°C	5 min
35 cycles	94°C	30 sec
	61°C	45 sec
	72°C	45 sec
	72°C	10 min

Table 3.2: GLP-1rCre PCR cycles

	95°C	5 min
39 cycles	95°C	1 min
	58°C	1 min
	72°C	1 min
	72°C	10 min

Table 3.3: Primers used

	oIMR0013 (10 μM)	CTTGGGTGGAGAGGCTATTC	Invitrogen U0772 (C05)

GLP-1rKO	oIMR0014 (10 μM)	AGGTGAGATGACAGGAGATC	Invitrogen U0772 (C06)
	GLP1R F2 (10 μM)	TACACAATGGGGAGCCCCTA	Invitrogen U0772 (C07)
	GLP1R R2 (10 μM)	AATGTCATGGGATGTGTCTGGA	Invitrogen U0772 (C08)
GLP-1rCre	iCre 1 (10 μM)	GTAGTCCCTCACATCCTCAGG	Invitrogen A0616 (G07)
	GLP-1 (10 μM)	CAGCGCCGAACATCTCCTGG	Invitrogen A0616 (G06)
	iCre 2 (10 μM)	GACAGGCAGGCCTTCTCTGAA	Invitrogen A0616 (G08)
	iCre 3 (10 μM)	CTTCTCCACACCAGCTGTGGA	Invitrogen A0616 (G09)
	β-catenin Rm41 (10 μM)	AAGGTAGAGTGATGAAAGTTGTT	Invitrogen A0616 (G10)
	β-catenin Rm42 (10 μM)	CACCATGTCCTCTGTCTATTC	Invitrogen A0616 (G11)

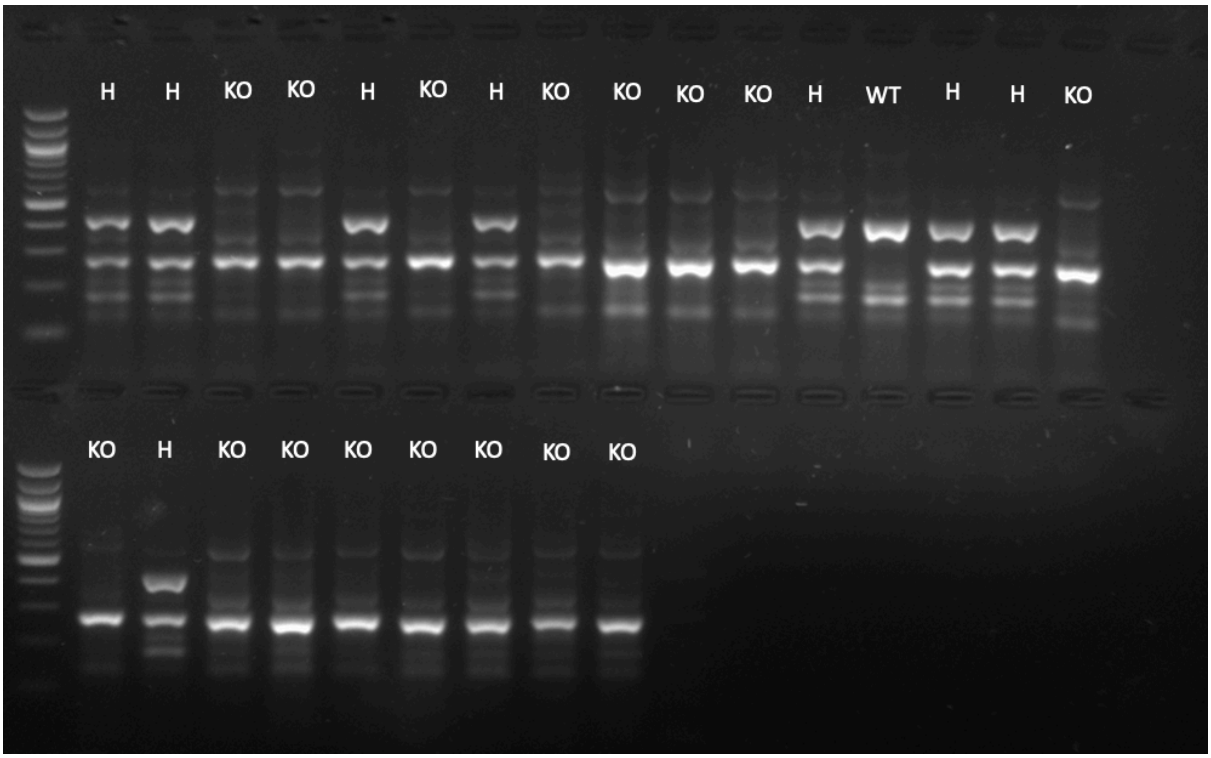

Figure 3.1: GLP-1rKO genotyping. Lanes labelled as WT (wild type), H (heterozygous), or KO (knock out). WT = 437 bp, KO = 280 bp.

3.1.2. *Trichuris muris* passage

Caecum and large intestine collected from high dose infected RAG-/- mice in 50ml falcon tube containing pre-warmed (37°C) RPMI with 5x pen strep. Gut was carefully cut open and gently washed in RPMI to remove faecal content. Adult worms were gently removed using forceps and placed into 6 well plates containing RPMI-Penstrep and kept warm on heat mats. Plates were then incubated at 37°C in a humidity box for 4 hours then overnight. At each time point, medium containing eggs was removed and centrifuged at 2000rpm for 15 mins. Excretory/secretory (E/S) supernatant was poured into a new 50ml falcon tube and the pellet containing eggs was resuspended in 40ml qH2O then poured through a 100μM sieve into a cell culture flask. Eggs were wrapped in tin foil and stored in the dark at room temperature for 8 weeks before being moved to 4°C. The collected E/S antigen was concentrated via repeated centrifuging before dialysing (in 12-14 kDa MWCO dialysis tubing) against PBS at 4°C overnight. Concentration of E/S was determined using the Nanodrop 2000c (Fisher Scientific).

3.1.3. *T. muris* Infection and Determination of Worm Burden

Mice were infected with low dose *Trichuris muris* infection (approximately 30 embryonated eggs in 200μL PBS) via oral gavage with a blunt needle. Worm burdens were determined by counting the worms present in the caecum and proximal large intestine.

3.1.4. GLP-1r Agonist Treatment

Exendin-4 or lixisenatide were administered via intraperitoneal injection with a 25g 5/8" needle either daily throughout infection or once chronic infection had established (32-34 days post infection) (Fig. 3.2). Exendin-4 was used at 2μg/200μL PBS and lixisenatide was used at 1μg, 5μg, or 10μg in 200μL PBS.

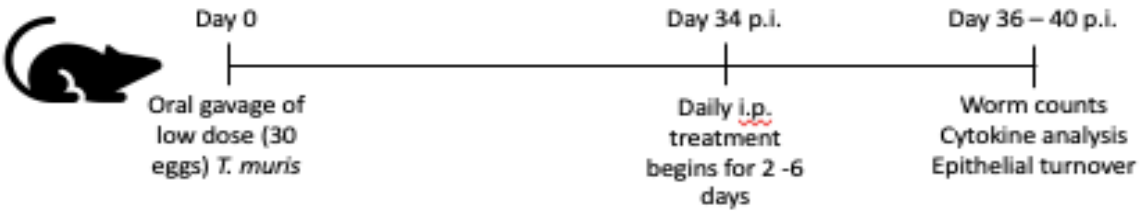

Figure 3.2: Schematic to show timeline of infection and GLP-1r agonist treatment of mice. Mice were infected with low dose (30 eggs) *T. muris* at day 0 and left for 34 days to allow infection to establish. At day 34 post infection, adult worms would be successfully embedded in the crypts. On day 34, daily intraperitoneal injection with GLP-1 receptor agonist began and continued for 2-6 days. After treatment, mice were sacrificed to obtain caecum and proximal large intestine, mesenteric lymph nodes, and blood.

3.2. Histology

Caecal tip samples obtained at the time of sacrifice and fixed in Carnoys (60% absolute ethanol, 30% chloroform, 10% glacial acetic acid) solution for 24 hours before being stored in 70% ethanol. Samples dehydrated through an ethanol-xylene gradient (90% ethanol for 1 hour, 100% ethanol for 1 hour, xylene for 45 minutes, then fresh xylene for another 45 minutes before storing in paraffin wax overnight) then embedded in paraffin wax. Once cooled, ribbons of 5μM sections were taken using a microtome and these were placed in the waterbath and transferred to microscope slides.

3.2.1. Alcian Blue-PAS for Goblet Cells

Slides were dewaxed in xylene for 10 minutes and moved through an alcohol gradient (100%, 90%, 70%, 50%, H2O). Slides were then stained for goblet cells in alcian blue [Sigma] for 5 minutes, 1% periodic acid [Thermo Scientific] for 5 minutes, Schiff's reagent [Thermo Scientific] for 15 minutes, then counterstained with haematoxylin [Thermo Scientific] for 1 minute, with thorough wash steps between each stain. Once stained, slides were dehydrated via an ethanol-xylene gradient (70% ethanol for 30 seconds, 100% ethanol for 30 seconds, xylene for 1 minute). Slides were then coverslipped using DPX.

Images were captured using Nikon Eclipse E600 LED microscope and analysed using ImageJ Fiji software. Crypt length and basement membrane thickness were measured using the measure tool and goblet cells per 20 randomly selected caecal crypt units (CCUs) were counted.

3.2.2. Immunofluorescence for Tuft Cells

Slides were briefly cleared and dewaxed in xylene then through an alcohol gradient. After washing in PBS, slides were blocked in 5% donkey serum for 1 hour at 4°C. Slides were washed again in PBS, then incubated in primary antibody (anti-DCAMKL1, 1:500, AbCam Ab31704) overnight at 4°C. The next day, slides were washed in PBS and incubated in secondary antibody (donkey anti-rabbit IgG alexafluor647, 1:800 in PBS) for 1 hour at room temperature. Slides were then washed in PBS, mounted using DAPI Fluoroshield, and tuft cells per crypt enumerated using Zeiss AxioScope A1 fluorescence microscope.

3.2.3. BrdU Pulse-Chase Experiment for Epithelial Turnover

BrdU (10mg per mouse, Sigma) was administered via intraperitoneal injection with a 25g needle at 22:00hrs and mice were culled after a 12-hour period at 10:00hrs and caecal tip samples collected. Slides were prepared as previously described.

For staining, slides were dewaxed in xylene for 1 hour and washed in 100% ethanol. Peroxidase activity was then blocked using peroxidase block (180ml methanol + 3ml H_2O_2) for 20 minutes at room temperature. Slides were then hydrolysed in 1M hydrochloric acid

for 8 minutes at 60°C before being neutralised in boric acid buffer for 6 minutes. After washing in PBS, slides were then blocked in 5% rabbit serum in PBS for 30 minutes at room temperature. Slides were then washed in PBS before incubating in anti-BrdU (1:20) for 1 hour at room temperature. After another wash in PBS, sections were incubated in rabbit anti-rat peroxidase (1:100 in 10% mouse serum-PBS) for 1 hour at room temperature. Slides were washed in PBS and sections developed in DAB for 2-3 minutes before being washed again in PBS. Sections were counterstained with haematoxylin (1:5) for 1 minute before being washed through an alcohol gradient into xylene and coverslipped using DPX.

3.3. Gut Digest for Intraepithelial Lymphocytes and Large Intestine Lamina Propria

Small and large intestine samples were collected in HBSS, and all mesenteric fat was trimmed, and faeces removed. Intestines were cut open and washed in HBSS in before being put into 10ml IEL strip media and shaken in Stuart Orbital Incubator for 20 minutes at 37°C. Samples were poured through 100μM sieves into clean falcon tubes to collect intraepithelial lymphocyte fraction. At this point, tissue was moved into new 50ml falcon tubes containing 10ml digest media and placed in Stuart Orbital Shaking Incubator for 30 minutes at 37°C. Strip media and digest media are made up as 50ml according to table 3.4.

Table 3.4: Epithelial strip media and lamina propria digest media recipes.

Epithelial Strip Media	47ml Hanks Balanced Salt Solution (HBSS)
	1ml Hepes (1M)
	500μL Ethylenediaminetetraacetic acid (EDTA) (0.5M)
	0.0073g Dithiothreitol (DTT)
	1.5ml Fetal Calf Serum (FCS)
Lamina Propria Digest Media	47ml Hanks Balanced Salt Solution (HBSS)
	4.7ml Fetal Calf Serum (FCS)
	0.024g Dispase
	0.024g Collagenase

3.3.1. Isolation of Intraepithelial Lymphocytes

Intraepithelial lymphocyte fractions were centrifuged at 400g for 5 minutes. Supernatants were removed and pellets resuspended in 8ml 30% Percoll (GE Healthcare) in 15ml centrifuge tubes and centrifuged at 600g for 20 minutes with slow acceleration (2) and deceleration (4). Pellets were resuspended in 4ml PBS to wash cells and centrifuged at 400g for 5 minutes. Pellets were then resuspended in 1ml full media (RPMI with 10% FCS (Sigma), 1% pen-strep (Sigma), 1% L-glutamine (Sigma), 1% MEM non-essential amino acids 100X (Sigma), 1% 1M Hepes (Gibco), and 50μM β-mercaptoethanol). Cells were counted using Invitrogen Countess automated cell counter.

3.3.2. Isolation of Large Intestine Lamina Propria Cells

Upon removal from the shaking incubator, samples were poured through 100μM sieves into clean 50ml falcon tubes. These were then centrifuged at 400g for 5 minutes and pellets resuspended in 1ml full media to count cells using Invitrogen Countess automated cell counter.

3.3.3. *In vitro* Stimulation of IELs and LILP

Once isolated, 1×10^6 cells were plated out in a 96 well plate to be stimulated with concanavalin A (ConA) (Sigma), Cell Stimulation Cocktail (1X, Invitrogen) only, or Cell Stimulation Cocktail with Exendin-4 (Sigma). For those to be stimulated with Cell Stimulation Cocktail and Exendin-4, 10nM exendin-4 was added and the plate was incubated for 30 minutes at 5% CO2 and 37°C. After 30 minutes, 1μL cell stimulation cocktail with protein transport inhibitors was added and the plate collected and stained for flow cytometry. To the wells without exendin-4 either 1μL cell stimulation cocktail or 7.5μL ConA were added and stimulated overnight before preparing for flow cytometry.

3.3.4. Cell Sorting and RNA Extraction of GLP-1r+ IELs

Isolated IELs were centrifuged at 400g for 5 minutes and pelleted cells resuspended in 500μL sorting media (DMEM without phenol red + 10ml FCS + 5.5ml Penstrep). FC block (anti-mouse CD16/CD32) was added at a 1:200 dilution and samples were left on ice for 15 minutes before staining. Staining antibodies were added (Table 3.5) in directly and samples

were left on ice for 25 minutes. Samples were then centrifuged at 400g for 5 minutes and pelleted cells resuspended in 500μL sorting media ready for sorting using the Sony MA900 Cell Sorter to collect GLP-1r+ and GLP-1r- immune cells. RNA was eluted from sorted cell samples using the Qiagen RNeasy Mini Kit according to manufacturer's instructions. Nanodrop 2000c (Fisher Scientific) was used to quantify the RNA in each sample at 260/280 ratio.

Table 3.5: Antibody panel used to stain cells for sorting.

Antibody	Manufacturer	Fluorophore	Clone
CD45	eBioscience	e506	30-F11
CD3	eBioscience	AF700	145-2C11
CD4	eBioscience	e450	GK1.5
CD8α	BD Horizon	Texas Red	53-6.7
CD8β	BD Pharminogen	PE	H35-17.2
TCRβ	Biolegend	PercP-Cy5.5	H57-597
TCRγδ	Biolegend	PE-Cy7	GL3

3.4. Isolation and Restimulation of Mesenteric Lymph Node Cells

Mesenteric lymph nodes (mLNs) were gently disaggregated through a 100μm sieve and plated out at $5x10^6$ cells per well of a 48 and 96 well plate. Cells were stimulated with cell stimulation cocktail with protein transport inhibitors (1X, Invitrogen), ConA, or ES antigen overnight. Cells stimulated with cell stimulation cocktail were then prepared and stained for flow cytometry. Cell-free supernatant of those wells stimulated with ConA or ES antigen were analysed via ELISA for cytokine production as described in section 3.7.

3.5. Flow Cytometry

All samples have been centrifuged to pellet cells prior to the following steps. Pellets were resuspended in 50μL fix-perm solution (Invitrogen) and left on ice for 30 minutes. Samples were then centrifuged at 400g for 5 minutes and pellets resuspended in 25μL FC block (1:200, diluted in 1x perm buffer) and left on ice for 15 minutes. Antibody mix (prepared using 1x perm buffer and individual antibodies according to tables 3.6 - 3.9) was then added

to each sample, and these were left on ice for 25 minutes. After staining was complete, samples were centrifuged at 400g for 5 minutes and pellets resuspended in 200µL 1% PBS-BSA before running using the Beckman Coulter CytoFlex flow cytometer and analysing on FlowJo (Version 10.7.2) using the gating strategies shown in Fig3.3 – Fig3.5.

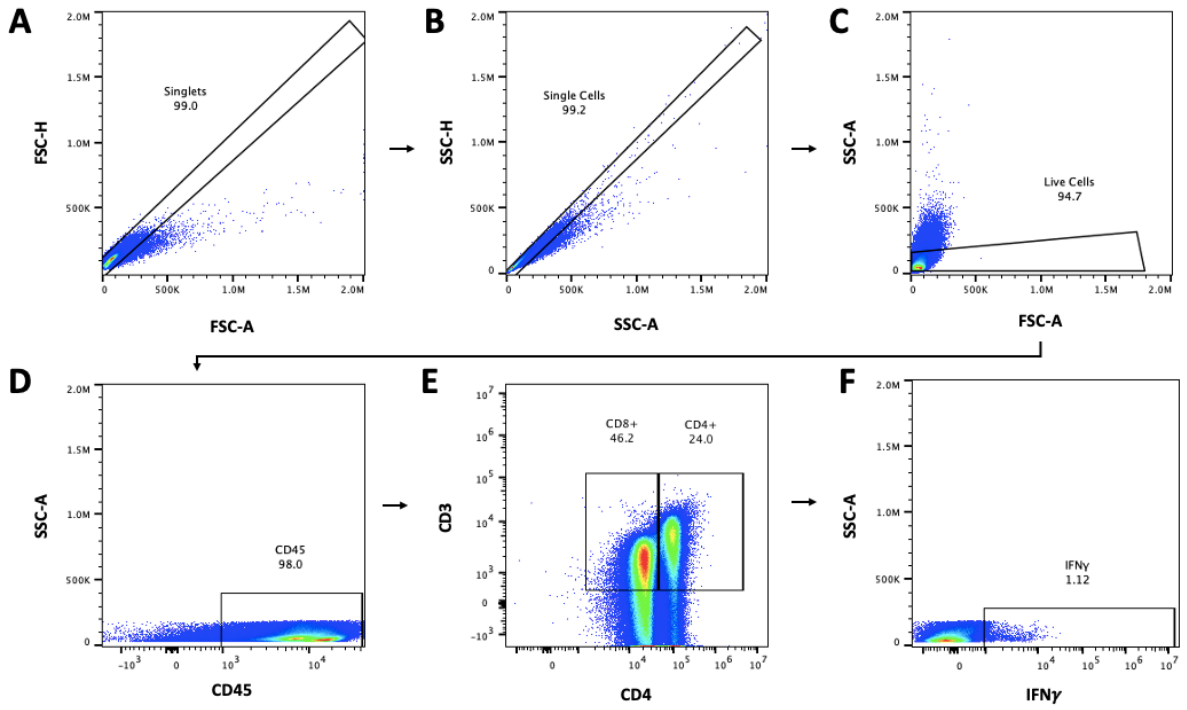

Figure 3.3: Gating strategy of mesenteric lymph node data. (A) Singlets were gated using FSC-H against FSC-A. (B) Single cells were then gated using SSC-H against SSC-A. (C) Live cells were gated using FSC-H against SSC-H. (D) Immune cells (CD45+) were gated using e506 (KO525-A) against SSC-A. (E) From the gated immune cells, cytotoxic (CD8+) and helper (CD4+) T-cells were gated using AF700 (APC-A700-A) for CD3 as a T-cell marker against PercP-710 (PC5.5-A) for CD4. Helper (CD4+) T cells are those positive for both CD3 and CD4. Cytotoxic T-cells express CD8 rather than CD4, therefore will only be positive for CD3. (F) Cytokines produced by CD8 and CD4 T-cells were analysed using the antibodies shown in tables 3.6 and 3.7 against SSC-A.

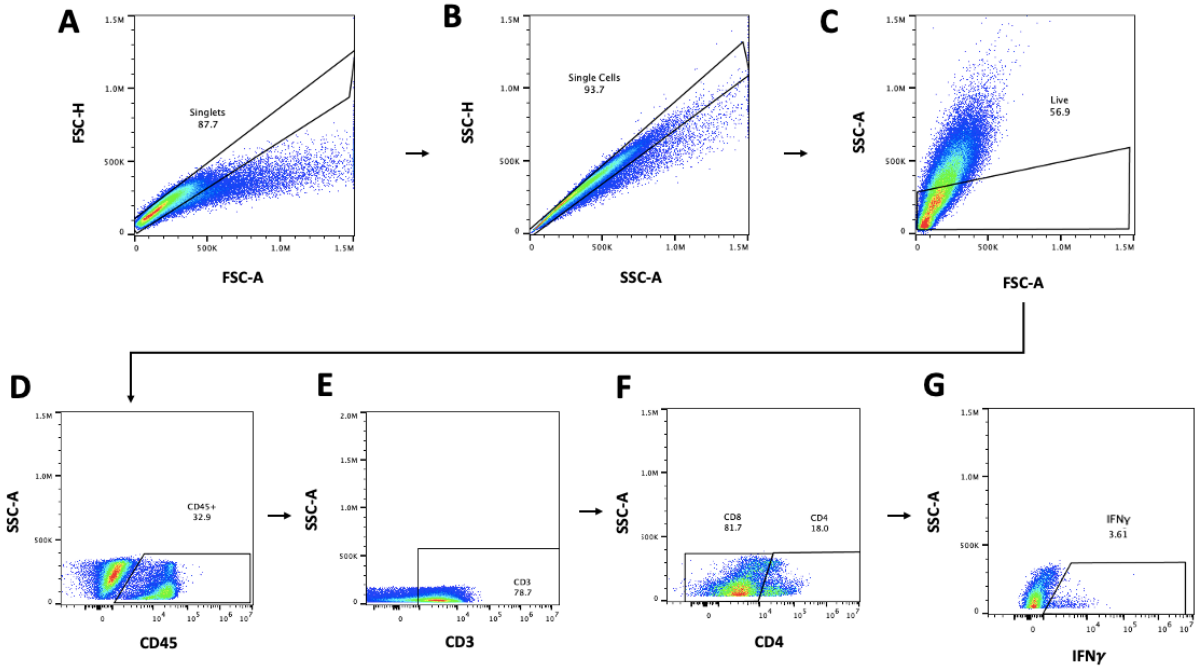

Figure 3.4: Gating strategy of lamina propria data. (A) Singlets were gated using FSC-H against FSC-A. (B) Single cells were then gated using SSC-H against SSC-A. (C) Live cells were gated using FSC-H against SSC-H. (D) Immune cells (CD45+) were gated using e506 (KO525-A) against SSC-A. (E) From the gated immune cells, CD4+ helper T-cells and CD8+ cytotoxic T-cells were gated from pre-gated CD3+ T-cells using APC-Cy7 (APC-A750) as a marker for CD4 against SSC-A. (F) Cytokines produced by CD4+ and CD8+ T-cells were analysed using the antibodies shown in table 3.9 against SCC-A.

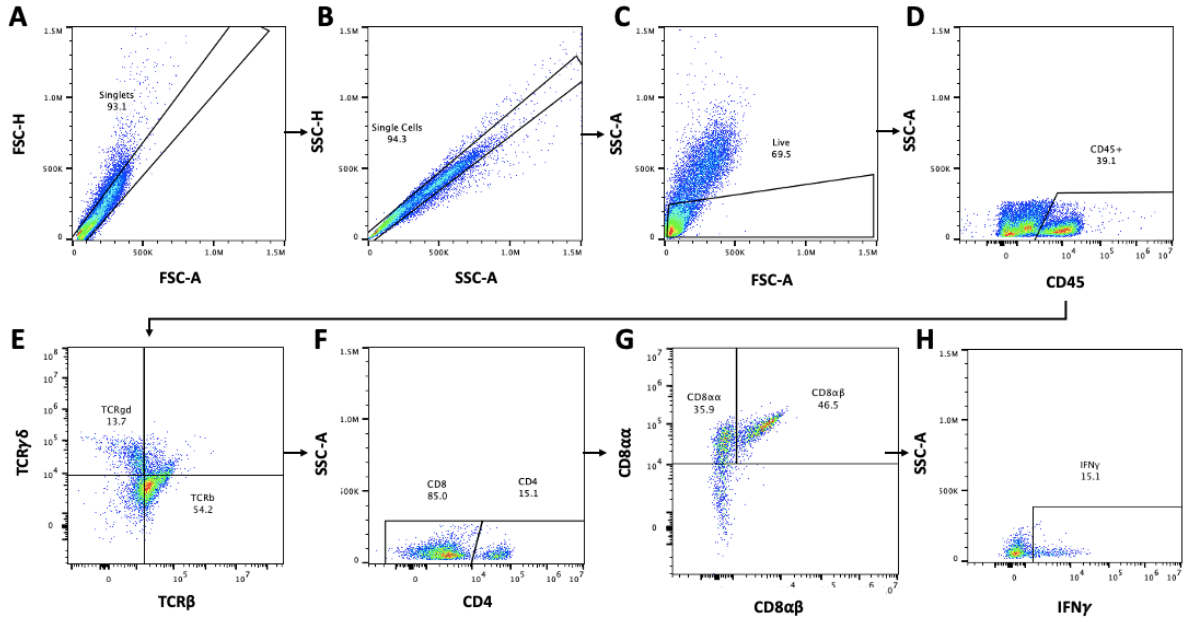

Figure 3.5: Gating strategy of intraepithelial lymphocytes. (A) Singlets were gated using FSC-H against FSC-A. (B) Single cells were then gated using SSC-H against SSC-A. (C) Live cells were gated using FSC-H against SSC-H. (D) Immune cells (CD45+) were gated using e506 (KO525-A) against SSC-A. (E) From the gated immune cells,

TCRβ and TCRγδ cells were gated using PercP-Cy5.5 (PC5.5-A, as a marker for TCRβ) against PE-Cy7 (PC7-A, as a marker for TCRγδ). (F) CD4+ helper T-cells and CD8+ cytotoxic T-cells were gated from TCRβ using APC-Cy7 (APC-A750-A) as a marker for CD4 against SSC-A. (G) CD8αα and CD8β cells were gated from CD8+ T-cells using PE-A (as a marker for CD8α) against Texas Red (ECD-A, as a marker for CD8β). (H) Cytokines produced by the different IEL subsets were gated using the antibodies shown in table 3.9 against SSC-A.

Table 3.6: Mesenteric lymph node flow cytometry antibody panel 1

Antibody	Manufacturer	Fluorophore	Clone
CD45	eBioscience	e506	30-F11
CD3	eBioscience	AF700	145-2C11
CD4	eBioscience	PercP-710	GK1.5
IFNγ	eBioscience	FITC	XMG1.2
IL-5	eBioscience	PE	TRFK5
IL-17	eBioscience	APC	17B7
IL-13	eBioscience	e450	13A

Table 3.7: Mesenteric lymph node flow cytometry antibody panel 2

Antibody	Manufacturer	Fluorophore	Clone
CD45	eBioscience	e506	30-F11
CD3	eBioscience	AF700	145-2C11
CD4	eBioscience	PercP-710	GK1.5
IL-13	eBioscience	FITC	13A
IL-9	eBioscience	PE	RM9A4
IL-22	eBioscience	APC	IL22JOP

Table 3.8: Mesenteric lymph node flow cytometry antibody panel to look at innate lymphoid cells

	Antibody	Manufacturer	Fluorophore	Clone
Dump Gate	CD3	eBioscience	APC-Cy7	145-2C11
	CD5	eBioscience	APC-Cy7	53.7.3
	CD11b	eBioscience	APC-Cy7	M1/70
	CD8	eBioscience	APC-Cy7	53-6.7
	Ly6G	eBioscience	APC-Cy7	RB6-8C5

	NK1.1	eBioscience	APC-Cy7	PK136
	B220	eBioscience	PE-Cy7	RA3-6B2
	CD11c	eBioscience	PE-Cy7	N418
	CD45	eBioscience	e506	30-F11
	CD127	eBioscience	e450	A7R34
	CD90.2	BioLegend	AF700	30-H12
	IL-13	eBioscience	FITC	13A
	IL-5	eBioscience	PE	TRFK5
	GATA3	eBioscience	PercP-710	TWAJ
	KLRG1	eBioscience	APC	2F1

Table 3.9: Large intestine lamina propria (LILP) and intraepithelial lymphocyte (IEL) flow cytometry antibody panel

Antibody	Manufacturer	Fluorophore	Clone
CD45	eBioscience	e506	30-F11
CD4	Life Technologies	APC-Cy7	GK1.5
CD8α	BD Biosciences	Texas Red	53-6.7
CD8β	eBioscience	PE	H35-17.2
TCRβ	eBioscience	PercP-Cy5.5	H57-597
TCR$\gamma\delta$	BioLegend	PE-Cy7	GL3
IFNγ	eBioscience	FITC	XMG1.2
IL-13	eBioscience	e450	13A
IL-22	eBioscience	APC	IL22JOP

3.6. Cytometric Bead Array (CBA)

IL-4, IL-5, IL-10, IL-13, IL-17, IFNγ, and TNFα were measured using a BD CBA flex kit (BD Biosciences) following the instructions in the kit. Supernatants were incubated for 1 hour at room temperature with capture beads coated with a capture antibody specific for one of the above cytokines. Bound cytokine was then detected using PE-conjugated detection reagent and compared to known standards. Fluorescence intensity was measured using BD FACScanto II and data analysed using FCAP Array™ (Version 3) Software.

3.7. Parasite-Specific Enzyme Linked Immunosorbent Assay (ELISA)

Flat bottomed 96 well plates were coated with 50μL ES antigen (5μg/ml in Carbonate Bicarbonate buffer (pH 9.6)) and kept in tinfoil at 4°C overnight. Plates were washed x5 in PBS-Tween20 (Sigma Aldrich) before being blocked in 50μL 3% PBS-BSA for 1 hour at room temperature. Sera were double diluted 1/20 – 1/2560 in PBS Tween and 50μL of each dilution placed into a well and incubated for 90 minutes at room temperature. Bound parasite-specific antibody was detected using 50μL of biotinylated rat anti-mouse IgG1 (BioRad, 1/500 in PBS Tween) or 50μL biotinylated rat anti-mouse IgG2a (BD Pharminogen, 1/500 in PBS Tween) and incubated for 1 hour at room temperature. Bound biotinylated antibody was detected by 50μL streptavidin peroxidase (Roche, 1/40 in PBS Tween), incubated for 1 hour at room temperature. Plates were then developed using 50μL peroxidase substrate (Sigma) and the reaction stopped after approximately 2 minutes using H_2SO_4. Plates were read at 450nm with 540nm reference on a Tecan Infinite pro plate reader.

3.8. Statistics

Figures were created using GraphPad Prism and results presented as mean ± SEM. Statistical analysis was also performed using GraphPad Prism where two experimental groups were compared via Student's t-test and three or more groups were analysed via ANOVA with Bonferroni's multiple comparisons. A p value of <0.05 was considered to be statistically significant ($p<0.05$ = *; $p<0.01$ = **; $p<0.005$ = ***).

4. Results

4.1. GLP-1r+ IELs appear in large intestine in response to *T. muris* infection

Intestinal intraepithelial lymphocytes of the murine intestine have been found to possess mRNA transcript for the receptor for GLP-1 (Yusta et al., 2015). We looked at protein expression of the GLP-1r in murine intestine of naïve and *T. muris* infected mice.

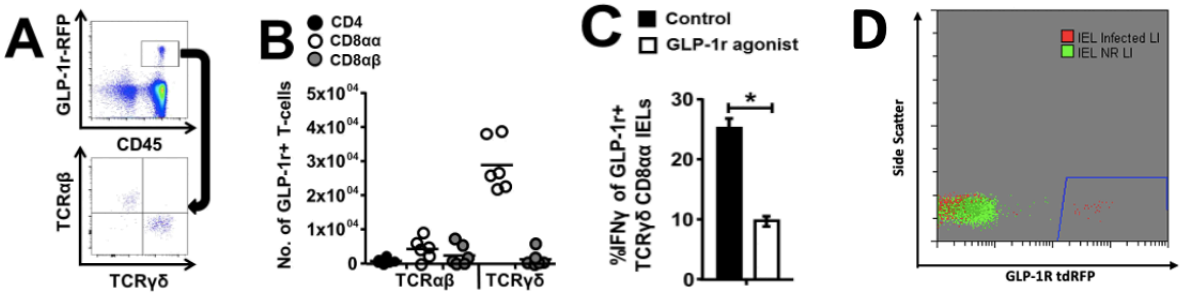

Figure 4.1: GLP-1r+ IELs appear in the large intestine in response to *T. muris* infection. (A) Representative flow cytometry plots of small intestinal IELs from GLP-1r-RFP mice, demonstrating IEL specific expression of the GLP-1 receptor in the murine intestine. (B) Numbers of IEL populations expressing the GLP-1r in small intestinal compartment assessed via flow cytometry. Data (n = 6) from two independent experiments. (C) %IFNγ expression in PMA/ionomycin stimulated GLP-1r+ IELs treated with GLP-1r agonist. Data (n = 6) from two independent experiments. (D) Representative dot plot overlay showing GLP-1r expression in the TCRγδ+ CD8αα+ IEL subpopulation from the large intestine of infected reporter mice (red) and naïve reporter mice (green). Data (n = 4-5) from 5 independent experiments.

We found expression of the GLP-1r to be exclusive to CD45 cells of the small intestine, specifically TCRαβ and TCRγδ IELs (Fig. 4.1 A). We then found the majority of GLP-1r+ T-cells to be CD8αα+ TCRγδ cells (Fig. 4.1 B). Therefore, we restimulated these TCRγδ CD8αα+ cells *in vitro* with PMA/ionomycin and determined the amount of IFNγ produced. We saw a significant decrease ($p = 0.03$) in IFNγ production in cells treated with a GLP-1rA in comparison to vehicle treated (Fig. 4.1 C). We then looked for GLP-1r protein expression in the TCRγδ CD8αα+ subpopulation in the large intestine of naïve and infected reporter mice. Interestingly, we saw no expression of GLP-1r in TCRγδ CD8αα+ cells (or any other CD45+ immune population) in naïve mice but infection with *T. muris* induced the appearance of GLP-1r+ IELs in the large intestine of reporter mice (Fig. 4.1 D). Collectively, this indicates immune cells with the GLP-1r are responsive to GLP-1r agonist treatment and these cells are

present during a chronic *T. muris* infection making them a potential target for therapeutic treatment.

4.2. Short-term GLP-1RA treatment induces expulsion of an established *T. muris* infection

Humans with *Trichuris trichiura* infection will normally have an established adult worm infection burden before treatment begins. We therefore infected C57BL/6 mice with the murine parasite *T. muris* and allowed this low-dose infection to establish for 34 days to allow the worms to reach patency and embed within the intestinal epithelium. We then gave a short-term treatment with the GLP-1rA, lixisenatide, for 2-6 days to determine whether this altered worm burden.

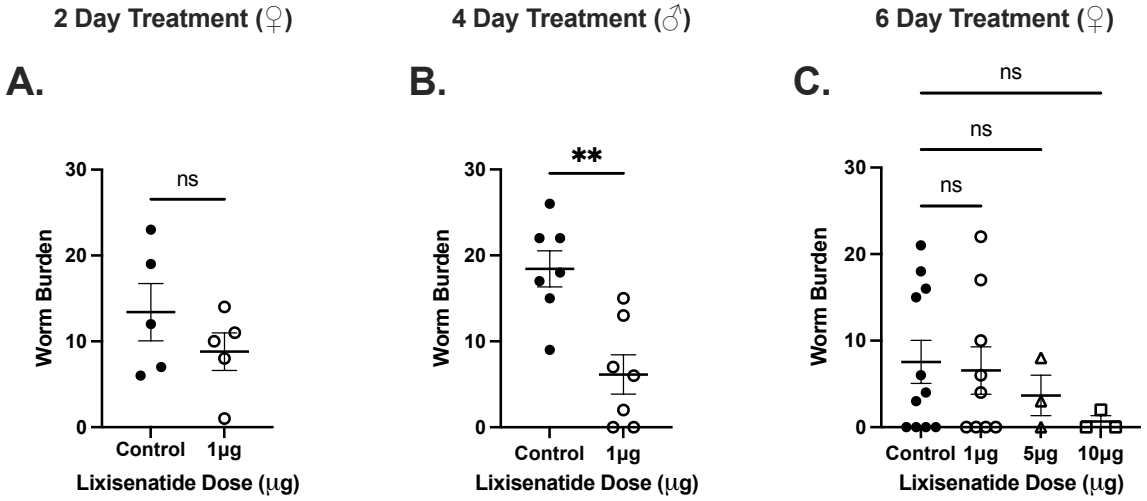

Figure 4.2: Short-term lixisenatide treatment induces expulsion of chronic *T. muris* infection in C57BL/6 mice. C57BL/6 mice were infected with low dose *T. muris* (approximately 30 eggs) at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of lixisenatide was started on day 34 post infection and treatments continued for either (A) 2 days (female mice), (B) 4 days (male mice), or (C) 6 days (female mice). Mice were sacrificed after treatment and worm burden determined by counting worms present in the caecum and proximal large intestine. Data (n = 3 – 11) from 4 independent experiments and presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We found no significant difference in worm burden between PBS controls and lixisenatide treated female mice after 2 days of treatment (Fig. 4.2 A) but following 4 days of treatment

we did see a significant decrease (p = 0.0019) in worm burden between the PBS control and lixisenatide treated groups in male mice (Fig. 4.2 B). However, we then found no significant difference between PBS controls and lixisenatide treated female mice after 6 days of treatment at 1μg (Fig. 4.2 C). These data led us to believe that the difference in response to lixisenatide driven worm expulsion could be based on the sex of the mice; since male mice were able to expel the worms after 4 days of treatment, but females could not at the comparable 1μg dose. We therefore decided to increase the daily dose of lixisenatide, which resulted in a dose dependent reduction in overall worm burdens, with worm burden reduced to almost zero after 6 days of treatment at 10μg/day lixisenatide.

Still, there is no significant difference in worm burden between the PBS controls and lixisenatide treated mice after 6 days of treatment with either 5μg/day or 10μg/day lixisenatide (Fig. 4.2 C).

These data strongly indicate that GLP-1rA treatment is able to induce expulsion of an established adult *T. muris* infection within the male mice, and suggests higher doses may be functional in the female cohort following further experimentation.

4.3. The adaptive immune system and a functional GLP-1 receptor are necessary for GLP-1 receptor agonist induced expulsion of *T. muris*

RAG-/- mice lack an adaptive immune system (Mombaerts et al., 1992), including TCRγδ T-cells (Mombaerts, 1995), so do not possess the identified GLP-1r+ intraepithelial lymphocytes. Treatment of RAG-/- mice with a GLP-1rA will therefore determine whether the adaptive immune system is involved in GLP-1rA-induced expulsion of a chronic *T. muris* dose.

We treated C57BL/6 and RAG-/- mice on the same background strain daily throughout infection (34 days) with a GLP-1rA and worm burden was determined. Daily treatment following infection was used to give as large a dose of GLP-1rA as possible to encourage parasite expulsion and fully assess if the adaptive immune response was involved in GLP-1RA induced expulsion.

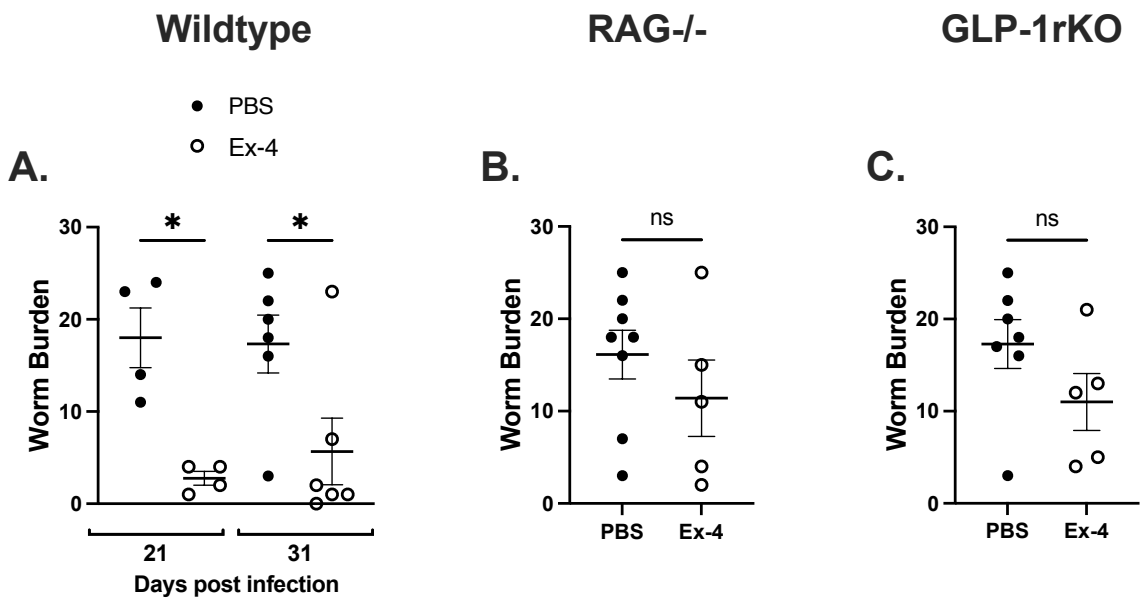

Figure 4.3: GLP-1rA agonist treatment does not cause expulsion of *T. muris* in RAG-/- or GLP-1R KO mice. (A) Worm burden of C57BL/6 mice after daily treatment with either PBS vehicle or 2μg Exendin-4. (B) Worm burden of RAG-/- mice after daily treatment with either PBS vehicle or 2μg Exendin-4. (C) Worm burden of GLP-1rKO mice after daily treatment with either PBS vehicle or 2μg Exendin-4. Mice were infected with a low dose *T. muris* infection at day 0 and treated daily for 34 days with PBS vehicle or Exendin-4. At day 34, mice were sacrificed, and worm burden was determined by counting adult worms in the caecum and proximal large intestine. Data (n = 4 – 8) from 4 independent experiments and presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We first confirmed daily GLP-1RA treatment following infection was able to expel *T. muris* infection and found a significant decrease (p = 0.0243) in worm burden in GLP-1rA treated mice in comparison to PBS controls at day 34 post infection (p.i.) with daily treatment. This significant decrease in worm burden is also seen at day 21 p.i in GLP-1rA treated mice (p = 0.0164) (Fig. 4.3 A). Interestingly, this significant difference in worm burden between PBS control and GLP-1rA treated mice at day 34 p.i. after daily treatment was not observed in RAG-/- mice (Fig. 4.3 B).

In order to determine whether GLP-1rA treatment causes expulsion of *T. muris* by acting through the GLP-1 receptor or whether it is directly toxic to the parasite we utilised GLP-1rKO mice that lack the receptor for GLP-1 (Scrocchi et al., 1996). We again saw no

significant difference in worm burden between PBS control and Ex-4 treated GLP-1rKO mice at day 34 p.i. after daily treatment (Fig. 4.3 C).

Collectively, these data demonstrate that daily GLP-1RA treatment can prevent the establishment of a chronic *T. muris* infection and requires a functional GLP-1 receptor rather than being toxic to the parasite. Furthermore, this expulsion mechanism is dependent on a functional adaptive immune system.

4.4. Antibody subtypes are unaltered following GLP-1RA induced *T. muris* expulsion

A Th2 immune response is associated with clearance of a helminth parasite, while a Th1 response is associated with susceptibility, allowing the infection to become chronic (Klementowicz et al., 2012). To determine whether lixisenatide treatment alters the immune response to chronic *T. muris* infection, we first looked at parasite specific antibody responses to parasite E/S antigen via sandwich ELISA using serum obtained upon sacrifice of the mice. Serum antibodies offer strong reflection of the type of immune response

occurring, with increased IgG1 and IgG2c being indicative of Th2 and Th1 responses, respectively (Else et al., 1993).

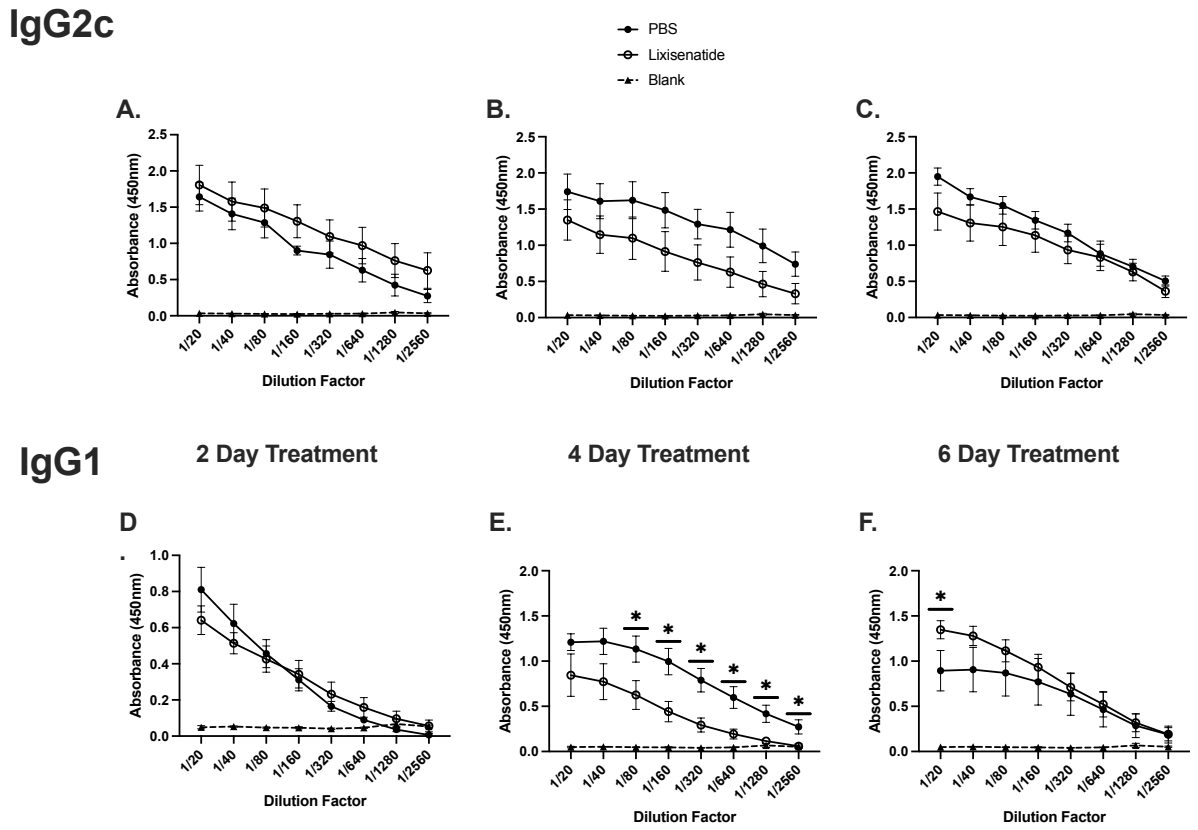

Figure 4.4: Parasite specific IgG1 and IgG2c antibody responses of mice with chronic *T. muris* infection. Mice were infected with low dose (approximately 30 eggs) *T. muris* and infection was allowed to establish for 34 days before treatment began. Treatment by intraperitoneal injection with either PBS (control) or lixisenatide began on day 34 and continued once daily for 2, 4, or 6 days. Upon completion of treatment, mice were sacrificed, and serum obtained. IgG1 and IgG2c levels were determined via sandwich ELISA using serum from 3 independent experiments. Plotted data show IgG2c levels of mice treated for (A) 2 days, (B) 4 days, and (C) 6 days and IgG1 levels of mice treated for (D) 2 days, (E) 4 days, and (F) 6 days. Data from 3 independent experiments and presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis performed using unpaired T-test at each dilution factor (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

No significant difference was found in either IgG2c or IgG1 between PBS control and treated mice after 2 days of GLP-1RA injections (Fig 4.4 A and D). Interestingly, mice given a 4-day course of lixisenatide appear to have significantly decreased IgG1 in comparison to the control mice), indicating a potential decreased Th2 response (Fig 4.4 E). However, no

significant difference in IgG2c was observed between 4-day treatment groups (Fig 4.4 B). After 6 days of treatment with GLP-1RA, there was no significant difference in IgG2c between control and treated mice. We do see a significant increase in IgG1 in the lixisenatide treated mice at the 1/20 dilution, but this does not carry through to the other dilution factors (Fig 4.4 C and F).

Collectively, these data indicate no alteration in parasite specific antibody production following short term GLP-1RA treatment of an established chronic *T. muris* infection.

4.5. Cytokines produced from E/S restimulation by the mesenteric lymph node are not altered by lixisenatide treatment

Given, the short term GLP-1RA treatment regime, we next assessed cytokine production which would give a more indicative indication of the active immune response as opposed to antibody production. A Th2 response including cytokines such as IL-4, IL-5, IL-10 and IL-13 has been shown to be involved in expulsion of *T. muris* in resistant mice (Klementowicz et al., 2012). IFNγ and TNFα are Th1 cytokines usually associated with susceptibility to a chronic infection. IL-17 has been found to increase the Th2 immune response in the lung in response to *Nippostrongylus brasiliensis* (Ajendra et al., 2020). Therefore, we looked at the cytokines produced by mLN cells restimulated with either E/S antigen or concanavalin A (ConA) using a cytometric bead array analysis to analyse the cytokines produced either in response to the parasite (E/S antigen) or in general (ConA).

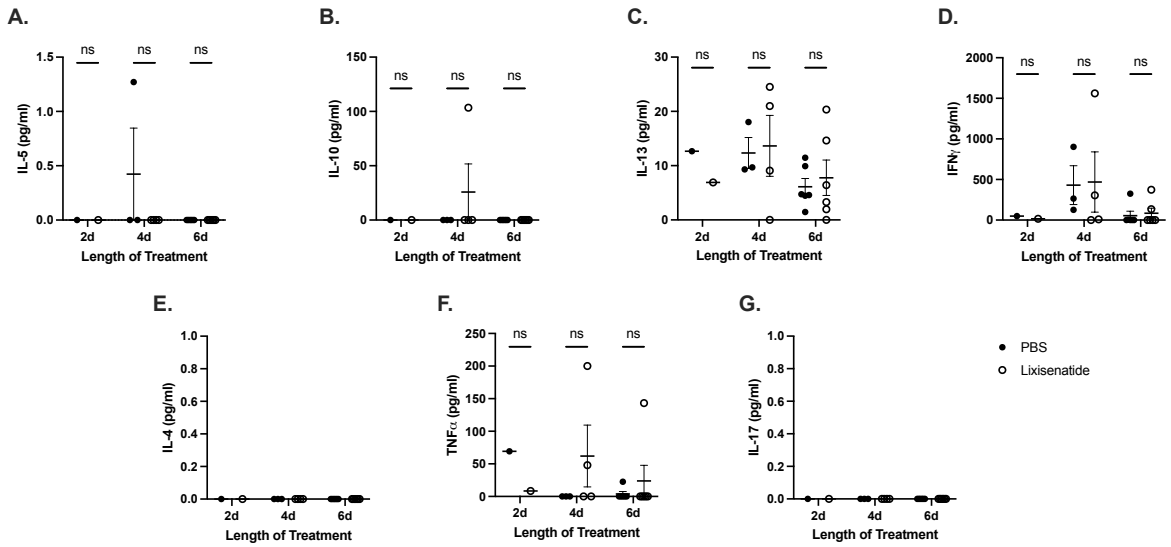

Figure 4.5: Cytokines produced by the mesenteric lymph node in response to restimulation with ES antigen are unaffected by lixisenatide treatment. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection and treatment was continued for either 2, 4, or 6 days. Mice were sacrificed after treatment and mLN were obtained for analysis. mLN were stimulated with ES antigen overnight (16 hours) and supernatant collected and analysed via cytometric bead array (CBA). Levels of (A) IL-5, (B) IL-10, (C) IL-13, (D) IFNγ, (E) IL-4, (F) TNFα, and (G) IL-17 were determined. Data (n = 2-6) are from 2 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We saw no significant difference in levels of IL-5, IL-10, IL-13, IFNγ, or TNFα between PBS controls and lixisenatide treated mice after 2, 4, or 6 days of treatment when restimulating the mesenteric lymph node with *T. muris* E/S antigen. No IL-4 or IL-17 were detected at any of the three timepoints when the mLN was restimulated with ES antigen (Fig. 4.5 A-G).

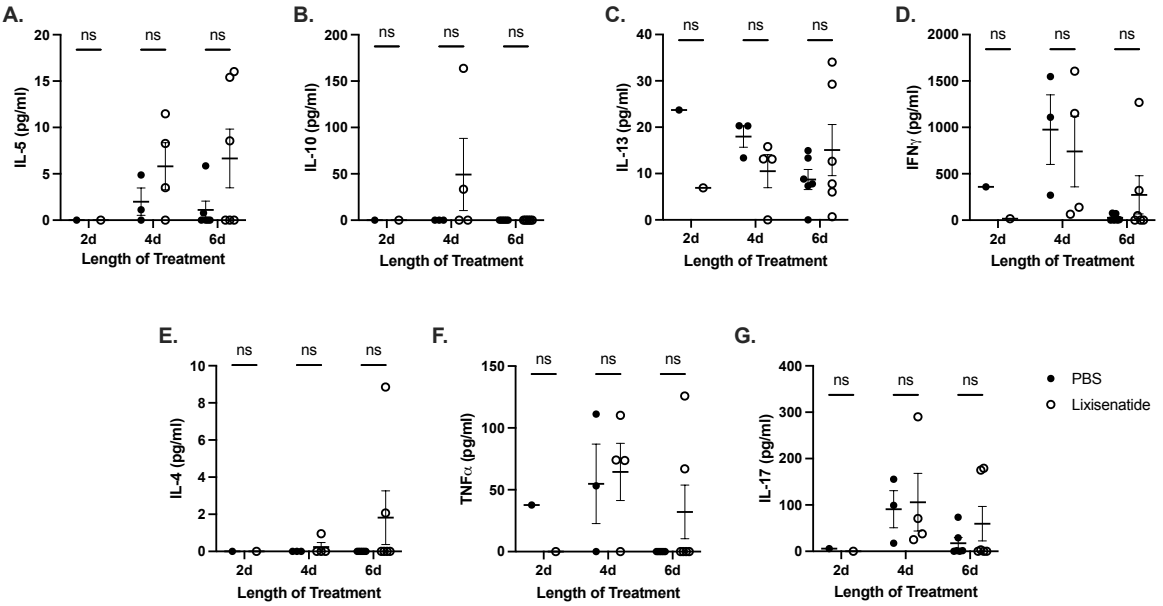

Figure 4.6: Cytokines produced by the mesenteric lymph node restimulated with concanavalin A are unaffected by lixisenatide treatment. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection and treatment was continued for either 2, 4, or 6 days. Mice were sacrificed after treatment and mLN were obtained for analysis. mLN were stimulated with ConA overnight (16 hours) and supernatant collected and analysed via CBA. Levels of (A) IL-5, (B) IL-10, (C) IL-13, (D) IFNγ, (E) IL-4, (F) TNFα, and (G) IL-17 were determined. Data (n = 2-6) are from 2 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We saw no significant difference in levels of IL-5, IL-10, IL-13, IFNγ, IL-4, TNFα, or IL-17 produced by the mesenteric lymph node restimulated with ConA between PBS controls and lixisenatide treated mice after 2 days, 4 days, or 6 days of treatment. We found that IL-4 and IL-17 were detected when the mLN was restimulated with ConA, but not with ES antigen. (Fig. 4.6 A-G).

Since a Th2 immune response is associated with expulsion of the parasite (Klementowicz et al., 2012), but we saw no differences in cytokines produced using a cytometric bead array, we utilised an alternative approach of flow cytometry to look at immune cell subsets of the mesenteric lymph node and the cytokines they were producing after overnight restimulation (16 hours) with Cell Stimulation Cocktail to determine whether GLP-1rA

treatment induces *T. muris* expulsion through alteration of immune cell subsets or cytokine response.

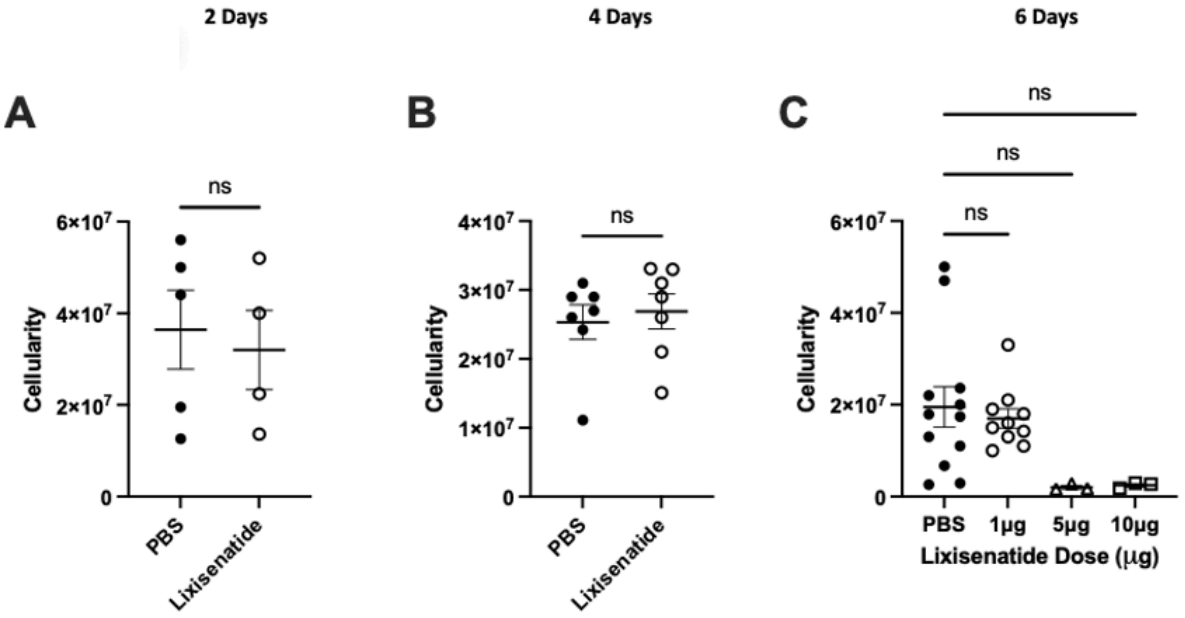

Figure 4.7: Cellularity of the mesenteric lymph node is unaffected by GLP-1r agonist treatment. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1µg lixisenatide was started at day 34 post infection and treatment was continued for either (A) 2 days, (B) 4 days, or (C) 6 days. Mice were sacrificed after treatment and mLN were obtained for analysis. Cell counts were obtained using Invitrogen Countess automated cell counter. Data (n = 2-6) are from 2 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We observed no differences in the cellularity of the mesenteric lymph node between control and treated groups at any timepoint (Fig. 4.7. A-C).

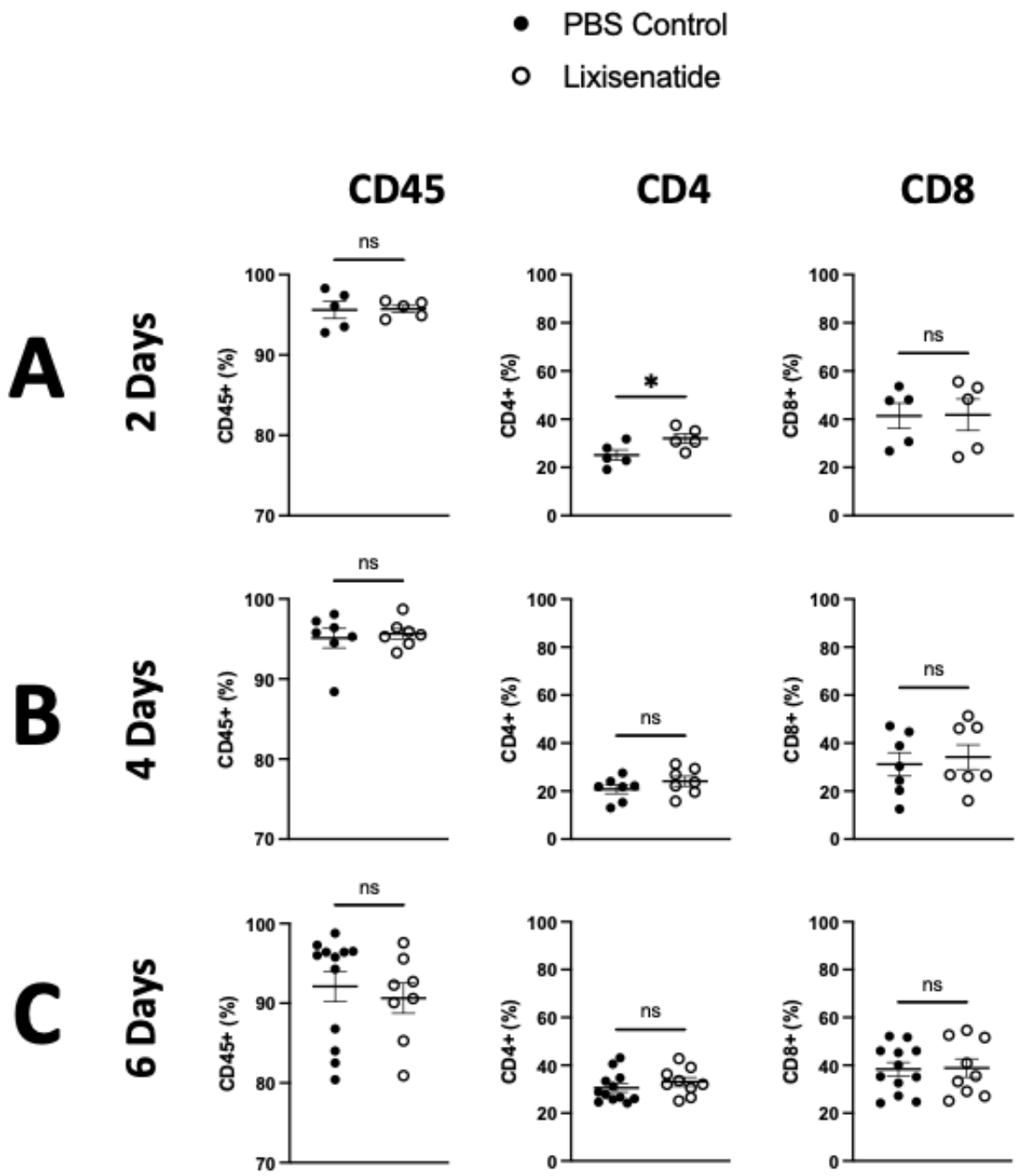

Figure 4.8: Percentage of CD4+ T Cells quickly increases following lixisenatide treatment, but this increase is not sustained. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for (A) 2 days, (B) 4 days, or (C) 6 days. Mice were sacrificed after treatment and mLN were obtained for analysis. mLN were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Data (n = 5-12) are from 4 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

There were no significant differences in the percentage of CD45+ immune cells in the mesenteric lymph node between the control and lixisenatide mice. We found a slight but significant increase (p = 0.0474) in the percentage of CD4+ T-helper cells in lixisenatide treated mice after 2 days of treatment in comparison to the control mice (Fig. 4.8. A). After 4 and 6 days of lixisenatide treatment, there was no significant difference in the percentage of CD4+ T-cells between control and lixisenatide treated groups. There were also no significant differences in the percentage of CD8+ cytotoxic T-cells between the control and treated groups at any timepoint (Fig. 4.8 A-C).

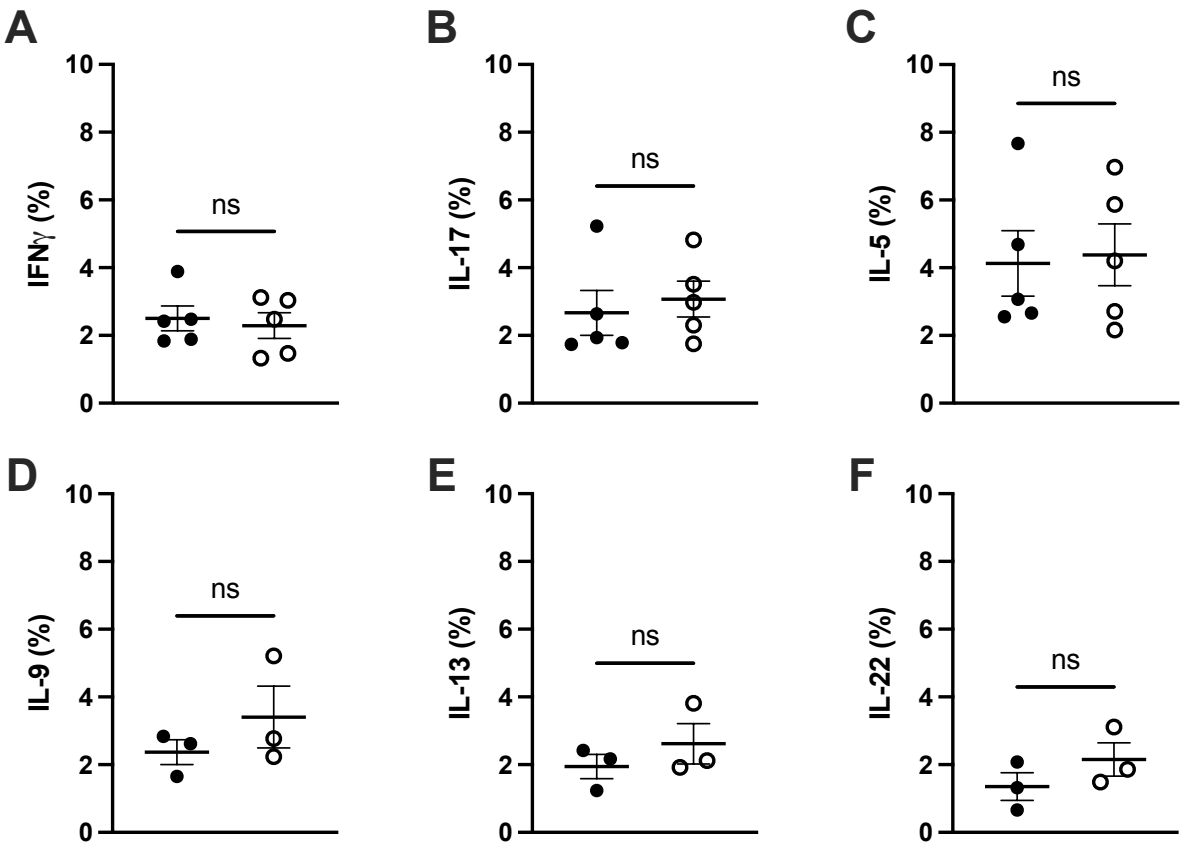

Figure 4.9: Cytokines produced by CD4+ T-helper cells in the mesenteric lymph node are unaffected by a 2-day treatment with lixisenatide of *T. muris* infected mice. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 2 days. Mice were sacrificed after treatment and mLN were obtained for analysis. mLN were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Plotted data show percentage of CD4+ T- cells in the mLN producing (A) IFNγ, (B) IL-5, (C) IL-17, (D) IL-9, (E) IL-13, and (F) IL-22. Data (n = 3-5) are from 3 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We next assessed individual cytokine profile in the CD4+ T-cells. There were no significant differences in IFNγ, IL-17, IL-5, IL-9, IL-13, or IL-22 produced by CD4+ T-helper cells of the mLN between the PBS controls and the lixisenatide treated mice after 2 days of treatment (Fig. 4.9 A-F).

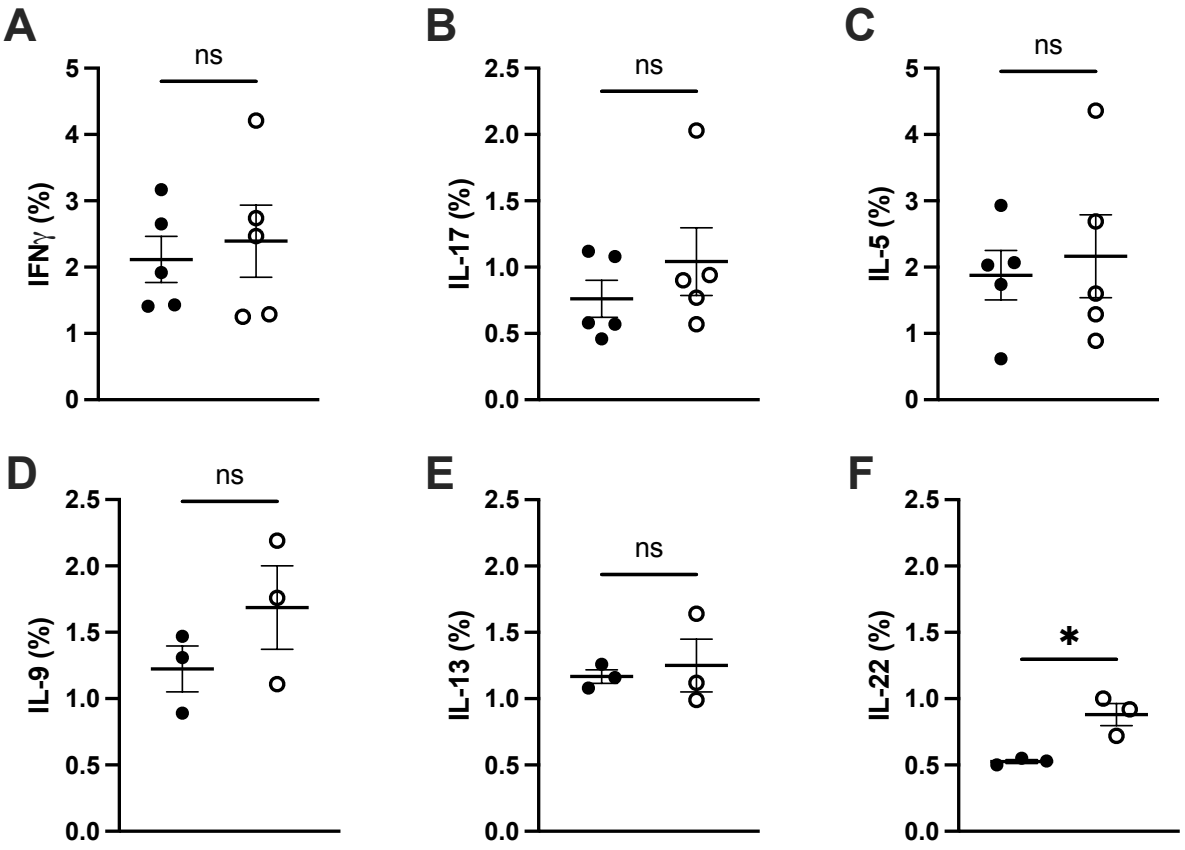

Figure 4.10: IL-22 produced by CD8+ T cells increases shortly following lixisenatide treatment in *T. muris* infected mice. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 2 days. Mice were sacrificed after treatment and mLN were obtained for analysis. mLN were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Plotted data show percentage of CD8+ T-cells in the mLN producing (A) IFN, (B) IL-5, (C) IL-17, (D) IL-9, (E) IL-13, and (F) IL-22. Data (n = 3-5) are from 3 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = p<0.05, ** = p<0.01, *** = p<0.001.

There were no significant differences in IFNγ, IL-17, IL-5, IL-9, or IL-13 produced by the CD8+ T-cells between the control and lixisenatide treated mice after 2 days of treatment from CD8+ cytotoxic T-cells of the mLN (Fig. 4.10 A-E). However, we did see a significant increase

(p = 0.0139) in IL-22 from these CD8+ T-cells in the lixisenatide treated mice compared to the PBS controls at this timepoint (Fig. 4.10 F).

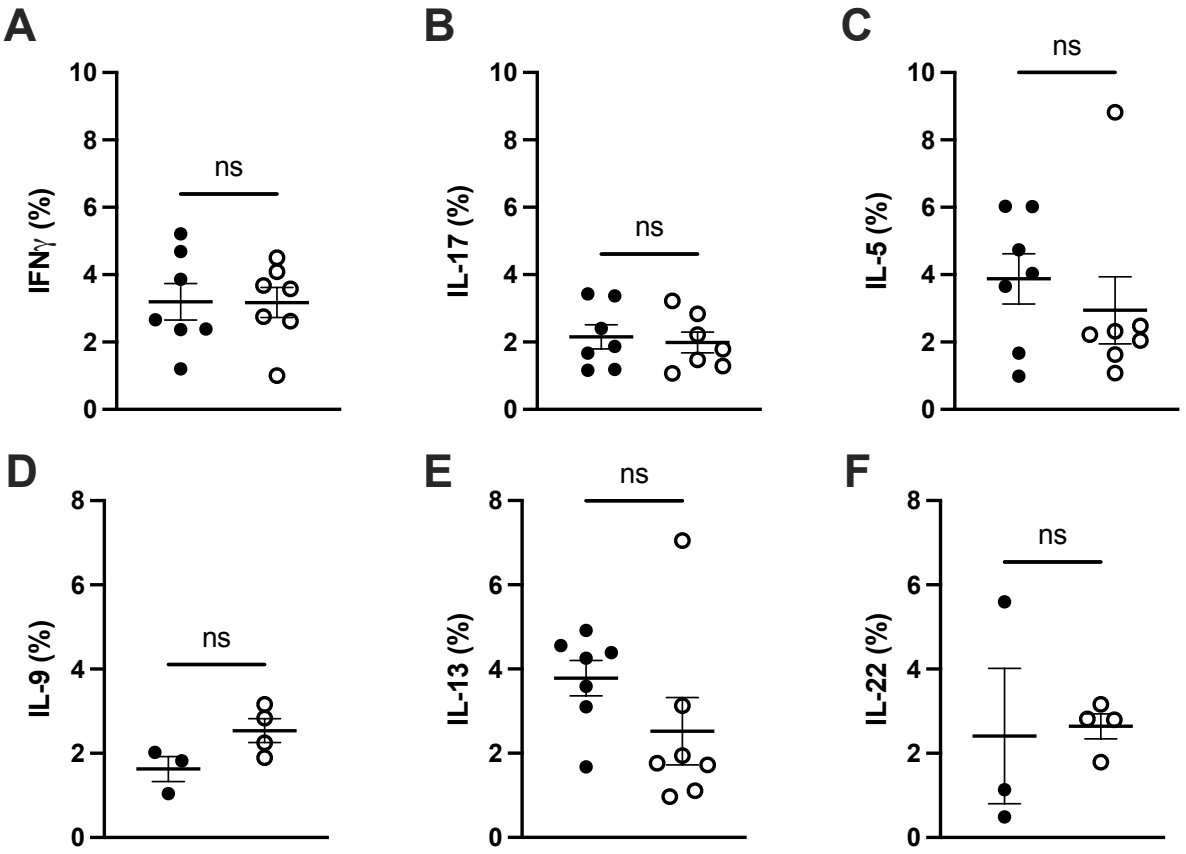

Figure 4.11: Cytokines produced by CD4+ T-cells in the mesenteric lymph node are unaffected by a 4-day course of lixisenatide treatment of *T. muris* infected mice. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 4 days. Mice were sacrificed after treatment and mLN were obtained for analysis. mLN were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Plotted data show percentage of CD4+ T-cells in the mLN producing (A) IFN, (B) IL-5, (C) IL-17, (D) IL-9, (E) IL-13, and (F) IL-22. Data (n = 3-7) are from 4 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We found no significant difference in levels of IFNγ, IL-17, IL-5, IL-9, IL-13, or IL-22 from CD4+ T-helper cells of the mLN between the control and lixisenatide treated mice after 4 days of treatment (Fig. 4.11 A-F).

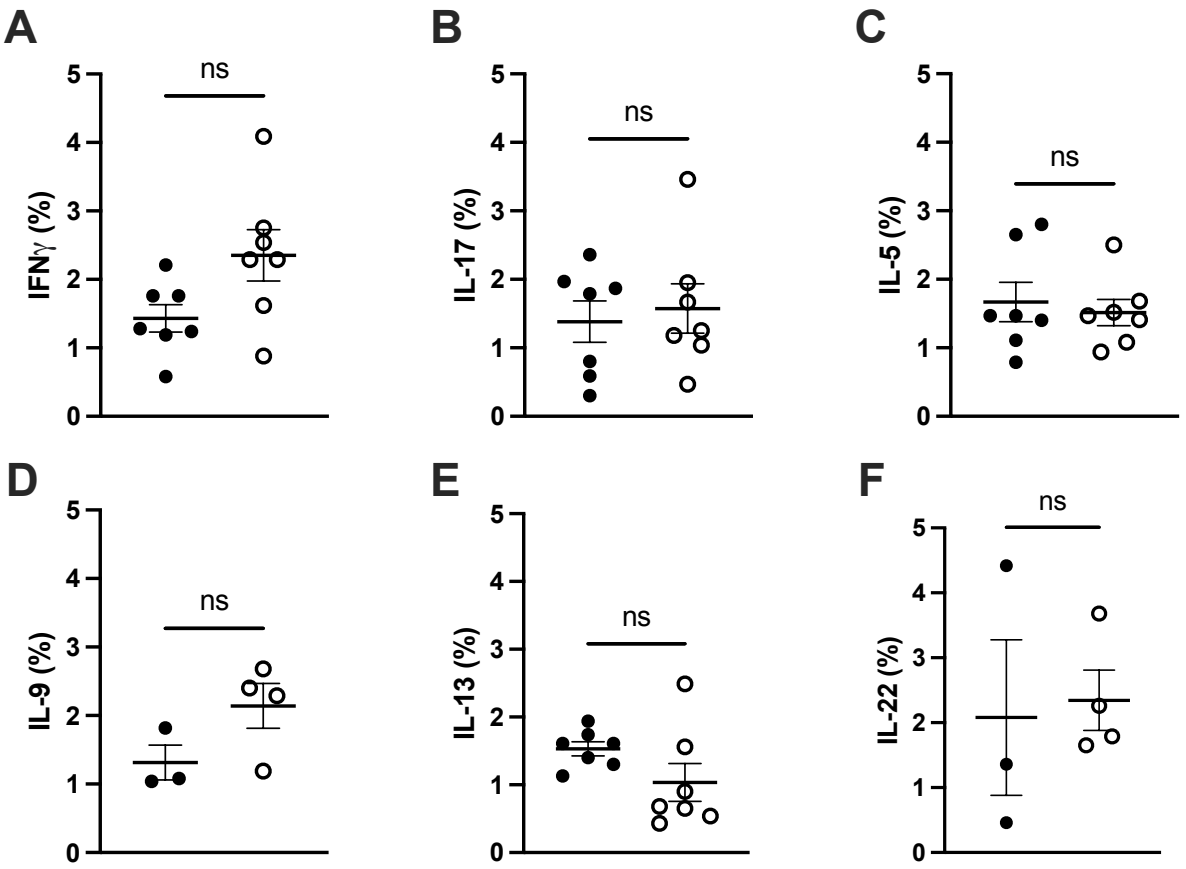

Figure 4.12: Cytokines produced by CD8+ T-cells in the mesenteric lymph node are unaffected by a 4-day course of lixisenatide treatment in *T. muris* infected mice. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 4 days. Mice were sacrificed after treatment and mLN were obtained for analysis. mLN were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Plotted data show percentage of CD8+ T-cells in the mLN producing (A) IFN, (B) IL-5, (C) IL-17, (D) IL-9, (E) IL-13, and (F) IL-22. Data (n = 3-7) are from 4 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

There were no significant differences in levels of IFNγ, IL-17, IL-5, IL-9, IL-13, or IL-22 from CD8+ cytotoxic T-cells of the mLN between PBS control and lixisenatide treated mice after 4 days of treatment (Fig. 4.12 A-F).

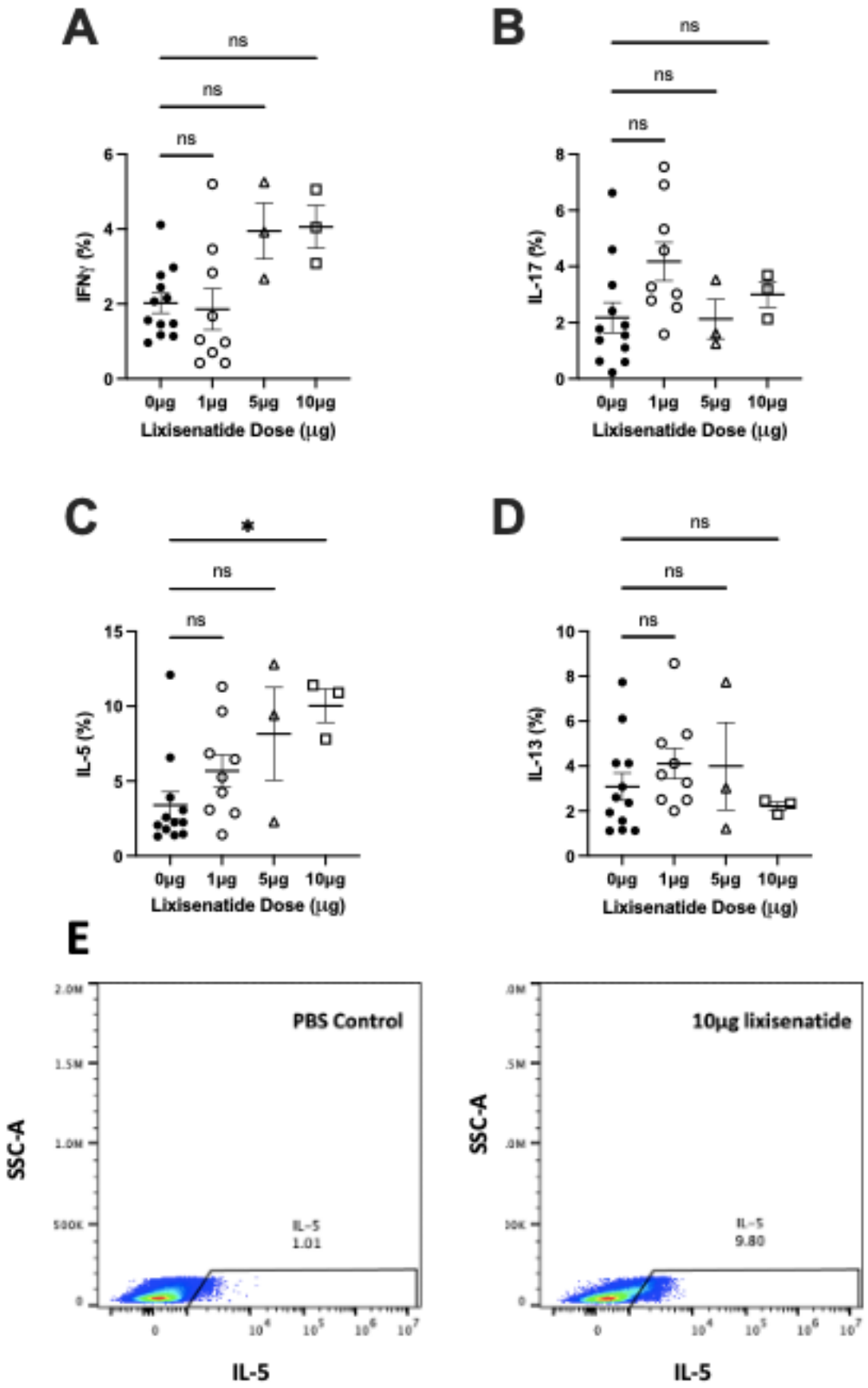

Figure 4.13: Daily treatment with 10µg lixisenatide appears to increase IL-5 produced by CD4+ T-cells of the mesenteric lymph node. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was

allowed to establish for 34 days. Daily intraperitoneal injection of 1μg, 5μg, or 10μg lixisenatide was started at day 34 post infection for 6 days. Mice were sacrificed after treatment and mLN were obtained for analysis. mLN were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Data plotted show the percentage of CD4+ T-cells in the mLN producing (A) IFN, (B) IL-5, (C) IL-17, and (D) IL-13. Data (n = 3-12) are from 4 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We found no significant difference in IFNγ, IL-17, or IL-13 from CD4+ T-cells of the mLN between PBS controls and mice treated with any of the six day time course doses of lixisenatide used (Fig. 4.13 A-B and Fig. 4.13 D). There was no significant difference in IL-5 between PBS control mice and the mice treated with 1μg or 5μg lixisenatide daily. However, mice treated with 10μg lixisenatide for 6 days have significantly increased ($p = 0.0150$) IL-5 from CD4+ T-helper cells in comparison to PBS control mice (Fig. 4.13 C).

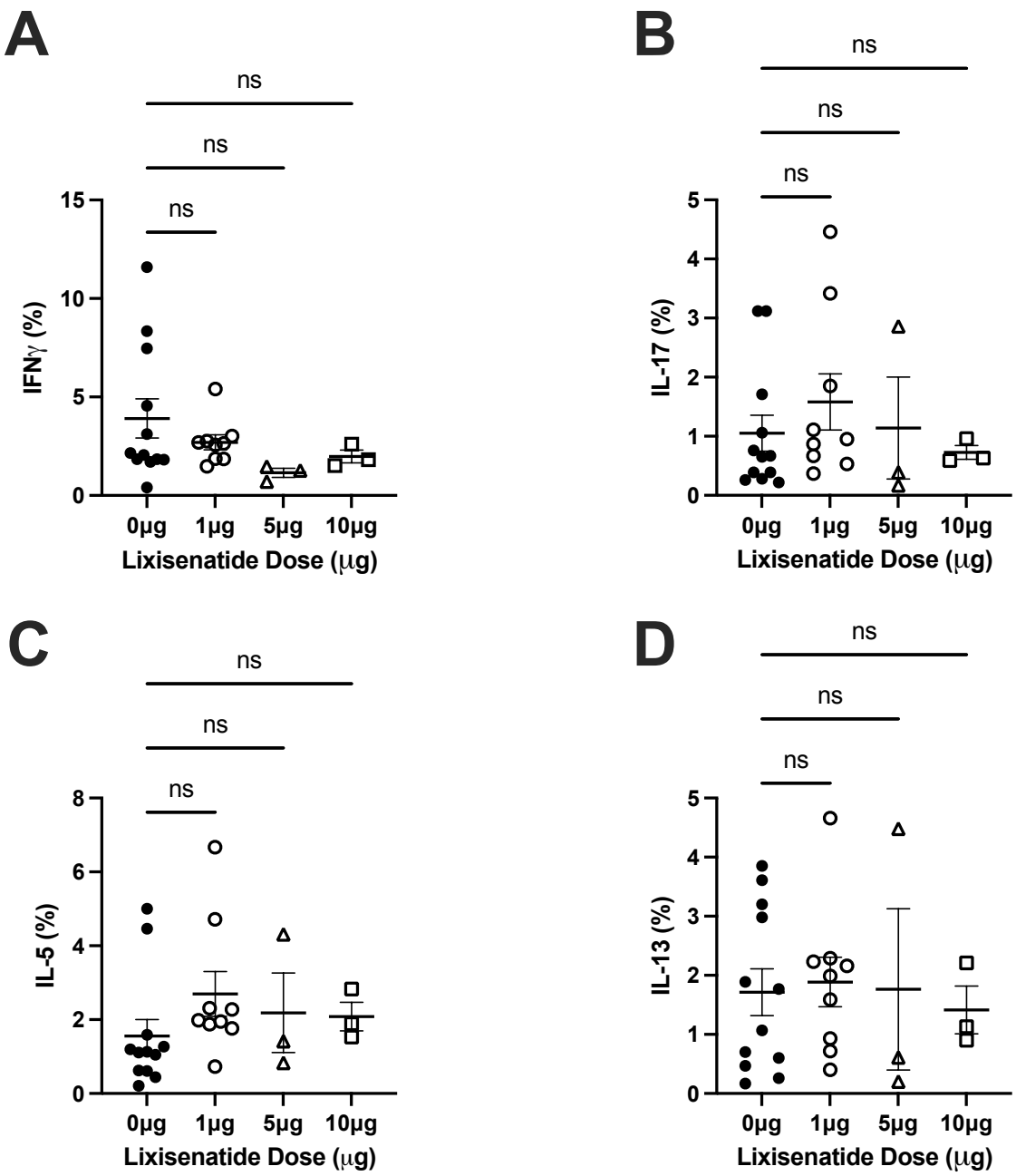

Figure 4.14: Cytokines produced by CD8+ T-cells in the mesenteric lymph node are unaffected by a 6-day course of lixisenatide treatment of *T. muris* infected mice. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg, 5μg, or 10μg lixisenatide was started at day 34 post infection for 4 days. Mice were sacrificed after treatment and mLN were obtained for analysis. mLN were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Plotted data show the percentage of CD8+ T-cells in the mLN producing (A) IFN, (B) IL-5, (C) IL-17, and (D) IL-13. Data (n= 3-12) are from 4 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We found no significant difference in IFNγ, IL-17, IL-5, or IL-13 from CD8+ T-cells of the mLN between PBS controls and mice treated with 1μg, 5μg, or 10μg lixisenatide after 6 days of treatment (Fig. 4.14 A-D).

Collectively, these data indicate that IL-22 or IL-5 may be involved in GLP-1rA induced *T. muris* expulsion, since treatment with lixisenatide was found to cause a significant increase in these cytokines from CD8+ cytotoxic and CD4+ T-cells respectively.

4.6. Immune cell subsets of the large intestine lamina propria, and cytokines produced, are largely unaffected by GLP-1r agonist treatment

After finding alterations in IL-22 and IL-5 in the mesenteric lymph by treatment with lixisenatide, we decided to look at the immune response in the large intestine and caecum to determine whether a more localised change in immune response was occurring. We stripped the epithelium from the intestine and then digested the lamina propria to obtain cells for analysis via flow cytometry after restimulation with cell stimulation cocktail overnight.

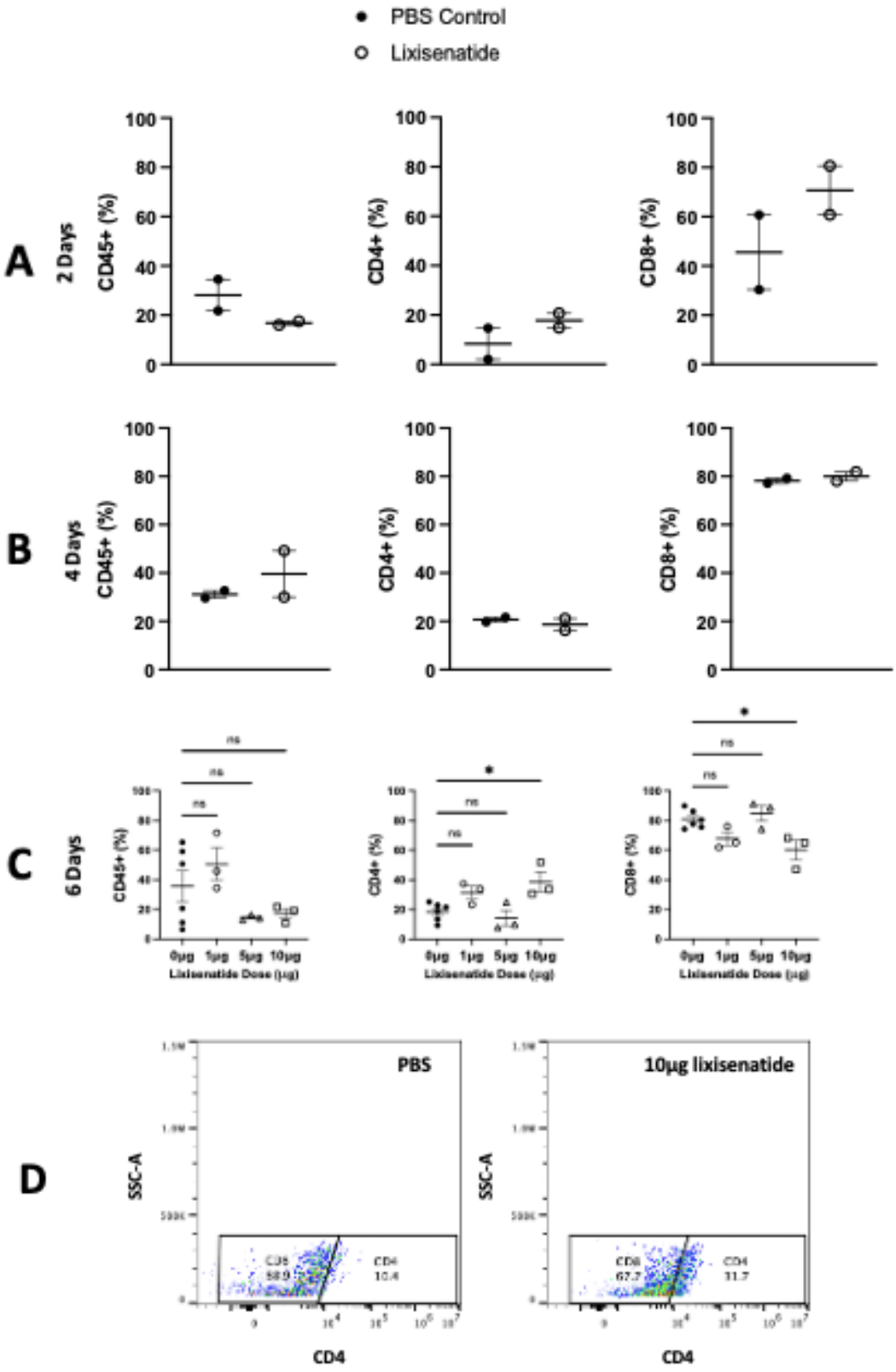

Figure 4.15: The percentage ratio of CD4+ and CD8+ T-cells are altered by high dose lixisenatide treatment.

C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1µg, 5µg, or 10µg lixisenatide was started at day 34 post infection for (A) 2

days, (B) 4 days, or (C) 6 days. Mice were sacrificed after treatment and large intestines were obtained and digested for lamina propria cells for analysis. Lamina propria cells were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Data (n = 2-6) are from 2 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We found no difference in the percentage of CD45+ immune cells, CD4+ helper T-cells, or CD8+ cytotoxic T-cells in the lamina propria between the PBS controls and lixisenatide treated mice after 2 days or 4 days of treatment (Fig 4.15 A-B). We found no significant difference in the percentage of CD45+ immune cells between PBS controls and lixisenatide treated mice after 6 days of treatment. We found no significant difference in the percentage of CD4+ or CD8+ T-cells after 6 days of treatment between the PBS controls and mice treated with 1μg or 5μg lixisenatide. However, we do see a significant increase ($p = 0.0143$) in the percentage of immune cells that are CD4+ helper T-cells in mice given high dose (10μg) lixisenatide daily for 6 days in comparison to PBS controls (Fig. 4.15 C). We also see a significant decrease ($p = 0.0120$ in the percentage of immune cells that are CD8+ cytotoxic T-cells in mice given high dose (10μg) lixisenatide daily for 6 days in comparison to PBS controls (Fig. 4.15 C).

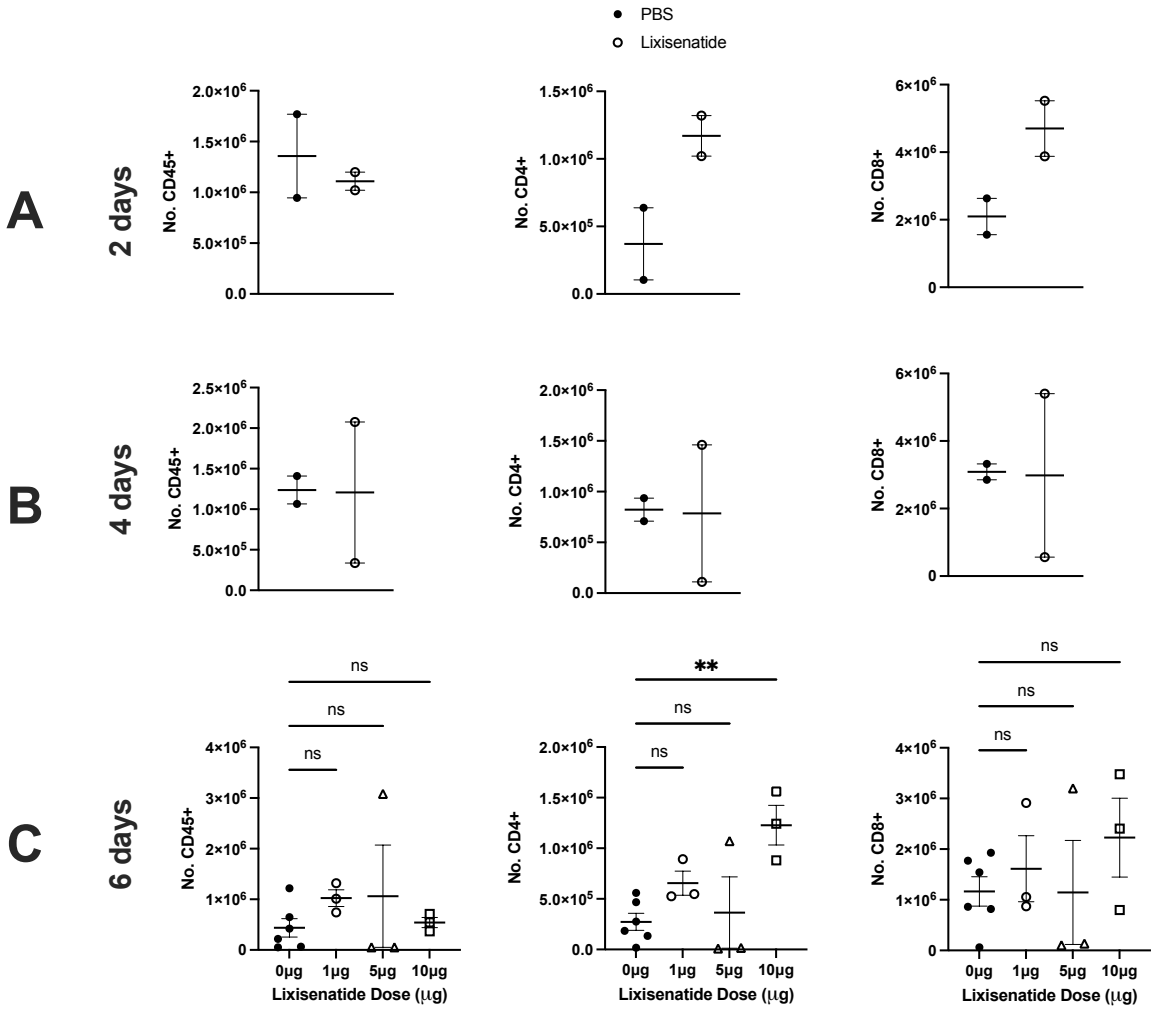

Figure 4.16: Number of CD4+ T-cells increases with high dose lixisenatide treatment. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1µg, 5µg, or 10µg lixisenatide was started at day 34 post infection for (A) 2 days, (B) 4 days, or (C) 6 days. Mice were sacrificed after treatment and large intestines were obtained and digested for lamina propria cells for analysis. Lamina propria cells were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Data (n = 3-6) are from 2 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We found no differences in the number of CD45+ immune cells, CD4+ T-cells, or CD8+ T-cells between PBS controls and lixisenatide treated mice at the 2 day or 4 day timepoint (Fig. 4.16. A-B). We found no significant difference in the number of CD45+ immune cells at the 6 day timepoint. We found a significant increase ($p = 0.0062$) in the number of CD4+ T-cells in mice treated with high dose (10µg) lixisenatide after 6 days of treatment in comparison to

PBS controls. Interestingly, we saw no significant difference in the number of CD8+ T-cells between lixisenatide treated mice and PBS controls at the 6 day time point, despite seeing a significant decrease in the percentage of these cells (Fig 4.16. C).

Collectively, this indicates that high dose lixisenatide treatment may alter the ratio of CD4/CD8 T-cells in the lamina propria of *T. muris* infected mice, increasing the proportion of T cells that are CD4+, the subset known to be crucial for expulsion of *T. muris. (*Koyama et al., 1995).

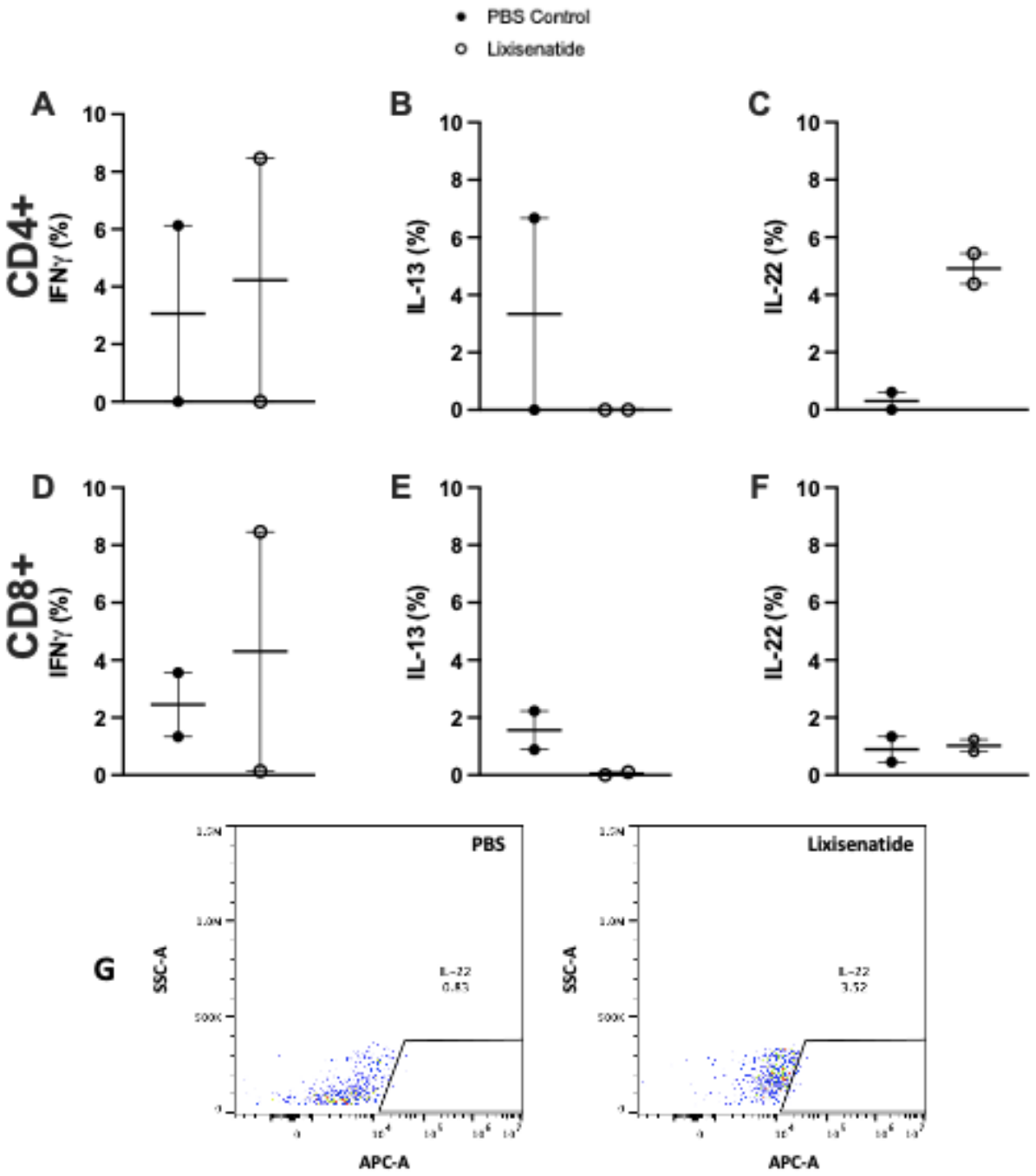

Figure 4.17: IL-22 produced by CD4+ T cells increases shortly following GLP-1RA treatment in *T. muris* infected mice. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 2 days. Mice were sacrificed after treatment and large intestines were obtained and digested for lamina propria cells for analysis. Lamina propria cells were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. We analysed (A) IFNγ, (B) IL-13, and (C) IL-22 from CD4+ helper T cells and (D) IFNγ, (E) IL-13, and (F) IL-22 from CD8+ cytotoxic T cells. (G) A representative image of IL-22 levels from CD4+ cells. Data (n = 2) are from 2

independent experiments and are presented as mean ± SEM. Statistical analysis was completed by unpaired t-test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We found no difference in the levels of IFNγ or IL-13 produced by CD4+ T-cells of the lamina propria between PBS controls and lixisenatide treated mice after 2 days of treatment (Fig. 4.17 A-B). However, we do see an increase in IL-22 produced by the CD4+ T-cells in lixisenatide treated mice compared to PBS controls (Fig. 4.17 C). This is preliminary data (n = 2) and therefore we cannot perform statistical analysis, so this should be repeated to confirm. We saw no difference in the levels of IFNγ, IL-13, or IL-22 produced by the CD8+ T-cells of the lamina propria between PBS controls and lixisenatide treated mice after 2 days of treatment (Fig 4.17 D-F).

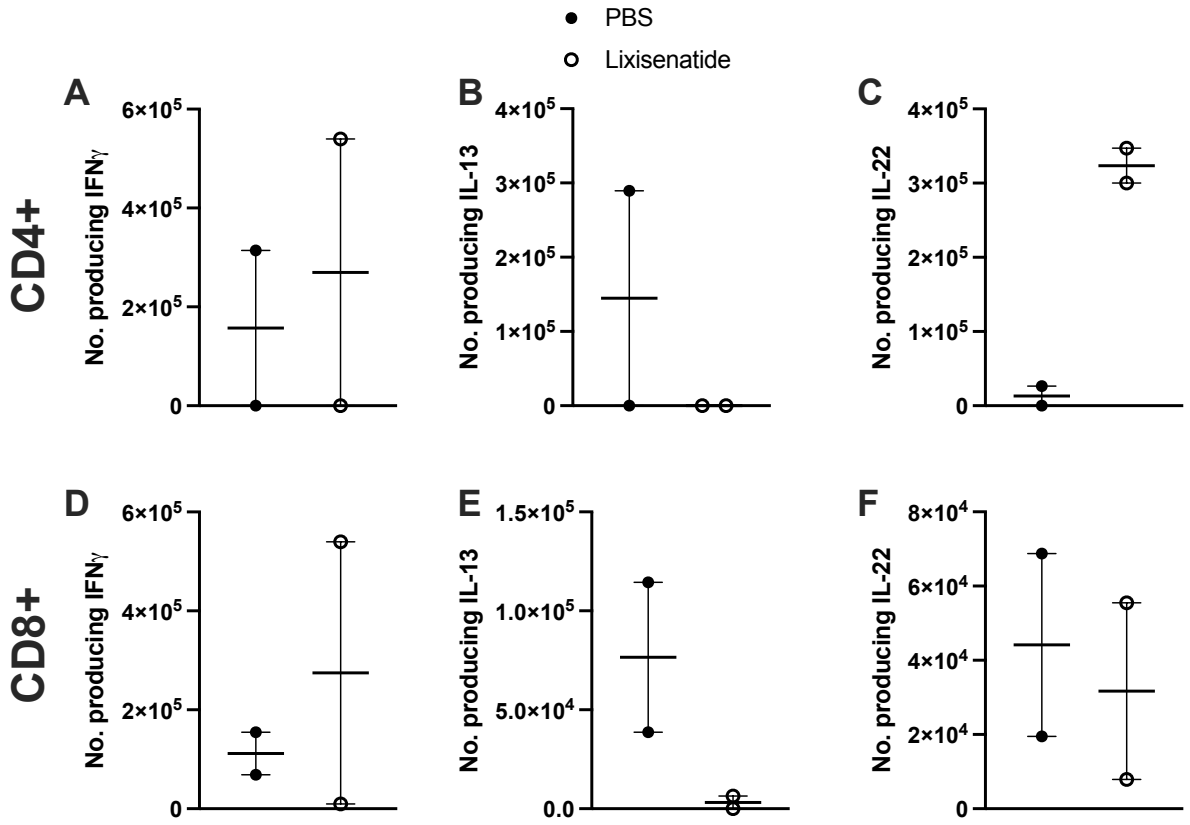

Figure 4.18: Number of IL-22 producing CD4+ T-cells increases following GLP-1RA treatment. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 2 days. Mice were sacrificed after treatment and large intestines were obtained and digested for lamina propria cells for analysis. Lamina propria cells were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. We analysed (A) IFNγ, (B) IL-13, and (C) IL-22 from CD4+ helper T cells and (D) IFNγ, (E) IL-13, and (F) IL-22 from CD8+ cytotoxic T cells. Data

(n = 2) are from 2 independent experiments and are presented as mean ± SEM. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We saw no difference in the number of CD4+ cells producing IFNγ or IL-13 between lixisenatide treated mice and PBS controls after 2 days of treatment (Fig. 4.18. A-B). We found an increase in the number of CD4+ cells producing IL-22 in lixisenatide treated mice compared to PBS controls (Fig. 4.18. C). Again, this is preliminary data (n = 2) and therefore we cannot perform statistical analysis, so this should be repeated to confirm. We saw no significant differences in the number of CD8+ cells producing IFNγ, IL-13, or IL-22 between lixisenatide treated mice and PBS controls at this timepoint (Fig. 4.18. D-F).

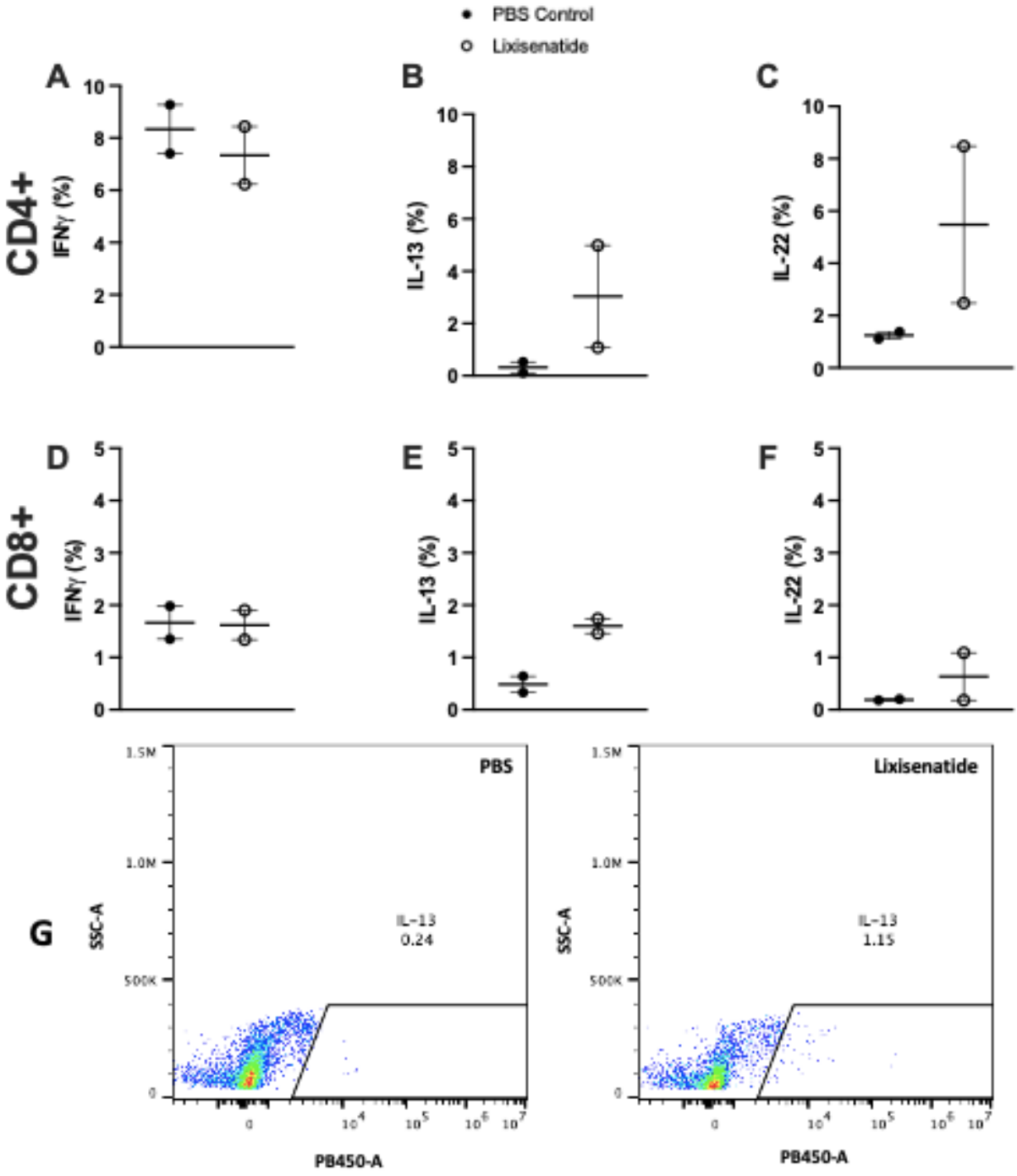

Figure 4.19: IL-13 produced by CD8+ T cells increases following lixisenatide treatment in *T. muris* infected mice. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 4 days. Mice were sacrificed after treatment and large intestines were obtained and digested for lamina propria cells for analysis. Lamina propria cells were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. We analysed (A) IFNγ, (B) IL-13, and (C) IL-22 from CD4+ helper T cells and (D) IFNγ, (E) IL-13, and (F) IL-22 from CD8+ cytotoxic T cells. (G) A representative image of IL-13 produced by CD8+ T cells. Data (n = 2) are from 2 independent experiments and

are presented as mean ± SEM. Statistical analysis was completed by unpaired t-test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We saw no difference in the levels of IFNγ, IL-13, or IL-22 produced by CD4+ T-cells of the lamina propria between PBS controls and lixisenatide treated mice after 4 days of treatment (Fig. 4.19 A-C). We found no difference in the levels of IFNγ or IL-22 produced by CD8+ T-cells of the lamina propria between PBS controls and lixisenatide treated mice after 4 days of treatment (Fig. 4.19 D and F). We saw an increase in the levels of IL-13 produced by CD8+ T-cells in lixisenatide treated mice compared to PBS controls, after 4 days of treatment (Fig. 4.19 E). However, this is preliminary data (n = 2) and therefore we cannot perform statistical analysis, so this should be repeated to confirm.

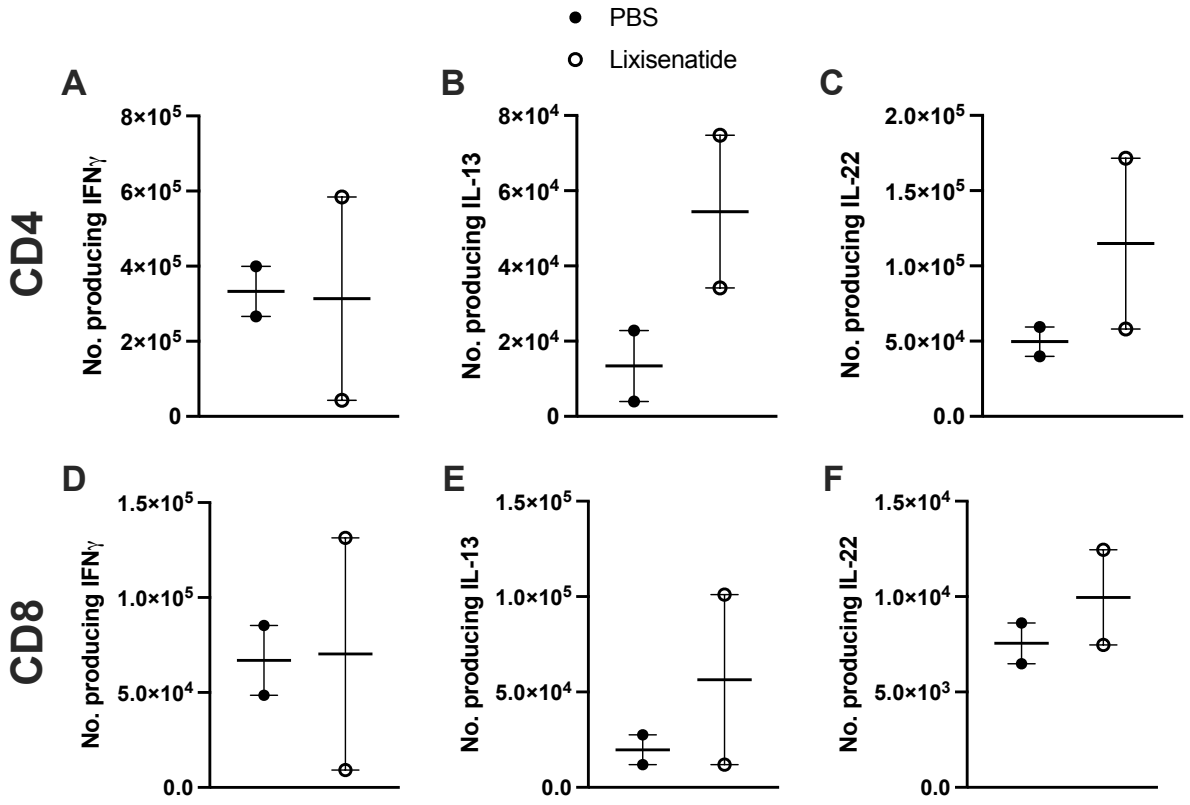

Figure 4.20: Number of CD4+ and CD8+ T-cells producing IFNγ, IL-13, or IL-22 unaffected by GLP-1RA treatment. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 4 days. Mice were sacrificed after treatment and large intestines were obtained and digested for lamina propria cells for analysis. Lamina propria cells were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors)

overnight and cells were collected and analysed the next day via flow cytometry. We analysed (A) IFNγ, (B) IL-13, and (C) IL-22 from CD4+ helper T cells and (D) IFNγ, (E) IL-13, and (F) IL-22 from CD8+ cytotoxic T cells. Data (n = 2) are from 2 independent experiments and are presented as mean ± SEM. Statistical analysis was completed by unpaired t-test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We saw no difference in the number of CD4+ T-cells producing IFNγ, IL-13, or IL-22 between lixisenatide treated and PBS control mice after 4 days of GLP-1RA treatment (Fig. 4.20. A-C). We also saw no difference in the number of CD8+ T-cells producing IFNγ, IL-13, or IL-22 between the lixisenatide treated and PBS control mice after 4 days of treatment (Fig. 4.20. D-F).

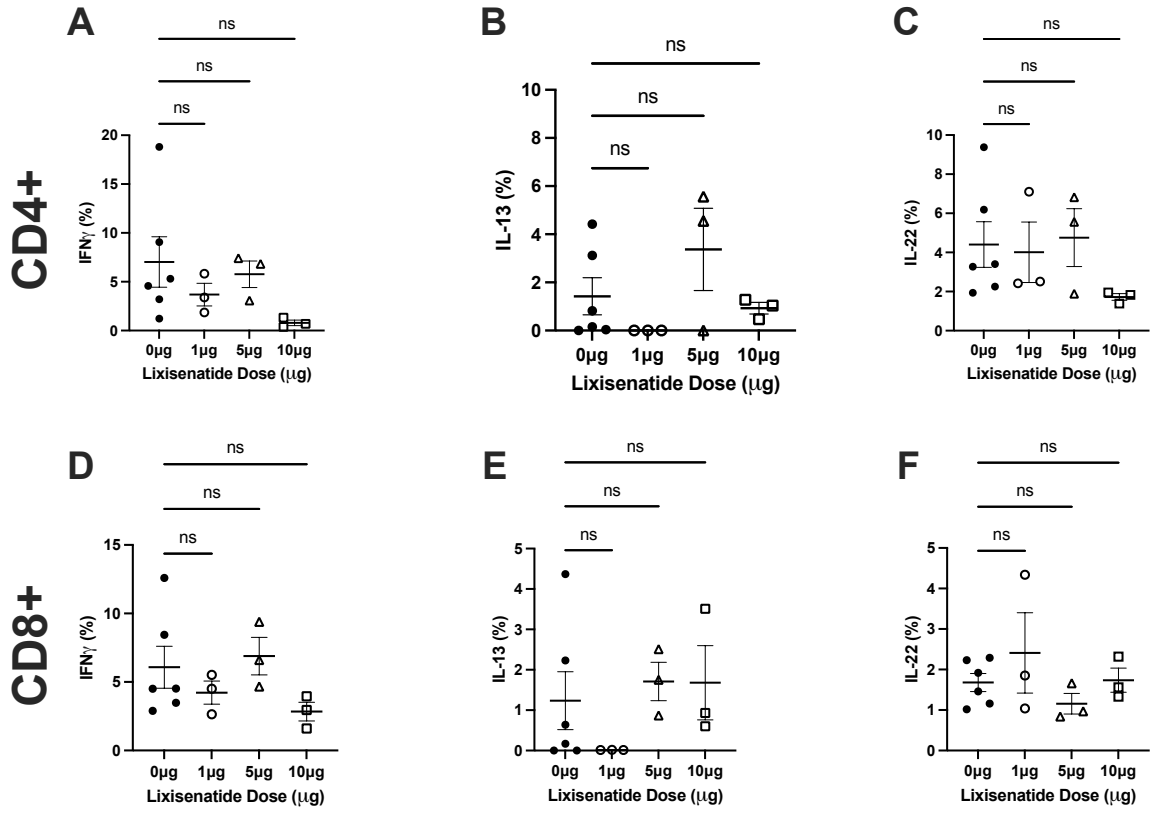

Figure 4.21: There are no differences in cytokines produced by CD4+ or CD8+ T-cells after 6 days of lixisenatide treatment at any of the doses used. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 6 days. Mice were sacrificed after treatment and large intestines were obtained and digested for lamina propria cells for analysis. Lamina propria cells were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. We analysed (A) IFNγ, (B) IL-13, and (C) IL-22 from CD4+ helper T cells and (D) IFNγ, (E) IL-13, and (F) IL-22 from CD8+ cytotoxic T cells. Data (n = 3-6) are from 2 independent experiments

and are presented as mean ± SEM. Statistical analysis was completed by one way ANOVA with Dunnett's multiple comparisons test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We saw no significant difference in the levels of IFNγ, IL-13, or IL-22 produced by CD4+ T-cells of the lamina propria between PBS controls and lixisenatide treated mice (1μg, 5μg, and 10μg) after 6 days of treatment (Fig. 4.21 A-C). We saw no significant difference in the levels of IFNγ, IL-13, or IL-22 produced by CD8+ T-cells of the lamina propria between PBS controls and lixisenatide treated mice (1μg, 5μg, and 10μg) after 6 days of treatment (Fig. 4.21 D-F).

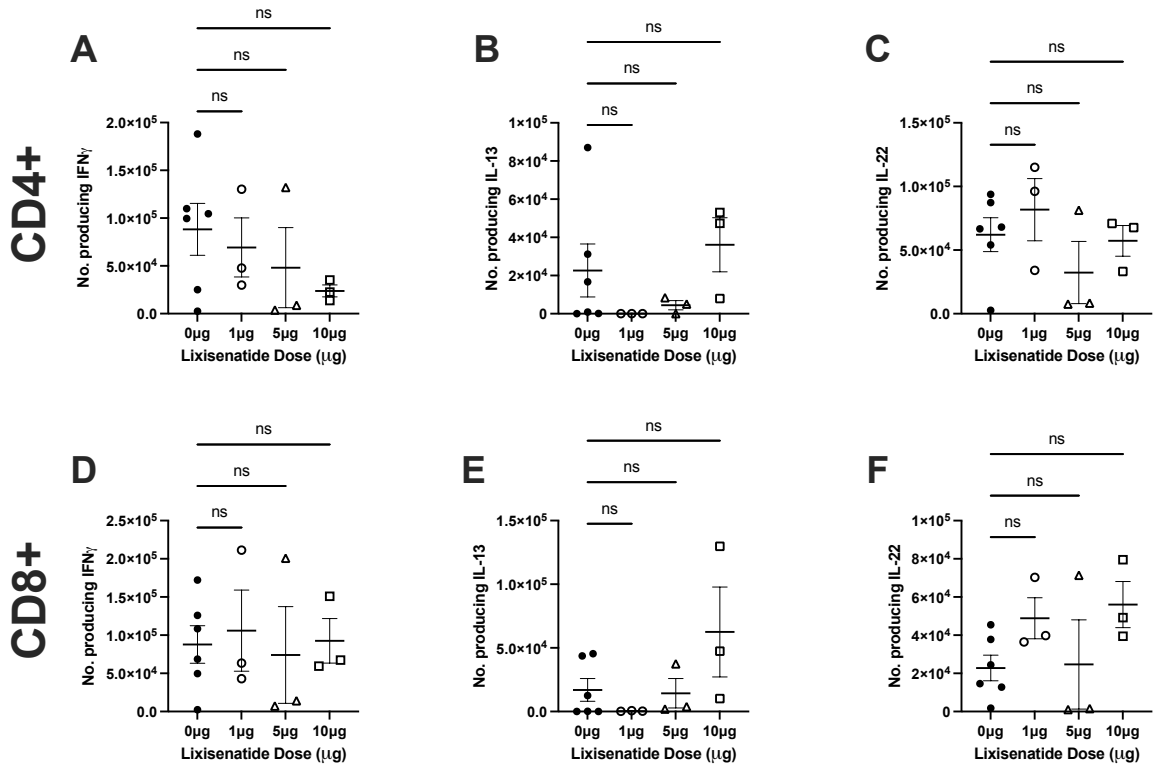

Figure 4.22: There are no differences in cytokines produced by CD4+ or CD8+ T-cells after 6 days of lixisenatide treatment at any of the doses used. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 6 days. Mice were sacrificed after treatment and large intestines were obtained and digested for lamina propria cells for analysis. Lamina propria cells were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. We analysed (A) IFNγ, (B) IL-13, and (C) IL-22 from CD4+ helper T cells and (D) IFNγ, (E) IL-13, and (F) IL-22 from CD8+ cytotoxic T cells. Data (n = 3-6) are from 2 independent experiments

and are presented as mean ± SEM. Statistical analysis was completed by one way ANOVA with Dunnett's multiple comparisons test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We saw no significant difference in the number of CD4+ cells producing of IFNγ, IL-13, or IL-22 between PBS controls and lixisenatide treated mice (1μg, 5μg, and 10μg) after 6 days of treatment (Fig. 4.22 A-C). We saw no significant difference in the number of CD8+ T-cells producing IFNγ, IL-13, or IL-22 between PBS controls and lixisenatide treated mice (1μg, 5μg, and 10μg) after 6 days of treatment (Fig. 4.22 D-F).

Collectively, these data indicate that there is an early alteration in cytokines IL-13 and IL-22 in response to GLP-1RA treatment. However, this appears to be a quick and short-lived response as we see no difference in these cytokines between treated mice and PBS controls after 6 days of treatment at any dose.

4.7. *In vitro* restimulation of lamina propria cells

To further examine any differences in cytokines produced by cells of the large intestine lamina propria, we restimulated the cells *in vitro* with added GLP-1RA. We stimulated cells from infected mice of the PBS control groups at three timepoints of infection: days 36, 38, and 40 days p.i.

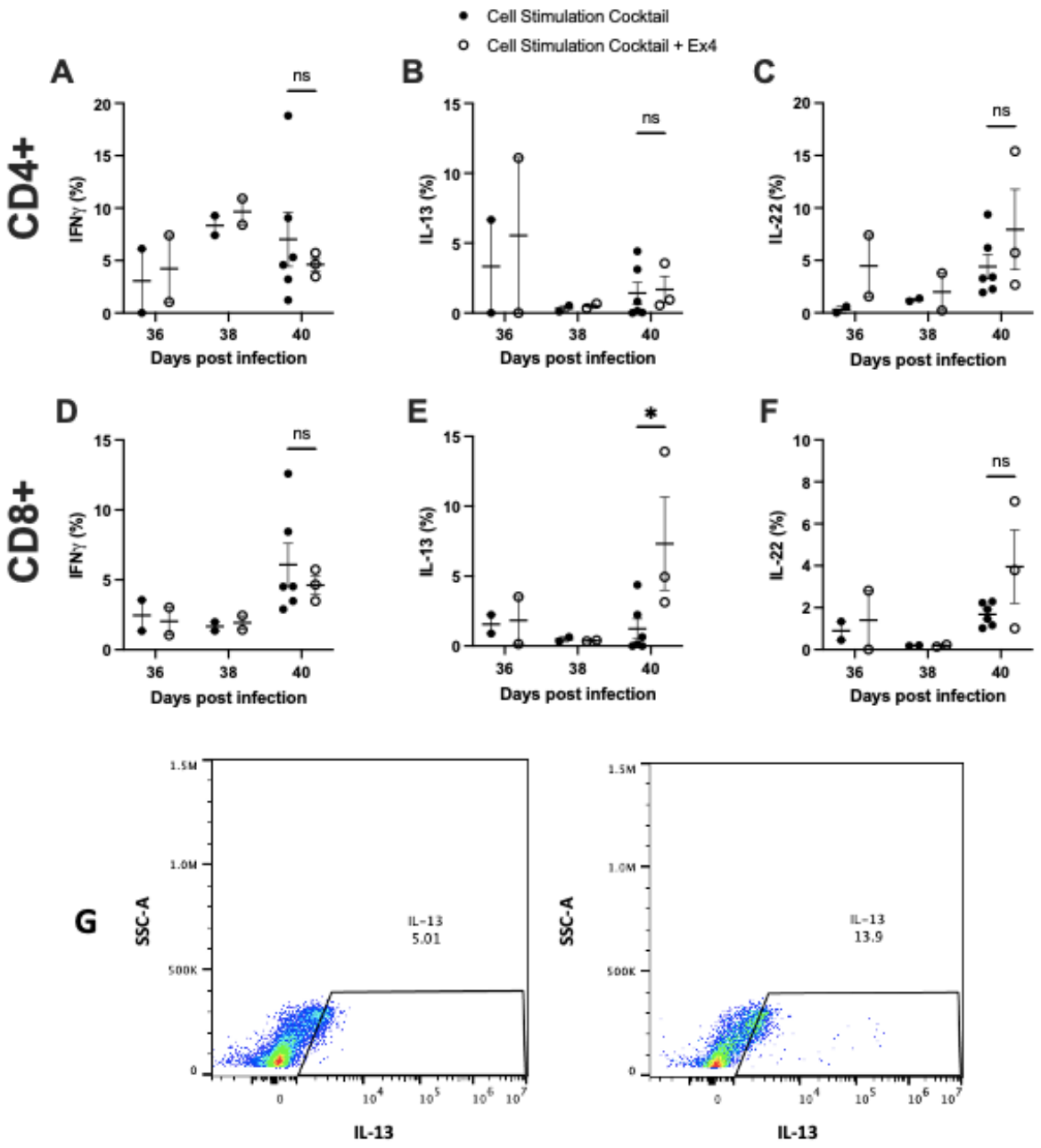

Figure 4.23: *In vitro* restimulation with GLP-1rA causes an increase in IL-13. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34-40 days. Mice were sacrificed after treatment and large intestines were obtained and digested for lamina propria cells for analysis. Lamina propria cells were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) and 10 nM exendin-4 overnight and cells were collected and analysed the next day via flow cytometry. We analysed (A) IFNγ, (B) IL-13, and (C) IL-22 from CD4+ helper T cells and (D) IFNγ, (E) IL-13, and (F) IL-22 from CD8+ cytotoxic T cells. Data (n = 2) are from 2 independent experiments and are presented as mean ± SEM. Statistical analysis was completed by two-way ANOVA with Šidák's multiple comparisons test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We saw no difference in the levels of IFNγ, IL-13, or IL-22 produced by CD4+ T-cells of the lamina propria at any of the three timepoints when restimulated with cell stimulation cocktail alone in comparison to those stimulation with cell stimulation cocktail plus a GLP-1rA (Fig. 4.23 A-C).

We also saw no difference in the levels of IFNγ or IL-22 produced by CD8+ T-cells of the lamina propria at any timepoint when restimulated with cell stimulation cocktail alone in comparison to those stimulation with cell stimulation cocktail plus GLP-1rA (Fig. 4.23 D and F). There was no difference in levels of IL-13 produced by CD8+ T-cells of the lamina propria in cells from mice 36 and 38 days p.i., but a significant increase in IL-13 was seen from cells from mice 40 days p.i. (Fig. 4.23. E).

These data show that in a reductionist approach of adding GLP-1rA to isolated lamina propria immune cells, we still see an alteration in IL-13 produced by CD8+ T-cells, indicating that GLP-1rA treatment can alter levels of IL-13 and this may play a role in GLP-1rA induced *T. muris* expulsion.

4.8. Intraepithelial lymphocytes and the cytokines they produce are largely unaffected by GLP-1RA treatment

Intraepithelial lymphocytes have been found to possess the GLP-1 receptor (Yusta et al., 2015). IELs are in close proximity to *T. muris* so we isolated these cells to examine the local immune response to *T. muris* when treated with GLP-1rA.

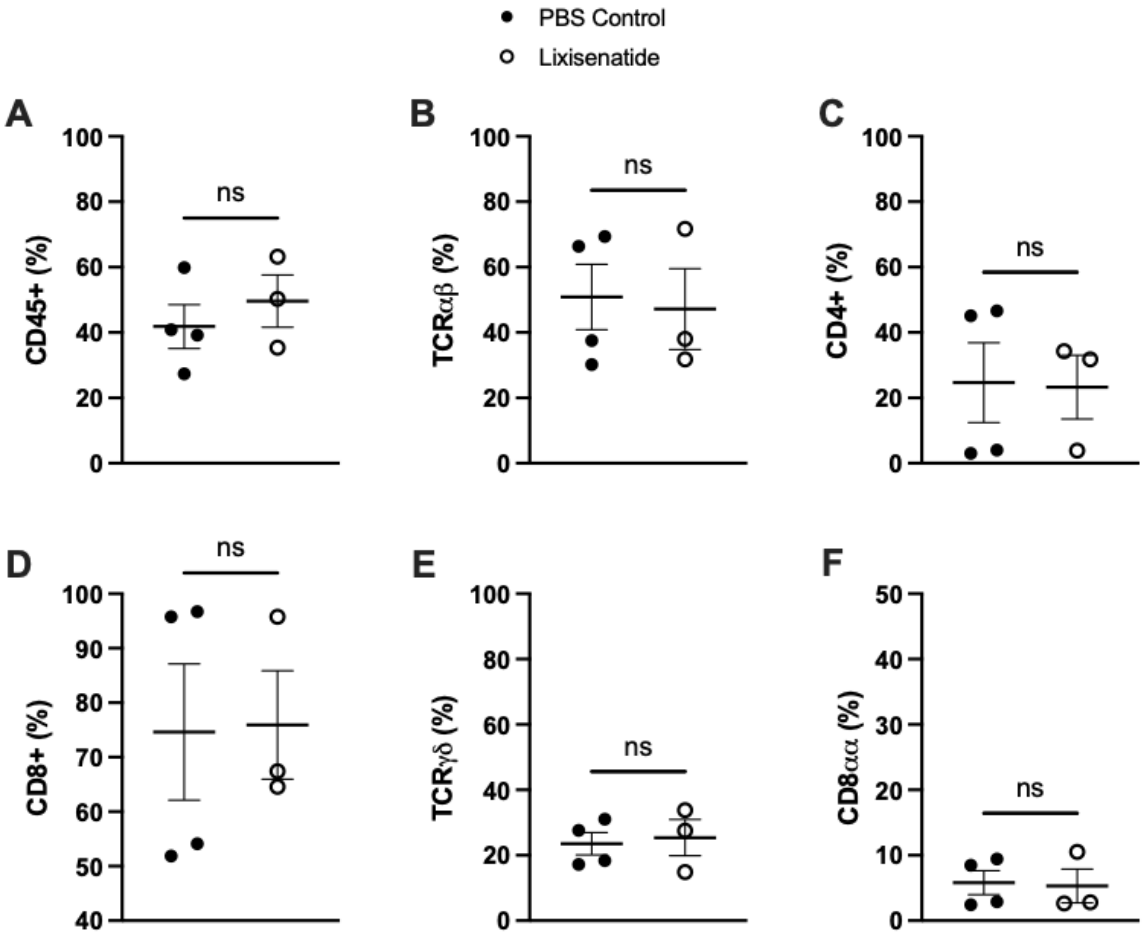

Figure 4.24: Immune cell subsets of the intestinal epithelium are unaltered by GLP-1rA treatment. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 4 days. Mice were sacrificed after treatment and large intestines were obtained and stripped to obtain intraepithelial lymphocytes (IELs) for analysis. IELs were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. We determined the percentage of (A) CD45+ immune cells, (B) TCRαβ+ cells, (C) CD4+ helper T cells, (D) CD8+ cytotoxic T cells, (E) TCRγδ+ cells, and (F) CD8αα+ cells. Data (n = 3-4) are from 2 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We found no significant difference in the percentage of CD45+ immune cells in the epithelium of the large intestine and caecum between PBS controls and lixisenatide treated mice after 4 days of treatment (Fig. 4.24 A). We found no significant difference in the percentage of these immune cells that were TCRαβ between PBS controls and lixisenatide treated mice (Fig. 4.24 B). We also found no significant difference in the percentage of TCRαβ that were CD4+ helper T-cells or CD8+ cytotoxic T-cells between PBS controls and

lixisenatide treated mice after 4 days of treatment (Fig. 4.24 C-D). We then looked globally at the percentage of TCRγδ+ immune cells. We found no significant difference in the percentage of immune cells that are TCRγδ+ between the PBS controls and lixisenatide treated mice (Fig. 4.24 E). We focused in on CD8αα+ cells, a subpopulation of TCRγδ+ cells, as we know that these possess the GLP-1r. We found no significant difference in the percentage of CD8αα+ between PBS controls and lixisenatide treated mice after 4 days of treatment (Fig. 4.24 F).

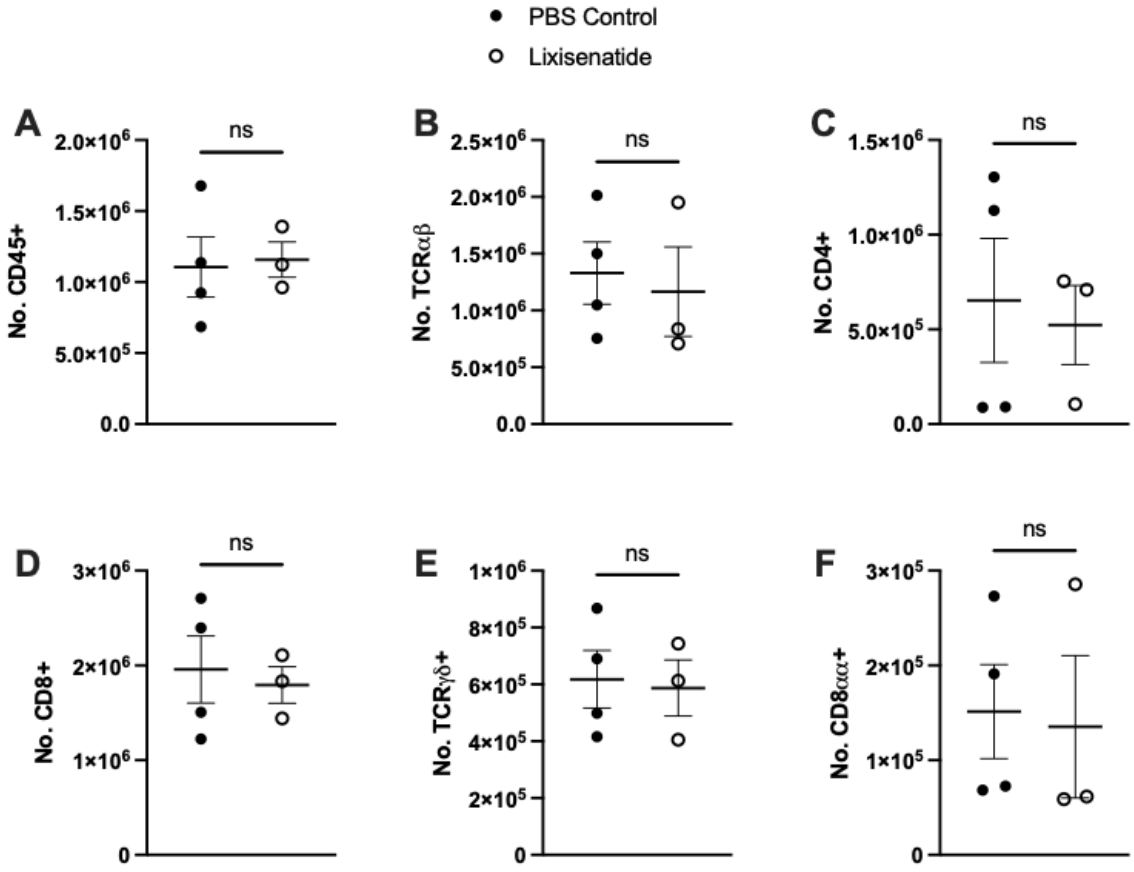

Figure 4.25: **Immune cell subsets of the intestinal epithelium are unaltered by GLP-1rA treatment.** C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 4 days. Mice were sacrificed after treatment and large intestines were obtained and stripped to obtain intraepithelial lymphocytes (IELs) for analysis. IELs were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. We determined the number of (A) CD45+ immune cells, (B) TCRαβ+ cells, (C) CD4+ helper T cells, (D) CD8+ cytotoxic T cells, (E) TCRγδ+ cells, and (F) CD8αα+ cells. Data (n = 3-4) are from 2 independent experiments and are presented as

mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We found no significant difference in the number of CD45+ immune cells in the epithelium of the large intestine and caecum between PBS controls and lixisenatide treated mice after 4 days of treatment (Fig. 4.25 A). We found no significant difference in the number of these immune cells that were TCRαβ between PBS controls and lixisenatide treated mice (Fig. 4.25 B). We also found no significant difference in the number of TCRαβ that were CD4+ helper T-cells or CD8+ cytotoxic T-cells between PBS controls and lixisenatide treated mice after 4 days of treatment (Fig. 4.25 C-D). We then looked globally at the number of TCRγδ+ immune cells. We found no significant difference in the percentage of immune cells that are TCRγδ+ between the PBS controls and lixisenatide treated mice (Fig. 4.25 E). We then focused in on CD8αα+ cells, a subpopulation of TCRγδ+ cells, as we know that these possess the GLP-1r. We found no significant difference in the number of CD8αα+ between PBS controls and lixisenatide treated mice after 4 days of treatment (Fig. 4.25 F).

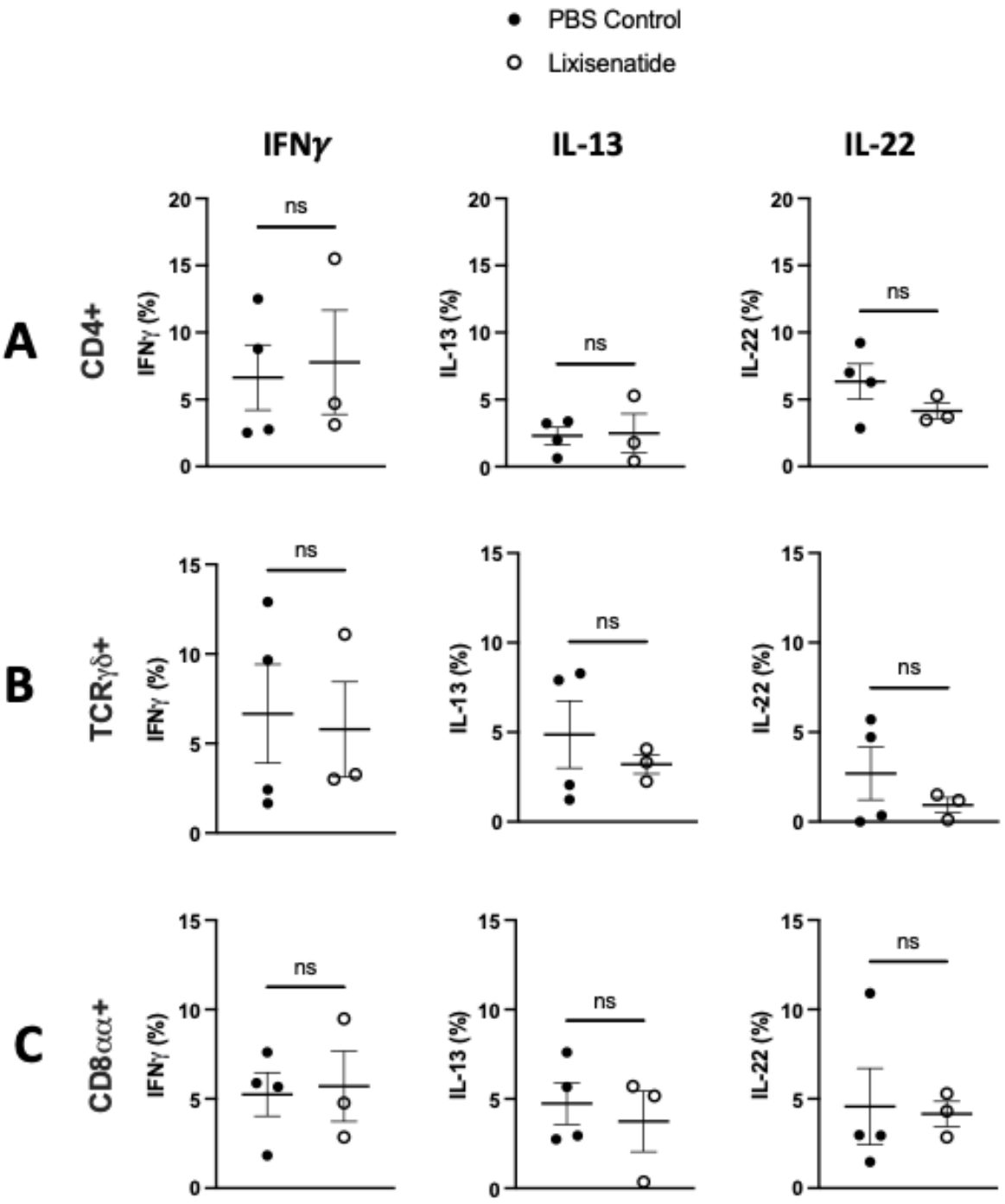

Figure 4.26: Cytokines produced by intraepithelial lymphocytes are unaffected by lixisenatide treatment. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 4 days. Mice were sacrificed after treatment and large intestines were obtained and stripped to obtain intraepithelial lymphocytes (IELs) for analysis. IELs were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Levels of IFN, IL-13, and IL-22 were determined from (A) CD4+ helper T cells, (B) TCRγδ+ cells, and (C) CD8αα+ cells. Data (n = 3-

4) are from 2 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We found no significant difference in the levels of IFNγ, IL-13, or IL-22 produced by CD4+ IELs between PBS controls and lixisenatide treated mice after 4 days of treatment (Fig. 4.26 A). We found no significant difference in the levels of IFNγ, IL-13, or IL-22 produced by TCRγδ+ IELs between PBS controls and lixisenatide treated mice after 4 days of treatment (Fig. 4.26 B). We saw no significant difference in the levels of IFNγ, IL-13, or IL-22 produced by CD8αα+ IELs between PBS controls and lixisenatide treated mice after 4 days of treatment (Fig. 4.26 C).

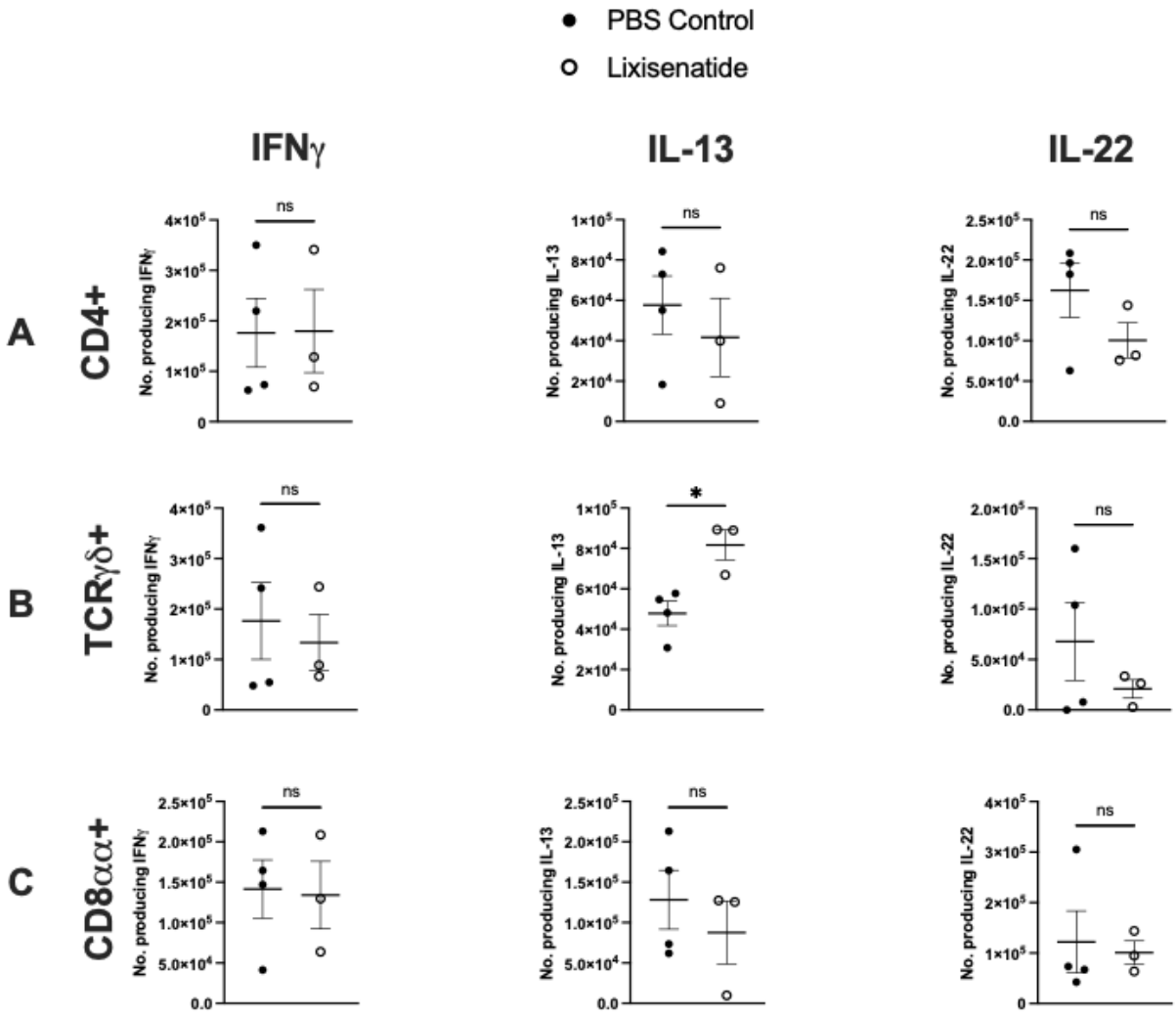

Figure 4.27: Number of TCRγδ+ cells producing IL-13 increases in response to GLP-1rA treatment. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily

intraperitoneal injection of 1μg lixisenatide was started at day 34 post infection for 4 days. Mice were sacrificed after treatment and large intestines were obtained and stripped to obtain intraepithelial lymphocytes (IELs) for analysis. IELs were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Levels of IFN, IL-13, and IL-22 were determined from (A) CD4+ helper T cells, (B) TCRγδ+ cells, and (C) CD8αα+ cells. Data (n = 3-4) are from 2 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We found no significant difference in the levels of IFNγ, IL-13, or IL-22 produced by CD4+ IELs between PBS controls and lixisenatide treated mice after 4 days of treatment (Fig. 4.27 A). We found no significant difference in the levels of IFNγ or IL-22 produced by TCRγδ+ IELs between PBS controls and lixisenatide treated mice after 4 days of treatment (Fig. 4.27 B). Interestingly, we found an increase in the number of TCRγδ+ IELs producing IL-13 at this timepoint (Fig. 4.27 B). We saw no significant difference in the levels of IFNγ, IL-13, or IL-22 produced by CD8αα+ IELs between PBS controls and lixisenatide treated mice after 4 days of treatment (Fig. 4.27 C).

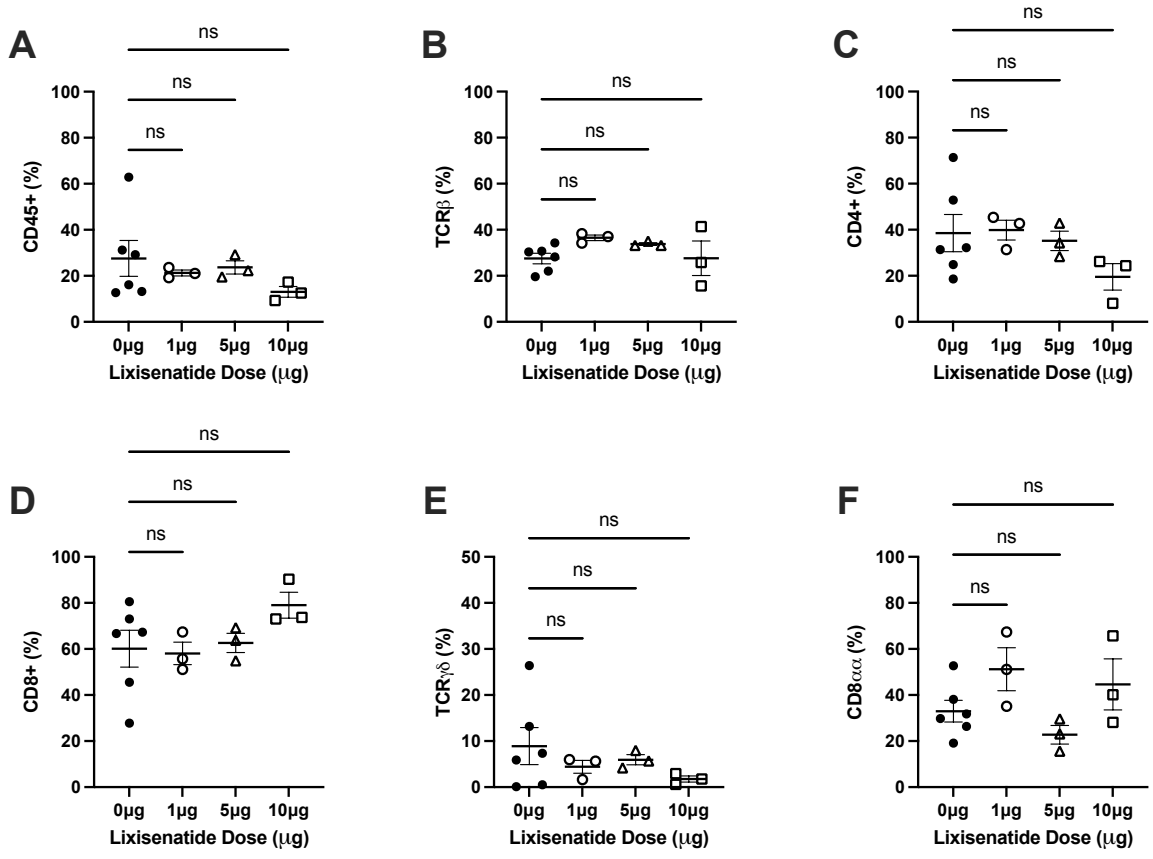

Figure 4.28: Immune subsets are unaffected by 6 days of lixisenatide treatment. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg, 5μg, or 10μg lixisenatide was started at day 34 post infection for 6 days. Mice were sacrificed after treatment and large intestines were obtained and epithelium stripped to obtain intraepithelial lymphocytes (IELs) for analysis. IELs were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. We determined the percentage of (A) CD45+ immune cells, (B) TCRαβ+ cells, (C) CD4+ helper T cells, (D) CD8+ cytotoxic T cells, (E) TCRγδ+ cells, and (F) CD8αα+ cells. Data (n = 3-6) are from 2 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by one-way ANOVA with Dunnett's multiple comparisons test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We found no significant difference in the percentage of CD45+ immune cells in the epithelium of the large intestine and caecum between PBS controls and lixisenatide treated mice after 6 days of treatment (Fig. 4.28 A). We found no significant difference in the percentage of immune cells that were TCRαβ+ between PBS controls and lixisenatide treated mice (Fig. 4.28 B). We also found no significant difference in the percentage of these TCRαβ+ cells that were CD4+ helper T-cells or CD8+ cytotoxic T-cells between PBS controls and lixisenatide treated mice after 6 days of treatment (Fig. 4.28 C-D). We found no significant difference in the percentage of immune cells that are TCRγδ+ between the PBS controls and lixisenatide treated mice (Fig. 4.28 E). We found no significant difference in the percentage of CD8+ T-cells that are CD8αα+ between PBS controls and lixisenatide treated mice after 6 days of treatment (Fig. 4.28 F).

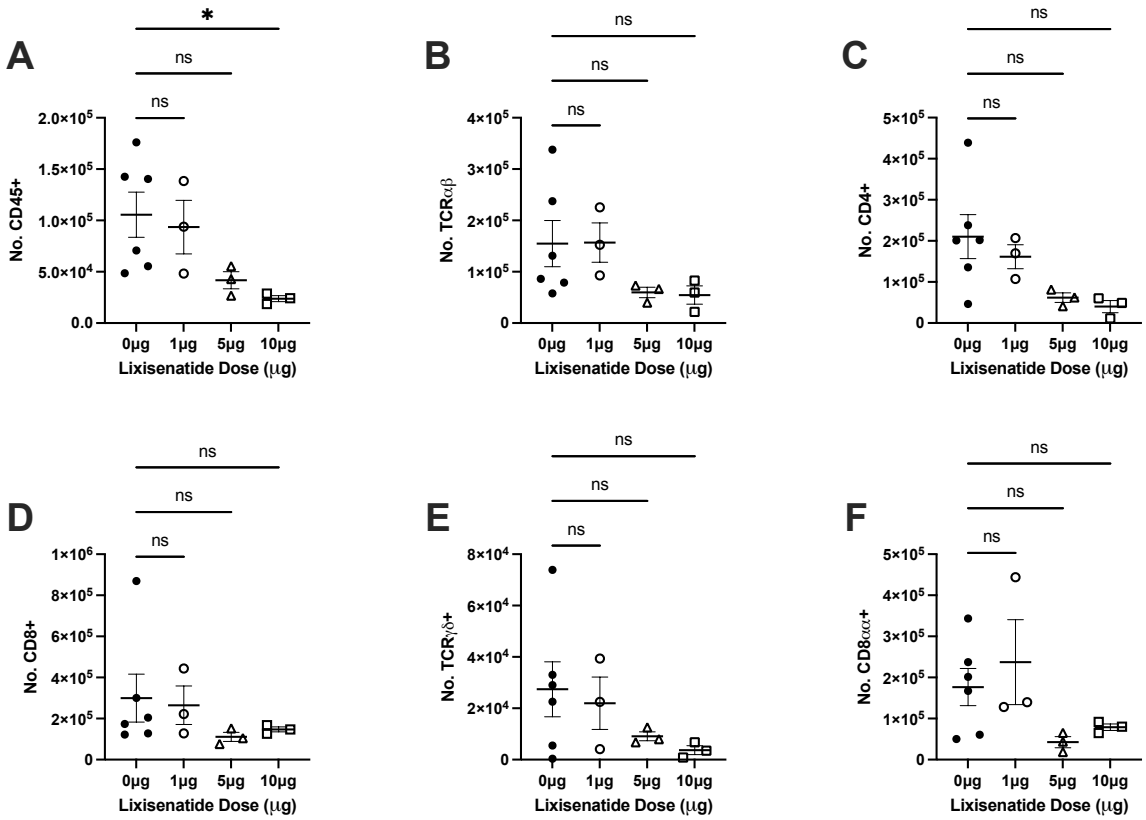

Figure 4.29: Number of CD45+ immune cells in the intestinal epithelium decreases in response to high dose GLP-1rA treatment. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1µg, 5µg, or 10µg lixisenatide was started at day 34 post infection for 6 days. Mice were sacrificed after treatment and large intestines were obtained and epithelium stripped to obtain intraepithelial lymphocytes (IELs) for analysis. IELs were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. We determined the number of (A) CD45+ immune cells, (B) TCRαβ+ cells, (C) CD4+ helper T cells, (D) CD8+ cytotoxic T cells, (E) TCRγδ+ cells, and (F) CD8αα+ cells. Data (n = 3-6) are from 2 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by one-way ANOVA with Dunnett's multiple comparisons test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We found no significant difference in the number of CD45+ immune cells in the epithelium of the large intestine and caecum between PBS controls and lixisenatide treated mice after 6 days of treatment (Fig. 4.29 A). We found no significant difference in the number of immune cells that were TCRαβ+ between PBS controls and lixisenatide treated mice (Fig. 4.29 B). We also found no significant difference in the number of these TCRαβ+ cells that were CD4+ helper T-cells or CD8+ cytotoxic T-cells between PBS controls and lixisenatide treated mice after 6 days of treatment (Fig. 4.29 C-D). We found no significant difference in

the number of immune cells that are TCRγδ+ between the PBS controls and lixisenatide treated mice (Fig. 4.29 E). We found no significant difference in the number of CD8+ T-cells that are CD8αα+ between PBS controls and lixisenatide treated mice after 6 days of treatment (Fig. 4.29 F).

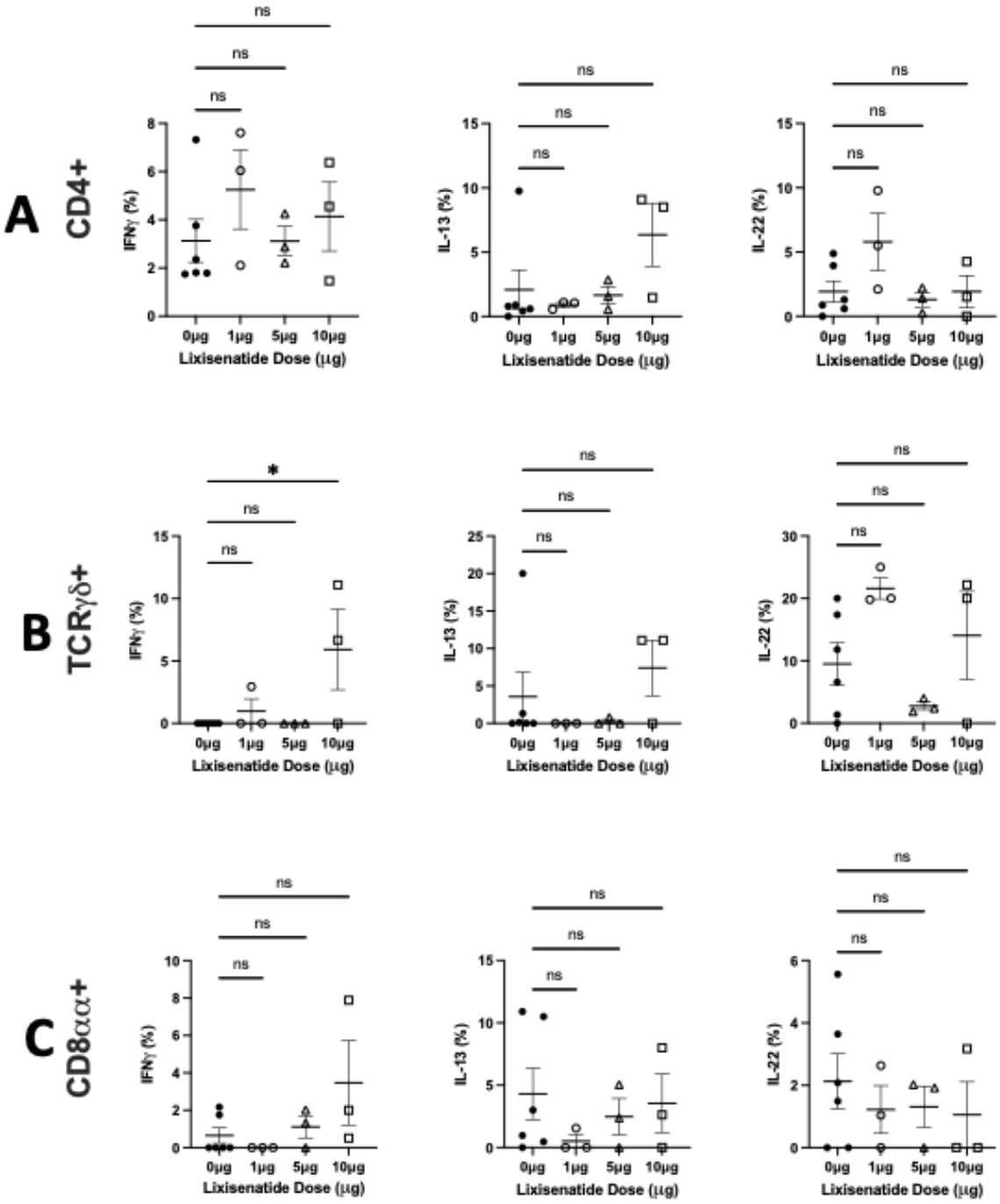

Figure 4.30: High dose lixisenatide appears to increase IFNγ production from TCRγδ+ cells. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg, 5μg, or 10μg lixisenatide began on day 34 post infection for 6 days. Mice

were sacrificed after treatment and large intestines were obtained and stripped to obtain intraepithelial lymphocytes (IELs) for analysis. IELs were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Levels of IFN, IL-13, and IL-22 were determined from (A) CD4+ helper T cells, (B) TCR$\gamma\delta$+ cells, and (C) CD8$\alpha\alpha$+ cells. Data (n = 3-4) are from 2 independent experiments and are presented as mean $\pm$ SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We found no significant difference in the percentage of CD4+ T-cells producing IFNγ, IL-13, or IL-22 between PBS controls and lixisenatide treated mice after 6 days of treatment with any dose (Fig. 4.30 A). We found no significant difference in the percentage of TCR$\gamma\delta$+ IELs producing IFNγ, IL-13, or IL-22 between PBS controls and lixisenatide treated mice (1μg or 5μg) after 6 days of treatment. However, we did see a significant increase ($p = 0.0174$) in IFNγ produced by TCR$\gamma\delta$+ IELs in mice treated daily with 10μg lixisenatide for 6 days compared to PBS controls (Fig 4.30 B). We found no significant difference in the percentage of CD8$\alpha\alpha$+ IELs producing IFNγ, IL-13, or IL-22 between PBS controls and lixisenatide treated mice (1μg, 5μg, or 10μg) after 6 days of treatment (Fig. 4.30 C).

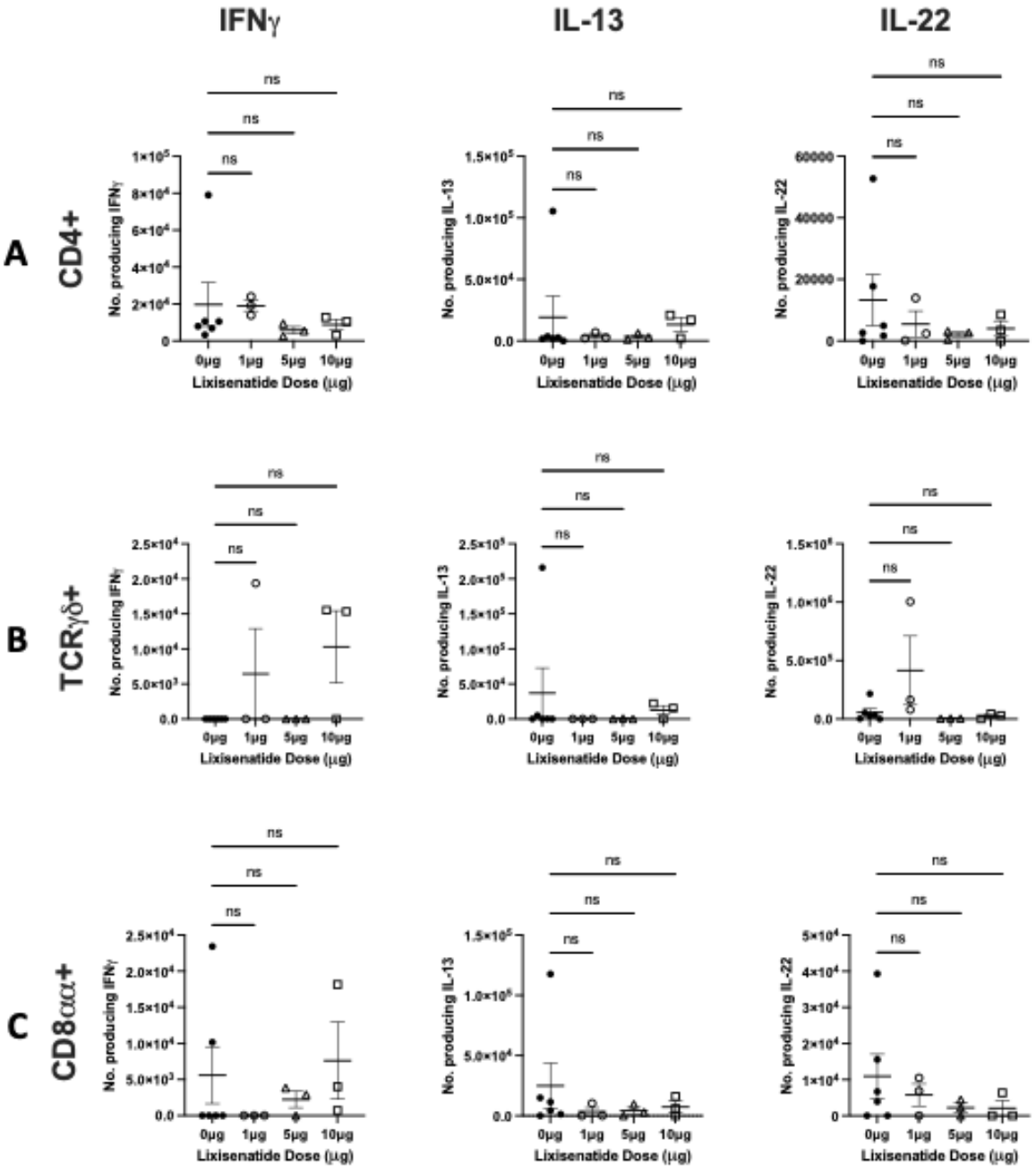

Figure 4.31: Six day GLP-1rA treatment does not affect cytokines produced by IELs. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34 days. Daily intraperitoneal injection of 1μg, 5μg, or 10μg lixisenatide began on day 34 post infection for 6 days. Mice were sacrificed after treatment and large intestines were obtained and stripped to obtain intraepithelial lymphocytes (IELs) for analysis. IELs were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Levels of IFN, IL-13, and IL-22 were determined from (A) CD4+ helper T cells, (B) TCRγδ+ cells, and (C) CD8αα+ cells. Data (n = 3-4) are from 2 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We found no significant difference in the number of CD4+ T-cells producing IFNγ, IL-13, or IL-22 between PBS controls and lixisenatide treated mice after 6 days of treatment at any dose (Fig. 4.31 A). We found no significant difference in the number of TCRγδ+ IELs producing IFNγ, IL-13, or IL-22 between PBS controls and lixisenatide treated mice after 6 days of treatment at any dose (Fig. 4.31 B).We found no significant difference in the number of CD8αα+ IELs producing IFNγ, IL-13, or IL-22 between PBS controls and lixisenatide treated mice after 6 days of treatment at any dose (Fig. 4.31 C).

Collectively these data indicate that minor alterations in cytokines may play a role in GLP-1rA induced *T. muris* expulsion. There may be potential involvement of IL-13, a cytokine known to be important in the weep and sweep response to intestinal helminth infection, as we once again see alterations to this cytokine.

4.9. *In vitro* restimulation of IELs with GLP-1RA has little effect on the cytokines produced

After looking at cytokines produced by IELs when restimulated with cell stimulation cocktail (1X) and seeing very little change in cytokines produced, we decided to restimulate the cells with added GLP-1rA to determine whether *in vitro* GLP-1rA treatment would alter the cytokines produced.

We first used IELs stripped from the large intestine and caecum of PBS control and lixisenatide treated mice with chronic *T. muris* infection after 4 days or 6 days of *in vivo* treatment.

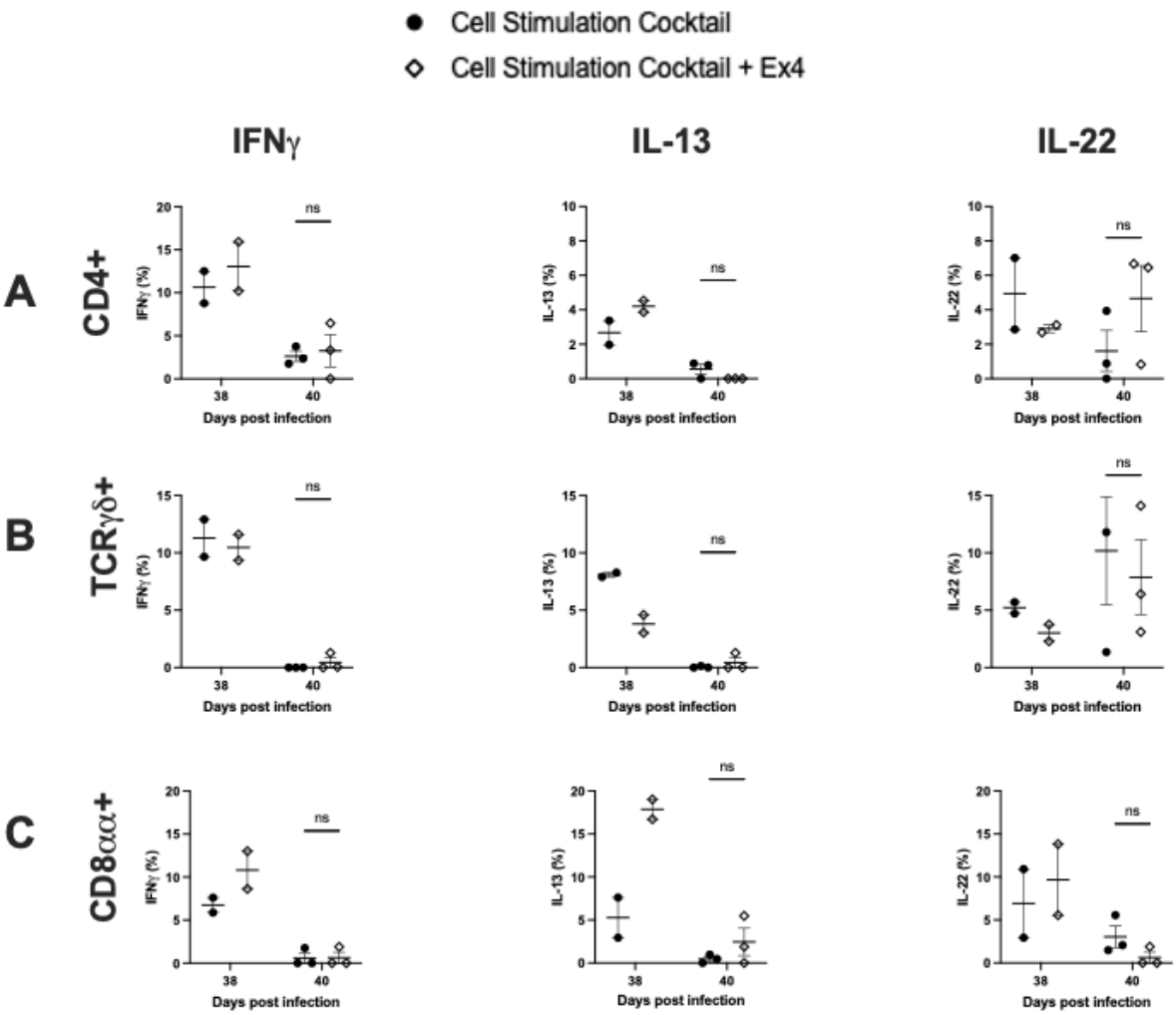

Figure 4.32: Restimulation of intestinal intraepithelial lymphocytes in vitro with GLP-1rA increases levels of IL-13 produced by CD8αα+ and TCRγδ cells. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection was allowed to establish for 34-40 days. Mice were sacrificed after treatment and large intestines were obtained and stripped to obtain intraepithelial lymphocytes (IELs) for analysis. IELs were stimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) and 10 nM exendin-4 overnight and cells were collected and analysed the next day via flow cytometry. Data (n = 2) are from 2 independent experiments and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We saw no difference in the levels of IFNγ or IL-22 produced by TCRγδ+ cells between IELs restimulated with cell stimulation cocktail alone and those restimulated with cell stimulation cocktail and GLP-1rA. In cells from mice 38 days post infection (p.i.), we saw a decrease in production of IL-13 by TCRγδ+ IELs when restimulated with GLP-1rA in

comparison to those restimulated without it. There was no difference in levels of IL-13 in cells from mice 40 days p.i. (Fig. 4.32 A).

We saw no difference in the levels of IFNγ or IL-22 produced by CD8αα+ cells between IELs restimulated with cell stimulation cocktail alone and those restimulated with cell stimulation cocktail and GLP-1rA) at either timepoint. In cells from mice 38 days p.i., we did see an increase in production of IL-13 by CD8αα+ IELs when restimulated with exendin-4 in comparison to those restimulated without it. There was no significant difference in cells from mice 40 days p.i. (Fig. 4.32 B).

We saw no significant difference in the levels of IFNγ, IL-13, or IL-22 produced by CD4+ T-cells between IELs restimulated with cell stimulation cocktail alone and those restimulated with cell stimulation cocktail and GLP-1rA (Fig. 4.32 C).

Collectively, these data indicate an involvement of Th2 cytokine, IL-13, although interestingly we see conflicting changes in IL-13 produced by different IEL subsets.

4.10. Innate lymphoid cell subsets are unaffected by GLP-1RA treatment

RAG-/- mice lack an adaptive immune system, but do possess innate lymphoid cells (ILCs) (Bando and Colonna, 2016). ILCs have been found to be important in other helminth infections (Oliphant et al., 2014; Pelly et al., 2016), although not in *T. muris* (Glover et al., 2019). We used RAG-/- mice to determine whether GLP-1r agonist alters the innate lymphoid cells during *T. muris* infection.

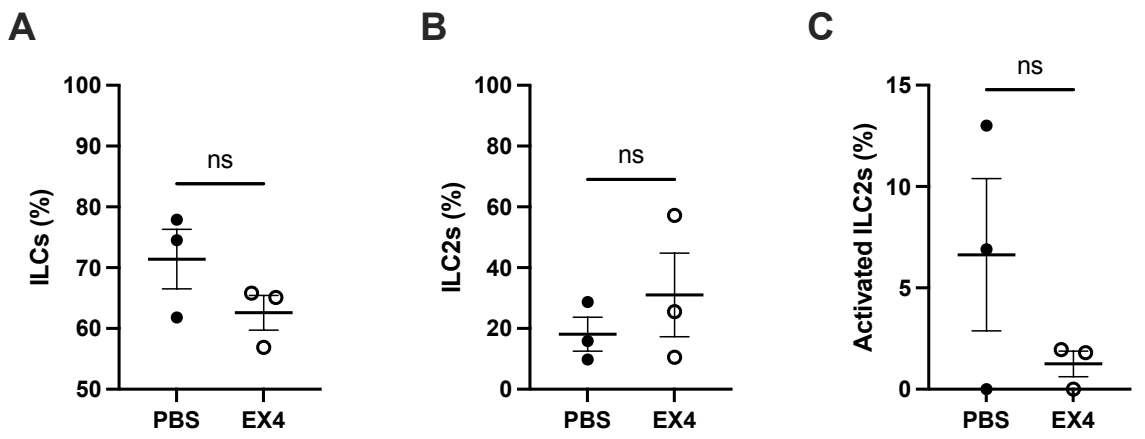

Figure 4.33: Innate lymphoid cells are unaffected by treatment with GLP-1r agonist. RAG-/- mice were infected with a low dose *T. muris* infection at day 0 and treated daily for 34 days with PBS vehicle or Exendin-4. At day 34, mice were sacrificed, and mLN were obtained for analysis. mLN were restimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Plotted data show (A) Percentage of immune cells that are innate lymphoid cells (ILCs), (B) Percentage of ILCs that are ILC2s, and (C) Percentage of ILC2s that are activated. Data (n= 3) are from 1 independent experiment and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We saw no significant difference in the percentage of CD45+ immune cells (Fig. 4.33 A). We also saw no significant difference in the percentage of immune cells that were ILCs (Fig. 4.33 B). We found no significant difference in the percentage of ILCs that were ILC2s, and the percentage of those ILC2s that were activated (Fig. 4.33 C).

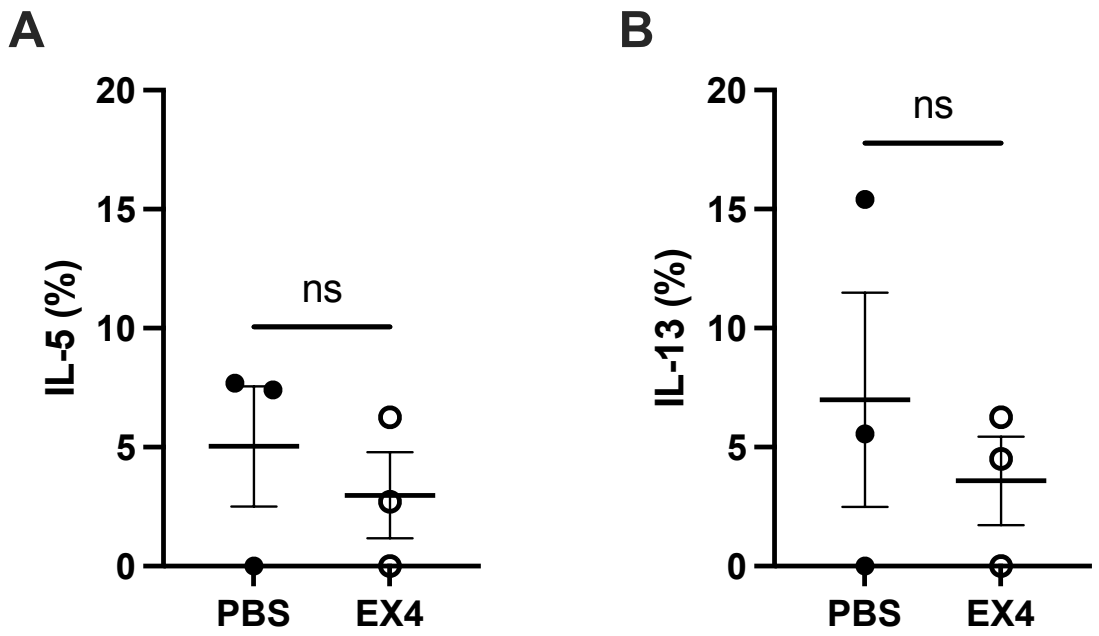

Figure 4.34: Levels of IL-5 and IL-13 produced by ILC2s are unaffected by treatment with GLP-1r agonist, exendin-4. RAG-/- mice were infected with a low dose *T. muris* infection at day 0 and treated daily for 34 days with PBS vehicle or Exendin-4. At day 34, mice were sacrificed, and mLN were obtained for analysis. mLN were restimulated with Cell Stimulation Cocktail (with Protein Transport Inhibitors) overnight and cells were collected and analysed the next day via flow cytometry. Plotted data show (A) Levels of IL-5 from ILC2s, and (B) Levels of IL-13 from ILC2s. Data (n= 3) are from 1 independent experiment and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We saw no significant difference in the levels of IL-5 and IL-13 produced by ILC2s of restimulated mesenteric lymph node cells (Fig 4.34 A-B). There is potentially a downwards trend in the percentage of these two cytokines in the exendin-4 treated mice compared to PBS controls, however this is not statistically significant.

4.11. Cytokines produced by the mLN of RAG-/- mice are unaffected by GLP-1RA treatment

We looked at the cytokines produced by mLN cells of RAG-/- restimulated with either ES antigen or concanavalin A (ConA) using a cytometric bead array analysis to analyse the cytokines produced either in response to the parasite (ES antigen) or in general (ConA).

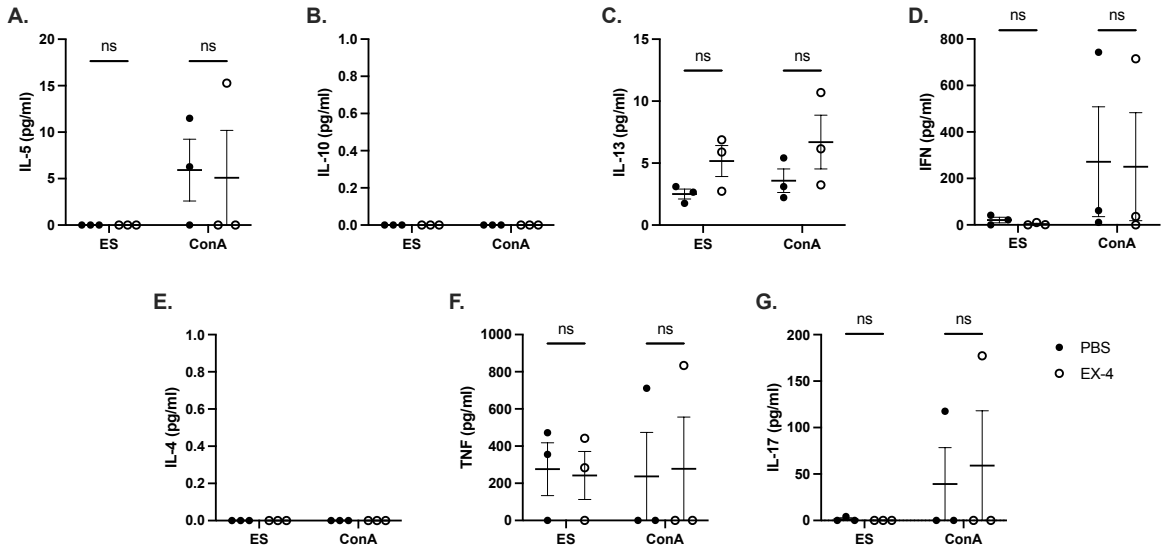

Figure 4.35: Cytokines produced by the mesenteric lymph node of RAG-/- mice are unaffected by GLP-1rA treatment. RAG-/- mice were infected with low dose *T. muris* at day 0 and daily intraperitoneal injection of 2μg exendin-4 in 200μL PBS or 200μL PBS control were administered. Mice were sacrificed after treatment and mLN were obtained for analysis. mLN were stimulated with ES antigen or ConA overnight (16 hours) and supernatant collected and analysed via CBA. Data (n= 3) are from 1 experiment and are presented as mean ± SEM. Each plotted point represents 1 mouse. Statistical analysis was completed by unpaired t-test. * = p<0.05, ** = p<0.01, *** = p<0.001.

We saw no significant difference in levels of IL-5, IL-10, IL-13, IFNγ, IL-4, TNFα, or IL-17 produced by the mesenteric lymph node restimulated with ConA between PBS controls and GLP-1rA treated mice (Fig. 4.35. A-G). We did not detect any IL-10 or IL-4 when stimulated with either ES antigen or ConA (Fig. 4.35. B and E).

Collectively, these data indicate that ILCs do not play a role in GLP-1rA induced *T. muris* expulsion as we see no expansion of these cells and no difference in any cytokines examined.

4.12. GLP-1rA-induced *T. muris* expulsion is not induced by alterations in goblet cells

In order to assess what mechanism was responsible for GLP-1RA induced expulsion of *T. muris* and given increases in Th2 cytokines and IL-22 we next examined established TH2 driven expulsion mechanisms for helminths. Goblet cell hyperplasia and changes in mucins have been identified as key mechanisms of expulsion of *Trichuris muris* (Gerbe et al., 2016; Hasnain et al., 2011b; Hasnain et al., 2010). To determine whether these changes occur and

play a role in lixisenatide-induced worm expulsion, caecal tip sections were stained using alcian blue-PAS staining for goblet cells. Caecal crypt length and membrane thickness were measured, and number of goblet cells counted for 20 randomly selected caecal crypt units.

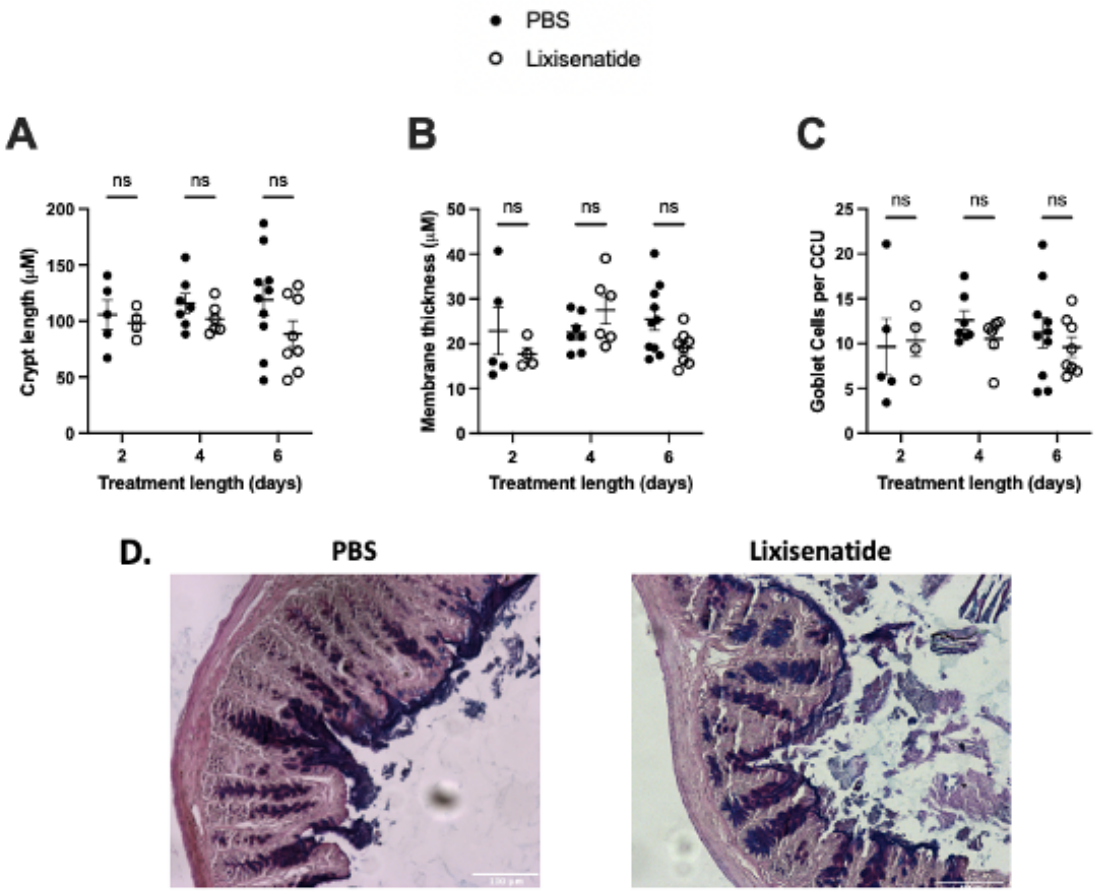

Figure 4.36: GLP-1rA treatment does not affect structure or goblet cells of the caecal crypt. C57BL/6 mice were infected with low dose *T. muris* and infection allowed to establish for 34 days before treatment. Treatment with either PBS control or GLP-1rA lixisenatide began on day 34 p.i. and continued for 2-6 days before sacrifice. Caecal tip samples from infected mice were stained using alcian blue-PAS. Crypt length, membrane thickness, and number of goblet cells per caecal crypt unit (CCU) were analysed using ImageJ. (A-C) Quantification for each mouse was performed by measuring 20 CCUs and determining the mean (A) crypt length (μM), (B) membrane thickness (μM), and (C) number of goblet cells per CCU. Each plotted point represents one mouse. (D) Representative images of alcian blue-PAS-stained caecal tissue sections to visualise crypts and goblet cells in PBS controls and lixisenatide treated mice. Data (n = 4-10) are from 4 independent experiments and are presented as mean ± SEM. Statistical analysis was completed by one way ANOVA. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We saw no significant difference in crypt length between PBS controls and lixisenatide treated mice after 2, 4, or 6 days of treatment (Fig. 4.36 A). We found no significant difference in thickness of the basement membrane between PBS and lixisenatide treated mice after 2, 4, or 6 days of treatment (Fig. 4.36 B). There was no significant difference in the number of goblet cells per caecal crypt unit (CCU) after 2 days, 4 days, or 6 days of lixisenatide treatment in comparison to PBS controls (Fig. 4.36 C).

Despite seeing a significant decrease in worm burden in male mice treated with lixisenatide for 4 days, we saw little difference in female mice treated for as long as 6 days. We decided to increase the dose in female mice and again, treat for 6 days with lixisenatide (Fig. 4.37A-C). I have collated this data with the previous data for mice treated for 6 days, to look at any histological changes in the caecum as the dose of lixisenatide increases.

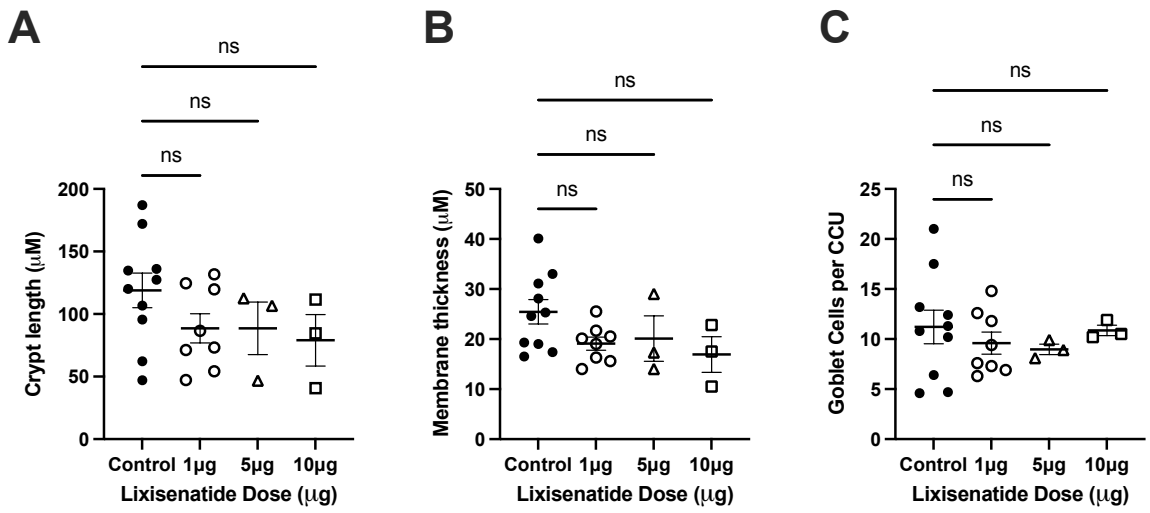

Figure 4.37: An increasing dose of GLP-1rA treatment does not affect structure or goblet cells of the caecal crypt. Female C57BL/6 mice were infected with low dose *T. muris* and infection allowed to establish for 34 days before treatment. Treatment with either PBS control or GLP-1rA lixisenatide began on day 34 p.i. and continued for 6 days before sacrifice. Caecal tip samples from infected mice were stained using alcian blue-PAS. Crypt length, membrane thickness, and number of goblet cells per caecal crypt unit (CCU) were analysed using ImageJ. (A-C) Quantification for each mouse was performed by measuring 20 CCUs and determining the mean (A) crypt length (μM), (B) membrane thickness (μM), and (C) number of goblet cells per CCU. Each plotted point represents one mouse. Data (n = 3-10) are from 4 independent experiments and are presented as mean ± SEM. Statistical analysis was completed by one way ANOVA. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We found no significant difference in the crypt length between any of the groups after 6 days of treatment. Although not statistically significant, we can see a trend in which crypt length decreases as the lixisenatide dose increases (Fig 4.37 A). We found no significant difference in the membrane thickness between any groups after 6 days of treatment. Again, we saw a trend in which the membrane thickness decreases as the lixisenatide dose increases (Fig 4.37 B), although this is statistically insignificant. We found no significant difference in the number of goblet cells per CCU between any groups after 6 days of treatment (Fig. 4.37 C).

Collectively, these data indicate that goblet cell hyperplasia does not play a role in GLP-1rA induced *T. muris* expulsion.

4.13. Tuft cells appear to decrease in response to GLP-1RA treatment

Previous studies found a small but significant increase in tuft cells in the caecum in response to trickle infection with *T. muris* (Glover et al., 2019). We decided to look at whether treatment of C57BL/6 mice with a low dose, chronic infection of *T. muris* with GLP-1r agonist, lixisenatide, altered the number of tuft cells present, as tuft cells have been shown to produce IL-25 and are important mechanisms of expulsion in other intestinal helminth infections (Gerbe et al., 2016; Grencis and Worthington, 2016; Howitt et al., 2016b; von Moltke et al., 2016).

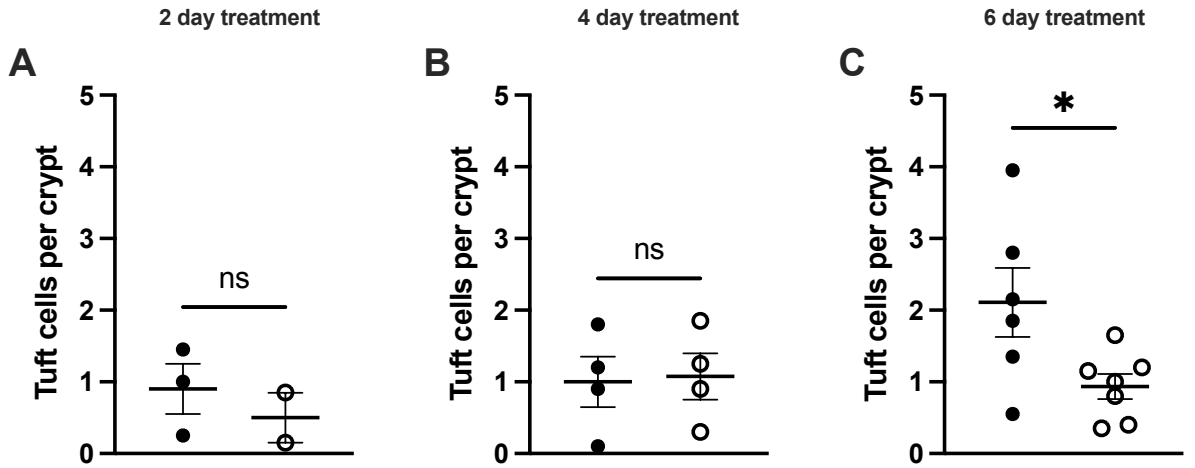

Figure 4.38: Tuft cell numbers decrease in response to GLP-1rA treatment. C57BL/6 mice were infected with low dose *T. muris* and infection allowed to establish for 34 days before treatment. Treatment with either PBS

control or GLP-1rA lixisenatide began on day 34 p.i. and continued for 2-6 days before sacrifice. DCAMKL1 antibody was used to identify tuft cells. Quantification for each mouse was performed by counting tuft cells in 20 CCUs and determining the mean number per crypt. Data (n = 3 - 7) are from 3 independent experiments and are presented as mean ± SEM. Statistical analysis was completed by one way ANOVA. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

We found no significant difference in the number of tuft cells per crypt between PBS control and lixisenatide treated mice after both 2 and 4 days of treatment (Fig. 4.38 A-B). However, we found a significant decrease ($p = 0.0329$) in the number of tuft cells per crypt in lixisenatide treated mice in comparison to PBS controls following 6 days of treatment (Fig 4.38 C).

Collectively this indicates that tuft cells are not required for GLP-1rA induced *T. muris* expulsion as we see no significant increase between control and treated mice.

4.14. Intestinal epithelial turnover increases in response to lixisenatide treatment

Increased epithelial turnover is known to be a key mechanism in expulsion of *T. muris* (Cliffe et al., 2005; Oudhoff et al., 2016a; Zaph et al., 2014). Therefore, we used a bromodeoxyuridine (BrdU) pulse-chase experiment over a 12-hour period to determine the rate of epithelial turnover.

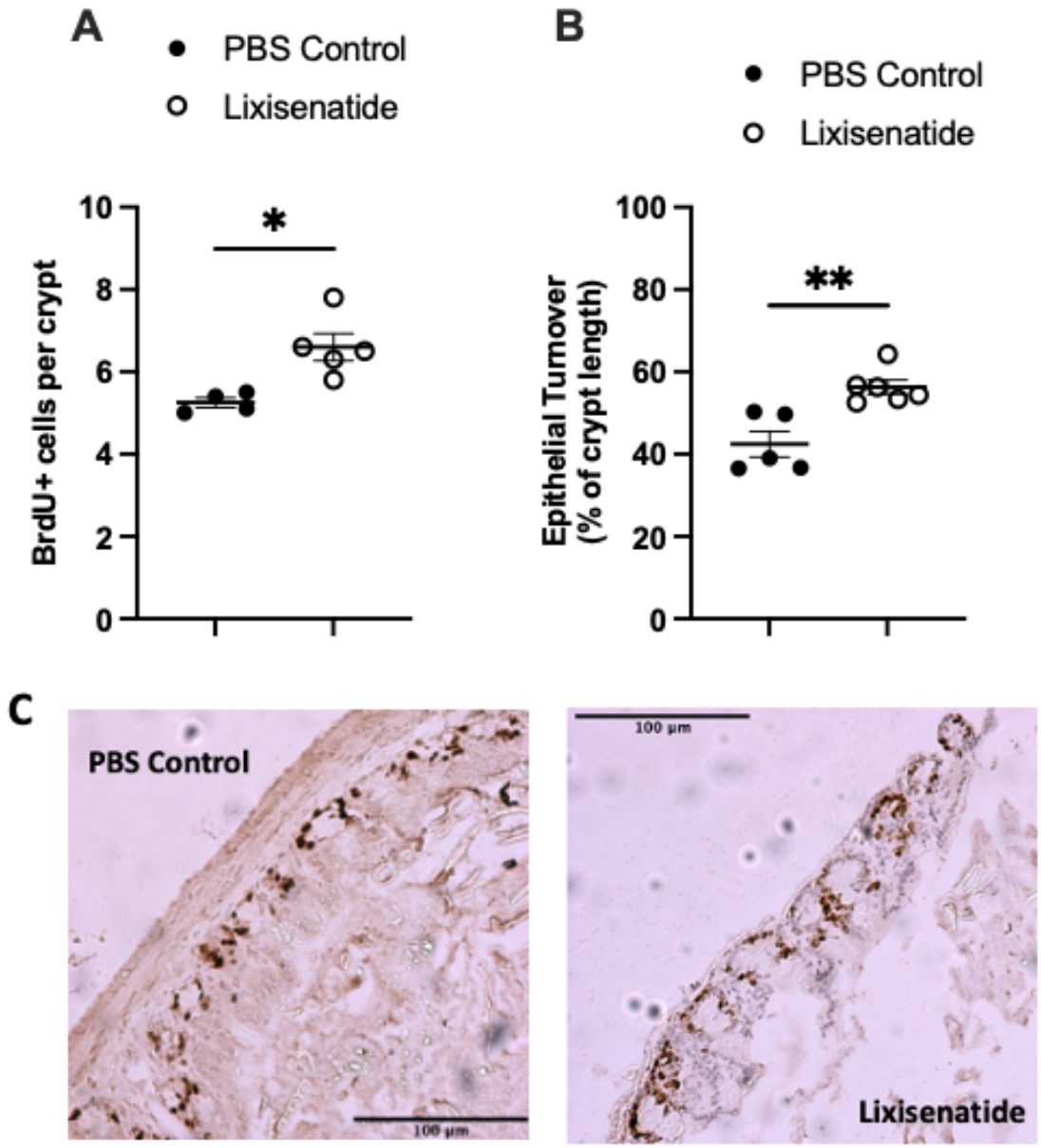

Figure 4.39: Epithelial turnover is significantly increased in lixisenatide treated mice. C57BL/6 mice were infected with low dose *T. muris* at day 0 and infection allowed to establish for 34 days. Daily intraperitoneal injection with either PBS vehicle or lixisenatide began on day 34 and continued for 6 days before mice were sacrificed. BrdU was injected 12 hours before sacrificed. Caecal tip was collected at the point of sacrifice and stained with anti-BrdU and migration distance and number of proliferating cells per crypt were determined under a microscope. (A) Average number of BrdU positive cells per crypt. (B) Rate of epithelial turnover assessed by movement of BrdU+ cells up the crypt. (C) Representative images of BrdU stained caecal tissue of PBS controls and lixisenatide treated mice. Data shown (n = 5-6) are from 4 independent experiments and are presented as mean ± SEM. Statistical analysis was completed by one way ANOVA. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Interestingly, the number of proliferating cells, as measured by positively stained BrdU cells, was significantly increased ($p = 0.0104$) following GLP-1RA treatment (Fig. 4.39 A). We next examined the rate of epithelial turnover and strikingly, this was significantly increased ($p = 0.0029$) in lixisenatide treated mice, with an increase in the migration distance of these cells over the 12-hour period (Fig. 4.39 B).

These data suggest that GLP-1 is modulating the local intestinal immune response through s. currently unknown mechanism to increase epithelial turnover, allowing expulsion of the parasite by forcing it out from its niche.

5. Discussion

Enteroendocrine cells are potent producers of peptide hormones, including GLP-1, and have a role in orchestrating mucosal immunity (Worthington et al., 2018), with recent studies revealing a GLP-1-IEL-GLP-1r axis with potential to modulate enteric immune responses (Yusta et al., 2015). With helminth infection being in such close proximity to these GLP-1r+ IELs, we wanted to investigate the potential of GLP-1r agonists for the treatment of helminth infection. This study is the first to investigate the effect of GLP-1r agonists on helminth infection and has potential to provide a new therapeutic option for such infections, which currently are proving difficult to treat due to increasing resistance to current drugs (Adegnika et al., 2015; Hotez, 2017; Keiser and Utzinger, 2010).

We found GLP-1r agonist treatment to be effective in inducing worm expulsion in male C57BL/6 mice with an established chronic *T. muris* infection. This requires the GLP-1r and the adaptive immune system. We saw minor changes in cytokines produced by lamina propria immune cells and intraepithelial lymphocytes. Interestingly, we saw a significant increase in epithelial turnover, a known mechanism of intestinal parasite expulsion (Cliffe et al., 2005). These data suggest that GLP-1 is modulating the local intestinal immune response through GLP-1r+ IELs to increase epithelial turnover and allow expulsion of *T. muris*. Although we saw some alteration in cytokines produced, further experimentation is required to fully define the mechanisms that GLP-1r agonist treatment is able to expel chronic infection via increased epithelial turnover.

5.1. GLP-1r+ IELs appear in large intestine in response to *T. muris* treatment

Previous research found intraepithelial lymphocytes in the small intestine to possess transcript for the GLP-1 receptor (Yusta et al., 2015). We found within the small intestine that the CD8$\alpha\alpha$ TCR$\gamma\delta$ were the majority expressors of GLP-1r protein. Interestingly these GLP-1r+ IELs appear in the large intestine in response to *T. muris* infection with the receptor not being present at the protein level in naïve animals. Post translational effects may be regulating the expression of the GLP-1r on these cells, as we know that the RNA is present in large intestine cells (Yusta et al., 2015). This also suggests that DSS colitis induces expression of the receptor at protein level since both low dose *T. muris* infection and DSS induced colitis are associated with a Th1 response. GLP-1r+ IELs also increase in response to *Trichinella spiralis* infection (Worthington, unpublished). *T. spiralis* is associated with a Th2 response indicating that a Th2 immune response also induces expression of the GLP-1r. A possible explanation would be that the detection of bacteria or other factors may be inducing expression of the GLP-1r during these infections and intestinal injury. To determine why these GLP-1r+ IELs appear during *T. muris* infection, we could sort IELs from the large intestine of reporter mice and treat them *in vitro* with different factors before analysing the cells for presence of the GLP-1r. Given the presence of these GLP-1r+ IELs that appear in the niche of *T. muris* during infection, it is possible that they have a role in the immune response during infection.

5.2. Short-term lixisenatide treatment clears *T. muris* infection

We found short-term treatment with GLP-1r agonist, lixisenatide, to induce worm expulsion in C57BL/6 mice with a chronic *Trichuris muris* infection. We saw a significant reduction in worm burden in male C57BL/6 mice after treatment with lixisenatide for 4 days in comparison to PBS-treated controls. Unfortunately, we saw no significant differences in worm burden of female C57BL/6 mice with a chronic *T. muris* infection, even when given up to a 6-day treatment with lixisenatide. There appears to be differences in response to GLP-1r agonist treatment dependent on sex of the mice. We found male mice able to expel worms sooner and with a lower dose of lixisenatide than female mice. After 6 days of treatment, there was still no significant difference in worm burden between the lixisenatide treated mice and PBS controls. This is contrary to previous research that found female mice

(AKR and BALB/c) to expel worms when males couldn't, due to a heightened Th2 response in the females (Bancroft et al., 2000; Hepworth et al., 2010). Female BALB/c IL-4-KO mice can expel worms, although later than wildtype controls, while males develop a chronic infection (Bancroft et al., 2000). Notably, these previous observations were in AKR and BALB/c mice, while our studies were carried out on C57BL/6 mice. Yet, similar differences were observed in TNFα-receptor KO mice on a C57BL/6 background, with females clearing infection and males developing a chronic infection (Hayes et al., 2007).

Studies on sex steroid hormones revealed that male-associated dihydrotestosterone can hinder the ability of dendritic cells to activate T cells and may also skew T-cell differentiation towards a Th1 response and hence, allow chronic infection to develop. Furthermore, female-related hormone 17-β-estradiol was found to encourage generation of a Th2 response *in vitro* (Hepworth et al., 2010). Furthermore, the composition of gut microbiota is significantly different between female and male mice, independent of diet (Peng et al., 2020). We know that the gut microbiota is important for hatching of *T. muris* eggs (Hayes et al., 2010) and therefore may lead to differences in establishment of infection between male and female mice. These previous data show that sex can influence the immune response to *T. muris* and subsequent parasite expulsion, providing a potential explanation for the differences in response to treatment observed in these experiments.

By increasing the dose used, we did manage to get the worm burden of female, lixisenatide treated mice to almost zero when treated with 10μg/day lixisenatide for 6 days. However, this was still statistically insignificant in these pilot studies as we only used a small number of mice (3 mice) in each group of increased doses, to determine whether an increase in dose would increase the effect of the drug. These experiments should be repeated to confirm the exciting results seen here. Some of the female control mice in the 6-day treatment experiment managed to clear the infection, despite not receiving GLP-1r agonist treatment. We used a sandwich ELISA to determine whether these mice were ever infected based on their parasite-specific antibody levels. Only one of these mice had never been infected, and was removed from all subsequent analysis, including the worm burden data. However, the other 4 control mice with no worms all showed parasite-specific antibody responses and therefore must have been infected. These experiments should be repeated with a new

batch of eggs. However, establishment experiments did show these to be viable eggs. It is possible that the female mice are simply unresponsive to the treatment and therefore the experiment should be repeated to investigate this further. Previous studies have found GLP-1rA treatment to work better in males than females (Anichini et al., 2013; Vallöf et al., 2020).

We would like to work towards using one single dose of lixisenatide to clear a *T. muris* infection. This may require a higher dose to be used, or a longer time to be left between treatment and sacrifice of the mice. We started our experiments with a low dose of lixisenatide. Baggio et al. (2017) used lixisenatide at 10μg/kg and found this to be an effective dose (Baggio et al., 2017). If we assume the average weight of the mice to be approximately 30g, this indicates that as low as 0.3μg lixisenatide would be an effective dose. We chose to start with 1μg as our treatments were short-term. Other studies have used higher doses of lixisenatide in mice, including Werner et al. (2010) who treated daily with 500μg/kg, which would be around 17μg based on the average weight of a mouse (Werner et al., 2010), so we were confident that our doses (1μg-10μg) were safe and effective for use. We still have room to increase the dose of lixisenatide we are using, and therefore have potential to work towards a one-dose treatment. It may also be interesting to see how lixisenatide works in combination with existing anthelmintic drugs or whether it would work against other intestinal helminth infections. It is possible that this treatment would work specifically against *Trichuris* as an increase in epithelial turnover, as seen in this study, may not be enough to shift other parasites from their differing niches. Given the specific drug resistance of *Trichuris,* this new therapy will still be very beneficial even if it is unable to clear other helminth infections.

5.3. GLP-1rA induced *T. muris* expulsion requires the GLP-1r and the adaptive immune system

Some peptide hormones have been found to be toxic to parasites. Vasoactive intestinal peptide (VIP), urocortin (UCN), adrenomedullin (AM), corticotropin-releasing hormone, ghrelin, and melanocyte-stimulating hormone have been found to act as potent antitrypanosome agents, killing the infective bloodstream forms of *T. brucei brucei* (Delgado

et al., 2009). We decided to treat GLP1r-/- mice every day throughout infection (34 days) to give the treatment every opportunity to kill the worms or to induce expulsion using the readily available GLP-1r agonist, Exendin-4, and found GLP-1rKO mice able to maintain worm burdens whereas treated C57BL/6 wild-type littermate mice did not. This in combination with the failure of Ex-4 treatment to clear worms in RAG-/- mice also strongly suggests that the drug is acting through the GLP-1r and is not directly toxic to the worms. To confirm whether GLP-1rA treatment is toxic to *T. muris,* we would want to perform *in vitro* screening, treating adult worms in a plate to determine whether they are able to survive and produce eggs.

We know that T cells are important in expulsion of *T. muris.* Transfer of T-cell populations, without B-cell populations, from *T. muris* infected donors into naïve recipients was found to transfer immunity to infection into the naïve mice (Lee et al., 1983). Nude mice, which lack T cells, are susceptible to *T. muris* infection, but expulsion of worms can be induced by transfer of splenocytes. Transfer of mesenteric lymph node cells or thymocytes into these mice was also able to partially restore a resistant phenotype (Ito, 1991). Further studies found depletion of CD4+ T cells to result in susceptibility to infection and adoptive transfer of CD4+ T cells into severe combined immunodeficiency (SCID) mice, which lack an adaptive immune system, was able to induce expulsion, proving the importance of CD4+ T cells in protective immunity to *T. muris* (Else and Grencis, 1996). Our RAG-/- mice Ex-4 treatments also indicated that the adaptive immune system is necessary for GLP-1r agonist-induced *T. muris* expulsion which would suggest that GLP-1r+ CD8 cells may be the initial target of treatment.

This project naturally focused on intraepithelial lymphocytes of the intestine that possess the GLP-1 receptor. However, we do know that other cells in the body also possess this receptor. There is an abundance of the GLP-1r in pancreatic β-cells, but the receptor is also widely expressed on cells of the central nervous system, heart, kidneys, and the stomach (Holst, 2007). Since treated RAG-/- mice were unable to clear the infection, we suspect that the adaptive immune system must play a role in GLP-1rA induced worm expulsion. However, with GLP-1 receptors being widely expressed throughout the body, it is possible that exendin-4 treatment may not solely rely on the adaptive immune response, with some

involvement from other cell types to initiate the response in adaptive cells not possessing the GLP-1r. To further investigate this, we could deplete individual populations of T cells to determine their role in GLP-1rA induced *T. muris* expulsion. We could confirm the reliance on GLP-1r+ IELs through adoptive transfer of the GLP-1r+ IELs into RAG-/- mice. Adoptive transfer of IELs could be carried out following previously published protocols, such as by Ostanin et al. (2010). IELs would be isolated and sorted before being injected intraperitoneally into recipient RAG-/-/GLP-1rKO mice and allowed to reconstitute for 2 weeks (Ostanin et al., 2010). We could then treat these mice daily with exendin-4 and compare worm burdens with RAG-/-/GLP-1rKO mice that did not receive the cells. If adoptive transfer of GLP-1r+ IELs can successfully restore GLP-1r agonist-induced worm expulsion, we can conclude that they are necessary for this response. If expulsion was absent, we could transfer CD4+ helper cells in combination in each case measuring epithelial turnover in tandem. SCID mice, lacking B and T cells, have been found to have significantly decreased worm burden when epithelial turnover was elevated by *in vivo* blockade of CXCL10 (Cliffe et al., 2005). Based on this, it is possible that adoptive transfer of just IELs into RAG-/-GLP-1rKO mice may be enough to restore the GLP-1rA induced expulsion seen in C57BL/6 mice.

Treatment with GLP-1rA has been found to promote intestinal growth directly by increasing the number of crypts through an increase in fibroblast growth factor 7 (*Fgf7*). GLP-1rA treatment was also found to induce components of the IGF-1 and ErbB signalling pathways, although these were not essential for intestinal growth (Koehler et al., 2015).
Studies have found serum IGF-1 to be significantly decreased in *Trichuris* infection (Duff et al., 1999; Kurniawan et al., 2020). Anthelmintic treatment was found to increase serum IGF-1, with this potentially being mediated by alterations in insulin levels during *Trichuris* infection (Kurniawan et al., 2020) suggesting multiple potential mechanisms of GLP-1 treatment. However, our RAG-/- data suggests that some aspect of the adaptive immune system is required for GLP-1r agonist induced *T. muris* expulsion.

GLP-1rA treatment is also known to affect the intestinal gut microbiota (Wang et al., 2016; Zhang et al., 2018; Zhao et al., 2018). We know that hatching of *T. muris* eggs requires contact with type 1 bacterial fimbriae and disruption of these bacteria prevents hatching

(Hayes et al., 2010). If GLP-1rA treatment is altering the microbiome, this could prevent hatching of *T. muris* eggs and therefore prevent the infection from establishing within the gut. However, our studies found the worms to be able to establish and a chronic infection to develop. Furthermore, our short-term treatments were carried out on mice with an already established *T. muris* infection (day 34 post infection), allowing us to rule this out as a cause of decreased worm burden in the treated male mice.

5.4. GLP-1rA treatment causes alterations to IL-13 and IL-22

The importance of B cells and antibody in *T. muris* infection has not yet been established. B cell deficient mice (μMT mice) are susceptible to *T. muris* infection and a resistant phenotype can be restored by adoptive transfer of B cells or through administration of IgG from resistant mice (Blackwell and Else, 2001). However, adoptive transfer of CD4+ T cells alone into SCID mice was able to induce expulsion of *T. muris,* proving that expulsion can occur in the absence of B cells (Else and Grencis, 1996). Serum antibodies offer a strong reflection of the type of immune response occurring during *T. muris* infection, with IgG1 and IgG2c being indicative of Th2 and Th1 responses, respectively. We know that a Th2 response is associated with resistance to *T. muris* while a Th1 response is associated with susceptibility and development of a chronic infection (Blackwell and Else, 2002). We found a significant decrease in serum IgG1 levels at the 1/160 dilution in mice treated with lixisenatide treated mice, indicating a very subtle decrease in a Th2 response at this point. Interestingly, these were the mice with significantly decreased worm burdens. However, although serum antibodies can give an indication of the type of immune response mounted, they offer no indication of infection status. Systemic and mucosal immunity operate largely independently, and serum antibody levels can continue to rise long after the worms have been expelled so serum antibodies do not directly indicate the current presence of infection (Blackwell and Else, 2002; Tomasi et al., 1965).

ILCs are known to play important roles in resistance to a number of helminth infections, including *N. brasiliensis* and *H. polygyrus* (Oliphant et al., 2014; Pelly et al., 2016). Previous research has found that ILCs, specifically ILC2s, do not have an important role in response to *T. muris* (Glover et al., 2019). We saw no significant differences in the percentage of ILCs,

ILC2s, or activated ILC2s. We also saw no significant difference in IL-5 or IL-13 produced by the ILC2s. Previous studies saw a decrease in ILCs, and the proportion of ILC2s, during trickle infection with *T. muris* (Glover et al., 2019) indicating that our mechanism is unlikely to involve the ILC arm of immunity.

Previous studies have found tuft cells to increase significantly during other helminth infections, including *N. brasiliensis* and *H. polygyrus* infection. These studies show tuft cells to have an important role during these infections, inducing expansion of ILC2s through secretion of IL-25 (Gerbe et al., 2016; Howitt et al., 2016a). Recently, other studies have found a small but significant increase in tuft cell numbers after trickle infection with *T. muris* (Glover et al., 2019). We saw a small but significant decrease in tuft cell number after 6 days of treatment with lixisenatide. This could be due to treated mice having lower worm burdens, however by this timepoint the female mice had not fully cleared the infection and therefore we would expect any responses involved in expulsion to continue.

Previous studies have proven T cells to be important in the immune response and expulsion of *T. muris.* CD8+ T cells have a role in the Th1 response to *T. muris* within the caecal mucosa but are not required for Th2 driven immunity and depletion of CD8α+ T cells has no significant effect on worm burden. However, depletion of CD8α+ T cells was found to alter the cytokines produced, with a fivefold increase in IL-5 seen in infected AKR mice but not C57BL/6. A significant increase in IFNγ was seen in both AKR and C57BL/6 mice depleted of CD8α T cells (Humphreys et al., 2004). In female mice treated with lixisenatide for 2 days, we observed a small but significant increase in percentage of CD4+ T-cells, but this was not accompanied by a decrease in CD8+ T-cells. In mice treated for 6 days with 10μg lixisenatide, we saw a significant increase in CD4+ T-cells and a significant decrease in CD8+ cytotoxic T-cells of the lamina propria. In previous studies, a depletion of CD8+ T cells was found in induce a significant increase in IFNγ in C57BL/6 mice. In AKR mice, an increase in IL-5 was also seen (Humphreys et al., 2004). We did not see any alterations to cytokines produced, despite seeing a decrease in CD8+ T cells, although this was based on full depletion of CD8 T-cells and not the subtle shift we observed. Previous studies have proven CD4+ T cells to be essential for resistance to *T. muris* infection, with depletion of these cells

resulting in susceptibility and development of a chronic infection (Koyama et al., 1995). Furthermore, adoptive transfer of CD4+ T cells from resistant BALB/c mice into susceptible SCID mice can rescue the immune response and allow expulsion of the worms (Else and Grencis, 1996). Interestingly, we only see this increase in the number of CD4+ T cells in the mesenteric lymph node after 2 days of treatment. By 4-6 days of treatment, there is no significant difference in number of CD4+ cells.

After 2 days of lixisenatide treatment in female mice, we found an increase in IL-22 from CD4+ T-cells. In male mice treated for 4 days with lixisenatide, we see a small increase in IL-22 produced by CD8+ T-cells, but all other cytokines produced remain unchanged by the treatment. Although IL-22 is usually associated with CD4 T-cells, or ILCs, CD8 T cells are capable of producing IL-22 (Kondo et al., 2009; Res et al., 2010). However, the presence of these CD8 T cells capable of producing IL-22 in the gut is not known. Our CD8 population was determined from CD3+ T cells and not distinguished via TCR$\gamma\delta$/TCR$\alpha\beta$, so it is possible that this production of IL-22 could be from infiltrating TCR$\gamma\delta$ cells and this should be further confirmed. IL-22 induces multiple and diverse mechanisms to employ a strong defensive barrier in the intestine. IL-22 has a role in clearance of pathogens that have penetrated the intestinal barrier. It is able to facilitate the production of chemokines and cytokines to mediate innate cell recruitment to the infection site and can also regulate the complement pathway (Muñoz et al., 2015; Sakamoto et al., 2017). IL-22 has been found to directly induce expression of mucin genes in mucosal epithelial cells and increase the number of goblet cells in the intestinal mucosa (Sugimoto et al., 2008). Previous work has found IL-22 to play a role in *T. muris* induced goblet cell hyperplasia, with *Il22-/-* mice exhibiting reduced goblet cell hyperplasia and increased worm burden (Turner et al., 2013). Despite this, we found no significant changes in the number of goblet cells, despite seeing an increase in IL-22.

Peptide hormones have previously been linked to mucus production. Peptide YY, another gut hormone produced by intestinal L cells, has previously been found to increase mucin secretion in rat colon. This same study found GLP-1 to have no effect on mucin release in the colon (Plaisancié et al., 1998). Another study found GLP-1 to significantly reduce both mucus secretion and goblet cell hyperplasia in the lungs of mice. Upregulation of Muc5ac

was inhibited by GLP-1 (Zhu et al., 2015). These data are interesting, as we expect to see goblet cell hyperplasia with upregulation of Muc5ac in mice resistant to *T. muris* (Cliffe and Grencis, 2004).

Previous studies have found Muc5ac to be critical for expulsion of *T. muris* and loss of Muc5ac resulted in susceptibility to infection. Absence of Muc5ac increases the viability of the worms (Hasnain et al., 2011a). While Muc2 plays a role in protection against *T. muris* infection, it is not essential as loss of Muc2 only delayed worm burden by a week, rather than allow a chronic infection to develop (Hasnain et al., 2010). We did not examine the specific mucins present in the gut in this study and therefore any future work should investigate this. Western blotting could be used, following a protocol such as that by Glover et al. (2019), to determine the specific mucins present and determine whether there is an increase in Muc5ac with GLP-1rA treatment.

In female mice treated with 10μg/day lixisenatide for 6 days, we see a significant increase in levels of IL-5. In mice, IL-5 controls eosinophilia, and has been found to play a key role in the response to other helminth infections, such as *N. brasiliensis* (Filbey et al., 2019). However, *T. muris* infected mice treated with anti-IL-5 antibody were found to still be able to expel all worms, indicating that IL-5 may not play an essential role in clearance of a *T. muris* infection (Betts and Else, 1999). However, future work should examine eosinophilia via histology and flow cytometry to rule out their involvement.

After 4 days of treatment, we found a significant increase in IL-13 produced by CD8+ T cells. When restimulated with exendin-4 *in vitro,* we found a significant increase in IL-13 produced by CD8+ T cells of the lamina propria. These data are interesting as IL-13 is usually primarily produced by CD4+ T cells (Bancroft et al., 1998). IL-13 plays a major role in resistance to *T. muris* and mice lacking this cytokine are susceptible to infection (Bancroft et al., 1998). IL-13 promotes maintained mucin sulphation in mice resistant to *T. muris*. Sulphation of mucins is an important component of the response to *T. muris,* with the intestinal crypts of resistant animals containing mostly sulphated mucins while susceptible animals have predominantly sialylated mucins (Hasnain et al., 2017). IL-13 has also been linked to tuft cell hyperplasia in other helminth infections, such as *N. brasiliensis* (von Moltke et al., 2016). However, tuft cell

hyperplasia is not thought to play a role in the immune response to *T. muris* infection (Glover et al., 2019). IL-13 has an important role in increasing intestinal smooth muscle contractility during helminth infection (Zhao et al., 2003). Furthermore, *Il13-/-* mice have decreased crypt length in the steady state gut, indicating a role for IL-13 signalling in regulation of intestinal stem cell renewal (McDermott et al., 2005). Overall, IL-13 appears to have an important role in many effector mechanisms associated with clearance of an intestinal helminth infection.

5.5. GLP-1rA treatment alters IL-13 produced by IELs

IELs can possess either the αβ T cell receptor (TCRαβ) or γδ T cell receptor (TCRγδ). IEL populations can acquire the expression of CD8αα homodimers (Hoytema van Konijnenburg and Mucida, 2017). The role of the CD8αα homodimer on intestinal IELs is poorly defined but it is thought to play a role in promoting cytokine production by IELs (Leishman et al., 2001). Antigen-experienced TCRγδ IELs were found to preferentially express IFNγ while antigen-naïve TCRγδ IELs produce IL-17. TCRαβ+CD8αα- IELs have both cytotoxicity and immune-regulation machinery, suggesting a role in maintenance of the mucosal barrier (Hoytema van Konijnenburg and Mucida, 2017).

We saw an increase in IFNγ produced by TCR$\gamma\delta$+ IELs after 6 days of treatment with 10μg lixisenatide for 6 days. This is interesting because an increase in IFNγ is associated with susceptibility to *T. muris* and development of a chronic infection (Else et al., 1994), but these mice had a worm burden of almost zero. IFNγ has been found to play a role in epithelial hyperproliferation during *T. muris,* but also reduces cell movement up the crypts through induction of CXCL10 from intestinal epithelial cells (Artis et al., 1999; Cliffe et al., 2005). The combination of crypt hyperplasia with slow cell movement up the crypt leads to a larger niche for worms to occupy, thus helping the worms to avoid expulsion (deSchoolmeester et al., 2006).

However, when restimulated with Ex-4, we saw a significant increase in IL-13 from CD8$\alpha\alpha$+ IELs and a significant decrease in IL-13 from TCR$\gamma\delta$ IELs. An increase in IL-13 is associated with resistance to infection and expulsion of worms, so it is interesting to see the different

IEL subsets with altered levels of this cytokine in response to Ex-4. IL-13 plays a major role in resistance to *T. muris* and mice lacking this cytokine are generally susceptible to infection (Bancroft et al., 1998).

IL-13 has an important role in increasing the rate of epithelial turnover, with IL-13-/- mice on a BALB/c background having a rate of turnover resembling that of susceptible AKR mice, rather than the increased epithelial turnover seen in wildtype BALB/c mice that are resistant to high dose *T. muris* infection (Cliffe et al., 2005). Recent studies also found IL-13 signalling to be essential for self-renewal of Lgr5+ intestinal stem cells (Zhu et al., 2019).

5.6. GLP-1rA treatment increases epithelial turnover

We know that increased epithelial turnover is a key mechanism in worm expulsion. Previous studies have found the rate of epithelial cell turnover to increase in both susceptible and resistant mice, however it was almost doubled in the resistant strain (BALB/c) day 14 post infection in comparison to susceptible AKR mice. Crypt hyperplasia has been observed in AKR mice, but not BALB/c. The slow rate of epithelial turnover seen in susceptible mice explains the development of crypt hyperplasia. The combination of these means that adult worms are unable to be pushed across the threshold for expulsion (Cliffe et al., 2005).

We found a significant increase in intestinal epithelial turnover in mice treated with lixisenatide for 6 days compared to PBS control mice. This is interesting, as at this point, the mice had not expelled their worms and we saw no significant difference in worm burden. However, these data indicate that lixisenatide treatment is increasing intestinal epithelial turnover, and given more time, or a higher dose, may be sufficient to induce clearance of the parasite. Although we observed no significant difference in crypt depth, we did see a trend in which the crypt length appears to decrease with lixisenatide treatment. It is possible that the combination of increased epithelial turnover with the decreased crypt length is allowing the worms to be pushed over the threshold for expulsion.

5.7. Importance of the Work

Soil transmitted helminth infection affects over 1.5 billion individuals in the most deprived countries, causing significant diseases and disability (Palmeirim et al., 2018). These infections are considered to be neglected tropical diseases by the World Health Organisation and they greatly impact the quality of life of infected individuals (Hotez, 2017; Hotez et al., 2014). These infections are common in countries with poor sanitation and can cause great morbidity in those with higher worm burdens, especially children (Hotez et al., 2014; Ngwese et al., 2020). Infection can cause stunted growth and cognitive impairment in infected children (Bethony et al., 2006).

Resistance of *T. trichiura* to current anthelminthic drugs is increasing, leading to the urgent need for new therapeutic options for Trichuriasis. With the recent discovery that a group of immune cells at the intestinal barrier, intraepithelial lymphocytes (IELs), possess receptors for the peptide hormone GLP-1 (Yusta et al., 2015), this work investigates the GLP-1-IEL-GLP-1r axis and its potential role in intestinal helminth infection. GLP-1r agonists are already approved for use in humans, allowing a quick and simple repurposing of the drug.

Current treatments are being used as a preventative chemotherapy in the countries most affected. Preventative chemotherapy involves one oral dose of albendazole or mebendazole once or twice yearly (Schulz et al., 2018). For effective treatment of soil transmitted helminth infection, the NHS state that mebendazole should be taken twice daily for 3 days (NHS., 2019). Mebendazole costs £1.34 for one course of treatment (6 doses) for whipworm, hookworm, or roundworm (NICE., 2022). GLP-1rA treatment for diabetes comes in the form of an injectable pen containing 3ml at 6mg/ml. The pens come in a pack of 3 for £117.72. The dose used for treatment of diabetes is 0.6-1.8mg/day. If we were to use the lower dose of 0.6mg/day, a 3 pack of pens would provide 90 doses, and therefore each dose would cost £1.308. This would mean a 4-dose course of treatment would cost £5.23, so approximately 5x the cost of current treatments (NICE., 2021b).

We would also like to determine whether this treatment would induce any immunological memory, allowing future infections to be cleared without the need for repeated treatment. To investigate this, we would infect C57BL/6 mice with a low dose (30 eggs) infection and

allow this to establish for 34 days before beginning treatment, as we did in our original experiments. We would then treat the mice and re-infect with another low dose infection. Infection would be allowed to establish before the mice would be sacrificed and worm burden determined.

5.8. Conclusions

We hypothesise that GLP-1rA treatment might have a direct effect on CD8$\alpha\alpha$ TCR$\gamma\delta$ IEL immune cells with the GLP-1r to alter some currently unknown immune factors to increase the rate of epithelial turnover in the intestine. We saw alterations to IL-13, a cytokine known to increase the rate of epithelial turnover, produced by intraepithelial lymphocytes and immune cells of the lamina propria, indicating a potential role for this cytokine in GLP-1rA induced *T. muris* expulsion.

Our findings indicate GLP-1r agonist treatment to have exciting potential as a new therapeutic for helminth infection, *Trichuris trichiura.* We know that GLP-1r agonists are safe for use in human because they are already approved for treatment of type 2 diabetes mellitus. Since the drugs are already approved for human use, we could simply repurpose them which would allow their use in helminth infection treatment to begin much quicker. With resistance of *T. trichiura* quickly increasing to current anthelmintics, we would want to get any new therapeutics into use as soon as possible. This treatment would greatly improve the quality of life of many children in developing countries.

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