Evaluating type 2 immune responses during gastrointestinal nematode infection using a STAT6 inhibitor

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ABSTRACT (English)

Approximately 20% of the world's population suffer from allergic airways diseases such as asthma. Present treatments, such as inhaled corticosteroids and β-agonists reduce symptoms; however, they are not curative. In addition, of those who suffer from severe asthma, present treatments do not alleviate symptoms and exacerbations continue to cause morbidity. Specifically, allergic airways disease is primarily mediated by a maladaptive Type 2 inflammatory condition that induces dyspnea, wheezing and intermittent airway obstruction. Therefore, therapeutics that target the underlying immunologic mechanisms of disease are of great clinical interest.

Abundant data from both murine models and human studies suggest that a key transcription factor involved in mediating allergen-induced Type 2 immune responses is signal transducer and activator of transcription 6 (STAT6). In allergy, STAT6 is responsible for mediating production of the canonical Type 2 cytokines (IL-4, -13, -5, -9), mucus production, smooth muscle contraction and IgE production.

STAT6 inhibitory peptide (STAT6-IP) is a cell-penetrating peptide, capable of inhibiting T helper 2-biased airwary inflammation in ovalbumin/ragweed allergy models and in respiratory synctitial virus infection models. In these models, intranasal STAT6-IP delivery potently reduces allergic inflammatory responses, including eosinophil levels in the bronchoalveolar lavage fluid and mucus production in airway epithelial cells. To date, it is not known whether STAT6-IP modulates protective Type 2 immune responses.

The overall goal of this M.Sc. was to investigate the potential of STAT6-IP to modulate protective Type 2 immune responses during helminth infection. *Heligmsomoides polygyrus bakeri* (*Hpb*) was used as the model helminth due to the requirement for effective Type 2 immunity in both primary and challenge infection.

In order to define potential modulation of Type 2 responses during helminth infection by STAT6-IP, STAT6-dependent immune responses were first assessed in STAT6-deficient mice. Our results indicate that STAT6 was required to reduce adult worm and egg burden formation of intestinal granulomas, and production of *Hpb*-adult worm specific IgG.

STAT6-IP was first tested in primary *Hpb* infection, which is characterized by a chronic infection and inefficient Type 2 responses. Our data suggest that neither intranasal nor intraperitoneal delivery of STAT6-IP at the time of initial infection was sufficient to modulate infection outcomes such as antibody responses and granuloma formation. However, three-day intranasal delivery of STAT6-IP resulted in reduced adult worm burden and fewer eggs per gram of intestinal feces. Moreover, upon repeated intraperitoneal delivery of STAT6-IP (nine doses over the course of 13 days), no detectable differences were obtained in most outcomes compared to PBS-treated animals, although adult-worm specific IgG1 was reduced at early times after primary infection. Altogether, our findings suggest that STAT6-IP, given as a short-term immunomodulator in allergic airways disease, is unlikely to alter protective Type 2 immunity in the gastrointestinal tract.

We also investigated whether STAT6-IP delivery at the time of primary *Hpb* infection altered the course of *Hpb* challenge infection, which is curative, due to the Th2-dependent induction of alternatively activated macrophages, which mediate larval killing within the granuloma. Our data demonstrate that three-dose delivery of STAT6-IP, via either the intraperitoneal or intranasal route, had no effect on worm expulsion and granuloma formation during challenge infection. However, three-dose intraperitoneal delivery of STAT6-IP-Arg9 reduced adult-worm specific IgG1 responses, suggesting that STAT6-IP may modulate humoural immunity.

Based on this work, STAT6-IP has the potential to act as a novel therapy for allergic lung diseases without potential side effects to protective Type 2 immune responses in the gastrointestinal tract.

ABSTRACT (French)

Environ 20% de la population mondiale souffre de maladies allergiques pulmonaires comme l'asthme. Les traitements actuels, tels que les corticostéroïdes inhalés et les β-agonistes, réduisent les symptômes; Cependant, ils ne sont pas curatifs. De plus, ces derniers ne soulagent pas les symptômes chez les patients souffrant d'asthme sévère et les exacerbations continuent de provoquer une morbidité. Parmi les facteurs génétiques et environnementaux inconnus, la maladie allergique des voies respiratoires est principalement une maladie inflammatoire de type 2 qui induit une dyspnée, une respiration sifflante et une obstruction intermittente des voies aériennes. Par conséquent, les thérapies visant les mécanismes immunologiques sous-jacents de la maladie sont d'un grand intérêt clinique.

Des données abondantes à la fois de modèles murins et d'études humaines suggèrent qu'un facteur de transcription clé impliqué dans la médiation des réponses immunitaires de type 2 induites par un allergène est le transducteur de signal et l'activateur de la transcription 6 (STAT6). En allergie, STAT6 est le médiateur de la production des cytokines canoniques Th2 (IL-4, -13, -5, -9), de la production de mucus, de la contraction des muscles lisses et de la production d'IgE.

Le peptide inhibiteur de STAT6 (STAT6-IP) est un peptide pénétrant dans les cellules, capable d'inhiber l'inflammation T-auxiliaire de type 2 des voies respiratoires dans les modèles d'allergie à l'ovalbumine / herbe à poux et dans les modèles d'infection du virus syncitial respiratoire. Dans ces modèles, la transmission intranasale de STAT6-IP réduit potentiellement les réponses inflammatoires allergiques, y compris les niveaux d'éosinophiles dans le liquide de lavage broncho-alvéolaire et la production de mucus dans les cellules épithéliales des voies respiratoires. À ce jour, le potentiel de modulation des réponses immunitaires protectrices de type 2 par STAT6-IP est encore inconnu.

L'objectif général de cette M.Sc. était d'étudier le potentiel de STAT6-IP pour moduler les réponses immunitaires protectrices de type 2 pendant l'infection helminthique. Heligmsomoides polygyrus bakeri (Hpb) est utilisé comme modèle d'helminthes en raison de l'importance d'une immunité efficace de type 2 à la fois contre l'infection primaire et secondaire de ce parasite.

Afin de définir la modulation potentielle des réponses de type 2 lors de l'infection helminthique par STAT6-IP, les réponses immunitaires dépendantes de STAT6 ont d'abord été évaluées chez des souris déficientes en STAT6. Nos résultats indiquent que STAT6 était nécessaire à la réduction de la charge parasitaire d'œufs et de vers adultes, la formation de granulomes intestinaux, et la production d'IgG1 spécifiques aux vers adultes d'Hpb.

STAT6-IP a d'abord été évalué dans l'infection d'Hpb primaire, caractérisée par une infection chronique et des réponses inefficaces de type 2. Nos données suggèrent que la délivrance intranasale et intrapéritonéale de STAT6-IP au moment de l'infection primaire était insuffisante pour réduire les réponses aux anticorps et la formation de granulomes suite à l'infection. Cependant, la livraison intranasale en trois jours de STAT6-IP a entraîné une réduction de la charge parasitaire de vers adultes et une diminution d'œufs par gramme de selles intestinales. De plus, après une administration intrapéritonéale répétée de STAT6-IP (neuf doses au cours de 13 jours), aucune différence détectable n'a été obtenue dans la plupart des résultats, bien que la quantité d'IgG1 spécifique au ver adulte ait été réduite au début de l'infection primaire. Dans l'ensemble, nos résultats suggèrent que STAT6-IP, administré en tant qu'immunomodulateur à court terme dans la maladie des voies respiratoires allergiques ne devrait pas modifier l'immunité protectrice de type 2 dans le tractus gastro-intestinal.

Nous avons également voulu déterminer si la délivrance de STAT6-IP au moment de l'infection primaire d'Hpb peut affecter le développement de l'infection secondaire d'Hpb. Cette

dernière est curative en raison de l'induction de macrophages alternativement activés qui sont responsables de la destruction des larves dans le granulome. Nos données démontrent que l'administration de trois doses de STAT6-IP, soit par voie intrapéritonéale, soit par voie intranasale n'a aucun effet sur l'expulsion des vers et la formation de granulomes lors de l'infection secondaire. Cependant, l'administration intrapéritonéale de trois doses de STAT6-IP-Arg9 a réduit les réponses d'IgG1 spécifiques aux vers d'adulte, ce qui suggère que STAT6-IP peut moduler l'immunité humorale.

Sur la base de ce travail, STAT6-IP a le potentiel d'agir comme une nouvelle thérapie pour les maladies pulmonaires allergiques sans effets secondaires potentiels sur les réponses immunitaires protectrices de type 2 dans le tractus gastro-intestinal.

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Although this thesis only has one name written on it, it would be a crime not to give credit where credit is due. I have been thinking about how I was going to write this section for a while because I wanted to find the right words to thank those who have helped me in the last two years.

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CONTRIBUTION OF CANDIDATE

Experimental conception, execution and design were performed by the candidate with guidance from co-supervisors, including review of the thesis. Throughout the work reported in the thesis, a number of mouse models and assays were established and performed in the laboratory by the author: *Hpb* and STAT6-IP delivery time course, *Hpb*-specific ELISAs and immunofluorescence for *Hpb* granulomas. Propagation of the *Hpb* lifecycle at the RI-MUHC was established with the kind help of the Dr. Irah King and Dr. Mary Stevenson and laboratories, both from McGill University.

Chapters/articles, co-author contribution to knowledge

The writing of the chapters was performed by the author, with significant editorial contributions from both co-supervisors. The contribution of co-authors is described below.

Chapter 1: The role of a novel STAT6 inhibitor in protective and maladaptive Type 2 immune responses

Alexia I De Simone, Brian J Ward and Elizabeth D Fixman

Author contributions: AIDS conceptualized and wrote the manuscript. EDF and BJW provided scientific direction and editorial guidance.

Chapter 2: STAT6 signalling modulated Type 2 immune responses during gastrointestinal nematode infection

Alexia I De Simone, Annie Beauchamp, Elizabeth D Fixman and Brian J Ward

Author contributions: AIDS, EDF and BJW designed experiments and conceptualized the manuscript. AIDS performed the experiments and wrote the manuscript. AB helped with infections and tissue harvesting and counted granulomas. EDF and BJW provided support, scientific direction and significant editorial guidance.

Chapter 3: Evaluating Type 2 Immunity during primary *Heligmosomoides polygyrus* infection using a STAT6 inhibitor

Alexia I De Simone, Annie Beauchamp, Brian J Ward and Elizabeth D Fixman

Author contributions: AIDS, EDF and BJW designed experiments and conceptualized the manuscript. AIDS performed the experiments and wrote the manuscript. AB helped with infections and tissue harvesting and counted granulomas. EDF and BJW provided support, scientific direction and editorial guidance.

Chapter 4: STAT6 inhibitory peptide administered at the time of primary *Heligmosomoides polygyrus bakeri* infection did not modulate Type 2 immunity during secondary infection

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Author contributions: AIDS, BJW and EDF designed experiments and conceptualized the manuscript. AIDS performed experiments and wrote the manuscript. AB performed granuloma counts. KR assisted in flow cytometric acquisition and analysis. SC provided the methodology for immunofluorescence. LC prepared intestinal tissue sections and performed histological staining. EDF and BJW provided support, scientific direction and editorial guidance.

LIST OF ABBREVIATIONS

AAM- Alternatively Activated Macrophage

Arg9- Arginine 9

BAL- Bronchoalveolar lavage fluid

CPP- cell-penetrating peptide

DC- Dendritic cell

GATA3- Transcription factor GATA 3

FI-RSV- formalin-inactivated Respiratory Synctitial Virus

H&E- Hematoxylin and Eosin

Hpb- Heligmosomoides polygyrus bakeri

IFN- Interferon

IgG- Immunoglobulin G

IgG1- Immunoglobulin G1

IgG2a- Immunoglobulin G2a

IgE- Immunoglobulin E

IL- Interleukin

IL-4Rα- IL-4 receptor alpha

IN- Intranasal

IP- Intraperitoneal

OVA- Ovalbumin

PBS- Phosphate buffered saline

PTD- protein transduction domain

RSV- Respiratory Synctitial Virus

Sal- Saline

STAT6- Signal Tranduscer and Activator of Transcription 6

STAT6-CP (or CP)- STAT6 control peptide

STAT6-IP (or IP)- STAT6 inhibitory peptide

STH- Soil transmitted helminth

Th2- T helper 2

TSLP- Thymic Stromal Lymphopoietin

INTRODUCTION

Allergic airways disease currently affects 250-300 million indivuals worldwide. To date, treatments such as inhaled corticosteroids and β-agonists primarily treat the symptoms; however, they do not address the underlying immunologic abnormalities associated with the disease and are insufficient to reduce morbidity in severe asthmatics. Therefore, developing therapeutics that target specific mechanisms contributing to disease manifestation and progression are of interest.

The first segment of this thesis is dedicated to a literature review. An overview of the clinical impact of allergic lung disease and asthma is provided, with a focus on epidemiology and present treatments. In addition, a brief summary is given on the novel biologics currently licensed and in clinical trials. Afterwards, a detailed description is provided for the basis of Type 2 immunity in mediating allergic airways diseases, in particular, the role of STAT6 in disease and immunopathology. In addition, this review introduces findings of a novel STAT6 inhibitor (STAT6-IP) capable of reducing Type 2-biased airway inflammation. The last portion of this segment is ascribed to the potential of STAT6-IP to modulate protective type 2 immune responses at a distal site, in this case, to enteric nematode infection. Therefore, Type 2 immunity in resistance and suscpetibility to helminth infection is summarized in this review.

The second chapter is a brief article summarizing studies on our helminth model of choice, *Heligmosomoides polygyrus bakeri (Hpb)*, in STAT6 deficient mice. The goal of this chapter is to elucidate a potential phenotype in STAT6$^{-/-}$ mice that may be compared to animals treated with STAT6-IP.

The third chapter describes the maintenance of Type 2 immune responses during primary *Hpb* infection following a three-dose intranasal delivery of STAT6-IP. We also study how a novel

route of STAT6-IP administration, in this case an intraperitoneal route, does not alter disease manifestations. Several mechanisms for this lack of responses are discussed.

The fourth chapter of this thesis focuses on the role of STAT6-IP in modulating secondary *Hpb* infection in which STAT6 is required to induce memory T helper 2 cells to promote alternatively activated macrophages, which kill *Hpb* larvae. Our findings support that three dose delivery of STAT6-IP at the time of primary infection does not alter host protection to challenge infection. Potential modulation of humoural immunity by STAT6-IP is discussed.

Immunomodulatory therapy is a promising avenue to alter maladaptive responses that result in allergic airways diseases. STAT6-IP is a potential candidate for immunomodulatory therapy for diseases such as asthma. In this thesis, we build on the existing literature that STAT6-IP reduces Type 2-driven immunopathology to investigate the effects of this inhibitory peptide in modulating infections where Type 2 immunity mediates protection. In this case, we chose to test the effects of STAT6-IP in modulating a gastrointestinal nematode infection that may be studied as a chronic (primary infection) or acute (secondary infection) infection model.

CHAPTER 1

Literature Review: The role of a novel STAT6 inhibitor in protective and maladaptive Type 2 immune responses

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LR 1. Clinical impact of allergic airways disease and asthma

1.1. Definition and Epidemiology

Chronic allergic diseases of the airways are defined by the presence of intermittent airway obstruction, dyspnea and mucus production (1). Such diseases, including asthma, hypersensitivity pneumonitis asthma and chronic eosinophilic pneumonia, are primarily associated with T helper 2 (Th2) inflammation (1, 2). Two-hundred to three-hundred million children and adults are burdened by allergic airways disease worldwide (3-6). According to the World Health Organization, asthma affects over 10% of North Americans and between 5-7% of Europeans (5, 7). Chronic allergic diseases contribute to a large proportion of healthcare spending: in the United States alone, $5322 per person is spent on direct and indirect medical costs while $2010 per person is spent each year on medication expenditures (8). For unknown reasons, the prevalence of wheezing, allergic rhinitis and severe asthma in children of low socioeconomic status is increasing each year, both in developing and industrialized countries (9, 10).

1.2. Current treatments for allergy and asthma

Presently, asthmatics may be treated with inhaled corticosteroids and long acting β-agonists, both of which target the symptoms of asthma: corticosteroids reduce inflammation, and β-agonists, which mimic the effects of epinephrine and norepinephrine, induce bronchodilation and inhibit the release of histamine by mast cells (11, 12). While most symptoms are well controlled with these medications, they are not allergen-specific, and do not target the underlying immunologic abnormalities associated with Th2 inflammation (10). In addition, a small cohort of asthmatics diagnosed with severe asthma require both high-dose inhaled corticosteroids and a second drug such as a systemic corticosteroid to treat symptoms (13). As a result, hospitalizations and emergency room visits of this group accounts for fifty percent of healthcare spending

associated with asthma (14).

The development of novel therapeutic approaches for asthma has been challenging due, in part, to the complex immunological mechanisms involved in disease development as well as diverse environmental triggers and unknown genetic factors that all contribute to disease pathogenesis (4, 10). Current therapeutic goals target the recruitment, expansion and maintenance of myeloid and lymphoid cell populations in diseased-tissues (15). Several novel therapeutics being investigated in clinical trials act as antagonists that block the Th2 maladaptive component of allergic inflammation. These include antisense oligonucleotides, microRNAs and humanized monoclonal antibodies (mAb) that block Th2-associated receptor signalling (16). For instance, biologics that block receptors (IL-4Rα, IL-13Rα), cytokines (IL-13, IL-5) or transcription factors (GATA binding protein 3 transcription factor [GATA3]) have all been successful at reducing immunopathology associated with asthma (6, 17, 18). These biologics are effective since they can block both the initiation of allergic responses and the positive feedback loops that mediate allergic inflammation (19). For instance, mepolizumab, an anti-IL-5 biologic, is currently approved for treatment of eosinophilic disorders in asthma by blocking the interaction of IL-5 with its cognate receptor, IL-5Rα (20). In addition, omalizumad, a recombinant humanized anti-IgE monoclonal antibody is currently marketed to treat moderate to severe persistent allergic asthma (21). In a phase-III double-blind, placebo controlled trial, omalizumab resulted in fewer asthma exacerbations and reductions in both β-agonist and inhaled corticosteroid use among pediatric asthmatics (14, 21). Therefore, the use of novel biologics alone or in combination with traditional medications may be the future for allergic airways disease management.

Several mAbs aimed at inhibiting 'early' mediators of allergic inflammation are also under investigation. For example, anti-thymic stromal lymphopoietin (TSLP) mAb, AMG 157, reduced

blood and sputum eosinophils as well as allergen-induced bronchoconstriction in patients with mild asthma (18, 22). In addition, CNTO 7160, an IL-33-specific mAb, is also being assessed in a phase I clinical trial. Altogether, the next generation of allergy therapeutics target immune mediators that induce early events in allergic inflammation.

1.3. Asthma prevalence in areas of the world endemic for helminth infection

Western lifestyles have been associated with increased prevalence of asthma and allergy (5, 23). For instance, in the United States alone, the prevalence of childhood asthma has increased by 38% between 1980-2003 (24). Among many unknown factors, the growing prevalence of asthma has been associated with indoor lifestyles, changes in diet, overuse of antibiotics, increases in obesity and overall improved sanitation (25). While the 'hygiene hypothesis' correlates increased cleanliness and urban lifestyles with greater rates of asthma, it does not explain all of the epidemiological evidence associated with improved hygiene and asthma morbidity. For instance, recent population-based studies suggest that rates of childhood asthma have begun to decline although household sanitation conditions and family sizes remain unchanged (26, 27). In addition, the highest prevalence of severe asthma is no longer in high income countries, but among children from middle and low-income countries (28). In accordance with increased sanitation, the hygiene hypothesis has also theorized that the absence of exposure to infectious organisms, including helminths, may contribute to rising rates of allergic disease worldwide (29). However, Latin American countries, which have higher incidences of microbial infection than European countries, report large incidences of asthma, contradictory to this expanded version of the hygiene hypothesis (30). Ultimately, the hygiene hypothesis does not encompass all the complexities associated with asthma and allergy and as a result, elucidation of molecular mechanisms that contribute to allergen-induced airway diseases remain of great interest.

LR 2. Type 2 immune responses mediate both allergic lung disease and protective immunity to helminths

2.1. The basis of Type 2 immune responses in allergy

The paradigm of T helper (Th) cell differentiation conveys a fundamental concept of adaptive immunity whereby a given naïve T cell, upon presentation of its cognate antigen, and in the presence of cytokines and co-stimulatory signals, becomes activated and differentiates into an effector T cell, whose ultimate phenotype is guided by the inflammatory milieu and co-stimulatory signals (31). Under conditions that favor allergic-type responses (e.g. the presence of TSLP or IL-33, induction of OX40L on antigen presenting cells, etc.), presentation of allergen-derived peptides by major histocompatibility complex II (MHC-II) on dendritic cells (DCs) results in differentiation of Th2 cells, which produce interleukin (IL)- 4, IL-5, IL-13, and IL-9 (31). IL-4 acts as an autocrine signal by binding the IL-4Rα in association with the common γ chain, thereby stimulating antigen-specific Th2 cell proliferation (19, 31). IL-4 and IL-13 also initiate allergen-specific B cell responses that produce Immunoglobulin (Ig) G1 opsonizing antibodies and mast-cell sensitizing IgE antibodies (31). Release of IL-5 by Th2 cells results in recruitment and activation of eosinophils that degranulate and promote Th2 responses (31). During allergic inflammation, IL-9 also stimulates the proliferation of T cells, induces mast cell differentiation and promotes IgE production by B cells (32). Basophils have also been shown to contribute to asthma pathogenesis upon activation of IgE and subsequent release of histamine (33). Innate lymphoid cells (ILCs)-2 have recently been identified as novel producers of IL-9, IL-5 and IL-13 and have been implicated in early events in allergic inflammation (32). Altogether, the concerted actions of the innate and adaptive effector cells mentioned above comprise Type 2 immunity.

Upon repeated exposure to an allergen, maladaptive Type 2 immune responses arise: mast

cells release histamine, eosinophils de-granulate, mucus production increases, and collagen deposition occurs in the lungs (34). The result of this maladaptive response mediated largely by innate effector cell types such as macrophages, eosinophils, basophils and mast cells results in bronchoconstriction, vasodilation, fibrosis, and airway remodeling of the lungs (34). Aberrant activation of Type 2 immunity is considered to be the main underlying pathological basis of airway inflammation, wheezing, and breathlessness (35).

2.2. Type 2 immunity in helminth infection

While Type 2 immune responses result in immunopathology in asthma and allergy, Type 2 mediated inflammation promotes expulsion and tolerance in helminth infection (15). Specifically, type 2-mediated helminth expulsion requires coordinated interactions between leukocytes, granulocytes and physiologic processes. While the exact mechanism is still debated, tissue injury by helminths and necrosis of epithelial cells induces release of innate cytokines such as TSLP and IL-33 (36-38). These innate cytokines induce activation of basophils that become early sources of IL-4 and ILC2s that produce large quantities of IL-5 and IL-13 needed to promote Th2 differentiation (39, 40). Th2 cells participate in clearing helminth infection via release of Th2 cytokines such as IL-4 and IL-13 that recruit effector cells such as B cells, alternatively activated macrophages (AAMs), and neutrophils to the site of infection (19). Parasite-specific IgE bound to mast cells induce release of histamine in the presence of helminths in the lung or other infected tissues (41). Other Type 2-mediated responses to helminth infection include mucus production by goblet cells as well as smooth muscle contraction in the gut (19, 29). Finally, collagen deposition, release of amphiregulin and tissue reconstruction promote wound healing (42). Ultimately, the coordinated actions of these cells/tissues typically result in either clearance of helminths or immune tolerance in the gut and lung (19, 43).

2.3. The role of STAT6 in Type 2 immune responses

Abundant data from both murine models and human studies indicate that a key transcription factor involved in mediating both allergen-induced immune responses and protection from helminth infection is signal transducer and activator of transcription 6 (STAT6) (19, 44). STAT6 is a regulator of many transcription factors required for Type 2 immunity and promotes the expression of Type 2 cytokines, including IL-4, IL-5, and IL-13 in $CD4^+$ T cells (19). In turn, STAT6 is activated by IL-4 and IL-13. Specifically, binding of IL-4 or IL-13 to their cognate receptors (each of which includes the IL-4Rα subunit) induces activation of the receptor-associated Janus family tyrosine kinases, which phosphorylates residues on the IL-4Rα subunit, leading to recruitment and subsequent phosphorylation of STAT6 (19). Phosphorylated STAT6 monomers homo-dimerize and translocate to the nucleus where they initiate transcription of numerous genes in a variety of cells including intestinal and alveolar epithelial cells, B cells, macrophages and smooth muscle cells (19). For example, STAT6-induces expression of mucin genes *muc5ac* and *gob5a,* which in turn produce mucus from goblet cells. STAT6 also induces IgE class-switching in B cells leading to production of IgE (19, 34). While these responses are beneficial for helminth expulsion, they are problematic in allergic diseases (19).

Based on the role that STAT6 plays in mediating successful host responses to helminths, it is not surprising that STAT6 also triggers aberrant Type 2 responses during allergic lung disease. STAT6-deficient animals have decreased IL-4 and IL-13 levels in the lung and regional lymph nodes, are unable to produce IgE and are largely protected from the induction of acute and chronic asthmas (19, 44, 45). Moreover, allergen-induced pulmonary eosinophilia, airway hyperresponsiveness and mucus production cannot be induced by simple adoptive transfer of differentiated Th2 $CD4^+$ cells into $STAT6^{-/-}$ hosts, providing evidence that Th2 cells alone are not

sufficient to drive allergic responses in the airways (46). On the other hand, mice expressing STAT6 selectively in airway epithelial cells exhibit modest inflammation, mucus production and airway hyperresponsiveness in response to IL-13 (43, 44). Given the varied roles for STAT6 in Th2 cell differentiation and Type 2 immune responses induced by IL-4 and IL-13, inhibition of this transcription factor prior to, during, or post allergen-exposure may be an attractive therapeutic approach.

LR 3. STAT6 inhibitory peptide (STAT6-IP) modulates maladaptive Th2-mediated conditions

3.1. Design of STAT6-IP and theoretical mechanism of action

STAT6 inhibitory peptide (STAT6-IP) is a novel therapeutic developed to inhibit aberrant Type 2-mediated immune responses (47). STAT6-IP acts as a cell-penetrating peptide whereby an effector domain is conjugated to a protein transduction domain (PTD). The STAT6-IP effector domain is comprised of the seven amino acids surrounding phospho-tyrosine 641 of murine STAT6 (47). PTDs allow the peptide of interest to enter cells (48). STAT6-IP has been designed using two distinct protein transduction domains, either Protein Transduction Domain 4 (PTD4) derived from HIV-TAT or with Arginine 9 (Arg9), a nine-residue arginine motif (47). Although the precise mechanism(s) of uptake have proved to be elusive, both the TAT PTD and Arg9 PTD likely enter cells via some combination of endocytosis and direct translocation across the plasma membrane (49).

Presently, inhibitors of the STAT6 pathway are being investigated in both academia and in the pharmaceutical sector. Such therapeutics include small molecules, non-SH2 domain dimerization inhibitors, JAK inhibitors, small-interfering RNAs, and phospho-peptides targeting the SH2 domain of STAT6 (including STAT6-IP) (50).

3.2. STAT6-IP inhibits airway-inflammation in ovalbumin and ragweed allergy models and respiratory syncytial virus infection/vaccine models

To date, STAT6-IP has been demonstrated to inhibit airway inflammatory responses in ovalbumin (OVA)- and ragweed-induced allergy models (47, 51). In a dose-dependent manner, intranasal (IN) STAT6-IP administration reduced OVA-induced eosinophilia in the bronchoalveolar lavage fluid (BAL) and IL-13 production in the lungs (47). In the OVA model, IN STAT6-IP administration also limited mucus production in the airways as well as IL-4 and IL-13 from ex-vivo OVA-stimulated splenocytes (47). Similar results were also obtained in a murine ragweed model of allergic inflammation: STAT6-IP reduced Th2 cytokines from splenocytes such as IL-13, IL-4 and IL-5 but increased IL-10 levels. Data from the ragweed model suggest that STAT6-IP may have an immunomodulatory effect rather than simply a Th2-inhibitory effect (51). In both OVA and ragweed models of allergic inflammation, there were no changes in IFN-γ levels from cultured splenocytes from mice treated with peptide suggesting that STAT6-IP is specific to Type 2 cytokine inhibition (51). Ultimately, the ability of STAT6-IP to modulate murine models of allergic inflammation is of clinical interest not only to allergists but also to those studying the role of STAT6 in other models of disease.

STAT6-IP also inhibits aberrant Th2-biased airway inflammatory responses in a formalin-inactivated (FI) Respiratory Synctitial virus (RSV) vaccine model (52). In the FI-RSV model, vaccination with FI-RSV in the hind limb with simultaneous treatment of STAT6-IP IN resulted in reduced airway inflammation following exposure to RSV four weeks later (52). This study provided evidence that STAT6-IP could have long-lasting, systemic effects by modulating the balance of IL-4^{+}CD4^{+} and increasing IFN-γ^{+}CD4^{+} and IFN-γ^{+}CD8^{+} T cell populations (52). In addition, STAT6-IP treatment resulted in a significant increase in neutrophils and macrophages

and a significant decrease in eosinophils in the BAL fluid (52). In a neonatal-adult double RSV infection model, in which RSV is administered in the neonatal period and then again in adult period, IN STAT6-IP delivery at the initial RSV exposure reduced type 2, alternatively activated macrophages (AAMs) in the neonatal mice as well as collagen deposition in the lungs of adult mice following re-infection (53). From both the FI-RSV and neonate/adult RSV infection models, it has been hypothesized that STAT6-IP modulates aberrant Type 2 immune responses at the time of early antigen exposure (e.g. AAM induction) and/or STAT6-IP modulates Th2 differentiation leading to diminished memory Th2-adaptive responses that promote airway inflammation upon secondary exposure (infection) (53).

While STAT6-IP reduces maladaptive Th2-biased responses to allergens and viral infections in the lung, it is currently unknown if STAT6-IP exposure may compromise protective Type 2 immune responses: as in, during helminth infection. In addition, based on the potential of STAT6-IP to have systemic activity (consistent with the finding of STAT6-IP inhibition in the FI-RSV model), elucidating whether the peptide can modulate immunity at distal sites is also of interest. Therefore, our objective is to examine the effects of intranasal/lung delivery of STAT6-IP, if any, on a model helminth infection restricted to the gut in which Type 2 immune responses are required for protection.

LR 4. *Heligmosomoides polygyrus bakeri* as a model to study the effects of STAT6-IP during helminth infection

4. 1. *Heligmosomoides polygyrus bakeri:* a murine model of roundworm helminth infection

Soil-transmitted helminths (STHs) such as *Ascaris lumbricoides, Trichuris trichiura* and various hookworms infect over one billion people worldwide (54). Interestingly, while these helminths infect individuals on a global scale, they rarely cause mortality (55). While STHs infect

humans and animals and remain as chronic infections in the host, drug treatment with anti-helminthic agents generally resolve infections. However, re-infection rates are high due to the ubiquity of these microorganisms in some environments with poor sanitation (55).

Heligmosomoides polygyrus bakeri (Hpb) is a murine gastrointestinal nematode commonly used in the laboratory to study chronic helminth infections and host-pathogen interactions (29, 56). It has a similar lifecycle to human trichostrongyle parasites since *Hpb* infects and remains in the small intestine for extended periods of time (57). One difference between *Hpb* and other human and murine nematodes is that *Hpb* has a completely enteric lifecycle (58). The rather simple parasite lifecycle begins when free-living larvae are ingested and adult worms localize to the lumen of the small intestine (56). Excretion of eggs in the feces occurs shortly thereafter, approximately ten days post-infection (56). In laboratory settings, eggs from feces can be cultured ex-vivo to obtain L3 larvae that can be stored at 4°C for four to six months (56). These infective L3 larvae, upon delivery via gavage, rapidly penetrate the duodenal wall, where they encyst and molt in the submucosa (muscle layer of the muscularis externa) of the small intestine and feed on host tissues (59). Adult worms emerge in the intestinal lumen approximately seven days post-gavage where they coil around intestinal villi, feed, and mate (58). Shortly thereafter, parasite eggs can be detected in the lumen of the small intestine and to a similar extent in the feces (59). Ex vivo, eggs develop into L1, L2 and then the infective L3 larvae seven days later (56). Autoinfection does not occur in this model so the number of infective larvae administered defines worm burden i.e. egg maturation into infective larvae occurs ex-vivo and not within the host (59).

4.2. Host-protective responses during primary *Hpb* infection

Hpb worm burden peaks fourteen days post-infection when a majority of infective larvae have successfully matured into adult worms in the lumen of the small intestine (60). Following

successful establishment in the lumen, adult worms begin to produce excretory/secretory (E/S) products that create an immunomodulatory environment that dampens host protective responses, in particular, Th2-mediated responses (56, 61). In addition, *Hpb* infection induces myeloid-derived suppressor cells that result in reduced AAMs in the lamina propria and spleen as well as decreased CD4+GATA3+ Th2 cells in the spleen (61). As host Th2 responses are diminished, parasite fitness increases and egg burden peaks, normally around day fourteen post-infection (62). Worm expulsion is gradual following the peak of infection at fourteen days, at which time adult worm recoveries of ~50-80% are typically obtained in primary infection (60, 62). While it is unknown whether adult worms are ultimately cleared alive by a 'weep and sweep' method, whereby mucus production and smooth muscle contraction expel the worm, or are damaged/killed by the host immune response, expulsion of adult worms occurs approximately fifteen weeks post-infection in Balb/c mice (63). Overall, *Hpb* is a successful murine pathogen that has both an acute phase with larval maturation and transformation into adult worms between eight and fourteen days and a chronic phase of infection characterized by immunomodulation of the host immune response.

Classical pathology associated with *Hpb* infection in Balb/c mice includes the formation of small intestinal granulomas or 'foci' that have an organized structure predominated by Type 2-driven immunity (59). In primary *Hpb* infection, neutrophils are the first innate cells that surround larva in the submucosa, as early as day four post-infection (29, 64-66). Shortly thereafter, AAMs surround the neutrophils and induce the production of a fibrotic extracellular matrix around the mature larvae (65). Very few lymphocytes, such as CD4+ T cells, are recruited to the site of infection during primary *Hpb* infection (65). However, unlike many helminth infections, larvae do not remain trapped in foci during primary infection and as a result, *Hpb* granulomas typically 'heal' over time (62).

As with other helminth infections, successful host resistance to *Hpb* requires the induction of Type 2 immunity and down-regulation of Type 1 immunity (58, 67). For instance, during *Nippostrongylus barasiliensis* and *Trichinella spiralis* infections, large quantities of IL-4, IL-13 and IL-10 promote worm expulsion and decrease parasite fecundity (58). In contrast, active production of IFN-γ is associated with elevated worm burdens and increased pathology (58, 62). However, blockade of Type 2 responses during *Hpb* infection does not default to a Type 1 response, as occurs in *Schistosoma mansoni* infection (29, 60, 62). Host 'balance' of Type 1 and Type 2 immunity is critical in many helminth models, including *Hpb*.

Protective host responses following primary infection with *Hpb* require IL-4, IL-13 and the IL-4Rα subunit (56, 68, 69). Mice lacking IL-4Rα signalling have higher worm burden over time as well as greater parasite fecundity (62). Strains of mice that more effectively clear *Hpb* also express increased levels of markers of AAMs - *Arg* and *RELM-a* - in duodenal tissues (62). IL-4 and IL-13 also maintain intestinal wall integrity and promote smooth muscle contraction (43, 70). Mice lacking IL-4 have diminished intestinal granulomas suggesting that the recruitment of innate effectors to the site of larval maturation requires IL-4 signalling (62). It has been recently been reported that ILC2s are early sources of IL-4 in primary *Hpb* infection. In studies that selectively depleted IL-4 in ILC2s, Th2 differentiation did not occur and immunity to *Hpb* was compromised (71).

Humoural immunity also contributes to protection in *Hpb* infection. Specifically, polyclonal Immunoglobulin G1 (IgG1) increases in *Hpb*-infected mice as infection becomes chronic (72). IgG1 is the major isotype produced and clearly assists in worm expulsion since partial protection is afforded by transferring IgG1 sera from chronically infected mice into naïve mice prior to infection (72). However, the IgG1 antibodies produced during primary *Hpb* infection mainly target

E/S products rather than adult worms and larvae (56, 72). IgG1 targeted against adult worms increases during secondary *Hpb* infection and contributes to host protection (56, 73). Simultaneously, IgE antibodies are produced during primary and secondary *Hpb* infection and correlate with changes in parasite fecundity; peak IgE levels occur at 21 days post-infection (68). Early studies using anti-CD4 monoclonal antibodies (mAb) demonstrated that $CD4^+$ T cells are required to maintain reduced worm burdens (68). Not surprisingly, IgE production also requires CD4+ T cells as delivery of anti-CD4 antibodies to *Hpb*-infected mice abrogates total serum IgE and increases egg numbers (68). Other studies investigating the role of IL-4 (and IL-13) using anti-IL-4Rα mAbs, or using IL-4 complexes elucidate a pivotal role for IL-4 whereby mice can be 'cured' following chronic infection when treated with IL-4 (62, 74, 75). While IL-4 plays a role in smooth muscle contractility, it does not regulate the rate of worm penetration into the gut lumen during primary *Hpb* infection (58).

Studies designed to assess the role of eosinophils in primary *Hpb* infection have used anti-IL-5 mAb and eotaxin-1/CCL11-deficient mice. Both the cytokine IL-5 and the chemokine eotaxin-1/CCL11 play critical roles in eosinophil activation and recruitment to the site of antigen exposure respectively (76). As a result, mice deficient in either IL-5 or eotaxin-1/CCL11 provide evidence that eosinophils play a dispensable role during *Hpb* and *N. brasiliensis* infection (76, 77). However, it has been recently elucidated that eosinophils supress Th2 responses in order to promote gut homeostasis and IgA production during *Hpb* infection (78). The use of eosinophil-deficient ΔdblGATA-1 mice highlights a novel role for eosinophils, since the absence of eosinophils in this study resulted in increased levels of GATA-3, IL-4 producing Th2 cells in Peyer's patches and increased IgG1 titres leading to host resistance (78). Although the mechanism is still unclear, it has been speculated that eosinophils reduce Th2 differentiation via interaction

with CD103+CD11b- DCs in Peyer's patches (79).

OX40L/OX40 receptor-ligand interactions play a significant role in the development of IL-4 producing Th2 cells via i) priming by OX40L on antigen presenting cells and ii) OX40 signalling on T cells that contributes to survival of effectory memory T cells homing to germinal centers (80, 81). Data from studies using OX40L$^{-/-}$ mice infected with *Hpb* demonstrate that OX40L/OX40 signalling is critical in reducing worm burden and parasite fecundity, and for production of both IL-4 and IgE (82). Nevertheless, the OX40L/OX40 interaction is dispensable for producing E/S-specific IgG1 and antigen-specific CD4+ Th2 cell expansion (82). Together, these data suggest that OX40/OX40L interactions are required for some, but not all Th2 responses to primary and secondary *Hpb* infection. Overall, IL-4, IL-13, IL-4-Rα, CD4+ T cells, and OX40/OX40L signalling all participate in the protective host responses during *Hpb* infection.

4.3. Host-protective responses during secondary *Hpb* infection

While mice infected with *Hpb* remain chronically infected, drug-treatment with an anti-helminthic agent and subsequent re-infection several weeks later results in rapid parasite elimination in Balb/c mice (66). Protection to *Hpb* infection is mediated by IL-4/13-producing memory Th2 cells that induce AAMs within the granulomas (66). Induction of arginase-producing AAMs is also IL-4 and STAT6-dependent during *Hpb* re-challenge (66). The concerted effort of AAM, neutrophils, dendritic cells and Th2 cells mediate larval killing within the granulomas (29). Basophils, known to act as the main source of IL-4 during *N. brasiliensis* infection, are insufficient to mediate efficient Th2 differentiation during *Hpb* infection (83). For example, in studies utilising basophil-deficient mice, basophil-derived IL-4 and IL-13 was necessary for protection during challenge infection in an IgE dependent manner (84).

Ultimately, *Hpb* is a useful helminth model to study the delicate balance between the host immunity and parasite survival with the establishment of chronicity in primary infection but the rapid development of a protective response during secondary infection.

4.4. Sex differences in gastrointestinal helminth infection

Sex plays a role in both the rates of allergic diseases and susceptibility to helminth infection (85-87). Specifically, abundant epidemiological studies indicate that males have greater susceptibility to viral, bacterial and parasitic infections than women (88). For instance, in humans, females are more resistant to *Leishmaniasis, Trypanosoma cruzi* and *Trypanosoma brucei* infections (88). In rodents, female Balb/c mice expel *Hpb* adult worms more rapidly and have less morbidity than their wild-type male counterparts (85, 89-91).

Sex differences in susceptibility to infectious diseases may be directly related differences between immune responses. Females, whether adult or children, have greater CD4/CD8 T cell ratios than age-matched males while males have greater CD8+ T cell frequencies (92). A number of diseases have been linked to sex: autoimmune diseases and infectious diseases such as Human Immunodeficiency Virus, Malaria and Zika are female-biased while non-reproductive cancers and infectious diseases such as Schistosomiasis and Hepatitis B infection are male-biased (92). In asthma models, it has been postulated that sex differences mediate changes in T effector cell populations due to larger numbers of myeloid dendritic cells (DCs) that migrate to the lymph nodes from the lungs (93). In addition, increases in myeloid DC populations are correlated with increased AAM populations that drive allergic inflammation in females (93). Given that the precise mechanism that underlies differences at the level of innate and adaptive immunity is not well understood, it is important to consider the relationship between sex and susceptibility/resistance to infection in experimental studies (91, 92, 94).

LR 5. Modulating mucosal immune responses in the gut via intranasal immunization

5.1. STAT6-IP as a potential lung-gut axis modulator of host immune responses

Mucosal immunity underlies the unique balance between tolerance and induction of immune responses at barrier sites where the host is constantly exposed to microorganisms, allergens, and pollutants from the environment (34). The lung and gut are the two most well-characterized tissues that demonstrate how mucosal immunity at one site may be modulated by mucosal responses at the other site (95). Given that STAT6-IP is to be used as an intranasal therapeutic, it is of interest to investigate the modulation of host gut responses via the lung-gut axis. Specifically, it has been well documented that immune effector cells activated during infection in the gut have direct immunomodulatory effects on responses to allergens or pathogens in the lung (95). For instance, helminths modulate allergic responses to house dust mite and RSV infection in the lungs: i) via E/S products that induce myeloid-derived suppressor cells; and ii) via alterations of the intestinal microbiota that alter short-chain fatty acid content in the bloodstream, which promotes T regulatory cell suppressor activity (96, 97). In addition, *Hpb* infection reduces inflammation in other models of disease such as arthritis and colitis (7, 98, 99).

Recent data suggests that T cells may home to the gut following interaction with antigen-stimulated dendritic cells in the lungs (100). For instance, intranasal delivery of salmonella antigens protected mice from a pathogenic Salmonella infection via dendritic cell priming of T cells to Peyer's patches (100). Moreover, other studies have shed light on the establishment of memory CD8+ T cell pools in the small intestinal epithelia following intranasal immmunisation with influenza virus (101). Ultimately, these data provide evidence of a mucosal cross talk between

the gut and the lungs with the potential to be altered following therapeutic intervention.

5.2. Intranasal immunization confers systemic and mucosal immunity

Intranasal immunizations have shown promise as a novel route of delivery in humans due to its potential to generate systemic and mucosal immunity (102). Systemic immunity is generally characterized by IgG production, neutralizing antibody responses and cytokine production by splenocytes (103, 104). On the other hand, mucosal immunity is generally the arm of the immune responses responsible for protection and tolerance at mucosal surfaces via the production of IgA and induction of antigen-specific memory $CD4^+$ or $CD8^+$ T cells at the mucosal surface (105). Vaccine antigens delivered IN are of pharmaceutical interest since they are more likely to reach lymphoid follicles than intramuscular vaccines and because they can be administered without a healthcare professional (102). A limited number of vaccines that generate mucosal immunity have been developed and include those targeting polioviruses, *Salmonella typhi* and rotaviruses (106). Homing of DCs to lymph nodes and migration of T cells to the site of infection at barrier sites is also of therapeutic interest. For instance, the 'prime-pull' approach has been shown to confer protection to viruses at barrier sites. In this method, a parenteral vaccine is given first to elicit systemic T cell responses (prime) followed by a topical chemokine treatment to promote T cell recruitment to the mucosal site (pull). 'Prime-pull' immunization has been successful in protecting mice against genital Herpes simplex virus via production of tissue-resident memory CD8+ T cells and has shown modest effects on local and systemic antibody responses to Human Immunodeficiency Virus gp140 enveloppe protein (105, 107). These studies suggest the potential to generate effective vaccines to protect at mucosal barrier sites using IN immunization strategies. Whether the immune response to an enteric infection can be altered by IN exposure to an immunomodulator, such as STAT6-IP, has yet to be investigated.

LR 6. Hypotheses and Aims

6.1. Hypotheses

Given i) the importance of STAT6 in promoting IL-4/IL-13-mediated Th2 responses in allergy models and ii) the protective role of IL-4/IL-13 during *Hpb* infection, we hypothesize that primary *Hpb* infection in mice deficient in STAT6 will have compromised Type 2 immunity resulting in reduced host protection. Specifically, we expect that STAT6-deficient ($STAT6^{-/-}$) mice will have higher worm burdens and fewer CD4+ T cell and macrophages within foci compared to their wildtype (WT) counterparts. The results of these experiments will define the maximum immunomodulatory effects of STAT6-IP in subsequent STAT6-IP experiments.

Given i) the immunomodulatory role of STAT6-IP in the lungs and spleens in allergy and RSV models and ii) the potential modulation of gut responses via IN administration, we hypothesize that IN STAT6-IP delivery will reduce protective Type 2 immune responses in both the primary and secondary *Hpb* infection models based on the role of STAT6 in host responses to helminths. We suspect that mice treated with IN STAT6-IP will have a phenotype that is characterized by a Type 2 response that is intermediate between WT mice and mice completely lacking STAT6.

6.2. Aims

I will test whether mice receiving STAT6-IP retain the ability to develop robust Type 2 immune responses and expel *Hpb*. Furthermore, I will determine if STAT6-IP-treated mice have similar infection outcomes as those observed in $STAT6^{-/-}$ mice. The specific objectives for this M.Sc. thesis are:

1) To evaluate STAT6-dependent responses in primary *Hpb* infection by assessing worm and

egg burdens as well as antibody produtcion in STAT6$^{-/-}$ versus WT mice

2) To define STAT6-IP effects via IN administration in primary and secondary *Hpb* infection models

3) To compare inhibitory activity of STAT6-IP peptides with different protein transduction domains: Arg9 or PTD4

6.3. Conclusions

Asthma and allergy-associated diseases are responsible for great morbidity and costs to healthcare in developed and developing countries. Of the various factors contributing to allergic airways disease, STAT6 is responsible for mediating Th2 activation, IgE production as well as mucus production leading to immunopathology in the lungs. STAT6-IP is a dominant-negative, cell-penetrating peptide, that was designed to inhibit homodimerization of STAT6 and downregulate maladaptive Th2-associated immunopathology. STAT6-IP inhibits Th2-biased airway inflammatory responses in allergy and infection models. To date, modulation of protective Type 2 responses in the gut by STAT6-IP has not been assessed. In this thesis, the immunomodulatory effects of STAT6-IP are investigated in a strictly enteric nematode infection that requires a STAT6-dependent mechanism for protection.

In conclusion, given its immunomodulatory potential and the burden of allergic airways disease worldwide, STAT6-IP is likely to be used in combination with current asthma therapeutics among children and adults from industrialized countries. However, the genesis of this project began with the concern that while Th2 immunomodulatory therapy or related therapies that target Type 2 immune responses may reduce asthma morbidity, it may also result in side effects that alter protective immunity to gastrointestinal helminth infections. This is of particular importance if STAT6-IP were to be used in regions where both asthma and helminth infections are prevalent.

Ultimately, the knowledge acquired from this project will provide a better understanding of STAT6-dependent responses during primary *Hpb* infection and further the understanding of the effects of STAT6-IP administration on modulation of host responses in the gut. Defining whether delivery of STAT6-IP can disrupt a protective Type 2 response to a gastrointestinal helminth infection will provide a more complete understanding of the mechanism of action of STAT6-IP and the potential safety of this inhibitor as an asthma therapeutic. Moreover, this information may provide further insight into the potential for STAT6-IP to influence the balance between protective and maladaptive Type 2 responses.

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2.1. PREFACE TO CHAPTER 2

STAT6 inhibitory peptide (STAT6-IP) has been previously used in a respiratory syncytial virus infection model whereby maladaptive Th2 inflammatory responses in the lung are reduced. In order to test the effects of STAT6-IP during *Heligmosomoides polygyrus bakeri* infection in the gut, an established phenotype of host responses is required in STAT6$^{-/-}$ animals.

STAT6$^{-/-}$ mice have been implemented in models of allergic inflammation and helminth infection to identify STAT6-dependent host responses in Type 2 immunity. For instance, in models of OVA-induced allergy, absence of STAT6 completely reduced airway hyperresponsiveness and eosinophilia in the bronchoalveolar lavage. In addition, in the helminth model *Nippostrongylus basiliensis,* STAT6 deficiency resulted in reduced eosinophil recruitment to the skin and increased the number of lung larvae upon primary infection.

Our findings presented in Chapter 2 highlight the requirement for STAT6 in mediating host resistance. In STAT6$^{-/-}$ mice, adult worm burden, parasite fecundity and production of IgG1 were all increased. We also validate the *Hpb* infection model as an appropriate tool to study the potential modulation of protective Type 2 immune responses following STAT6-IP treatment.

CHAPTER 2

STAT6 signalling modulated Type 2 immune responses during gastrointestinal nematode infection

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2.2. ABSTRACT

STAT6 is a key transcription factor required for the induction of Type 2-mediated responses that mediate both protection from helminth infections and allergic inflammation. In this study, we sought to determine what role STAT6-dependent responses play in a primary infection model with a strictly enteric nematode, *Heligmosomoides polygrus bakeri* (*Hpb)*, by comparing the responses of wild-type mice and STAT6-deficient animals. Our findings reveal that STAT6 has a protective effect: reducing adult worm burden and parasite fecundity; inducing the formation and maintenance of intestinal granulomas; and stimulating the production of total IgG as well as adult-worm specific IgG1. Together, our data support the canonical Th2 paradigm whereby STAT6 is involved in parasite clearance and maintenance of protective Th2 responses during helminth infection. These findings define the 'maximum' STAT6 signalling effect in the *Hpb* infection model in order to assess the impact of a novel STAT6 inhibitor.

2.3. INTRODUCTION

STAT6 is a key transcription factor involved in the induction and maintenance of T helper 2 (Th2) immunity (1, 2). As a result, STAT6 plays a central role in both adaptive (eg: helminth infection) and maladaptive responses (eg: allergy, asthma). In asthma, STAT6 signalling is involved in the induction of allergen-induced airway inflammation and maintenance of airway hyper-responsiveness (AHR) and airway remodeling (3).

STAT6 is a cytoplasmic protein that becomes activated upon binding of interleukins (IL)-4 and -13 to their cognate receptors, which each contain the IL4-Rα subunit, on a variety of immune cells, including T cells, B cells and macrophages (2, 4). Once ligated, IL-4Rα induces Janus kinase (JAK) activation and STAT6 monomers are recruited and phosphorylated (2). Once phosphorylated, two STAT6 monomers homodimerize and translocate into the nucleus, to mediate expression of genes responsible for inducing and maintaining Type 2 adaptive immunity. Effector functions induced by STAT6 signalling include: goblet cell differentiation and secretion of mucus, production of chemokines to recruit eosinophils and Th2 cells to the site of allergen exposure, Th2 cell activation and release of IL-4,-13,-5, -9, secretion of IgE and IgG1 from cognate B cells and transcription of *Arg-, Ym-1* and *Fzz-1* genes that define the alternatively activated macrophage (AAM) phenotype (2). STAT6 also mediates up-regulation of genes responsible for smooth muscle contraction, such as *RhoA* (2, 5, 6).

The importance of STAT6 in allergic airways disease has been best demonstrated in mouse models in which STAT6 signalling is reduced or eliminated. For instance, in ovalbumin (OVA)-induced models of allergic inflammation, STAT6-deficient mice fail to develop antigen-induced AHR and eosinophils are not found in the bronchoalveolar lavage fluid (3, 4, 7).

The role of STAT6 has also been investigated in nematode models including *Nippostrongylus brasiliensis* and *Trichinella spiralis*. In studies of *N. brasiliensis* in Balb/c mice, STAT6 is responsible for IL-13-mediated hypercontraction of the jejunum, leading to parasite expulsion (5). In a *T. spiralis* infection model in Balb/c mice, STAT6-dependant signalling is required for the production of IL-4 and IL-13 leading to increased mastocytosis in the gut and ultimately worm expulsion (8). Another helminth, *Heligmosomoides polygyrus bakeri* (*Hpb),* is commonly used in laboratory settings to study chronic enteric helminth infections and adaptive Type 2 immune responses (9).

In this study, our objective was to investigate the role of STAT6 in protective Type 2 immune responses during primary *Hpb* infection. We hypothesized that, given the role of STAT6 in mediating Type 2 immune responses, and in accordance with observations in other enteric helminth models, STAT6$^{-/-}$ mice infected with *Hpb* would be unable to control infection. Our findings provide evidence for a role of STAT6-signalling in mediating adult worm expulsion, production of intestinal granulomas and reduced parasite fecundity as well as the production of both adult-worm- specific IgG and IgG1.

2.4. MATERIALS AND METHODS

Larval stock preparation

To obtain larvae for experiments, six to eight-week-old male Balb/c mice, bred in-house (originally purchased from Jackson Laboratories, Bar Harbour, ME), were inoculated by gavage with 200 L3 larvae (kind gift from Dr. Irah King, McGill University). 14 days post-infection, fecal pellets were collected from mice placed in cages lined with moist paper towels for six hours. Animal studies were approved by the McGill University Animal Care Committee and were performed following the guidelines of the Canadian Council on Animal Care.

Culture of *Hpb* eggs has been described previously (10). Briefly, fecal pellets were moistened with sterile water approximately twice the volume (in mL) of the weight of the feces. The moistened fecal pellets were blended into a paste and placed in the center of a round piece of 125mm Whattman filter paper (Whatman, Missasauga, ON) in a 150x15mm Petri dish for seven to ten days at 25°C. The edges of the filter paper were kept clean of feces. The filter paper was kept moist via drop-wise addition of approximately 2mL sterile water every other day. Starting on day seven, larvae were collected in a 50mL Falcon tube by washing the clean edges of the filter paper with sterile water, allowing the larvae to settle by gravity. Most of the supernatant was removed, leaving ~5mL. Viable larvae were counted and stored for up to six months at 4°C (10). Prior to infection, larvae were re-counted and the parasites were kept on ice.

Hpb infection and quantification of worm burden

Six-eight week-old male and female wild-type (WT) Balb/c and STAT6 knockout (KO) mice on a Balb/c background were infected with 200 L3 *Hpb* larvae by gavage. 15 WT mice (eight male, 7 female) and 15 STAT6$^{-/-}$ mice (seven male, eight female) were euthanized at 14 days post-

infection (dpi). 12 WT mice (four male and eight female) and 15 STAT6$^{-/-}$ mice (seven male, eight female) were euthanized at 28 dpi. At both time points, blood as well as intestinal feces were collected. Intestinal wall granulomas were counted by visual inspection under a dissecting microscope (Zeiss, Oberkochen, Germany). Quantification of adult worms was performed as described previously (10). Briefly, the intestine from the stomach to the cecum, was dissected and placed in a petri dish containing phosphate-buffered saline (PBS). With the use of a dissection microscope, adult worms were teased from the intestinal lumen using forceps (Fine Science Tools, North Vancouver, BC).

The assessment of parasite fecundity, using the collected intestinal fecal pellets, was adapted from Valamparambil et al., 2014 (10). Two to three fecal pellets were placed in a 1mL saturated salt solution for quantification. The fecal pellets were weighed and collected in a 1mL saturated sodium chloride solution (~400g/L) and vortexed at room temperature until pellets were broken apart. Homogenized feces were allowed to settle overnight at room temperature. The following day, three visible layers could be observed. The cloudy middle layer, where *Hpb* eggs settle, was collected and egg counts were performed in triplicate at 100x magnification. Egg counts are expressed as number of eggs/gram of feces.

Preparation of Adult worm homogenate (AWH)

Adult worm homogenate can be used as an *Hpb* adult worm-specific antigen for cell stimulation and for enzyme-linked immunosorbent assays (ELISAs) (10). Preparation of AWH has been described previously (10). Briefly, adult worms were removed from the intestines of 14 day WT *Hpb*-infected mice and washed ten times at room temperature with PBS to remove eggs and intestinal debris. Worms were allowed to sediment by gravity each time before the next wash.

Worms were transferred to a 7mL Dounce-style glass tissue grinder (Wheaton, Millville, NJ) and homogenized in 1mL PBS manually for ten minutes at room temperature. The homogenate was centrifuged for ten minutes at 800xg at room temperature and supernatants were collected to obtain *AWH*. Protein concentration was measured using the Pierce bicinchoninic acid assay according to manufacturer's instructions (Thermo Scientific, Ville Mont-Royal, QC) and AWH was stored in aliquots at -80°C prior to use.

Anti-AWH IgG1/IgG2a ELISAs

Antibody ELISAs were performed according to a protocol modified from Thermo-Scientific. 96-well U-bottom plates (Corning, NY) were incubated at 4°C overnight with 1ug/mL AWH or with a purified mouse IgG1 or IgG2a diluted in carbonate-bicarbonate buffer (Sigma-Aldrich, St. Louis, MO). Following overnight incubation, wells were washed with wash buffer (PBS + 0.05% Tween 20) (Sigma-Aldrich) and blocked for one hour with PBS + 2% bovine serum albumin (Sigma-Aldrich) at 37°C. 50µL of serum samples were administered in duplicate wells. Serum samples from infected mice were diluted 1:10 for animals euthanized at 14 dpi and 1:20 for animals euthanized at 28 dpi and heat inactivated at 56°C for 30 minutes. Serum from uninfected animals were applied to wells undiluted. Following a one hour incubation at 37°C, wells were washed six times with wash buffer. Plates were incubated for thirty minutes with 75µL of anti-IgG1-HRP or 75µL of anti-IgG2a-HRP (Southern Biotech, Birmingham, AL) diluted in carbonate-bicarbonate buffer for a dilution of 1:20000 and 1:10000 respectively. After washing wells six times with wash buffer, 100µL of Strepavidin was applied to wells for fifteen minutes. 50µL of sulfuric acid were used as a stop solution and ELISAs were read immediately at an optical density (OD) of 450nm. Final concentrations of each antibody titres were obtained by comparing ODs of

samples to a standard curve while accounting for blank wells and dilution factors. Samples that were below the range of detection of the standard curve were given a value 50% of the lowest detectable OD value on the standard curve.

Statistical Analysis

Statistical significance was obtained using an unpaired student's T-test or by two-way ANOVA (Version 6; GraphPad, La Jolla, CA). Differences were considered significant when $p < 0.05$.

2.5. RESULTS

STAT6-dependent responses influence granuloma formation and parasite fecundity but not adult worm expulsion at 14 days post-*Hpb* infection

While adult *Hpb* worm recovery in the lumen of the small intestine was similar between WT and STAT6$^{-/-}$ mice at 14 dpi (Fig. 1A), STAT6$^{-/-}$ mice had a significantly lower number of intestinal granulomas compared to WT mice ($p<0.0005$: Fig. 1B). While worm burden numbers were equal at this time point, STAT6$^{-/-}$ mice had a significantly elevated egg burden compared to their WT counterparts ($p<0.05$: Fig. 1C), suggesting that lack of STAT6 signalling may increase host parasite fitness leading to earlier egg development and production.

STAT6-signalling contributes to granuloma maintenance as well as reduced parasite burden and fecundity at 28 dpi

The adult worm burden decreased in WT animals by 28 dpi but remained at levels similar to 14 dpi in the STAT6$^{-/-}$ group (Fig. 1A, 2A). Egg burdens in the STAT6$^{-/-}$ infected animals were significantly higher compared to WT animals at 28 dpi ($p<.0005$: Fig. 2A, 2C). While the reduced egg burden trend may appear to parallel trends in adult worm burdens, the ratio of eggs per gram per adult worm demonstrates a significant STAT6-dependent effect in reducing adult worm fitness at 28 dpi ($p<.001$; Fig. 2D) At 28 dpi, WT mice still had significantly more granulomas than the STAT6-deficient animals ($p<.0005$: Fig. 2B) most likely as a result of maintenance of foci that is abrogated in the absence of STAT6 signalling.

STAT6$^{-/-}$ mice have lower serum anti-*AWH* IgG1and total IgG levels

Uninfected animals had no detectable serum AWH-specific IgG or IgG1 at either 14 or 28 dpi (Fig. 3). Although AWH-specific IgG and IgG1 concentrations were relatively low at 14 dpi, WT mice still produced significantly more AWH-specific IgG1 ($p<0.0005$; Fig. 3A), but not IgG than the STAT6$^{-/-}$ animals (ns; Fig. 3B). By 28 dpi, antibody responses were much higher in all animals and differences between the WT and STAT6$^{-/-}$ animals were more evident. At the later time-point, both AWH-specific IgG and IgG1 levels were significantly higher in the WT compared to the STAT6$^{-/-}$ group ($p<0.0005$ and $p<0.01$ respectively: Fig. 3C, D). No detectable IgG2a antibodies were detected amongst wildtype or STAT6$^{-/-}$ animals (data not shown).

2.6. DISCUSSION

In this study, we measured both host and parasite outcomes to assess STAT6-dependent responses during primary infection with the gastrointestinal helminth, *Hpb*. Our findings indicate an important role for STAT6 signaling in adult worm clearance, granuloma formation/maintenance, parasite fecundity and *Hpb*-specific IgG1 and total IgG antibody production during both initial (14 dpi) and more chronic stages of infection (28 dpi).

Although the exact mechanism of adult worm clearance in primary *Hpb* infection is unclear, our findings show reductions in worm burden over time, a trend that was not observed in the STAT6$^{-/-}$ mice (Fig. 2A, 3A) (9). Worm numbers fell from approximately 100 to 25 in two weeks in WT mice, data that is similar to those published by Filbey et al. using the same inoculum as well as other studies utilizing 50-100 L3 larvae: each group reported an approximate 75% reduction in adult worm numbers by 14 dpi (11-13). Although we did not continue to assess worm burden after 28 dpi, we expect that, similar to others (14), worm burdens would have stabilized with time, resulting in complete expulsion approximately 100 dpi in WT Balb/c mice.

Maintenance of elevated worm burdens during primary *Hpb* infection has also been reported in studies using IL-4 receptor deficient (IL-4R$\alpha^{-/-}$) mice, which lack STAT6 activation. In a study published by Filbey et al, IL-4R$\alpha^{-/-}$ mice on a Balb/c background had equal adult worm burdens compared to WT mice at 14 dpi, similar to our STAT6$^{-/-}$ cohort (Fig. 2A). At 28 dpi, adult worm burdens in STAT6$^{-/-}$ mice were comparable to those reported in IL-4R$\alpha^{-/-}$ mice. Together, our data suggest that worm expulsion by the host requires the canonical IL-4 receptor signalling by IL-4 and/or IL-13 and downstream STAT6 activation (2).

Parasite fecundity is an important outcome to assess adult worm fitness of nematodes over time. Our studies revealed a role for STAT6 in the decrease in adult worm fitness during the first

28 days following infection (Fig. 2C, 3C). These results are similar with trends on parasite fecundity at 14 and 28 dpi assessed by Knott et al: while their findings report differing egg burdens between WT and STAT6$^{-/-}$ mice at 14 and 28 dpi, their data show normalized differences among WT and STAT6$^{-/-}$ when following parasite fecundity over 116 days (14). Whether or not our findings represent a STAT6-mediated decrease in egg burden over the entire course of primary infection, our data clearly demonstrate a role for STAT6-signalling in the reduction in parasite fecundity at 14 and 28 dpi.

The ability to form granulomas is a key indicator of the host immune response despite the chronic nature of primary *Hpb* infection in Balb/c mice (15). Although granulomas in primary *Hpb* infection are thought to be ineffective in limiting infection, differences in host susceptibility to infection can be observed when comparing responses in different mouse strains. In particular, SJL mice, that expel adult worms more rapidly than Balb/c or C57BL/6 mice, have greater numbers of granulomas and greater frequencies of IL-4Rα-rich macrophages within the granulomas (11). Therefore, while granulomas do not successfully kill larvae in primary *Hpb* infection in Balb/c mice, macrophages recruited to intestinal foci may damage adult worms and reduce adult worm fitness, leading to more rapid expulsion in SJL mice (11). In our studies, very few (approximately ten) intestinal granulomas were observed in STAT6$^{-/-}$ mice at both time points suggesting that STAT6 could, in fact, promote the recruitment of macrophages to granulomas leading to reduced parasite fitness (Fig. 2B, 3B).

Given that IL-4Rα$^{-/-}$ mice fail to produce granulomas at both 14dpi and 28dpi, we suspect that the formation of foci in our model (albeit very few) could have occurred via STAT6-*independent* mechanisms (11, 16). For instance, the STAT1 and STAT3 signalling pathways can induce AAM recruitment in helminth and allergy models: STAT1 is activated by IL-13 binding to IL-4Rα and/or

IL-13R-α1 and STAT3 is activated by IL-4 and IL-13 binding to IL-4Rα (16). Future studies to quantify AAMs and other IL-4/IL-13-producing effector cells within granulomas in the presence or absence of STAT6 signaling may shed light on mechanisms by which STAT6 regulates granuloma formation in primary Hpb infection.

Polyclonal IgG1 produced during primary infection largely play an ineffective role for protection since they target *Hpb* excretory/secretory products (HES) that act as decoy molecules (17). For instance, C57BL/6 mice are not protected when antibodies specific to the HES products VAL-1, -2, -4 are transferred to naïve mice infected with *Hpb* (17). Interestingly, while sera from mice infected once with *Hpb* do not confer protection, passive transfer of IgG from mice infected several times effectively reduces adult worm burden thus highlighting the importance of IgG in secondary, rather than primary *Hpb* infection (18). Whether or not STAT6 plays a role in protective IgG responses, such as the case for secondary *Hpb* infection, remains to be investigated.

Our data demonstrate the significance of STAT6 signaling in maintaining elevated adult worm-specific IgG1 titres since STAT6$^{-/-}$ mice produced limited AWH-specific IgG1 at both time points (Fig. 4A, C). We also suspect that reduced antibody responses in the absence of STAT6 signalling may be due to impaired germinal center formation: it has been reported that STAT6 is necessary for B cell production of IgG following stimulation of IL-4 and IL-13 by T follicular helper cells (19). Future experiments assessing the production of egg-specific IgG and IgG1 may shed light on role of STAT6 in reducing parasite fecundity.

Although the exact mechanism of worm expulsion has been hypothesized to function as a 'weep and sweep' mechanism, STAT6 signalling plays a critical role in intestinal smooth muscle contractility since STAT6 signaling is required for enteric nerve innervation during *Hpb* infection (5, 6). Coupled with significant differences in adult worm burden at 28 dpi (Fig. 3A), our results

are consistent with the findings that worm expulsion is subject to STAT6-dependent smooth muscle contraction (5, 6). Future studies aimed at defining goblet cell mucus production, and increases in mRNA levels of STAT6-dependent genes, *muc5ac* and *gob5*, would provide further confirmation of the role of STAT6 in adult worm clearance.

Several studies report that gender alters susceptibility/resistance to *Hpb* infection. When comparing worm burdens and intestinal granuloma levels, we did not find sex differences in our studies, a finding that is inconsistent with previously published studies (20-22). Host susceptibility based on sex has also been observed during *Schistosoma mansoni* and *Toxocara* species infection, in which males are more susceptible to disease (23). Given our small sample size (n=4-8 per sex, per genotype), our results at this time do not provide insights into potential links between sex, genotype and host susceptibility in STAT6 deficient and WT animals during *Hpb* infection.

STAT6 is critical in mediating both protective responses in helminth infection and maladaptive responses during allergic inflammation. The findings of this study indicate that STAT6 is required for adult worm clearance, granuloma formation, reduced parasite fecundity and production of antigen-specific IgG/IgG1 in Hpb-infected mice. These findings set the stage to explore which, if any, of these responses are modified in *Hpb*-infected mice treated with a STAT6 inhibitor.

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2.8. FIGURES AND LEGENDS

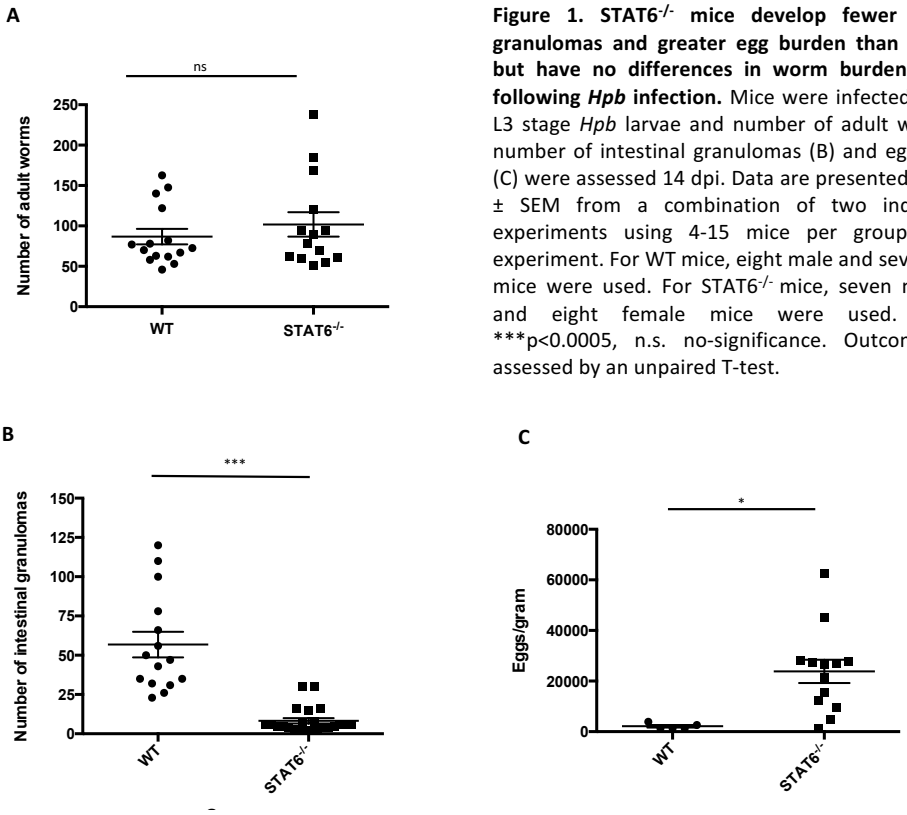

Figure 1. STAT6$^{-/-}$ mice develop fewer intestinal granulomas and greater egg burden than WT mice, but have no differences in worm burden, 14 days following *Hpb* infection. Mice were infected with 200 L3 stage *Hpb* larvae and number of adult worms (A), number of intestinal granulomas (B) and egg burdens (C) were assessed 14 dpi. Data are presented as means ± SEM from a combination of two independent experiments using 4-15 mice per group in each experiment. For WT mice, eight male and seven female mice were used. For STAT6$^{-/-}$ mice, seven male mice and eight female mice were used. *p<0.05, ***p<0.0005, n.s. no-significance. Outcomes were assessed by an unpaired T-test.

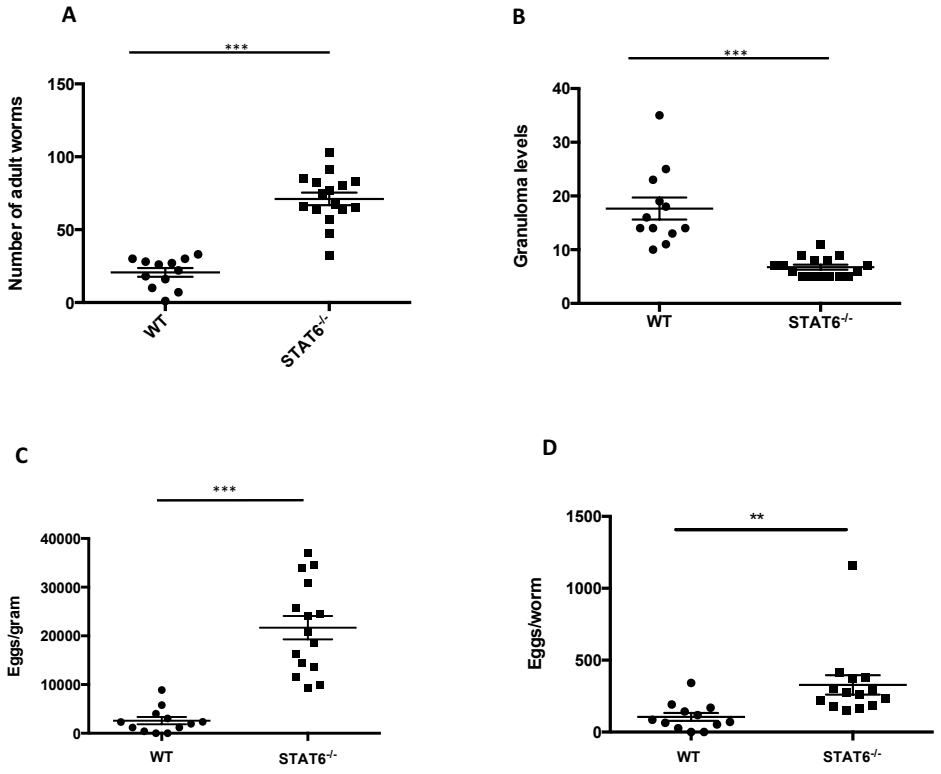

Figure 2. STAT6$^{-/-}$ mice maintain elevated worm burden, parasite fecundity and reduced intestinal granulomas 28 days-post *Hpb* infection. Mice were infected with 200 L3 stage *Hpb* larvae and number of adult worms (A), number of intestinal granulomas (B) and egg burden (C) were assessed 28 dpi. Figure D represents the ratio of eggs per gram per worm assessed at 28 dpi. Data are presented as means ± SEM from a combination of two independent experiments using 12-15 mice per group in each experiment. For WT mice, four male and eight female mice were used. For STAT6$^{-/-}$ mice, seven male mice and eight female mice were used. ***p<0.0005, **p<0.001, n.s. no-significance. Outcomes were assessed by an unpaired T-test.

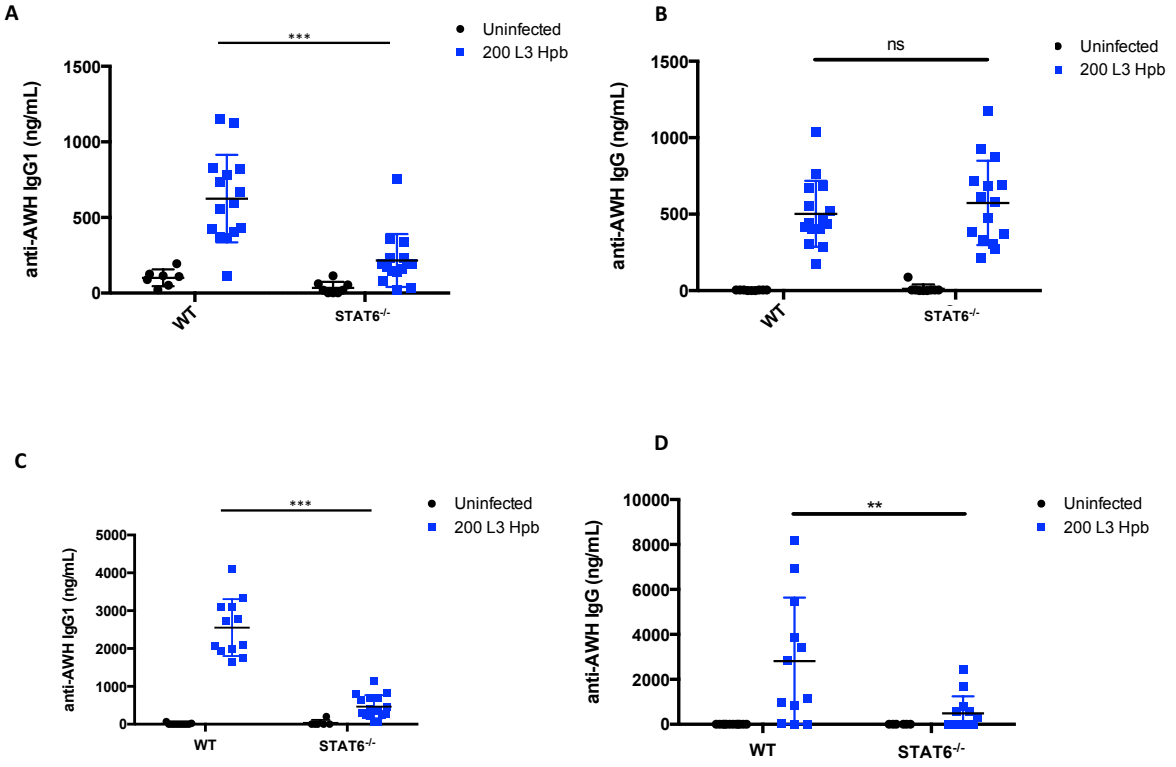

Figure 3. STAT6$^{-/-}$ mice fail to mount elevated anti-AWH IgG1 and total IgG titres during *Hpb* infection. WT and STAT6$^{-/-}$ mice were infected (or remained uninfected) with 200 L3 stage *Hpb* larvae and anti-AWH IgG1 serum titres were assessed as a measure of adult-worm specific antibodies via ELISA at 14 dpi (A) and 28 dpi (C). Serum was also assessed for total IgG titres at 14 dpi (B) and 28 dpi (D). Data are presented as means ± SEM from a combination of two independent experiments using 6-10 mice per group in each experiment. **p<0.001, ***p<0.0005, ns not-significant. Outcomes were assessed by a two-way ANOVA.

3.1. PREFACE TO CHAPTER 3

Our findings in Chapter 2 validated that Type 2 immune responses produced during primary *Hpb* infection are STAT6-dependent and thus are an ideal candidate for modulation by STAT6-IP.

In our first experiments examining STAT6-IP effects on *Hpb* infection, we chose to implement a three-dose STAT6-IP delivery schedule, similar to the FI-RSV 'Early Intervention' group where STAT6-IP delivery at the time of initial FI-RSV vaccination reduced maladaptive Th2-biased allergic inflammation upon infection four weeks later. We demonstrate that a similar intranasal delivery schedule of STAT6-IP at the time of primary *Hpb* infection transiently reduces adult worm and parasite fecundity but does not alter host protective immunity in the gastrointestinal tract when administered via an intraperitoneal (IP) route. We also show that an extended nine-dose delivery schedule via IP injection also had little effect on host immunity to *Hpb*.

This study is the first to provide insight into whether STAT6-IP can modulate helminth infections that rely extensively on host-pathogen balance to mediate chronic infection. We conclude that STAT6-IP use for allergic airways disease may be sufficient to treat allergy and RSV-induced immunopathology without impacting protective host responses to gastrointestinal nematode infection.

CHAPTER 3

Evaluating Type 2 Immunity during primary *Heligmosomoides polygyrus* infection using a STAT6 inhibitor

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3.2. ABSTRACT

Of the different types of asthma and chronic allergic airways diseases, intermittent airway obstruction induced by aberrant activation of Type 2 immunity affects 235-300 million individuals worldwide. In addition to biologics in clinical trials, Dr. Fixman has developed a novel chimeric peptide, STAT6 inhibitory peptide (STAT6-IP) to bind to and inhibit downstream activation of STAT6-dependent pathways that mediate allergic inflammation. STAT6 is a key transcription factor involved in Type 2-driven immunopathology and this peptide potently reduces lung inflammation in murine models of both Type 2-biased allergy and infection. It is not currently known if the use of STAT6-IP to prevent a maladaptive immune response in the lung will modulate protective Type 2 immune responses at distal sites. In this work, we investigate whether the 'standard' STAT6-IP treatment for respiratory allergy (intranasal (IN) delivery) can reduce protective Type 2 immunity in response to infection with the gastrointestinal nematode *Heligmosomoides polygyrus bakeri* (*Hpb*). We report that neither IN nor intraperitoneal (IP) delivery of STAT6-IP reduces the primary host response to *Hpb*. In a more extended exposure model (ie: IP delivery of STAT6-IP every other day for two weeks) a transient reduction in serum IgG1 was observed. Our findings suggest that STAT6-IP, given as a short-term immunomodulator in allergic airways disease, is unlikely to alter protective Type 2 immunity in the gastrointestinal tract.

3.3. INTRODUCTION

Allergic airway diseases, including asthma and allergic rhinitis, currently affect 5-10% of the world's population (1). Current treatments for asthma, including β-agonists and corticosteroids, primarily target the symptoms of asthma but do not address the underlying immunologic abnormalities and are less effective in severe cases (2). Allergic airway diseases commonly arise as a result of aberrant activation of Type 2 immunity in the lung associated with increases in interleukin (IL)-4 and IL-13, both of which activate the STAT6 transcription factor (2). Developing therapeutics that target IL-4 and/or IL-13, their receptors, and/or downstream signalling molecules is therefore of great interest.

STAT6 inhibitory peptide (STAT6-IP-PTD4) is a chimeric peptide, containing a phosphotyrosine sequence, conjugated to a protein-transduction domain termed PTD4, developed to bind the SH2 domain of STAT6 and thereby block STAT6-mediated gene transcription in a dominant-negative fashion (3). A negative control peptide, STAT6-CP, in which the phosphotyrosine was replaced by a phenylalanine residue, was also developed (3). STAT6-IP PTD4 (but not STAT6-CP) is protective in Type 2-biased murine models of ragweed- and OVA-induced allergic airway inflammation, reducing eosinophilia in bronchoalveolar lavage fluid and decreasing allergen-specific production of IL-4 and IL-13 by cultured splenocytes (3, 4). In addition, when young mice receive STAT6-IP at the time of exposure to Type 2-deviating respiratory syncytial virus (RSV) antigens, either in the form of a formalin-inactivated vaccine or by infection, they are protected from subsequent immunopathology upon re-exposure to the same antigens (eg: collagen deposition, airway hyperresponsiveness, eosinophil influx) (5, 6). Another STAT6-IP candidate, termed STAT6-IP Arg9 (whereby the phosphotyrosine containing portion

that was designed to target STAT6 was coupled to the Arg9 protein transduction domain) was also tested as a possible candidate for improved cell-penetrating ability compared to the PTD4 motif.

To date, modulation of immune responses following STAT6-IP application has been assessed only following intranasal (IN) administration and only using models in which Type 2 immune responses mediate local (ie: lung) immunopathology in the lungs. Not all Type 2 responses are maladaptive, however: for example, immunity to many helminth parasites is critically dependent on Type 2 immunity. If STAT6-IP is ever to be used as a therapeutic agent to prevent or modulate early-life allergies and asthma, it is important to determine whether or not such use has the potential to reduce protective Type 2 immune responses at distal sites. Therefore, our objective in this work is to evaluate possible STAT6-IP modulation of protective Type 2 immunity using the enteric nematode, *Heligmosomoides polygyrus bakeri* (*Hpb*).

Hpb is a murine helminth commonly used to assess host-parasite relationships and Type 2 immunity. In primary infection of Balb/c mice, *Hpb* larvae delivered orally penetrate the intestinal mucosa where they mature into adult worms. Adults are first detectable in the lumen of the small intestine of the host seven days post-infection (dpi) where they establish a chronic infection and remain for several weeks (7). Innate effector cells such as macrophages and neutrophils induce granuloma (or foci) formation at the site of larval maturation (8). Chronic *Hpb* infection is maintained by adult worm excretory/secretory (HES) products that induce myeloid-derived suppressor cells (MDSCs) (9, 10).

We report that IN delivery of STAT6-IP at the time of primary *Hpb* infection does not modulate the formation of granulomas or the levels of anti-worm-specific IgG1 antibodies. Paradoxically, a modest *reduction* in adult worm burden and parasite fecundity was observed in STAT6-IP treated animals at 28-days post-primary infection. No differences in host responses

were observed when STAT6-IP was delivered intraperitoneally (IP). When STAT6-IP was administered IP repeatedly over a two-week period however, adult-worm specific IgG1 was reduced 14 days post-infection. Ultimately, the results from this study suggest that IN administration of STAT6-IP is unlikely to significantly disrupt protective Type 2 immune responses to gastrointestinal helminth infection.

3.4. MATERIALS AND METHODS

Larval stock preparation

To obtain larvae for experiments, 6-8-week-old male Balb/c mice, originally purchased from Charles-River (St-Constant, QC) and bred in house, were inoculated by gavage with 200 *Hpb* L3 stage larvae. 14 days post-infection, fecal pellets were collected after placing mice in cages with moist paper towel for six hours. Animal studies were approved by the McGill University Animal Care Committee and were performed following the guidelines of the Canadian Council on Animal Care.

Culture of *Hpb* eggs into L3 infectious larvae has been described previously (7). Briefly, moistened fecal pellets were blended into a paste and placed in the center of round filter paper (Whattman, Mississauga, ON) for 7-10 days at 25°C. The edges of the filter paper were kept clean of feces and the filter paper was kept moist via drop-wise addition of approximately 2mL sterile water every other day. Following the incubation period, larvae were collected in a 50mL Falcon tube by washing the clean edges of the filter paper with sterile water. Viable larvae were counted and stored up to six months at 4°C in sterile water (7). Prior to infection, larval counts were performed and the parasites were kept on ice.

Peptides

STAT6-IP PTD4, STAT6-IP Arg9, and STAT6-CP were synthesized by Ontores, China and designed as described previously by Dr. Fixman and colleagues (3). Peptides were stored at -80°C in normal saline until used.

Hpb infection

Female Balb/c mice (6-8 weeks old), were inoculated with 200 L3 *Hpb* larvae via gavage. Mice in the "IN groups" were treated with phosphate-buffered saline (PBS) (Wisent, QC), STAT6-IP PTD4 ([100μg] Ontores), or STAT6-IP Arg9 ([100μg] Ontores, or STAT6-CP PTD4([100μg] Ontores), delivered in a 0.03mL volume, following brief isoflurane anesthesia. Mice in the "IP groups" were treated with the same quantity of peptide, delivered in a 0.2mL volume. Peptides (or control PBS) were delivered 24h before, the same day, and 24h after primary *Hpb* infection (see Figure 1A for a timeline). In the repeated delivery model (see Figure 1 B for a timeline), STAT6-IP was delivered IP every other day for 13 days. Mice were euthanized at either 14 or 28 dpi when blood was collected and serum was stored at -20°C for analysis of IgG1 levels. Intestinal wall granulomas were quantified by visual inspection under a dissecting microscope (Zeiss, Oberkochen, Germany). Quantification of adult worms was performed as described previously (7). Briefly, with the help of a dissection microscope, adult worms were teased from the intestinal lumen using forceps (Fine Science Tools, North Vancouver, BC) and quantified by visual inspection.

*Adult worm homogenate (*AWH*)*

Preparation of AWH has been described previously (7). Briefly, adult worms were removed from the intestines of infected mice 14 days post-infection (dpi), and washed ten times at room temperature (RT) with PBS to remove eggs and intestinal debris. Worms were allowed to sediment by gravity each time before the next wash. Worms were transferred to a 7mL Dounce-style glass tissue grinder (Wheaton, Millville, NJ) and homogenized in 1mL PBS manually for ten minutes at RT. The homogenate was centrifuged for ten minutes at 800xg at RT and supernatants

were collected to obtain AWH. The concentration of protein was quantified using the Pierce bicinchoninic acid assay according to manufacturer's instructions (Thermo Scientific, Ville Mont-Royal, QC) and AWH was stored at -80°C prior to use.

Anti-AWH IgG1/IgG2a ELISAs

Antibody ELISAs were performed according to a protocol modified from Thermo-Scientific. Briefly, 96-well U-bottom plates (Greiner, Bio One) were incubated at 4°C overnight with 1ug/mL AWH or with a purified IgG1 or IgG2a, both diluted in carbonate-bicarbonate buffer for the standard curve (Sigma-Aldrich, St. Louis, MO). Following overnight incubation, wells were washed with wash buffer (PBS + 0.05% Tween 20) and blocked for one hour with PBS + 2% bovine serum albumin (Sigma-Aldrich) at 37°C. Serum samples (50µL, 1:12800 dilution, previously heat inactivated at 56°C for 30 minutes) were then added to duplicate wells. Following a one hour incubation at 37°C, wells were washed six times with wash buffer. Plates were incubated for 30 minutes with 0.075mL of anti-IgG1-HRP or 0.075µL of anti-IgG2a-HRP diluted in carbonate-bicarbonate buffer for a dilution of 1:20000 and 1:10000 respectively. After washing wells as above, .01mL of TMB (Sigma-Aldrich) was applied to wells for fifteen minutes 0.05mL of sulfuric acid (Sigma-Aldrich) were used as a stop solution and ELISAs were read immediately at an optical density (OD) of 450nm using the EL 800 plate reader (BioTek, Winooski, Vermont). Final concentrations of each antibody were obtained by comparing logODs of samples to a standard curve, taking into account blank wells and dilution factors.

Statistical Analysis

Statistical significance was assessed using a one-way ANOVA with Prism software (Version 6; GraphPad, La Jolla, CA). Values were removed if they were considered outliers by the Grubb's Test (GraphPad, La Jolla, CA). Differences were considered significant when $p < 0.05$.

3.5. RESULTS

Short-term STAT6-IP delivery IN has no major impact on primary *Hpb* infection

Short-term IN treatment of mice with STAT6-IP at the time of *Hpb* infection (Fig. 1A) had no significant impact on adult worm burden, granuloma formation, parasite fecundity or *Hpb* adult worm-specific IgG1 levels at 14 dpi (Fig. 2). Surprisingly, at 28 dpi, STAT6-IP Arg9 and STAT6-IP PTD4, but not STAT6-CP treated mice, had reduced adult worm burdens compared to PBS-treated mice (Fig. 3A). In accordance with reduced worm burden, only STAT6-IP Arg9 treated mice had significantly reduced parasite fecundity compared to PBS-treated animals (Fig. 3C). While not statistically significant, the numbers of granulomas persisting at 28 days were slightly higher than the PBS animals in both the STAT6-IP PTD4 and STAT6-IP Arg9 groups and slightly lower in the STAT6-CP PTD4 group (Fig. 3B). Similar to 14 dpi outcomes, results were similar among groups for anti-AWH IgG1 titres (Fig. 3D). Adult-worm specific IgG2a was not detectable at either time point in any group (data not shown). Overall, reduced adult worm burden and parasite fecundity suggest that IN exposure to STAT6-IP may paradoxically improve infection outcomes (Fig. 3C).

Short-term STAT6-IP delivery IP also has no major impact on primary *Hpb* infection

Based on the effects of STAT6-IP on adult worm burden and parasite fecundity, albeit in the opposite way from originally anticipated, we wondered if intraperitoneal (IP) administration would have more obvious effects (ie: more reliable delivery, possible delivery to more relevant lymphoid tissue). At 14 dpi with or without STAT6-IP administered IP (Fig 1A), no significant differences were detected between groups with regard to worm burden, granuloma levels, parasite fecundity or parasite-specific IgG1 (Fig. 4). IgG2a levels were undetectable for all groups (data not shown).

At 28 dpi, no significant differences were obtained between groups (Fig. 5) although parasite fecundity varied considerably among the PBS and STAT6-IP Arg9 treated groups (Fig. 5C), despite similar adult worm burdens.

Repeated STAT6-IP Arg9 delivery IP results in lower adult-worm specific IgG1 at 14 dpi

Neither short-term regimen of STAT6-IP treatment (IN or IP) had damaging effects on primary *Hpb* infection. Thus, we next assessed whether or not repeated delivery of STAT6-IP would result in an altered host-parasite response (Fig 1B). Even with every other day administration, there were no significant differences in worm burden or granuloma levels between groups at 14 dpi (Fig. 6A, 6B). However, there was a statistically significant reduction in adult-worm specific IgG1 ($p<0.05$) in the STAT6-IP Arg9-treated group compared to the infected control animals (Fig. 6D). This difference was not observed in STAT6-IP PTD4-treated animals or in PBS or STAT6-CP treated animals (Fig. 6C). Even this difference in IgG1 levels disappeared at 28 dpi (Fig. 7C) and adult worm burdens and granuloma levels remained similar between groups (Fig. 7A, B).

3.6. DISCUSSION

In this study, we sought to determine whether or not a novel immunomodulator that may eventually be used in asthmatics to reduce Type 2-biased allergic airways disease could have a negative impact on protective Type 2 responses to enteric helminths. Neither short- nor longer-term treatment with STAT6-IP had any major impact on parasitologic (adult worm, egg burden, parasite fecundity) or host (granuloma formation) responses during primary *Helogmosomoides polygyrus bakeri* infection, changes in adult worm burden following short-term IN exposure and changes to the antibody response kinetics were observed in a repeated-dose model.

The strongest and most consistent result of this work is that short-term intranasal STAT6-IP treatment at the time of initial exposure to *Hpb* infection did not reduce the ability of the infected mice to respond to infection. Indeed, IN STAT6-IP delivery may have slightly improved host response (Figures 3-7). The paradoxical results obtained in this chapter do not mirror host responses in the STAT6$^{-/-}$/Hpb infection model (Chapter 2). Our original hypothesis was that STAT6-IP treatment would result in a phenotype intermediate between STAT6$^{-/-}$ and wild-type animals. To our surprise, we observed that IN STAT6-IP delivery maintained and even *promoted* protective Type 2 immunity during *Hpb* infection (Fig. 3A, C). Although our data do not provide a clear explanation for how IN STAT6-IP might improve host outcomes to *Hpb* infection, because there is a common mucosal immune system, it is possible that IN STAT6-IP administration alters worm antigen presentation in the lungs with subsequent T cell migration to the gastrointestinal tract leading to more protective host responses during primary infection (11). In addition, eosinophils, which are found within Peyer's patches that contribute to intestinal homeostasis and IgA production; ILC2s, which are early sources of Th2 cytokines; and neutrophils, which are the first mediators of granuloma formation, all operate via STAT6-independent mechanisms (12).

While STAT6-IP has been shown to reduce airway eosinophilia, and preliminary data suggest that STAT6-IP might modulate ILC2 populations in the lung (data not shown), our findings provide evidence that STAT6-IP may lead to subtle modulation of immunity, but does not impair protective host responses, to a helminth infection localized to the gastrointestinal tract. One additional reason that may explain the apparent promotion of worm expulsion at 28 dpi is the relatively elevated worm burden among PBS-treated mice (Fig. 3A). Our findings in Chapter 2 (Fig. 2A) and Fig. 5, 7 clearly demonstrate a decrease in adult worm burden over time suggesting an abnormal trend in Figure 3A. Thus, further experimental replicates are needed to confirm the relationship between IN STAT6-IP exposure and adult worm burdens.

To address the possibility that insufficient peptide delivery was responsible for the lack of effect, we administered STAT6-IP nine times over a fourteen-day period following primary infection (Fig. 1B). Although a modest reduction in IgG1 levels was observed at 14 dpi, no differences were seen 28 dpi suggesting a transient effect of the inhibitory peptide on antibody kinetics. These results are consistent with the STAT6-IP impact in the FI-RSV model since no differences in serum IgG1 were seen at later time points in either model (28 days following *Hpb* infection and 32 days post- FI-RSV inoculation) (5). Nevertheless, in the FI-RSV model, we reported an increase in serum anti-RSV IgG2a and a change in the Th1/Th2 balance in STAT6-IP treated animals after RSV challenge (5). Consistent with a prior report that *Hpb* rarely induces an IgG2a response (13), we saw no AWH-IgG2a and therefore no change in the balance of IgG1/IgG2a titres with STAT6-IP treatment. One reason for reduced antibody responses among the STAT6-IP Arg9 group compared to the PBS-treated group may be due to differences in adult worm burden (60 worms compared to 100, although not statistically significant). We suspect that the intensity of antibody titres does not mirror adult worm burden since all groups have increased

antibody responses irrespective of worm burden by 28 dpi (Fig. 8C). Overall, our findings provide evidence that even more prolonged delivery of STAT6-IP did not modulate host responses either early or late in primary *Hpb* infection. Studies investigating whether more prolonged exposure to STAT6-IP at the time of primary *Hpb* infection will alter immune responses in secondary *Hpb* infection are currently underway.

The prior work with STAT6-IP in the FI-RSV model is particularly interesting for the current studies. In both cases, the hypothesis was that STAT6-IP would be able to modulate immune responses 'at a distance'. In the FI-RSV model, STAT6-IP administered IN was able to change the balance of subsequent Th1/Th2 responses to the viral antigens first seen in the hind limb. In stark contrast, neither IN nor IP administration of STAT6-IP significantly changed host responses during primary *Hpb* infection in the gut. Of course, these models are dissimilar in many ways i) the organisms themselves are very different (simple microscopic-sized virus versus complex, macroscopic worm) ii) the duration of infection is very different (transient in the case of RSV versus chronic in *Hpb*) iii) the location of infectious challenge was the lung for RSV (same site as STAT6-IP administration) versus the gut for *Hpb* (distant from STAT6-IP administration site) and iv) the pattern of the infectious challenge also differed between models (vaccination-rest-challenge in RSV versus continuous infection with larvae-adult in *Hpb*. Any (or all) of these factors may have contributed to observed ability of STAT6-IP to modulate host responses at a distance. In *Hpb* infection for example, host responses are characterized by the induction of myeloid derived suppressor cells (MDSCs) that reduce GATA3+ Th2 effector cells via production of helminth excretory/secretory (HES) products and induction of tolerogenic dendritic cells (9, 14). Therefore, while STAT6-IP may effectively target aberrant Type 2 effector responses in allergy and infection

models in the lung, the presence of MDSCs and HES products may be sufficient to dampen host protective responses in the gut, thus negating any reduction of Type 2 immunity attributable to STAT6-IP. In retrospect, it would have been very interesting to include measurement of alternatively activated macrophages (AAMs) in these studies. In several different mouse strains, AAMs in the *Hpb* granulomas have been implicated in worm expulsion and AAM populations in the lung were clearly reduced by STAT6-IP exposure RSV-infected neonates (6, 9). Future studies assessing AAM populations within granulomas may provide a better understanding of the relationship between STAT6-IP delivery and altered host responses at distant sites.

STAT6-CP was designed with the same amino acid sequence as STAT6-IP although the effector domain of STAT6-CP contains a phenylalanine instead of a phosphorylated tyrosine (3). At 28 dpi, following IN delivery, the STAT6-CP treated mice had a reduction in granuloma numbers compared to STAT6-IP treated groups (Fig. 3B). Perhaps due to chance and the relatively small number of animals in this study, it is also possible that STAT6-CP has 'off-target' effects. Specifically, we suspect that STAT6-CP may, in some situations, act as an anti-microbial peptide. These peptides exert a wide range of both pro- and anti- inflammatory effects, including wound healing and mediating apoptosis (15). It is therefore at least plausible that STAT6-CP, operating as an anti-microbial peptide, may have promoted the involution of granulomas leading to the significant reduction in granuloma numbers observed at 28 dpi compared to the STAT6-IP animals. However, STAT6-CP did not influence total adult worm burden, parasite fecundity or IgG1 titres so it is also possible that this finding was due to chance alone.

PTDs derived from HIV-TAT protein (PTD4 in this work) have been used to deliver a range of therapeutic protein and DNA candidates in cancer and arthritis models (16, 17). As a cell-penetrating peptide (CPP), Arg9 is thought to be similar to TAT-derived peptides in some respects

but also destabilizes plasma membranes by creating transient pores in phospholipid bilayers, possibly by interacting with the actin cytoskeleton (18, 19). STAT6-IP has been designed to work with both the PTD4 and Arg9 protein transduction domains. In this work, most of the differences that achieved statistical significance were observed in the STAT6-IP Arg9 treated animals suggesting that the Arg9 peptide may act as a more efficient CPP (20). To date, most of the work with STAT6-IP has been done using the STAT6-IP PTD4 form because of concerns over the use of the more cationic Arg9 peptide in the lungs. Our observations suggest that the Arg9 form of STAT6-IP can be used IN safely for short periods at least, and that further work is needed to optimize STAT6-IP actions in vivo (including construction of a STAT6-CP Arg9).

In conclusion, we report that short-term administration of STAT6-IP, either IN or IP, did not significantly affect the development of protective Type 2 immunity in response to primary infection with the gastrointestinal nematode *Hpb*. Although we observed transient reductions in IgG1 levels after nine-dose IP delivery of STAT6-IP (consistent with our original hypothesis), we also observed modest reductions in worm burden and parasite fecundity 28 dpi after short-term IN treatment (contrary to our original hypothesis). Ultimately, these results suggest that IN STAT6-IP delivery, as might be given to a children and adults to modulate abnormal Th2 responses to allergens or infection, is unlikely to have negative impact on the development of protective Type 2 immune responses to chronic helminth infection in the gut.

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3.8. FIGURES AND LEGENDS

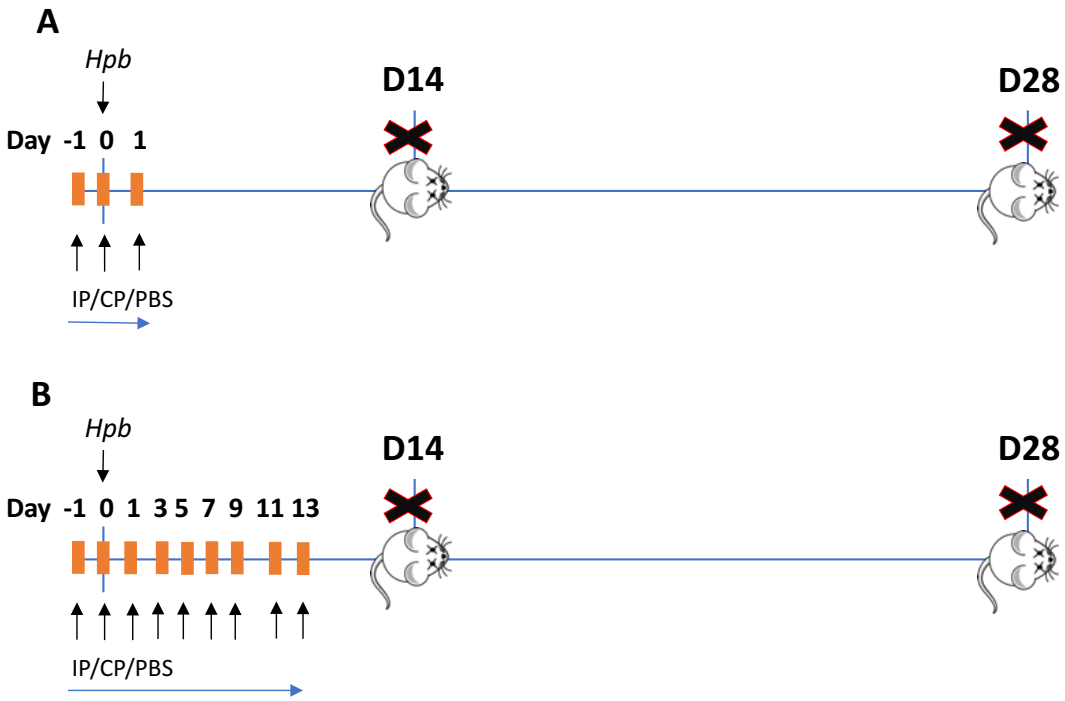

Figure 1. STAT6-IP administration and *Hpb* infection schedule. (A) Wildtype mice were infected with 200 L3 *Hpb* larvae at day 0 of infection. On days -1, 0, 1, mice were treated with either 5mM PBS, STAT6-CP-PTD4, STAT6-IP-PTD4 or STAT6-IP-Arg9 via intranasal (IN) or intraperitoneal (IP) injection. For mice treated via IP injection, an 'infected control' group was included that was not injected intraperitoneally (n=4). Mice were euthanized at 14dpi and 28dpi. Two independent experiments were conducted with a total of 5-10 mice per group for IN treatments (n=5 for STAT6-CP group) and one experiment with a total of 3-4 mice per group for IP treatments. **(B)** Wildtype mice were infected with 200 L3 *Hpb* larvae on day 0. On days -1, 0, 1, 3, 5, 7, 9, 11, & 13, mice were treated with either 100ug of, STAT6-CP PTD4, STAT6-IP PTD4, STAT6-IP Arg9, or PBS via IP injection. An 'infected control' group was infected with *Hpb* without any treatment. Five mice per group were euthanized at 14dpi and 28dpi and the experiment was repeated once. Outcomes were assessed by a one-way ANOVA, and Tukey's post-hoc test to define significant differences between groups.

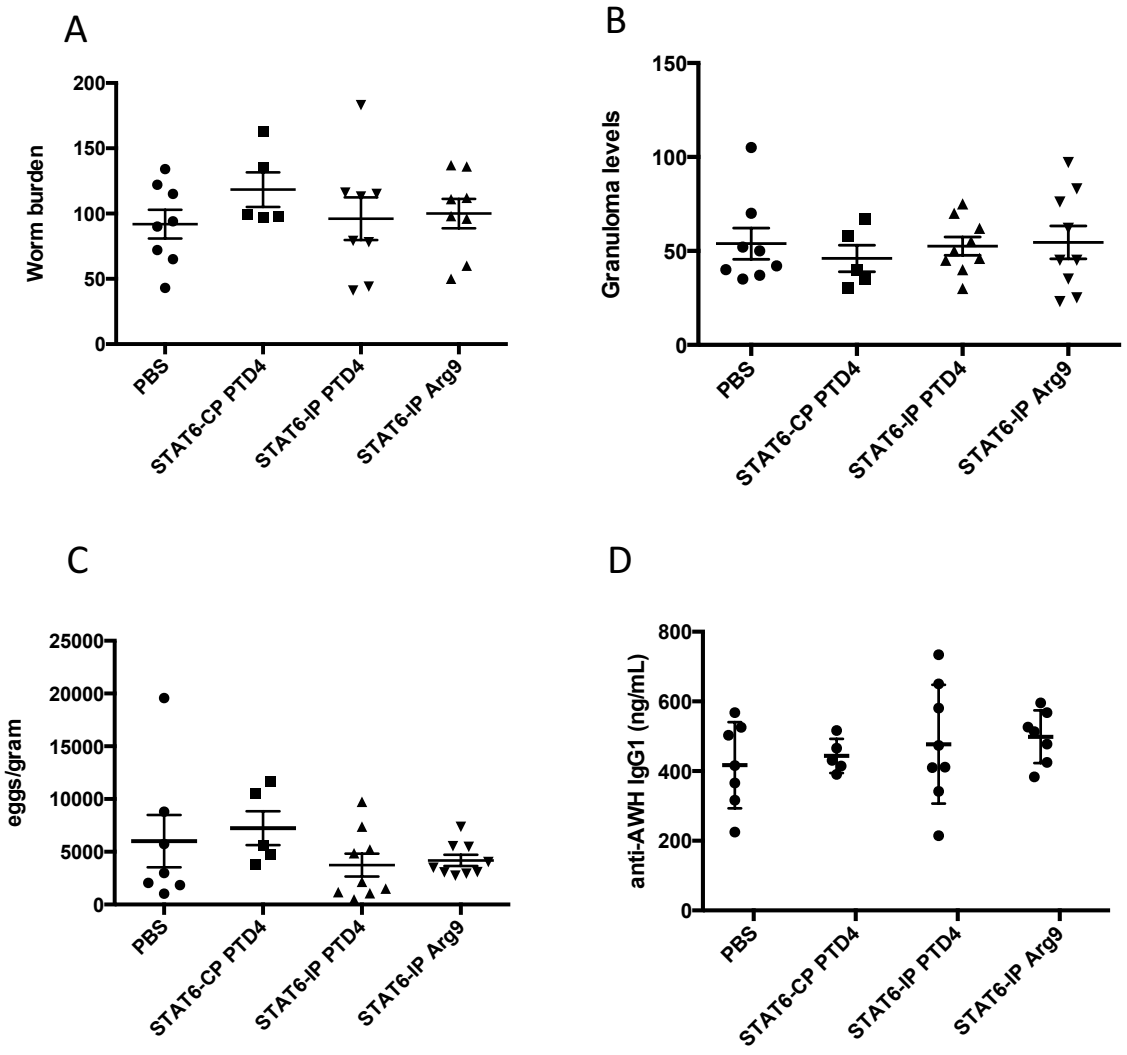

Figure 2. Three-dose intranasal STAT6-IP treatment does not alter adult worm burden, number of intestinal granulomas, parasite fecundity and anti-*AWH* IgG1 titres 14-days post *Hpb* infection. Mice were infected with 200 L3 stage *Hpb* larvae and treated IN with PBS or cell penetrating peptides as described in Fig. 1A. At 14dpi, **(A)** the number of adult worms, **(B)** the number of intestinal granulomas, **(C)** parasite fecundity as measured by eggs/gram and **(D)** anti-AWH IgG1 titres were quantified. Data are presented as means ± SEM from a combination of two independent experiments using five mice per group in each experiment. Outliers were removed following the Grubb's Outlier Test. Outcomes for STAT6-CP were measured once with n=5. Outcomes were assessed by a one-way ANOVA.

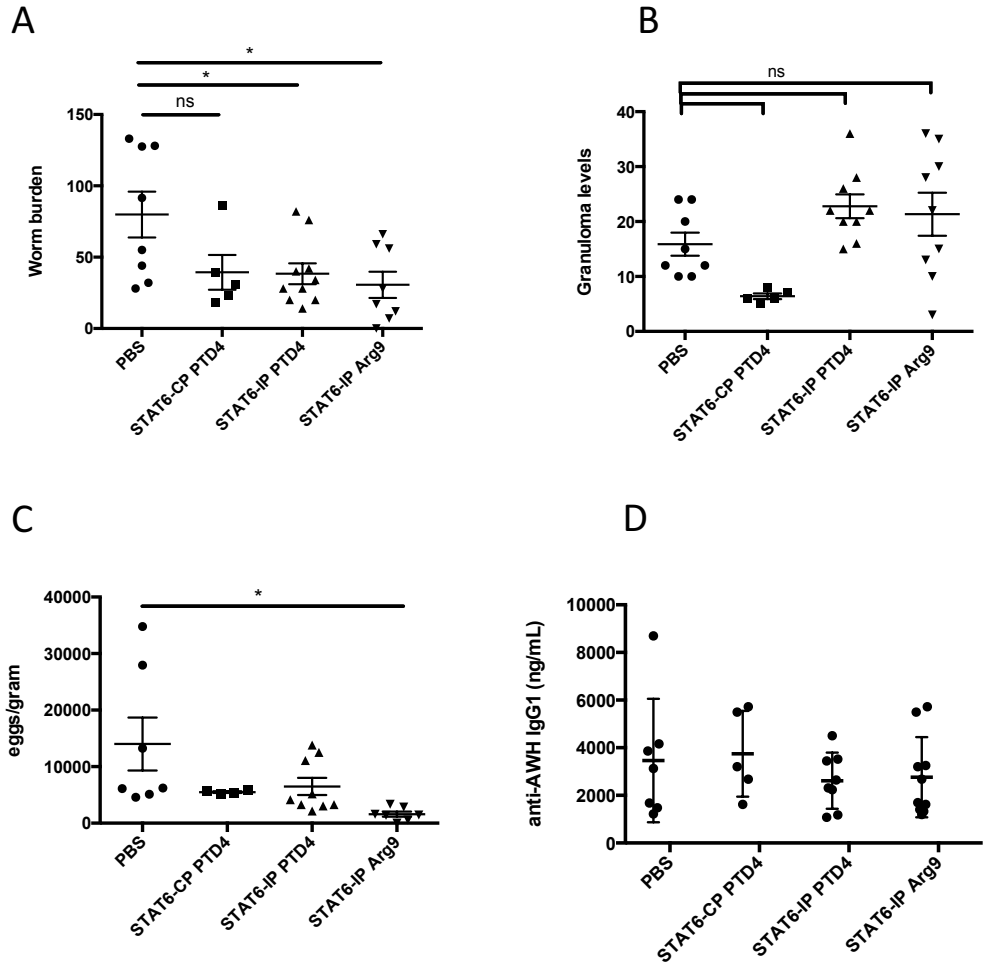

Figure 3. Intranasal STAT6-IP treatment does not alter the number of intestinal granulomas and anti-*AWH* IgG1 titres but STAT6-IP treatment reduces adult worm burden and STAT6-IP Arg9 treatment reduces parasite fecundity 28-days post *Hpb* infection. Mice were infected with 200 L3 stage *Hpb* larvae and treated IN with PBS or cell penetrating peptides as described in Fig. 1A. At 28dpi, **(A)** the number of adult worms, **(B)** the number of intestinal granulomas, **(C)** parasite fecundity as measured by eggs/gram and **(D)** anti-*AWH* IgG1 titres were quantified. Data are presented as means ± SEM from a combination of two independent experiments using five mice per group in each experiment. Outcomes for STAT6-CP were measured once with five mice per group. Outliers were removed following the Grubb's Outlier Test. Outcomes were assessed by a one-way ANOVA followed by Tukey's post-hoc test. *p<0.05, ns not-significant.

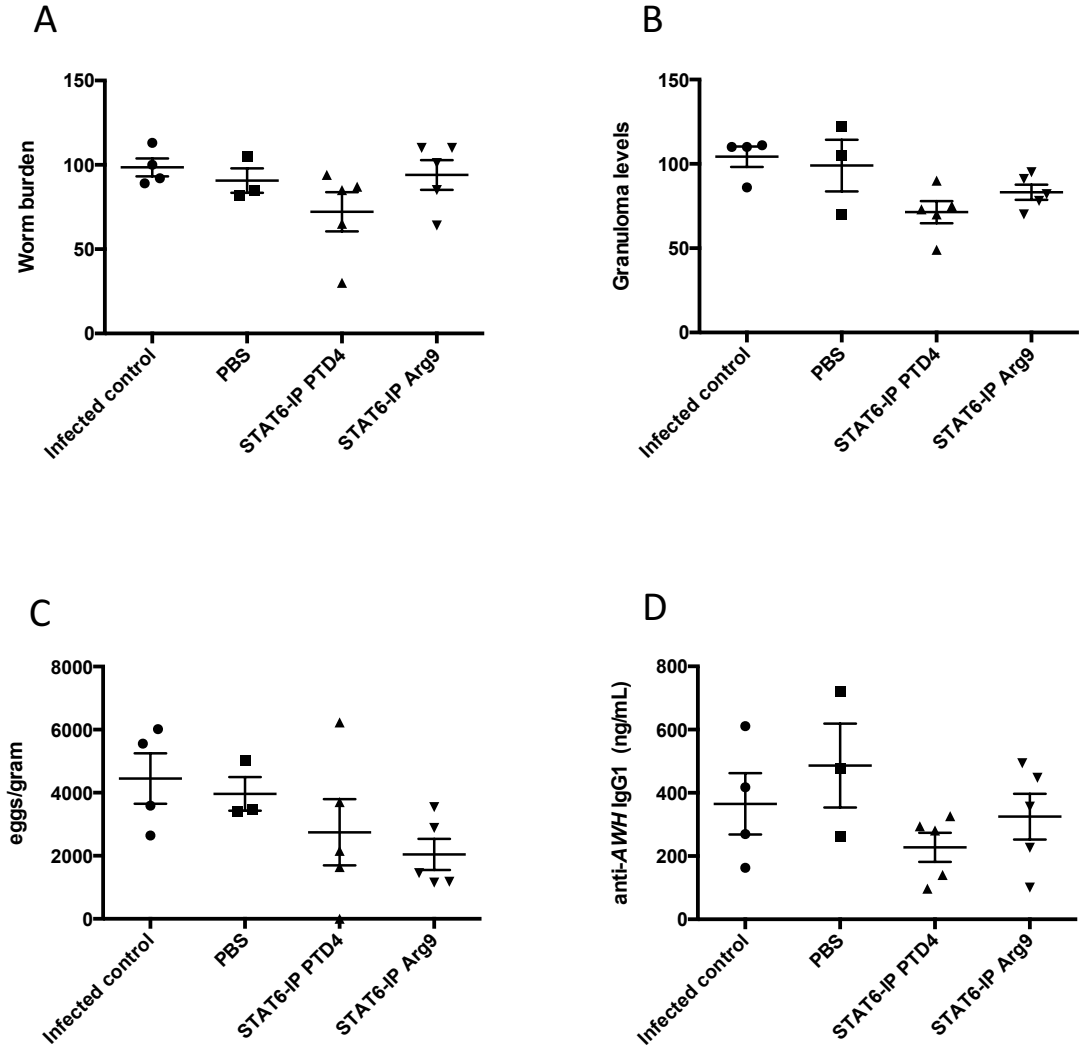

Figure 4. Intraperitoneal STAT6-IP treatment does not alter adult worm burden, number of intestinal granulomas, parasite fecundity and anti-*AWH* IgG1 titres 14-days post *Hpb* infection. Mice were infected with 200 L3 stage *Hpb* larvae and treated IP with with PBS or cell penetrating peptides as described in Fig. 1A. An additional 'infected control' group was included where mice were infected, in the absence of IP injection. At 14 dpi, **(A)** the number of adult worms, **(B)** the number of intestinal granulomas, **(C)** parasite fecundity as measured by eggs/gram and **(D)** anti-*AWH* IgG1 titres were quantified. Data are presented as means ± SEM from one experiment using three to four mice per group. Outcomes were assessed by a one-way ANOVA.

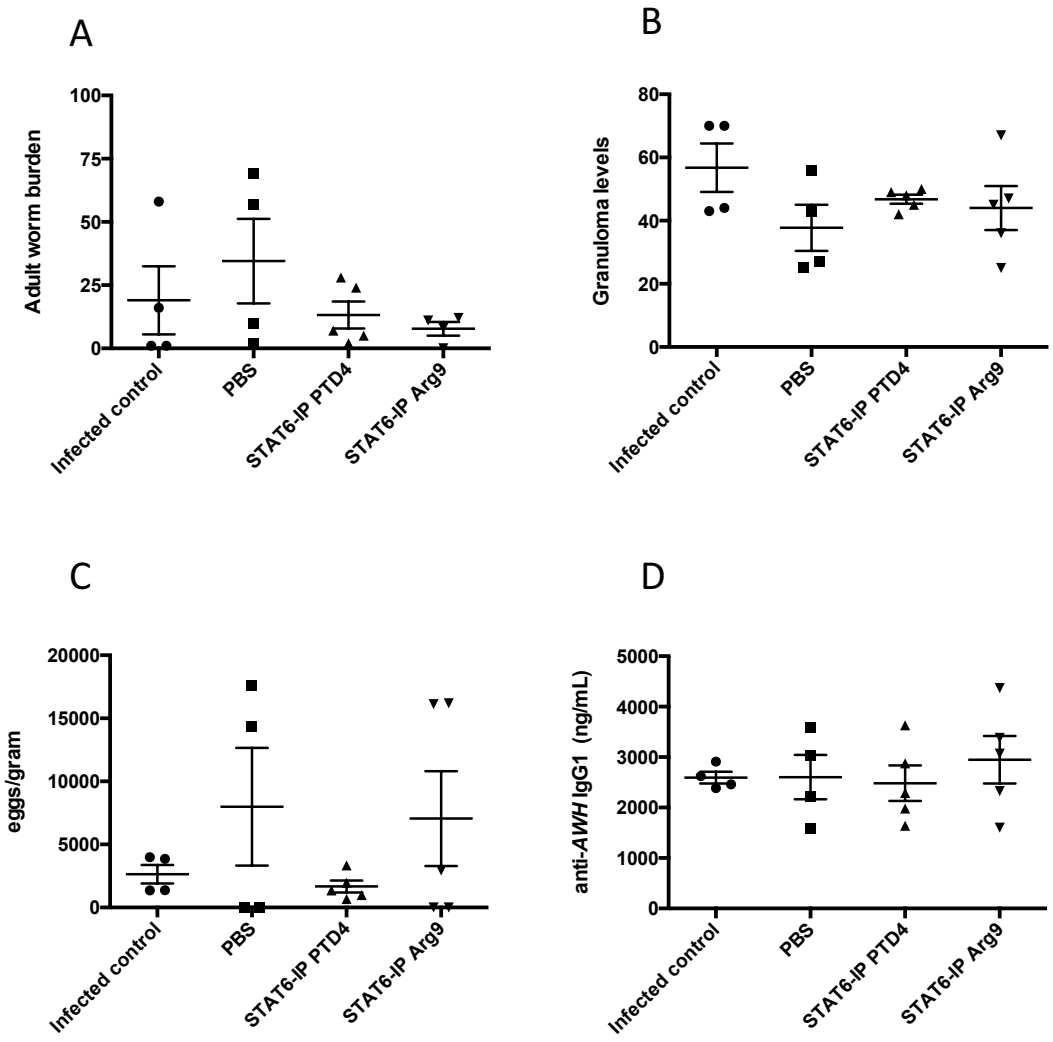

Figure 5. Intraperitoneal STAT6-IP treatment does not alter adult worm burden, number of intestinal granulomas, parasite fecundity and anti-*AWH* IgG1 titres 28-days post *Hpb* infection. Mice were infected with 200 L3 stage *Hpb* larvae and treated IP with with PBS or cell penetrating peptides as described in Fig. 1A. 28 dpi, (**A)** the number of adult worms, **(B)** the number of intestinal granulomas, **(C)** parasite fecundity as measured by eggs/gram and **(D)** anti-*AWH* IgG1 titres were assessed. Data are presented as means ± SEM from one independent experiment using three to four mice per group. Outcomes were assessed by a one-way ANOVA.

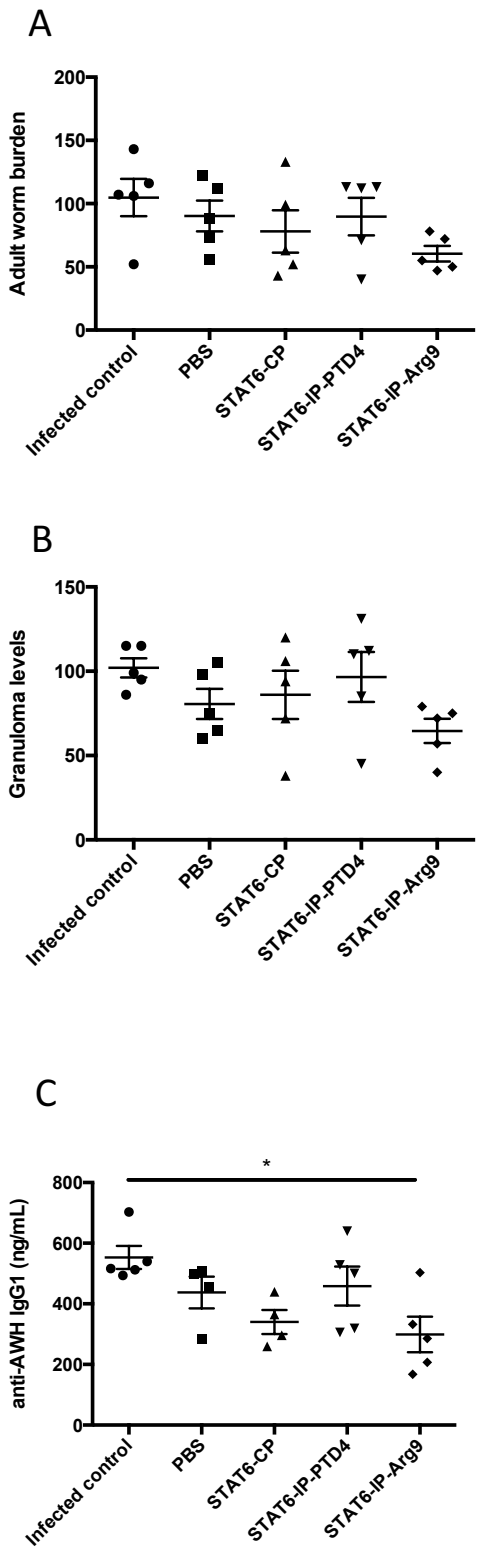

Figure 6. Repeated intraperitoneal STAT6-IP delivery does not alter adult worm burden, number of intestinal granulomas, or parasite fecundity but STAT6-IP-Arg9 treatment reduces anti-*AWH* IgG1 titres 14-days post *Hpb* infection. Mice were infected with 200 L3 stage *Hpb* larvae and treated IP with with PBS or cell penetrating peptides as described in Fig. 1B. At 14dpi, **(A)** the number of adult worms, **(B)** the number of intestinal granulomas and **(C)** anti-*AWH* IgG1 titres were quantified. Data are presented as means ± SEM from one experiment using five mice per group. Outcomes were assessed by a one-way ANOVA followed by Tukey's post-hoc test. *p<0.05, ns not-significant.

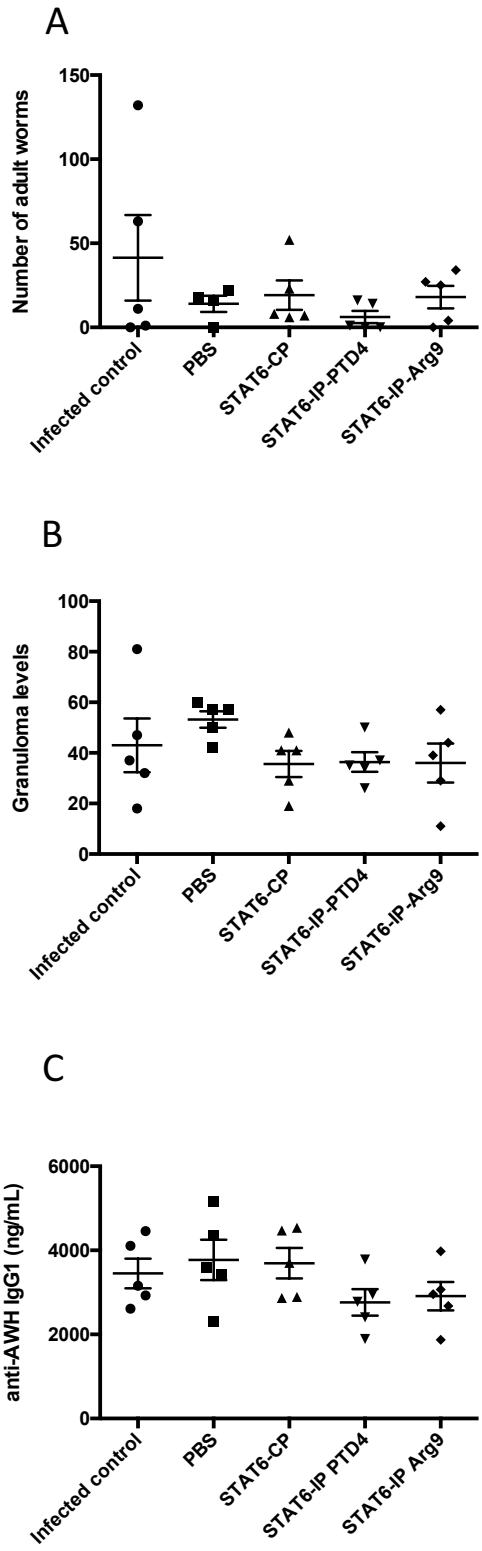

Figure 7. Repeated intraperitoneal STAT6-IP treatment does not alter adult worm burden, number of intestinal granulomas, parasite fecundity and anti-*AWH* IgG1 titres 28-days post *Hpb* infection. Mice were infected with 200 L3 stage *Hpb* larvae and treated IP with PBS or cell-penetrating peptides according to Fig. 1B. 14 dpi **(A)** the number of adult worms, **(B)** the number of intestinal granulomas and **(C)** anti-*AWH* IgG1 titres were assessed. Data are presented as means ± SEM from one experiment using five mice per group. Outcomes were assessed by a one-way ANOVA.

4.1. PREFACE TO CHAPTER 4

In Chapter 3, we demonstrate that STAT6-IP delivery at the time of primary infection does not significantly reduce host immunity to *Hpb* infection.

Secondary *Hpb* infection, that is, re-infection following natural expulsion of adult worms or pharmacological treatment, rapidly clears infection. Specifically, secondary *Hpb* infection inhibits larval maturation/adult worm transit through the intestinal wall via killing by alternatively activated macrophages (AAMs) that produce Arginase, Ym1 and Fizz-1 in a STAT6-dependent manner.

We show in Chapter 4 that three-dose delivery via IN or IP routes is insufficient to modulate helminth responses to secondary *Hpb* infection. Studies are underway to test whether repeated peptide delivery at the time of primary infection can modulate secondary *Hpb* infection. The findings presented here provide evidence that STAT6-IP administered early during infection, does not modulate STAT6-dependent protective Type 2 immunity during gastrointestinal nematode infection.

CHAPTER 4

STAT6 inhibitory peptide administered at the time of primary *Heligmosomoides polygyrus bakeri* infection did not modulate Type 2 immunity during secondary infection

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4.2. ABSTRACT

Chronic allergic diseases of the airways, mediated by aberrant activation of Type 2 immune responses, can neither be cured nor prevented. Current treatments such as corticosteroids and β-agonists only control the symptoms of the disease; they do not address the immunologic underpinnings of the disease. One key transcription factor involved in mediating allergic inflammation is Signal Transducer and Activator of Transcription 6 (STAT6). Dr. Fixman has designed a novel chimeric peptide termed STAT6 inhibitory peptide (STAT6-IP) that can reduce Th2-biased allergic inflammation in models of chronic and acute allergy as well as in respiratory virus infection. Here we report that peptide treatment, i.e. three-dose application of STAT6-IP at the time of initial antigen exposure, does not impair Type 2 immune responses associated with protection from infection with a gastrointestinal nematode, in this case, the murine helminth, from *Heligmosomoides polygyrus bakeri*. Specifically, STAT6-IP exposure at the time of primary infection does not modulate worm burden and granuloma levels 14 days post-secondary infection. However, intraperitoneal administration of STAT6-IP Arg9 reduced serum IgG1 titres compared to PBS-treated mice and intranasal STAT6-IP PTD4 delivery maintained alternatively activated macrophage populations within granulomas compared to PBS- and STAT6-IP Arg9 treated groups 14 days post-secondary infection. Our results provide reassurance that STAT6-IP could potentially be used to treat allergic airways diseases without interfering with protective Type 2 immunity within the gastrointestinal tract.

4.3. INTRODUCTION

Allergic airway diseases (ie: asthma) affects 235-300 million people worldwide (1). Present asthma treatments do not target the induction of aberrant Type 2 immunity in the lungs nor do they inhibit the positive feedback loops that mediate pulmonary immunopathology (2). Developing therapeutics that target mediators in Type 2 immunity, such as IL-4 and/or IL-13, their receptors, and/or downstream signalling molecules is therefore of great interest.

STAT6-inhibitory peptide (STAT6-IP) is a cell-penetrating peptide developed to transiently block the STAT6 transcription factor and its downstream processes linked to Type 2 immunity (3). Two STAT6-IPs have been designed based on differences to their protein transduction domain: STAT6-IP PTD4 used in previous studies, and a novel STAT6-IP Arg9 (3). STAT6 control peptide (STAT6-CP) was designed as a control for STAT6-IP and has been previously described (3). STAT6-IP, but not STAT6-CP, has been shown to protect against Th2-biased models of allergic airway inflammation in which it reduces eosinophilia in the bronchoalveolar lavage fluid and allergen-induced production of IL-4 and IL-13 (eg: ovalbumin (OVA), ragweed). Early-life treatment with STAT6-IP can also reduce CD206+ alternatively activated macrophage (AAM) populations in the lungs of neonatal mice infected with respiratory syncytial virus (RSV) (3-6). As a result, we are interested in developing STAT6-IP as a potential therapeutic for allergic airways diseases in humans.

To date, modulation of immune responses following STAT6-IP application have been assessed only following intranasal (IN) administration and only in models of aberrant Type 2 immunopathology in the lungs. In Chapter 3, we described studies investigating whether STAT6-IP can reduce protective Type 2 immune responses at distal sites in primary *Heligmosomoides polygyrus bakeri (Hpb)* infection. We found that neither short-term (three doses intranasally (IN)

or intraperitoneally (IP)) nor longer-term treatment (nine doses IP) were sufficient to significantly alter host immunity to this strictly gastrointestinal helminth.

Hpb is a murine helminth commonly used to assess host-parasite relationships and Type 2 immunity. While mice infected once with *Hpb* often remain chronically infected, either natural infection resolution or treatment with an anti-helminthic agent substantially alters the host-parasite interaction at the next exposure. Upon re-exposure to *Hpb*, previously-infected and cured mice rapidly control the infection by killing the larvae mediated primarily by memory Th2 cells and recruitment of alternatively activated macrophages (AAMs) to the granulomas (7). AAMs, which are characterized by the production and/or expression of arginase, Fizz-1 and Ym-1, are thought to kill the larvae as they mature into adult worms in the granulomas (7). In mice completely lacking STAT6 (ie: $STAT6^{-/-}$ mice), AAM killing of larvae is largely absent, consistent with the wealth of literature showing that STAT6 is a potent inducer of both Th2 and AAM differentiation (2, 7). Given that intranasal (IN) delivery of STAT6-IP reduces Type 2 immunity in the lung and has the potential to prevent the *induction* of Type 2 immunity at distal sites, we sought to investigate whether IN STAT6-IP delivery, at the time of primary *Hpb* infection, would reduce the Type 2 immunity required for protection from secondary *Hpb* infection (eg: type 2 cytokine production, granuloma AAMs) (3, 4, 6, 8).

In this study, we report that IN delivery of STAT6-IP at the time of primary *Hpb* infection did not alter protective responses to re-infection. Granuloma formation and parasite killing were intact and normal levels of anti-worm-specific IgG1 antibodies were produced at 14 days post-secondary infection. In addition, no changes were observed in the degree of inflammation assessed by hematoxylin and eosin staining. However, STAT6-IP-PTD4-treated mice may induce persistence of AAMs within granulomas, as assessed by immunofluorescence. When STAT6-IP

was delivered via the intraperitoneal (IP) route, the outcomes were similar, although adult-worm-specific IgG1 was slightly reduced. These results suggest that STAT6-IP has the potential to modulate the humoural response to *Hpb* and promote AAM persistence, but this effect does not significantly affect the outcome of infection. Ultimately, the results of this study suggst that IN delivery of STAT6-IP as a therapeutic for maladaptive Th2-biased airway inflammation is unlikely to disrupt protective Type 2 immune responses needed to clear helminth infections in the gastrointestinal tract.

4.4. MATERIALS AND METHODS

Larval stock preparation

To obtain larvae for experiments, 6-8-week-old male Balb/c mice, purchased from Charles-River (St-Constant, QC), were inoculated by gavage with 200 *Hpb* L3 stage larvae. 14 days post-infection, fecal pellets were collected after placing mice in cages with moist paper towel for six hours. Animal studies were approved by the McGill University Animal Care Committee and were performed following the guidelines of the Canadian Council on Animal Care.

Culture of *Hpb* eggs into L3 infectious larvae has been described previously (9). Briefly, moistened fecal pellets were blended into a paste and placed in the center of round filter paper (Whattman, Mississauga, ON) for 7-10 days at 25°C. The edges of the filter paper were kept clean of feces and the filter paper was kept moist via drop-wise addition of approximately 2mL sterile water every other day. Following the incubation period, larvae were collected in a 50mL Falcon tube by washing the clean edges of the filter paper with sterile water. Viable larvae were counted and stored up to six months at 4°C (9). Prior to infection, larval counts were performed and the parasites were kept on ice.

Peptides

STAT6-IP PTD4, STAT6-IP Arg9, and STAT6-CP PTD4 were synthesized by Ontores, China as described previously (3). STAT6-CP PTD4 was composed with the same peptide sequence as STAT6-IP, however the phosphorylated tyrosine residue in STAT6-IP was replaced with a phenylalanine residue. Peptides were stored at -80°C in normal saline until used.

Hpb infection

Female wild-type Balb/c mice (6-8 weeks old), were inoculated with 200 L3 *Hpb* larvae via gavage. Mice in the "IN groups" were treated with phosphate-buffered saline (PBS) (Wisent, QC), STAT6-IP PTD4 ([100μg] Ontores, China), or STAT6-IP Arg9 ([100μg] Ontores, China), diluted in PBS and delivered in a 0.03mL volume, following brief isoflurane anesthesia. Mice in the "IP groups" were treated with the same quantity of peptide, delivered in a 0.02mL volume. Peptides (or control PBS) were delivered 24 hours before, the same day, and 24 hours after primary *Hpb* infection (see Figure 1 for a timeline). 14 and 16 dpi, mice were drug-treated with 10mg/mL of the anti-helminthic agent pyrantel pamoate (Columbia Laboratories, Lexington, KY), delivered by gavage in a 0.025mL volume. Pyrantel pamoate was prepared in a 1:5 dilution with PBS supplemented with calcium and magnesium (Wisent). Mice were allowed to rest following drug treatment. 35 to 42 days following drug treatment, mice were inoculated with 200 L3 *Hpb* larvae, as above. An additional eight mice were infected as quality control i.e. primary infection. Mice were euthanized 14 dpi, blood was collected and serum was stored at -20°C for analysis of IgG1 levels. Intestinal wall granulomas were counted by visual inspection under a dissecting microscope (Zeiss, Oberkochen, Germany). Counting of adult worms was performed as described previously (9). Briefly, with the help of a dissection microscope, adult worms were teased from the intestinal lumen using forceps (Fine Science Tools, North Vancouver, BC) and quantified by visual inspection.

Adult worm homogenate (AWH)

Preparation of AWH has been described previously (9). Briefly, adult worms were removed from the intestines of WT mice, infected with *Hpb* 14 days prior, and washed ten times

at room temperature with PBS to remove eggs and intestinal debris. Worms were allowed to sediment by gravity each time before the next wash. Worms were transferred to a 7mL Dounce-style glass tissue grinder (Wheaton, Millville, NJ) and homogenized in 1mL PBS manually for ten minutes at room temperature. The homogenate was centrifuged for ten minutes at 800xg at room temperature and supernatants were collected to obtain AWH. The concentration of protein was quantified using the Pierce bicinchoninic acid assay method according to manufacturer's instructions (Thermo Scientific, Ville Mont-Royal, QC) and AWH was stored at -80°C prior to use.

Anti-AWH IgG1 ELISAs

Antibody ELISAs were performed according to a protocol modified from Thermo-Scientific. Briefly, 96-well U-bottom plates (Greiner, Bio One) were incubated at 4°C overnight with 1ug/mL AWH or with a purified IgG1, diluted in carbonate-bicarbonate buffer for the standard curve (Sigma-Aldrich, St. Louis, MO). Following overnight incubation, wells were washed with wash buffer (PBS + 0.05% Tween 20) and blocked for one hour with PBS + 2% bovine serum albumin (Sigma-Aldrich) at 37°C. Serum samples (0.05mL, 1:12800 dilution, previously heat inactivated at 56°C for 30 minutes) were then added to duplicate wells. Following a one hour incubation at 37°C, wells were washed six times with wash buffer. Plates were incubated for 30 minutes with 0.075mL of anti-IgG1-HRP diluted in carbonate-bicarbonate buffer for a dilution of 1:20000. After washing wells as above, 0.1mL of TMB (Sigma-Aldrich) was applied to wells for 15 minutes. 0.05mL of sulfuric acid (Sigma-Aldrich) were used as a stop solution and ELISAs were read immediately at an optical density (OD) of 450nm using the EL 800 plate reader (BioTek, Winooski, Vermont). Final concentrations of each antibody were

obtained by comparing logODs of samples to a standard curve, taking into account blank wells and dilution factors.

Intestinal tissue preparation using 'Swiss-rolling' technique

Swiss rolling of intestinal tissue sections was adapted from Bialkowka et al., 2016 (10). To prepare intestinal tissue sections as a 'Swiss-roll', small intestines were sectioned into ten centimeter portions and cut open longitudinally along the mesenteric line. After dissecting adult worms and counting the number of granulomas, intestinal segments were cut into four sections and placed with the luminal side facing upward on a Petri dish. Wrapping the edge of the proximal end of intestine with a toothpick, segments were 'Swiss-rolled' by rolling segments onto a toothpick using fingers. Forceps were used to slide the Swiss-roll off the toothpick and rolled sections were placed in a tissue-embedding cassette, noting the proximal end of the intestine on the cassette. Intestinal sections were rolled for immunofluorescence and for histology.

Preparation of frozen tissue blocks, sectioning and immunofluorescence

Swiss-rolls, for immunofluorescence, were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), frozen on dry ice-ethanol, and stored at -80°C. 4µm sections were cut from frozen blocks using Leica CM3050S cryostat (Wetzlar, Germany), mounted on positively-charged glass slides (Globe Scientific, Paramus, NJ) and stored at -20°C.

To obtain images for immunofluorescence, staining was performed as follows: slides were fixed with 4% paraformaldehyde (Sigma-Aldrich) for ten minutes and washed twice with PBS for five minutes each time. 0.2M glycine (Sigma-Aldrich) solution was applied to slides for 20 minutes to reduce aldehyde-induced auto fluorescence. Following a five-minute wash step with

PBS, samples were subjected to a 5% fetal-bovine serum (FBS) (Wisent) and 0.2% Triton (Sigma-Aldrich) in PBS for 40 minutes to reduce nonspecific binding and to permeabilize cell membranes. Antibodies were prepared in one percent FBS (Wisent) and slides were incubated overnight at 4°C. Slides from each group were stained with: anti-IL-4Rα-PE (1:40) (BioLegend, San Diego, CA), anti-CD206-FITC (1:100) (BioLegend), and anti-F4/80-Alexa-Fluor-647 (1:100) (BioLegend) or anti-CD4-Alexa-Fluor-647 (1:100) (BioLegend). 1:100 solutions were stained at a concentration of 5μg/mL and 1:40 dilutions were used at 12μg/mL. Following overnight incubation, slides were washed with PBS for five minutes, and then 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Thermo Fisher Scientific, Waltham, MA) was applied for five minutes at a concentration of 1:200 according to the manufacturer's instructions. Slow-fade mounting medium (Life Technologies, Carlsbad, CA) was added drop-wise to cover sections and a coverslip was added and sealed with nail polish. After drying, slides were analyzed using the Zeiss LSM780 Laser Scanning Confocal Microscope (Zeiss). Images were processed using ImageJ (National Institute of Health, USA).

Hematoxylin and eosin (H&E) staining

Following Swiss-rolling, intestines were placed in formalin (Fisher Scientific) in tissue-embedding cassettes. 4μm sections were sectioned using a microtome (Leica, Weltzlar, Germany) and sections mounted as above. To perform H&E staining, slides were heated for 20 minutes at 60°C and then immersed in Xylene (American Chemicals, Raleight, NC) twice for 10 minutes, then immersed in 100% ethanol (Sigma-Aldrich) twice for 5 minutes, once for 3 minutes with 80% ethanol, and once for 3 minutes with 70% ethanol. Slides were rinsed with distilled water for 5 minutes. Slides were immersed in Harris Hematoxylin (Sigma-Aldrich) for ten seconds and left

under soft running distilled water for five minutes. Hematoxylin was prepared with 200mL Harris hematoxylin (Sigma-Aldrich) and with 200mL distilled water. Slides were dipped 10 times in 80% ethanol and then submerged in eosin-phloxine (Sigma-Aldrich) for 1 minute. Eosin-phloxin solution was prepared with 100mL eosin, 10mL Phloxin B, 780mL 95% ethanol and 4mL glacial acetic acid (Fisher-Scientific). Slides were then subjected to 100% percent ethanol for ten minutes. Then, slides were run with 100% ethanol in a squeeze bottle to remove remaining eosin. Washes of 100% ethanol and xylene were subsequently followed, with incubations of 5 minutes each. Sections were fixed with three drops of acrytol (Leica) and allowed to dry and were imaged using Zeiss Axiocam Microscope (Zeiss) at 100x magnification.

Mesenteric lymph node (mLN) cell isolation and flow cytometry analysis

Following euthanasia, mLNs from mice were collected individually in 5mL RPMI 1640 medium (Wisent) supplemented with 2% FBS, and 10 000 International Units of penicillin (Wisent) and 10 000µg/mL streptomycin (Wisent). Organs were drained into a cell strainer and crushed. Cells were rinsed three times with 4mL of RPMI and then spun at 400xg for 5 minutes. After removing the supernatant, cells were re-suspended in 5mL RPMI supplemented with 10% FBS, 10 000 IU penicillin, 10 000µg/mL streptomycin and 1:1000000 β-mercaptoethanol (Sigma-Aldrich). Cells were plated at 0.5million cells/100uL in a 96-well U-bottom plate (Corning, NY) for flow cytometry. Cells were stimulated with phorbol-12-myristate 13-acetate (PMA) (1µg/mL) (Sigma-Aldrich), ionomycin (2µg/mL) (Sigma-Aldrich) and brefeldin A (1µg/mL) for three hours, at 37°C. Cells were washed with PBS and then viability dye at a concentration of 0.1µL dye/1 million cells and incubated for 20 minutes in the dark at 4°C. After a 5 minute spin at 360xg, cells were washed with 200µl PBS + 0.5% FBS 3 times. Next, cells were blocked with Fc block (BD

Life Sciences) at a concentration of 1µL/1e6 cells in PBS + 0.5% FBS for 10 minutes at 4°C in the dark. For extracellular stain, 1µg/mL of anti-CD3-v500 (BD Bioscience), anti-CD4-FITC (eBioscience) and anti-CD8-PerCP5.5 (eBioscience) were applied to cells for 10 minutes at 4°C in the dark. For intracellular staining, cells were fixed by re-suspending cells in Intracellular Fixation Buffer overnight. The following day, permeabilization (Perm) (BD Bioscience, Missauga, ON) buffer diluted in double-distilled water was added to cells, spun at 360xg for 5 minutes and washed with Perm buffer. 2µl/mL of intracellular antibodies (anti-IFNγ-BV650 (BioLegend), anti-IL-13-PE (eBioscience), anti-IL-4-APC (eBioscience)) were applied to cells for 45 minutes in the dark at 4°C. Following incubation, cells were washed once with perm buffer, once with PBS+ 1% FBS and then washed twice with PBS, and finally re-suspended with 100µL of PBS. Gating strategy is presented in Supplementary Figure 1. Samples were analyzed by flow cytometry (LSR Fortessa, BD Biosciences). Data were analyzed using FlowJo software (version 9.6.4.; Tree Star, Inc, Ashland, OR). 150 000 events were analyzed per sample.

Statistical Analysis

Statistical significance was assessed using a one-way ANOVA with Prism software (Version 6; GraphPad, La Jolla, CA). Values were removed if they were considered outliers by the Grubb's Test (GraphPad, La Jolla, CA). Differences were considered significant when $p < 0.05$.

4.5. RESULTS

Intranasal STAT6-IP delivery at the time of primary infection does not influence adult worm burden, number of intestinal granulomas and anti-*AWH* IgG1 titres 14 days after secondary *Hpb* infection

STAT6-IP was delivered in three doses during primary infection (Figure 1) to mimic the 'early intervention' timeline we had used previously in an FI-RSV infection model (5). 14 days after secondary infection, there were no significant differences in worm burden (Fig. 2A), number of intestinal granulomas (Fig. 2B), or anti-*AWH* IgG1 titres (Fig. 2C) whether treated with STAT6-IP Arg9, STAT6-IP PTD4 or PBS. As expected, control mice infected with *Hpb* for the first time (on days 35 and 42) had high worm burdens, abundant granuloma formation and low levels of adult-worm specific IgG1 (Fig. 2). These data demonstrate that three IN doses of STAT6-IP at the time of primary infection did not alter protective Type 2 immune responses during secondary infection.

Intranasal STAT6-IP delivery at the time of primary infection does not influence the structural organization of intestinal granulomas

We next wanted to verify whether STAT6-IP delivery at the time of primary *Hpb* infection modulated structural organization of intestinal granulomas that develop at the time of secondary infection. These granulomas are organized around maturing larvae/adult worms and typically consist of AAMs, neutrophils and $CD4^+$ T lymphocytes (7). The H&E stained sections of intestinal foci demonstrated that all groups had 'retained' adult worms within the granulomas with well-organized layers of cells surrounding the worms (Fig. 3B-D). As expected, in primary infection (Fig. 3A), no larvae or adult worms were found in granulomas (11). These data demonstrate that,

regardless of STAT6-IP treatment, all mice undergoing secondary *Hpb* infection were able to mount well-organized granulomas that effectively trapped larvae/worms to reduce parasite survival.

Intranasal STAT6-IP PTD4 treatment, but not STAT6-IP Arg9, at the time of primary *Hpb* infection maintains CD206+F4/80+IL4Rα+ AAMs and CD4+IL4-Rα+ Th2 cells within intestinal granulomas during secondary *Hpb* infection

While H&E staining provides information regarding the structural organization of granulomas, immunofluorescence has been widely used in the *Hpb* literature to define the cellular components of the granulomas (eg: lymphocytes, neutrophils and AAMs) (7, 12). Given that i) larval killing is mediated by STAT6-dependent memory Th2 cells that induce AAM differentiation and production of arginase and ii) previous studies elucidated a role for STAT6-IP in inhibiting CD206+ AAMs in the lungs, we assessed the impact of IN STAT6-IP on immune cell composition in granulomas (6, 7). Although a majority of larval killing occurs within days after secondary infection, we observed persistence of AAMs, defined by co-expression of F4/80 (red), CD206 (green) and IL-4Rα (blue), in STAT6-IP PTD4 treated mice (Fig. 4E) but not in the PBS (Fig. 4B) or STAT6-IP Arg9 treated groups (Fig. 4H). Consistent with the literature, there no apparent F4/80+ CD206+ IL-4Rα+ AAMs in the primary infection granulomas (Fig. 4J) (7). Thus, STAT6-IP PTD4, but not STAT6-IP Arg9 treatment may induce AAMs persistence within granulomas despite similarities in adult worm burden among all groups (Fig 2A).

While primary infection with *Hpb* results in recruitment of neutrophils, eosinophils and F4/80^{+} macrophages, but few AAMs, to the site of larval maturation, abundant CD4+ Th2 cells producing IL-4 are recruited to intestinal granulomas during secondary infection (7, 11). We

obtained similar results to the literature: only a small number of CD4+ Th2 cell were present in intestinal granulomas in mice experiencing primary *Hpb* infection (Fig. 6B), abundant Th2+ cells were present in granulomas of mice after secondary *Hpb* infection in mice treated with either PBS or STAT6-IP PTD4 at the time of primary infection (Fig. 5B-C). Therefore, IN STAT6-IP application does not impede Th2 cell activation required for protective immunity.

Intraperitoneal administration of three doses of STAT6-IP at the time of primary *Hpb* infection does not alter number of adult worms or granulomas but STAT6-IP Arg9 decreases adult-worm specific IgG1 titres 14 days post-secondary *Hpb* infection

Given that IN application of STAT6-IP at first exposure did not inhibit Type 2 immunity during *Hpb* infection, we next assessed the effects of administering STAT6-IP via intraperitoneal (IP) injection (Figure 1). Following drug treatment and re-infection, no significant differences in worm burden or the number of intestinal granulomas (Fig 6A-B) were found across all groups. However, IP STAT6-IP Arg9 treatment significantly reduced serum anti-*AWH* IgG1 titers (Fig. 6C), suggesting that IP STAT6-IP Arg9 delivery may modulate the humoural response to some degree. Although a similar trend for IgG1 was also observed in the STAT6-IP PTD4 treated mice, this difference did not reach statistical significance.

Consistent with the lack of impact on adult worm burden and intestinal granulomas following secondary *Hpb* infection, no structural differences were observed in the intestinal foci following H&E stain (Fig. 7). In summary, three doses of STAT6-IP delivered IP at primary *Hpb* infection did not alter STAT6-dependent granuloma formation or killing of larvae during secondary infection, though it did modestly reduce AWH-specific IgG1.

IP STAT6-IP delivery at the time of primary infection does not alter the percentage of IL-4+CD4+ and IL-13+CD4+ lymphocytes from mesenteric lymph nodes (mLNs)

IL-4 production by memory $CD4^+$ T cells during secondary *Hpb* infection is critical for successful larval killing (7). Following flow cytometric analysis of cytokine production by $CD4^+$ mLN cells, no significant differences were observed in the percentage of IL-4^+CD4^+ or IL-13^+CD4^+ T cells harvested from mice treated (or not) with STAT6-IP (Fig. 8). Moreover, in agreement with more robust responses following secondary *Hpb* infection, larger numbers of IL-4^+CD4^+ and IL-13^+CD4^+ T cells were present in the mLNs of these mice compared to those experiencing *Hpb* for the first time. Altogether, our data suggest that short-term exposure to STAT6-IP either IN or IP is not sufficient to significantly modulate protective immunity to *Hpb* infection.

4.6. DISCUSSION

STAT6 inhibitory peptide (STAT6-IP) was developed to reduce airway inflammation by targeting and inhibiting STAT6 in a dominant-negative fashion (3). STAT6-IP delivery to the lung reduces maladaptive Type 2 immune responses in murine models of OVA- and ragweed-induced asthma as well as FI-RSV vaccination and repeat RSV lung infection (3, 4, 6, 8). Here we show that STAT6-IP administered IN or IP at the time of primary *Hpb* infection, did not alter protective Type 2 immune responses during secondary infection (ie: adult worm burden). Although IN STAT6-IP PTD4 delivery appeared to induce slight increases in serum IgG1, this effect did not reach statistical significance (Fig. 2C). Intranasal delivery of STAT6-IP also tended to increase the persistence of AAMs within intestinal granulomas (Fig. 4E). Intraperitoneal delivery of STAT6-IP Arg9 also failed to significantly change the parasitologic outcomes of secondary infection but reduced worm-specific IgG1 antibody responses. Thus, despite subtle changes suggestive of very modest immunomodulation, three doses of STAT6-IP at the time of primary *Hpb* infection did not significantly affect the development of a protective response to secondary infection.

The main finding of this work is that three doses of STAT6-IP via either the IN or IP route at the time of primary *Hpb* infection did not significantly change the protective systemic and mucosal Type 2 immune responses to secondary infection. These findings suggest that the potential use of STAT6-IP as a therapeutic for allergic airways disease is unlikely to reduce protective immunity to gastrointestinal nematodes. These results differ from previous STAT6-IP studies using acute and chronic allergy models in which IN delivery at the time of antigen priming was found to have long-lived systemic effects, manifest as reduced IL-4 and IL-13 production from splenocyte cultures (3, 4). In our nematode model, any potential systemic effect of STAT6-IP that may have altered Th2 priming or differentiation did not significantly alter the course of

either primary (Chapter 3) or secondary infection. Given the complex nature of *Hpb* infection, and helminth infections in general, we suspect that more frequent doses of inhibitory peptide may be necessary to alter Type 2 immunity in the gut. Studies in which peptide is administered more frequently, i.e. every second day during primary infection are underway to assess whether greater exposure to the immunomodulatory effects of STAT6-IP will influence *Hpb* infection.

IL-4 producing $CD4^+$ Th2 cells are known to home to and persist for several weeks in the peritoneal cavity following primary *Hpb* infection via upregulation of $\alpha_4\beta_7$, an intestinal homing integrin (13). Given the acute nature of our STAT6-IP treatment timeline (-1, 0, 1 dpi), we suspect that early delivery of STAT6-IP may not have effected this unique effector T cell population that likely mediates IL-4 production during secondary infection. As mentioned above, it is possible that repeated delivery of STAT6-IP will shed light on potential modulation of these IL-4-producing $CD4^+$ Th2 cells.

We were surprised to observe no AAMs in PBS-treated mice compared to STAT6-IP PTD4 treated mice given that AAMs can be found at 4 dpi after secondary infection (Fig. 4B, 4E) (7, 12). While other groups have not reported, or commented on AAM populations beyond 6 days after secondary infection, we suspect that AAM populations may be reduced once larval killing is complete and that this does not occur in STAT6-IP PTD4 treated mice (ie: the AAM population persists). Future time-course studies to investigate changes in immune cell composition within granulomas during secondary infection may provide greater insight into the role of AAMs in larval/worm killing as well as granuloma wound-healing and involution. Flow cytometric analysis or Reverse-Transcription qPCR may also provide more quantitative results regarding changes in cellular composition or changes in AAM markers within the evolving granulomas.

B-cell class switching occurs primarily in Peyer's patches and mLNs during primary and secondary *Hpb* infection (14). Given that IP delivery of STAT6-IP Arg9 reduced anti-AWH IgG1 relative to PBS-treated animals suggests that STAT6-dependent B cell class-switching may be modulated by peptide treatment (Fig. 8). Further studies investigating B cell and T follicular helper cell populations in the Peyer's patches and mLNs may shed light on processes modulated by STAT6-IP treatment at this site.

While abundant evidence supports a detrimental role of eosinophils in allergic reactions, recent evidence indicates that eosinophils, which are dispensable in secondary *Hpb* infection, promote intestinal homeostasis and thus contribute to reduced Type 2 immunity during *Hpb* infection (15). In primary *Hpb* infection of ΔdblGATA-1 deficient mice, the absence of eosinophils increased IL-4 production by T follicular helper cells and germinal center B cells, increasing serum nematode-specific IgG1 and reducing parasite fecundity (14). While our data showed no significant relationships between worm burden and IgG1 levels, we hypothesize that the trend to increased AWH-specific IgG1 could be due, at least in part, to a reduction of eosinophils by IN STAT6-IP treatment at the time of primary infection (Fig. 2C). Interestingly, this raises the possibility that STAT6-IP application in the nose could improve host protection and promote parasite clearance. However, we found that IP STAT6-IP treatment reduced AWH-specific IgG1 suggesting that the immunomodulatory effects of STAT6-IP may be route-dependent (Fig. 6C). Given that IgG1 isotype switching may be altered depending on route of administration, it is possible that IP STAT6-IP delivery reduces protective immunity while IN STAT6-IP delivery improves protective immunity during helminth infection even though no significant differences were found in adult worm numbers between treatment arms.

In this work, our primary objective was to investigate whether three doses of STAT6-IP, given either IN or IP at the time of primary *Hpb* infection, could modulate protective immune responses to a secondary infection. Our results indicate that neither IN or IP delivery abrogated protective Type 2 immunity required for *Hpb* resolution. Ultimately, these studies are reassuring with regard to the potential use of STAT6-IP as an asthma therapeutic or as an immunomodulator for STAT6-dependent immune responses.

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4.8. FIGURES AND LEGENDS

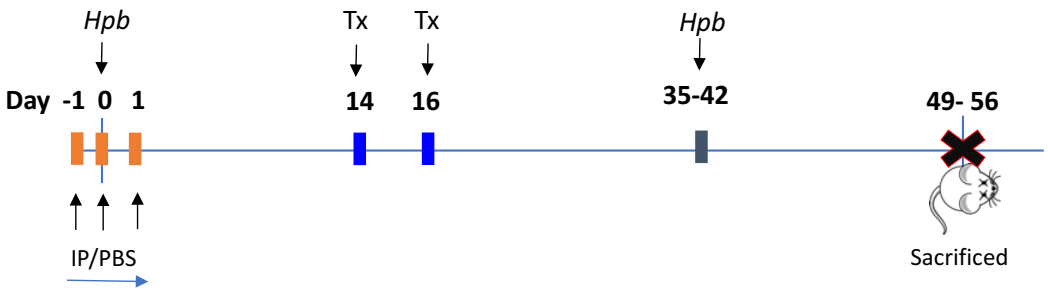


Figure 1. STAT6-IP administration and *Hpb* double infection schedule. Wild-type mice were infected on day 0 with 200 L3 *Hpb* larvae. On days -1, 0, 1, mice were treated with either 100ug STAT6-IP PTD4 or STAT6-IP Arg9 or PBS via intranasal (IN) or intraperitoneal (IP) injection. Mice were treated (Tx) with pyrantel pamoate at 14 and 16 days post-infection (dpi). 35-42 dpi, mice were re-infected with 200 L3 *Hpb*. An additional eight mice were infected at 35-42 dpi as a quality control (primary infection). Two-independent experiments were conducted with a total of 4-10 mice per group. Outcomes were assessed by a one-way ANOVA.

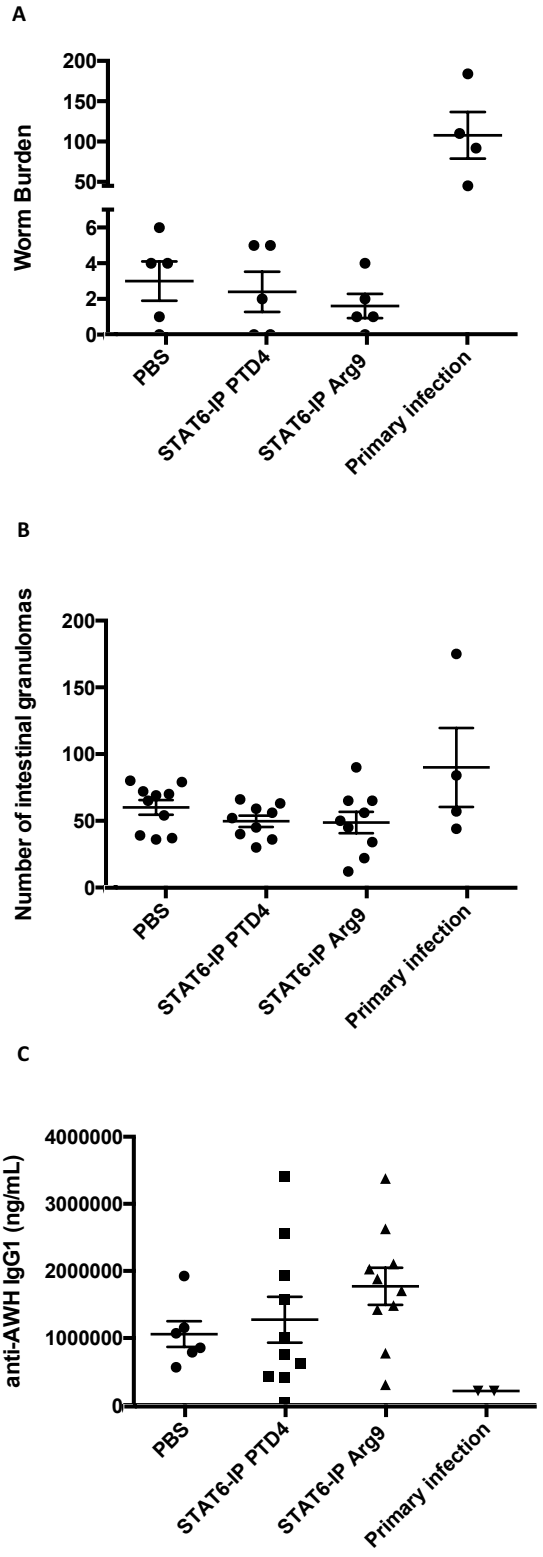


Figure 2. Intranasal STAT6-IP treatment does not alter adult worm burden, number of intestinal granulomas or anti-*AWH* IgG1 titres 14-days post-secondary *Hpb* infection. Mice were infected with 200 L3 stage *Hpb* larvae and treated IN with STAT6-IP (Arg9 or PTD4) or PBS according to Fig. 1. 14 days post-secondary *Hpb* infection, **(A)** the number of adult worms, **(B)** the number of intestinal granulomas and **(C)** anti-*AWH* IgG1 titres were quantified. Data from Figure A are presented means ± SEM of one experiment using 4-5 mice per group. Data from Figures B and C are presented as means ± SEM from two independent experiments with n=2-10 per group. Outcomes were assessed by a one-way ANOVA.

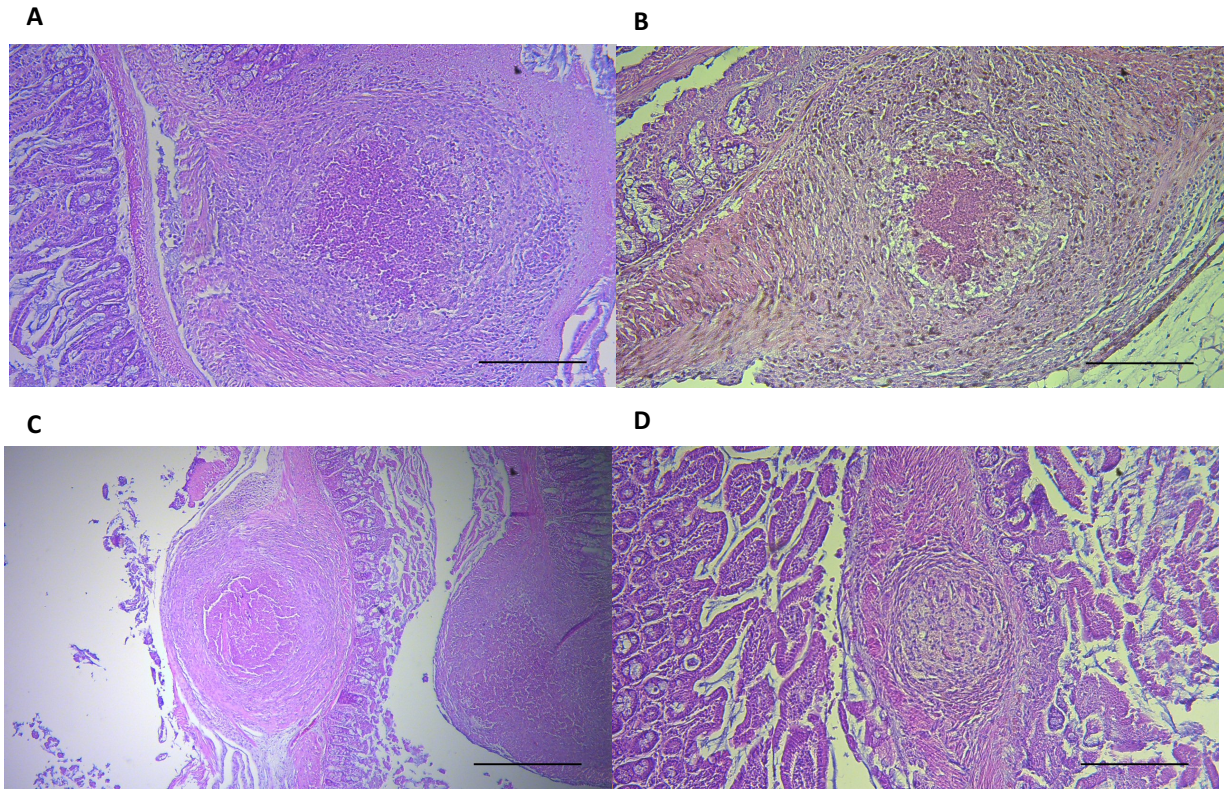


Figure 3. STAT6-IP treatment at the time of primary *Hpb* infection does not reduce structural organization of granulomas fourteen days post-secondary *Hpb* infection. Mice were treated IN with cell penetrating peptides or PBS and infected with *Hpb* according to Fig. 1. Structural organization of granulomas are presented as H&E staining of intestinal tissue sections of **(A)** mice that were infected once (primary), or **(B)** treated with PBS, **(C)** treated with STAT6-IP PTD4, **(D)** treated with STAT6-IP Arg9. Representative images from two independent experiments. Scale bar represents 10um.

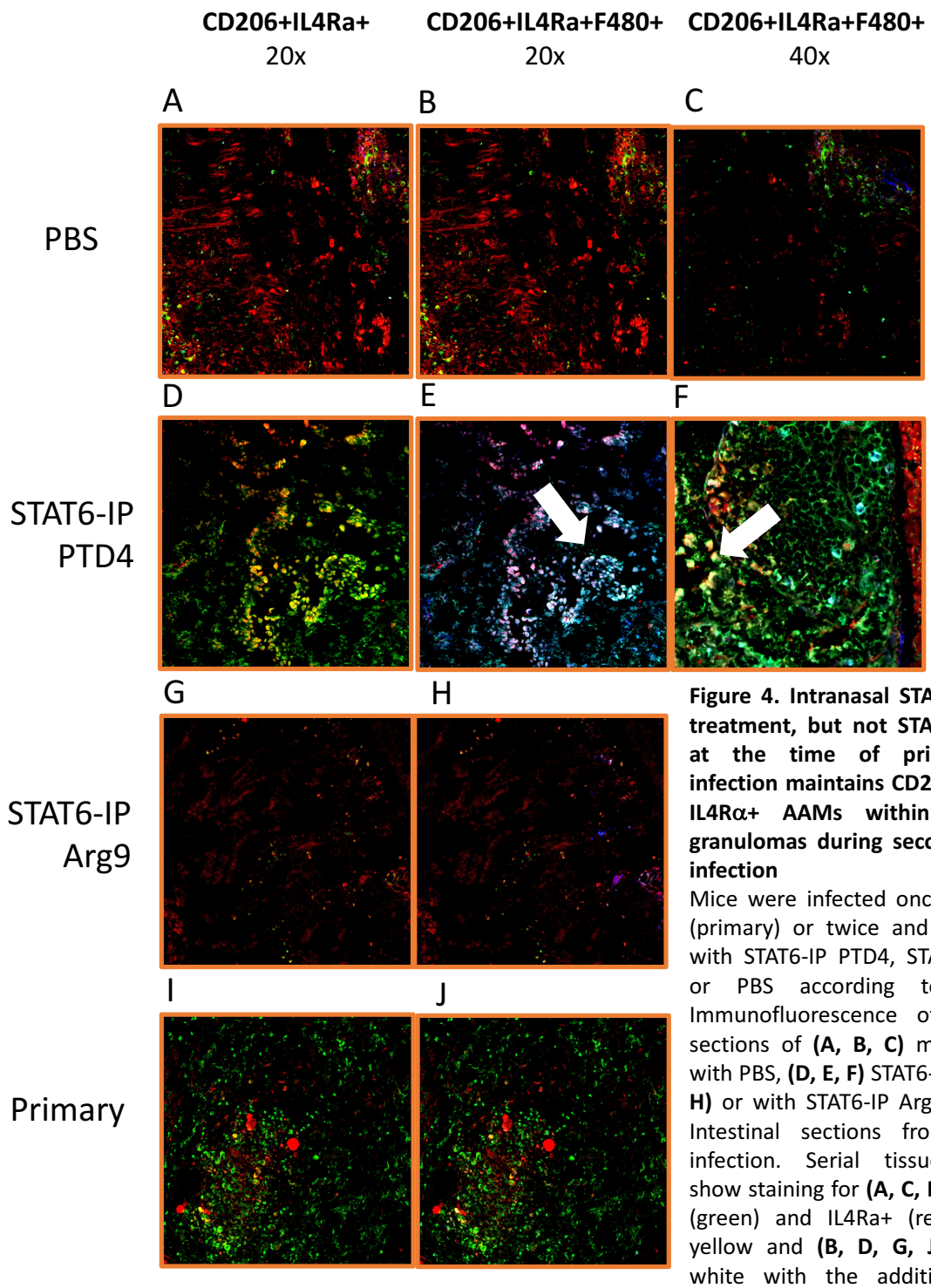


Figure 4. Intranasal STAT6-IP PTD4 treatment, but not STAT6-IP Arg9, at the time of primary *Hpb* infection maintains CD206+ F4/80+ IL4Rα+ AAMs within intestinal granulomas during secondary *Hpb* infection

Mice were infected once with *Hpb* (primary) or twice and treated IN with STAT6-IP PTD4, STAT6-IP Arg9 or PBS according to Fig. 1. Immunofluorescence of intestinal sections of **(A, B, C)** mice treated with PBS, **(D, E, F)** STAT6-IP PTD4 **(G, H)** or with STAT6-IP Arg9 and **(I, J)** Intestinal sections from primary infection. Serial tissue sections show staining for **(A, C, F, I)** CD206+ (green) and IL4Ra+ (red) cells in yellow and **(B, D, G, J)** AAMs in white with the addition F4/80+ (blue). Areas in white represent AAMs. Representative images **(A, B, D, E, G, H)** are shown at 20x magnification and **(C, F)** at 40x magnification.

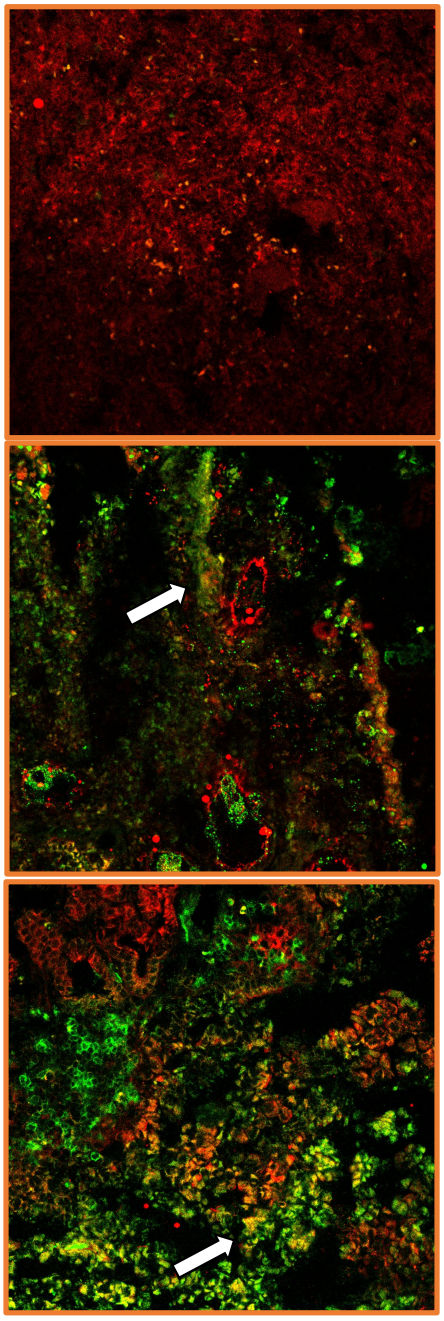


Figure 5. IL-4Ra+ CD4+ Th2 lymphocyte recruitment to foci 14 days post-secondary *Hpb* infection is not affected by IN STAT6-IP treatment at the time of primary *Hpb* infection. Mice were **(A)** infected once (primary) or treated IN with **(B)** PBS or **(C)** STAT6-IP PTD4 and infected with *Hpb* twice according to Fig. 1. CD4+ lymphocyte accumulation was assessed by immunofluorescence of intestinal tissue sections showing staining for CD4 (green) and IL-4Ra subunit (red). Co-localization of CD4+ IL4Ra+ (yellow) is indicated by white arrow. Representative images from two independent experiments.

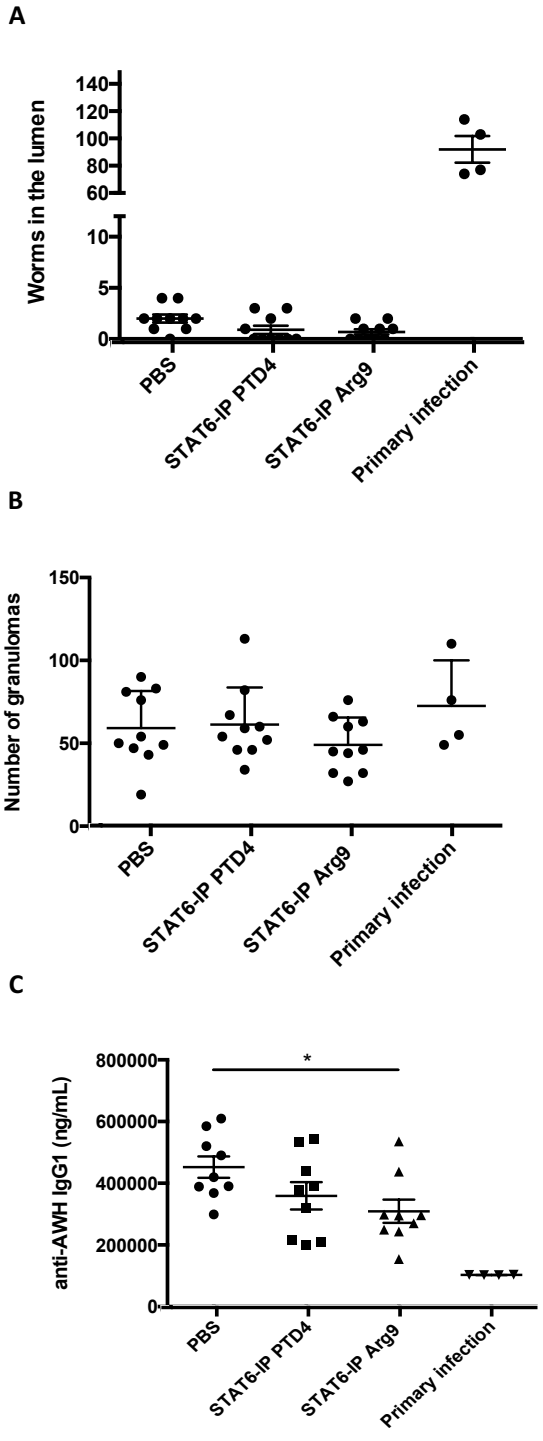


Figure 6. Intraperitoneal (IP) STAT6-IP treatment at the time of primary infection does not alter adult worm burden and number of intestinal granulomas but reduces anti-AWH IgG1 titres 14-days post-secondary *Hpb* infection. Mice were infected with 200 L3 stage *Hpb* larvae and treated IP with STAT6-IP Arg9, STAT6-IP PTD4 or saline according to Fig. 1. 14 days post-secondary *Hpb* infection, **(A)** the number of adult worms, **(B)** the number of intestinal granulomas and **(C)** anti-AWH IgG1 titres were assessed. Data are presented as means ± SEM from a combination of two independent experiments using five mice per group and two mice per group in the primary infection group in each experiment. Outcomes were assessed by a one-way ANOVA followed by Tukey's post-hoc test. *p<0.05.

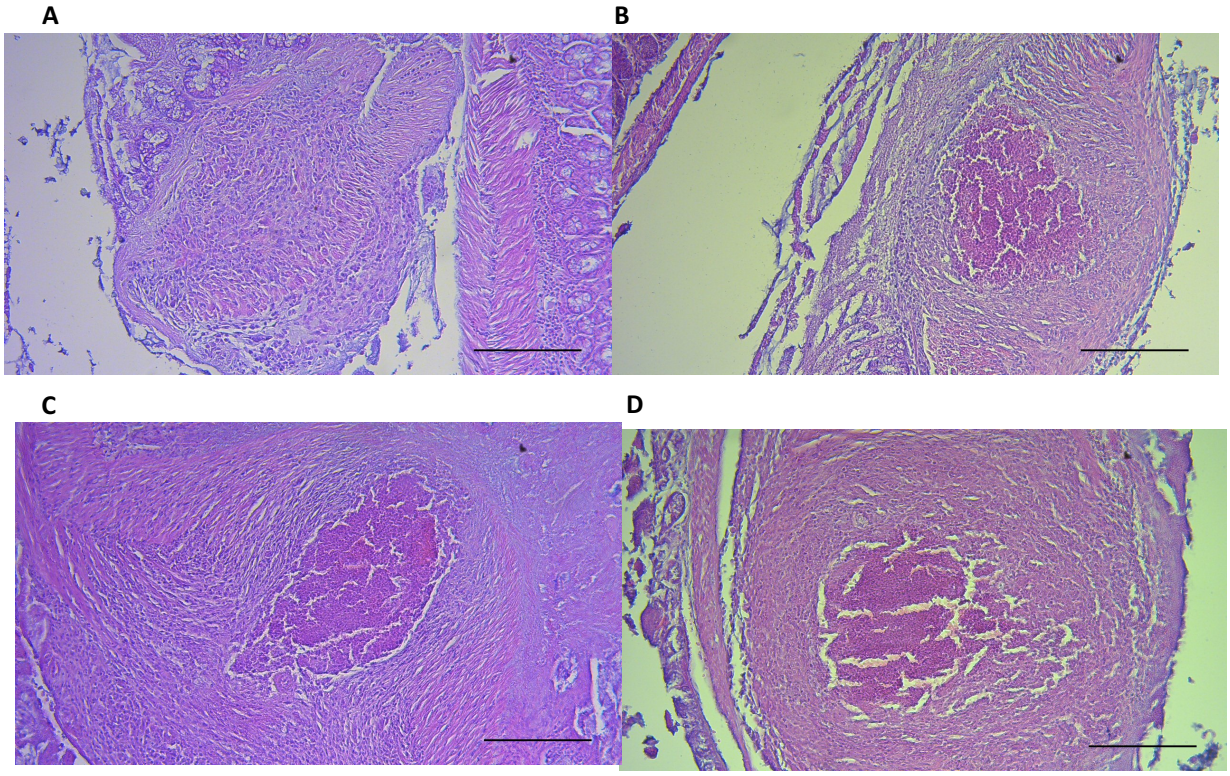


Figure 7. STAT6-IP treatment at the time of primary *Hpb* infection does not alter structural organization of granulomas 14 days post-secondary *Hpb* infection. Mice were infected with *Hpb* **(A)** once or challenged and treated with **(B)** PBS or **(C)** STAT6-IP PTD4 or with **(D)** STAT6-IP Arg9 IP according to Fig. 1. Granuloma formation was assessed by H&E staining of intestinal sections. Representative images from two independent experiments. Scale bars represent 10um.

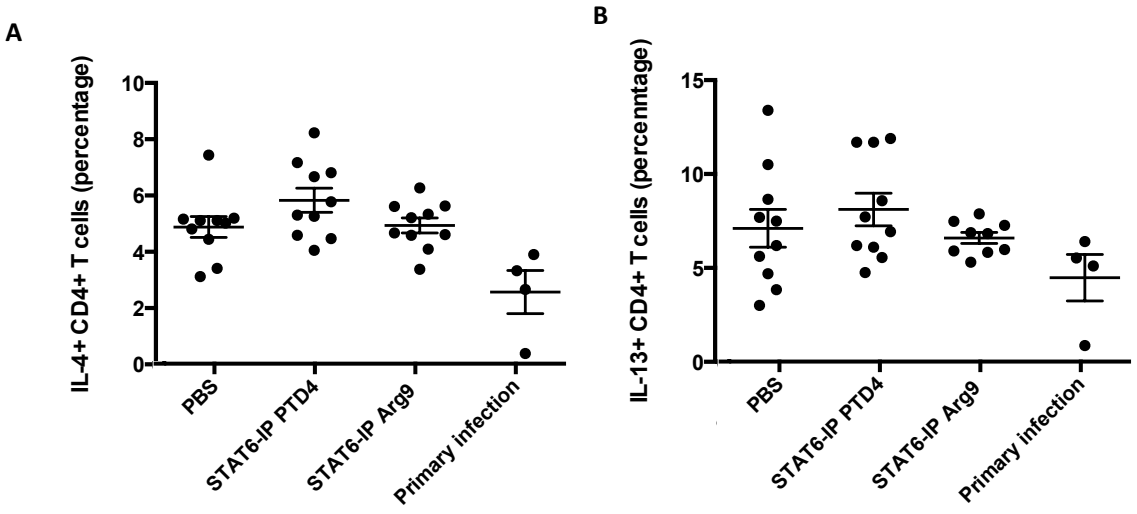
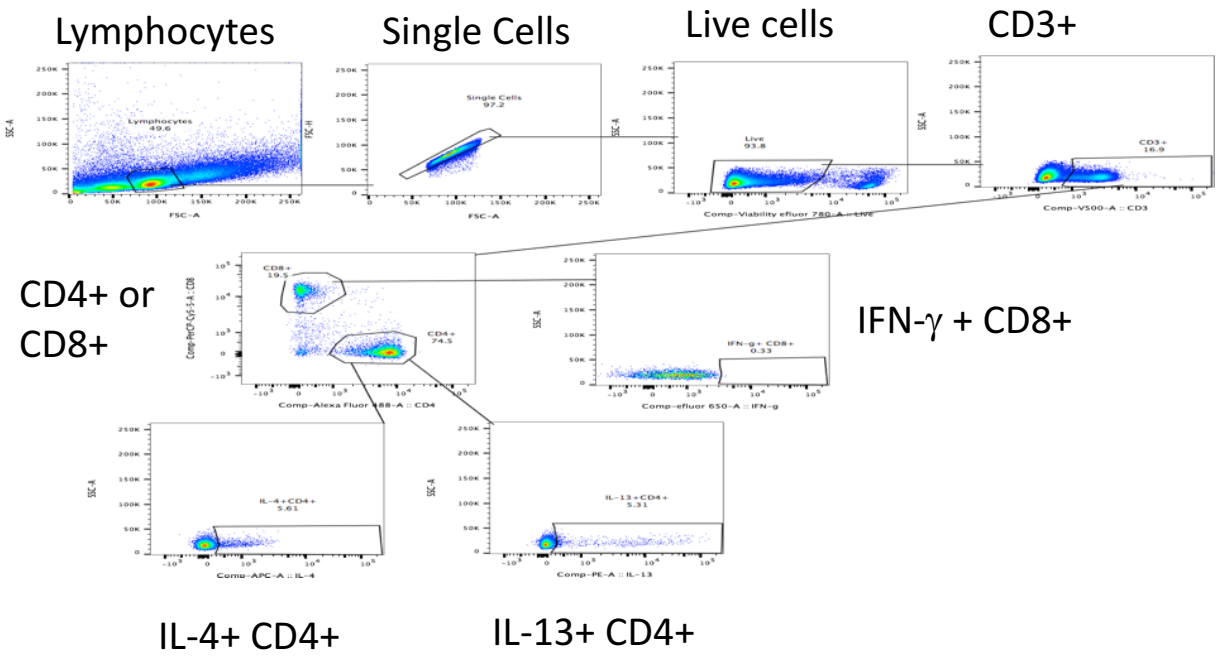


Figure 8: STAT6-IP treatment via intraperitoneal injection does not alter percentage of mesenteric lymph node IL-4+ CD4+ T cells and IL-13+ CD4+ T cells. Mice were treated with STAT6-IP PTD4, STAT6-IP Arg9 or saline via IP injections and infected with *Hpb* according to Fig. 1. (Supplementary Fig. 1. Gating strategy for IFNg+CD8+, IL-4+CD4+, IL-13+CD4+ was used to determine the **(A)** percentage of IL-4+CD4+ and the percentage of **(B)** IL-13+CD4+ T cells. Data are presented as mean ± SEM from five mice per group and from four mice per group for primary infection from two independent experiments. Outcomes were assessed using a one-way ANOVA.

4.8. FIGURES AND LEGENDS



Supplementary Figure 1: Gating strategy to determine the percentage of IFN-γ+CD8+, IL-4+CD4+, and IL-13+CD4+ T cells from mesenteric lymph nodes.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1. GENERAL DISCUSSION

Given that the last World Health Organization report published in 2013 claims between 235-300 million people are affected by asthma worldwide, we suspect, along with numerous reports of under-diagnosis, that the number of those affected by asthma is far greater in 2017 (1). There is abundant evidence in medical and scientific communities that allergic airways diseases, such as asthma, are highly prevalent in developing countries (1). In addition, new epidemiological data suggest that in developing regions of the world, such as Brazil, where helminth infections remain ubiquitous, rates of childhood asthma are increasing (2).

While environmental factors as well as underlying physiological and biological components of allergic airways diseases are now quite well understood, current marketed treatments still primarily target the symptoms of mild/moderate asthmatics (3). Importantly, of the 20% of patients who suffer from severe asthma, long-acting β-agonists and inhaled or systemic corticosteroids do not provide adequate symptom relief (4, 5). As a result, the morbidity of severe asthmatics generates enormous costs to healthcare: Over $5,000 per patient per year is spent on direct and indirect medical costs in the United States alone (6). Without therapeutics to target the underlying causes of allergic inflammation, medical costs for allergic airways diseases are not expected to decrease in the near future.

Recently, there is increased interest in biologics that target specific effector molecules, receptors or pathways that mediate immunopathology in the lungs (7). For instance, many biologics developed to target inflammatory processes in allergies, such as anti-IgE or anti-IL-5 monoclonal antibodies, have been successful in reducing asthma exacerbations as well as the use of inhaled-corticosteroids and β-agonists in paediatric populations (8, 9). Other biologics in

clinical trials target innate-type cytokines, such as TSLP, that promote Th2 immunity by a variety of mechanisms (10). Future asthma therapeutics will likely consist of current treatments (corticosteroids and β-agonists) in conjunction with novel biologics.

Chapter 1 of this thesis thoroughly reviews the available literature focusing on licensed treatments for lung allergy as well as therapeutic candidates currently undergoing clinical trials. Immune mechanisms involved in allergic inflammation, and the role of the transcription factor, STAT6, in mediating allergic inflammation are discussed. The canonical IL-4Rα/STAT6 signalling pathway as well as its role in inducing humoural immunity, alternatively activated macrophages, mucus production, and smooth muscle contraction is summarized (11). A substantial portion of the literature review in Chapter 1 bridges the link between maladaptive Type 2 immunity in allergic inflammation and the protective role of Type 2 immunity during helminth infection. Finally, the potential benefits of using immunomodulatory therapy to target STAT6 for treating allergic diseases is presented.

Dr. Fixman developed STAT6-IP. Data her laboratory obtained in collaboration with Dr. Christine McCusker, demonstrated that STAT6-IP delivered to the lung reduces Th2-biased allergic inflammatory responses, including AHR, mucus production, and eosinophil influx into the lung, in murine allergy models (12, 13). Srinivasa et al 2014, were the first to validate STAT6-IP in an infection model, using RSV (14). In this case, STAT6-IP reduced BAL fluid eosinophil levels and lung inflammation in the FI-RSV infection model (14). Data from these models provide evidence that airway STAT6-IP delivery can modulate local immunity (in the lung) as well as systemic immunity (in splenocyte cultures or distal hind limb vaccination) up to four weeks post-challenge. STAT6-IP likely interacts with antigen presenting cells and skews the balance of Th1/Th2 immunity, which can have long-lasting effects on inflammatory responses in these

models. Direct evidence that STAT6-IP interacts with cells of the innate immune system is provided by data from Srinivasa et al, who demonstrated that in neonatal mice exposed to RSV, STAT6-IP potently reduces CD206+ AAM; in adulthood, these mice have reduced airway hyperresponsiveness and eosinophilia in the lungs (15). Unpublished data from the Fixman lab indicate that STAT6-IP also reduces AAM differentiation in the lung as well as DC activation and migration to the lung draining lymph nodes in adult mice exposed to IL-33 ± OVA. Together, that data suggest that STAT6-IP targets DCs and macrophages, and possibly other cells of the innate immune system capable of influencing long-term events involved in either memory Th2 responses or effector responses at the time of initial exposure.

The findings obtained from these STAT6-IP data raised additional questions regarding: 1) the systemic effects of the inhibitor: 2) whether STAT6-IP could immunomodulate *protective* Type 2 immune responses; and 3) whether STAT6-IP could modulate immunity to infections at a site other than the lung. Thus, we set up a model whereby we could test all three concerns: our goal was to investigate whether an intestinal helminth infection, a model of protective Type 2 immunity, could be modulated by STAT6-IP administration. We used the murine enteric nematode, *Heligmosomoides polygrus bakeri (Hpb)*, since it induces robust IL-4 and IL-13-mediated Th2 responses, as well as potent IgG1 and IgE antibody responses (16). Primary *Hpb* infection induces a robust Type 2 immune response including the presence of macrophages within intestinal granulomas and production of IL-4 and IL-13 from $CD4^+$ Th2 cells (17). Despite mounting Type 2 immunity, primary infection is characterized by a chronic infection due to presence of myeloid-derived suppressor cells that dampen $CD4^+$ Th2 cells (18). Upon drug-treatment and re-infection, STAT6-dependent AAMs mediate killing of larvae within intestinal

granulomas (19). The *Hpb* infection lifecycle and host responses to primary and secondary *Hpb* infection are discussed in detail in Chapter 1 of this thesis.

To evaluate the potential effects of STAT6-IP administration during nematode infection, we first investigated the phenotype of STAT6$^{-/-}$ mice infected with *Hpb*. Our findings, presented and discussed in Chapter 2, support that STAT6 is critical for host protection by reducing adult worm burden, formation of intestinal granulomas and production of adult-worm specific IgG1 as well as total IgG during primary infection. These results were expected given existing literature describing the role of STAT6 in Type 2 immunity (11, 17). Although we did not study secondary *Hpb* infection in STAT6$^{-/-}$ animals and later time points beyond 28 days post-infection, we suspect that larval killing would be largely absent during secondary infection and parasite fitness would remain elevated compared to wild-type animals. Ultimately, we were successful in establishing a phenotype for primary *Hpb* infection in the absence of STAT6 and thus could model these findings with a potential phenotype following STAT6-IP administration to wild-type mice during *Hpb* infection.

Our goals for Chapters 3 and 4 were to test whether host responses were dampened following STAT6-IP treatment in primary (Chapter 3) and secondary (Chapter 4) *Hpb* infection. The rationale for a three-dose delivery schedule at the time of primary infection was to conduct a peptide administration schedule similar to what have proven effective in the FI-RSV 'Early Intervention' group, whereby mice treated to STAT6-IP following this schedule at the time of FI-RSV infection were protected from maladaptive Type 2 immunity in the lung following RSV infection four weeks later (14). Based upon our findings from both studies, we have concluded that three-dose IN or IP STAT6-IP delivery is insufficient to reduce host protection to *Hpb* infection, though changes to worm burden, humoural immunity and parasite fecundity were found.

For instance, we report that IP delivery of STAT6-IP significantly reduced IgG1 responses following secondary infection while IN delivery significantly reduced worm burden and parasite fecundity based on egg burden, 28 days post-primary infection. These results suggest that the IP injection may modulate host immunity such that humoural immunity is dampened while IN injection may improve infection outcomes. While it remains unclear why different routes of STAT6-IP administration induce opposing host responses, Mohrs et al, 2005 reported that $CD4^+$ Th2 cells accumulate in the airways and the peritoneal cavity during primary *Hpb* infection, providing evidence for initial contact among antigen-presenting cells targeted by STAT6-IP and Th2 cells (20). Given the different routes studied, we speculate that IN STAT6-IP delivery could *promote* T cell migration to the gastrointestinal tract, similar to work published by Ruane et al 2013, leading to increased Type 2 responses in the gut following infection and reduced parasite fitness (21). In contrast, an IP delivery route may reduce local B cell activation in the regional lymph node leading to decreased IgG1 responses during *Hpb* infection.

Limitations

It is important to note that several limitations exists in this thesis. The first: STAT6-IP had only modest effects in several readouts and only compared to PBS-treated animals. While STAT6-CP treated mice did not differ significantly from the PBS-treated controls, no significant differences were obtained between STAT6-IP- and STAT6-CP-treated mice in any outcomes. In some of our prior work, both published and unpublished, STAT6-CP, which differs from STAT6-IP by a single amino acid, has behaved similarly to STAT6-IP (13, 14). The reason(s) this occurs is under investigation, but we believe, as discussed in Chapter 4 that these peptides may, under some conditions, exhibit activity as antimicrobial peptides. Ongoing studies to address these potential off-target effects are underway.

Given that the majority of experimental outcomes from primary and secondary *Hpb* infection, presented in Chapters 3 and 4, did not demonstrate a phenotype intermediate between STAT6$^{-/-}$ and WT animals, we implemented a nine-dose delivery schedule via IP injection to address whether repeated exposure to STAT6-IP would diminish protective immunity to primary or secondary *Hpb* infection. Our initial findings suggest nine-dose IP delivery did not modulate protective immunity during primary *Hpb* infection; experiments to assess responses to secondary infection are ongoing. Therefore, this study suggests that repeated exposure of STAT6-IP does not alter local and systemic responses to gastrointestinal nematode.

Another reason why we chose a helminth infection model for Type 2 immunity, is the growing prevalence of allergy and asthma in areas of the world where infections by soil transmitted helminths are ubiquitous (2). If STAT6-IP were to be developed as a therapeutic for asthma and/or RSV lung infection, it is important to understand whether individuals would suffer from overt disease caused by helminth infection while using STAT6-IP to treat their asthma. Our findings indicate that IN administration of STAT6-IP largely does not alter the course of helminth infection localized to the gastrointestinal tract. The findings presented in this thesis raise two issues relevant for future work: 1) Does STAT6-IP modulate protective Type 2 immunity to helminth infections that transit through the lungs, the expected site of STAT6-IP delivery as an asthma therapeutic; and 2) What are the effects of STAT6-IP during concurrent helminth infection and Type-2 biased allergic inflammation in the lung such as during RSV infection or acute or chronic allergic lung diseases?

To address the first issue, other infection models that require Type 2 immunity for protection in the lungs will be implemented to better understand the scope of STAT6-IP. The murine helminth *Nippostrongylus brasiliensis* contains a lifecycle lung stage that requires Type 2

immunity for host protection and the trematode *Schistosoma mansoni* requires a Type 1 biased response for targeting lung-stage larvae. In particular, *N. brasiliensis* infection requires innate lymphoid cells 2 (ILC2s) and $CD4^+$ T cells for protection by stimulating IL-13 derived AAMs (22). Given that STAT6-IP can reduce AAM populations in the lung and modulate IL-4^+CD4^+ T cell responses in RSV-infected mice, *N. brasiliensis* might provide additional insight into STAT6-IP activity as a modulator of Type 2 immunity (23). Likewise, administering STAT6-IP intranasally during *S. mansoni* infection may also reduce infection outcomes given that STAT6-IP is capable of reducing Type 2 response and promoting Type 1 responses in the lungs (14, 15, 23). While *Hpb* was used to study the effects of STAT6-IP in modulating infections that require Type 2 immune responses for protection at distal sites of delivery, helminth models that travel through the lung and alveoli may help to elucidate whether IN STAT6-IP delivery modulates protective host immunity to parasites at local STAT6-IP delivery sites.

Another issue related to studies involving STAT6-IP as a potential therapeutic and disruption Type 2 protective immunity pertains to potential effects of STAT6-IP delivery during concurrent RSV and helminth infection. Recently published data suggest that *Hpb* chronic infection induces changes to the intestinal microbiota that promote Type 1 interferon production, leading to reduced inflammation and viral load in the lungs of RSV-infected mice (24). While these data demonstrate that *Hpb* infection can provide protection against RSV, we are interested in defining potential adverse effects of STAT6-IP in this type of co-infection. For example, does STAT6-IP delivery provide protection in RSV infection following *Hpb* infection? In particular, it would be of most interest to study this interaction in neonatal mice, given that RSV infection causes greater morbidity in infants (23).

STAT6-IP was designed as a cell-penetrating peptide comprised of a STAT6 binding domain coupled to a protein transduction domain (PTD). Cell-penetrating peptides have the potential to be potent therapeutics due to their ability to deliver various cargo, including both nucleic acid and proteins or peptides (amongst other molecules) into cells (25). The majority of our studies compared responses in mice treated with STAT6-targeting peptides using two different PTDs: PTD4 and Arg9. Our findings suggest that the Arg9 cell-penetrating peptide is most effective in modulating Type 2 immune responses in the *Hpb* infection model. In comparison, IN STAT6-IP PTD4 delivery slightly improved infection outcomes and promoted AAM persistence within granulomas two weeks post-secondary infection. Therefore, STAT6-IP PTD4 may be more promising as a future asthma or RSV therapeutic since it did not diminish protective Type 2 immunity in *Hpb*-infected mice.

5.2. CONCLUSIONS

Current treatments for asthma reduce symptoms but do not target the underlying immunologic abnormalities associated with immunopathology in the lungs. STAT6-IP is a novel, chimeric peptide, designed to transiently inhibit Type 2-biased allergic inflammation. STAT6-IP has demonstrated efficacy in models of allergic airways disease and RSV infection. These preclinical models support development of STAT6-IP as a therapeutic for human use. The findings presented in this thesis aim to provide increased understanding on potential adverse effects of STAT6-IP, in particular, whether STAT6-IP modulates protective Type 2 immunity. We find little evidence supporting that STAT6-IP administration to the lung (via IN delivery) or systemically (via IP injection) is sufficient to reduce host protective responses to a strictly enteric gastrointestinal nematode. Future work aiming to use other parasite models that require lung lifecycle stages and models of simultaneous helminth and RSV co-infection and/or allergic airway models will build on the scope of STAT6-IP as a novel therapeutic, specifically in regions of the world where helminth infections and rates of allergic diseases are prevalent. Overall, STAT6-IP is a promising candidate for immunomodulatory therapy of allergic airways diseases and a useful immunologic tool to study STAT6-dependent helminth responses.

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