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Helminth-derived inhibitors of the IL-33 pathway

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Declaration

I declare that this thesis is my own composition and the work presented in this thesis has been performed by myself if not otherwise stated.

The presented work has not been submitted for any other degree or professional qualification except as specified.

Date

Signature

Acknowledgments

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Abstract

Allergic asthma is a chronic inflammatory disease of the airways characterised by type 2 immune responses in the lungs. Increased mucus production, airway hyperresponsiveness and eosinophilia are the main features of the asthmatic lung, and they are caused by an increased production of IL-5 and IL-13. A strong inducer of these cytokines is the alarmin IL-33 that it is released upon necrotic death of epithelial cells. IL-33 can be released in the lung due to damage caused by inhalation of proteolytic allergens, parasite migration or respiratory viral infection. IL-33 activates several immune cell populations such as mast cells, T_H2 lymphocytes and type 2 Innate Lymphoid cells (ILC2s). Parasitic infection is associated with a decreased risk of developing allergic immune responses, and this prevention appears to be mediated by the release of immunomodulatory excretory/secretory products (ES).

The aims of this project are to study ES products from the intestinal murine nematode *Heligmosomoides polygyrus* (HES), and in particular identifying and characterising single proteins that interfere with the IL-33 pathway. HES administration has been shown to suppress both IL-33 and ST2 (the IL-33 receptor) in a mouse model of asthma. Therefore parasites may suppress the development of allergic asthma via the secretion of soluble mediators.

Firstly, before the beginning of this project, a single protein from HES was identified as suppressor of IL-33: the *H. polygyrus* Alarmin Release Inhibitor (HpARI). Through direct

binding assay, HpARI was shown to bind directly to the active cytokine, blocking IL-33-ST2 interaction. *In vitro* HpARI suppressed the release of IL-5 and IL-13 in response to IL-33. *In vivo*, administration of HpARI in an asthma model using the fungal allergen *Alternaria alternata* reduces ILC2s activation and eosinophilic inflammation.

Next, type 2 inflammation was analysed in a neonatal model of RSV infection. Respiratory viral infections during childhood have been associated with increased risk of asthma development later in life, especially in those infant hospitalised with severe RSV bronchiolitis. In a neonatal mouse model of RSV infection IL-33 play an important role for the development of type 2 immune responses. In our model, RSV infection in neonates induces activation of lung ILC2s 24h post-infection. When HpARI was co-administered with RSV, ILC2 activation was suppressed at primary RSV infection, and at RSV re-infection in later life. A trend for reduced viral titre was observed when HpARI was co-administered with RSV suggesting that HpARI might interfere with viral infectivity.

Finally, the next focus of the project was identifying a novel single protein involved in the suppression of ST2. Fractionation of HES was used to identify a novel protein which in recombinant form suppresses ST2. We named this novel protein *H. polygyrus* Binds Alarmin Receptor and Inhibits (HpBARI). HpBARI is a CCP domain-containing protein that suppresses IL-33 responses in vitro and in vivo. Using ELISA, direct binding assay and surface plasmon resonance, I showed that HpBARI binds directly to ST2 and that

this interaction prevents IL-33 from binding to its receptor, blocking initiation of type 2 immune responses.

These two newly identified parasite proteins, HpARI and HpBARI, both interfere with the IL-33 pathway. HpARI and HpBARI are related proteins as they each consist of 3 or 2 CCP domains, respectively, and they give insight into how parasites can immune modulate the host immune system.

Lay summary

Parasitic worm infections still affect much of the population of developing countries such as those in south-east Asia, sub-Saharan Africa, central and south Americas. Parasites establish long-term and recurrent infection in humans due to poor sanitation systems and the lack of access to clean drinking water. Long-term infections reflect the ability of the parasites to control the host immune system to increase the chances of survival. We believe that parasites suppress the immune system by producing soluble molecules.

Asthma has become far more common in the last century in developed nations such as those in Europe, USA, Canada and other industrialised areas, due to the improvement of sanitation system as well as changes in life style and changes in exposure to infection, especially parasites.

We think that molecules secreted by parasites can interfere with the mechanisms involved in the development of asthma, and that these secreted parasite molecules can provide a novel approach for the development of new medicines for allergies and asthma.

Human parasitic infections are difficult to study in a laboratory setting, but mouse models can closely mimic human infection. In this project we will use the secretions of the mouse parasite *Heligmosomoides polygyrus*, which has been shown to modulate asthmatic responses, to investigate the presence of single immunomodulatory proteins.

In this project, we characterised a previously-identified parasite protein called HpARI, and identified and characterised a previously unknown protein called HpBARI.

These proteins interfere with the IL-33 pathway involved in the initiation of the inflammatory responses associated with asthma. HpARI binds to IL-33 and HpBARI binds to its receptor.

HpARI has been widely studied both *in vivo* and *in vitro*, while the recently discovered HpBARI has been tested *in vitro* and *in vivo*. Both proteins are promising tools to develop new therapeutic to target this pathway in human disease.

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List of abbreviations

AChE – acetylcholinesterase

AEC – airway epithelial cells

AHR – airway hyperresponsiveness

AIP – anti-inflammatory protein

ALT – *Alternaria alternata*

APC – antigen presenting cells

BAL – bronchoalveolar lavage

BSA – bovine serum albumin

CBA – cytokine bead array

CCP – complement control protein

CLR - C-type lectin receptors

CPL – common lymphoid progenitor

DC - dendritic cells

DAMP – damage associated molecular pattern

EC – epithelial cells

EAE – experimental autoimmune encephalomyelitis

emPAI – exponentially modified protein abundance index

ELISA – enzyme-linked immunosorbent assay

EPO – eosinophil peroxidase

ES – excretory/secretory

EV – extracellular vescicles

FAK – Focal adhesion kinase

FCS – fetal bovine serum

Foxp3 – forkhead box P3

GI – gastrointestinal tract

GMCSF – Granulocyte macrophage colony stimulating factor

GSK3β - glycogen synthase kinase 3β

HDM – house dust mite

HES – *H. polygyrus* excretory secretory products

HpARI - *H. polygyrus* Alarmin Release Inhibitor

HpBARI - *H. polygyrus* Binds Alarmin Receptor and Inhibits

HpCPI – *H. polygyrus* Cysteine Protease Inhibitor

HpTGM - *H. polygyrus* TGFβ Mimic

ICS – intracellular cytokine stain

IFN – interferon

Ig – immunoglobulin

IL-1RAcP – interleukin 1 receptor accessory protein

IL-18RAcP – interleukin 18 receptor accessory protein

IL - interleukin

ILC – Innate lymphoid cells

iNOS – inducible nitric oxide synthase

L3 – stage 3 larvae

LPS - lipopolysaccharide

MC – mast cell

MHC – major histocompatibility complex

MOI – Multiplicity of infection

MPO - myeloperoxidase

MS – multiple sclerosis

MyD88- Myeloid differentiation factor 88

NF-κB – nuclear factor κB

NK – natural killer

NLR - NOD-like receptors

NmU – neuromedin U

NOD – nucleotide oligomerization domain

ORMDL3 – orosomucoid like 3

PAMP – pathogen associated molecular pattern

PFU – plaque forming units

PBS – phosphate buffered saline

PRR – pattern recognition receptor

RAG – recombinase activating gene

RELM – restin-like molecule

RIG-I – retinoic acid-inducible gene I

RORα - RAR-related orphan receptor alpha

RSV – respiratory syncytial virus

RU – responding unit

SEM – standard error of the mean

SPR – surface plasmon resonance

ST2 – suppressor of tumorigenicity 2

STAT – signal transduction and activator of transcription

TCR – T cell receptor

TGF – transforming growth factor

T_H – T helper cells

TIR – Toll-IL-1 receptor homology domain

TLR – toll-like receptor

TNF – tumor necrosis factor

T_{REG} – regulatory T cells

TRIF – TIR-domain containing adaptor inducing interferon-β

TRPM5 - Transient receptor potential cation channel subfamily M member 5

TSLP – thymic stromal lymphopoietin

UV-RSV – UV-inactivated RSV

VAL – venom allergen-like

Chapter 1

Introduction

1.1 Asthma

Asthma is a chronic inflammatory airway disease clinically characterised by wheeze, chest tightness, breathlessness, with reversible airflow limitations (Edwards et al. 2012; Barnes 2008; Holgate 2009). Asthma involves epithelial cells, smooth muscle cells, innate and adaptive immune cells that in different ways orchestrate asthmatic immunopathology (Global Asthma Network 2018; Whitsett & Alenghat 2015; Schatz & Rosenwasser 2014).

Around 5.4 million people currently suffer from asthma in the UK. The estimated cost of asthma is £5 billion/year in the United Kingdom alone (Edwards et al. 2012; Global Asthma Network 2018), associated not only with the cost of healthcare services and medicines, but also to the loss of work productivity.

Asthma is a heterogeneous disease with multiple different phenotypes, and the pathobiology is still unclear (Wenzel 2006; Holgate 2009). Allergic asthma is the most common phenotype of the disease, onset of which is observed usually during childhood, but can occur at any age (Holgate 2009). Asthma is typically characterised by T_H2 inflammation with eosinophilic and mast cell infiltrates, mucus production, and goblet cell hyperplasia (Wenzel 2006; Fahy 2015; Peters et al. 2018; Holgate 2009). The first-

line treatment for asthma is β_2-agonists, which act on the airway smooth muscle, so as to reverse the airway narrowing. Steroidal inhalers may be prescribed for severe or chronic asthma sufferers to target the inflammatory response in the airway (Global Asthma Network 2018; Holgate 2009). However, some forms of asthma are poorly controlled with the current treatment. Corticosteroids are usually effective in controlling T_H2 inflammation. Furthermore, a subpopulation of around 5%-25% of asthmatics do not respond to corticosteroid treatment. These corticosteroid-resistant sufferers account for around US$8 billion/year to the health system in Europe, Australia and the United States combined (Hansbro et al. 2017; Peters et al. 2018). Multiple mechanisms have been implicated in steroid-resistant asthma, and these sufferers are the target of biological therapeutics.

1.2 Helminth infection

Helminths are parasites that affect an estimated 1.5 billion people worldwide (Jourdan et al. 2018). Helminths are divided into the following three categories: trematodes (e.g. schistosomes); cestodes (e.g. tapeworms); and nematodes (e.g. hookworms). Helminth infections are common in developing countries, with soil-transmitted nematodes (e.g. *Ascaris lumbricoides, Trichuris trichiura* and *Ancylostoma duodenale*) being the most prevalent (Jourdan et al. 2018; Hotez et al. 2008). Helminthiasis are more frequent in young children, causing symptoms such as diarrhoea, anaemia, and impaired development. Anthelminthic therapy is effective in clearing the infection, although eggs

or larvae contaminate the environment, which allows for continuous reinfection (Sorobetea et al. 2018).

There is significant difference in the biology of helminths. For example, the intermediate host varies from species to species, as does the route of infection. Eggs, larvae and adult worms can all coexist in the human host, and the site of infection changes depending on the species (van Riet et al. 2007). Despite all these differences, most helminths elicit host type 2 immune responses, which are required for protection (van Riet et al. 2007; Allen & Maizels 2011). On the other hand, type 2 inflammatory responses have a detrimental role during asthma and allergic reaction.

1.3 Type 2 immune responses

The immune system evolved to protect our body from the external environment. The innate immune system offers rapid, but non-specific, responses, though it can activate the adaptive immune system to elicit antigen-specific responses. Antigen presenting cells (APCs) can polarise naïve T cells and generate effector T cells. Polarisation occurs through synergistic and antagonistic signals that lead to the generation of, for example, helper T type-1 (T_H1), helper T type-2 (T_H2) immune responses, as well as helper T type-17 (T_H17) and regulatory T cells (T_{REG}).

Type 1 immune responses are elicited by intracellular bacteria and viruses, and are also required to eliminate cancer cells. Type 1 responses are characterised by the production of IFNγ, IL-2, and TNFα. In contrast, Type 2 immune responses are triggered by parasites

and allergens. Type 2 responses can mediate allergic inflammation, rapid healing responses and ejection of parasites, and they are characterised by the secretion of IL-4, IL-5, and IL-13. Type 2 responses are associated with involvement of the entire mucosal tissue, the production of mucus by goblet cells, and smooth muscle contraction. T_H1 and T_H2 cells cross-regulate one another, for instance IFNγ suppresses production of IL-4 and type 2 immune responses, while IL-4 is associated with reduction of T_H1 responses and IFNγ (Pulendran & Artis 2012; von Moltke & Pepper 2017; Hammad & Lambrecht 2015; Kaiko et al. 2008). T_H1 and T_H2 responses were believed to be counter balance themselves, and being the major response to pathogens and allergens. This paradigm was valid until 2005 when the T_H17 subset was firstly described. T_H17 might have evolved for protecting the organism from microbes and fungi that cannot be eliminated by T_H1 and T_H2 (Tesmer et al. 2008). Another important T cell subset that is essential to maintain homeostasis and to prevent autoimmunity and reduce inflammation is the T_{REG}. T_{REG} are potently induced by TGFβ and have been involved in preventing the development of inflammatory disorder such IBD and asthma (Vignali et al. 2008).

In general, type 2 immune responses (Fig.1.1) have evolved to protect us from parasitic infection; they are an inflammatory response, which is accompanied by a phase of tissue repair and remodelling, in order to balance tissue damage induced by the migrating parasite (Lloyd & Snelgrove 2018; Pulendran & Artis 2012). However, when dysregulated, type 2 immune responses can cause asthma, allergies, dermatitis, and fibrosis, due to aberrant healing processes (Lloyd & Snelgrove 2018).

Molecular initiators of type 2 immune responses include epithelial-derived cytokines, such as TSLP, IL-25, and IL-33. For the purpose of this project, I will focus on IL-33, on which I shall expand in the next section. Epithelial-derived cytokines activate different immune cells to produce classical type 2 cytokines, including (but not exclusively) IL-4, IL-5, and IL-13. IL-5 is essential for the recruitment and survival of eosinophils, both during helminth infections and in allergic asthma. IL-4 is required for IgE class switching by B cells, while IL-13 acts on the effector phase of asthma and helminth expulsion due to the induction of mucus production and smooth muscle contraction (Allen & Maizels 2011; Lloyd & Saglani 2015; Hammad & Lambrecht 2015; Halim et al. 2012). While during parasitic infections, the initiation of type 2 inflammatory responses is required for an optimal parasite clearance, these responses are detrimental during allergic reactions and asthma. Allergic asthma is the most common phenotype of the disease, onset of which is usually observed during childhood, although it can occur at any age. Allergic asthma is characterised by T_H2 immune response (Holgate 2009).

In recent years, particular interest has been given to a subtype of asthma sufferers characterised by T_H17 responses. The T_H17 subset is elicited in response of extracellular bacteria and fungi, and it induces the recruitment of neutrophils. T_H17 differentiation is promoted by IL-6, IL-23 and the absence of both IFNγ and IL-4. Production of IL-17, IL-22, TNFα and GMCSF is a characteristic of the T_H17 subset. The role of the T_H17 subset has been controversial. Neutrophilic infiltrates have been observed in the lungs of asthmatic patients and T_H17 immune responses have been associated with those

asthmatics suffering from steroid resistant asthma (Chesné et al. 2014; Andersson et al. 2017; Eagar & Miller 2019; McCracken et al. 2016).

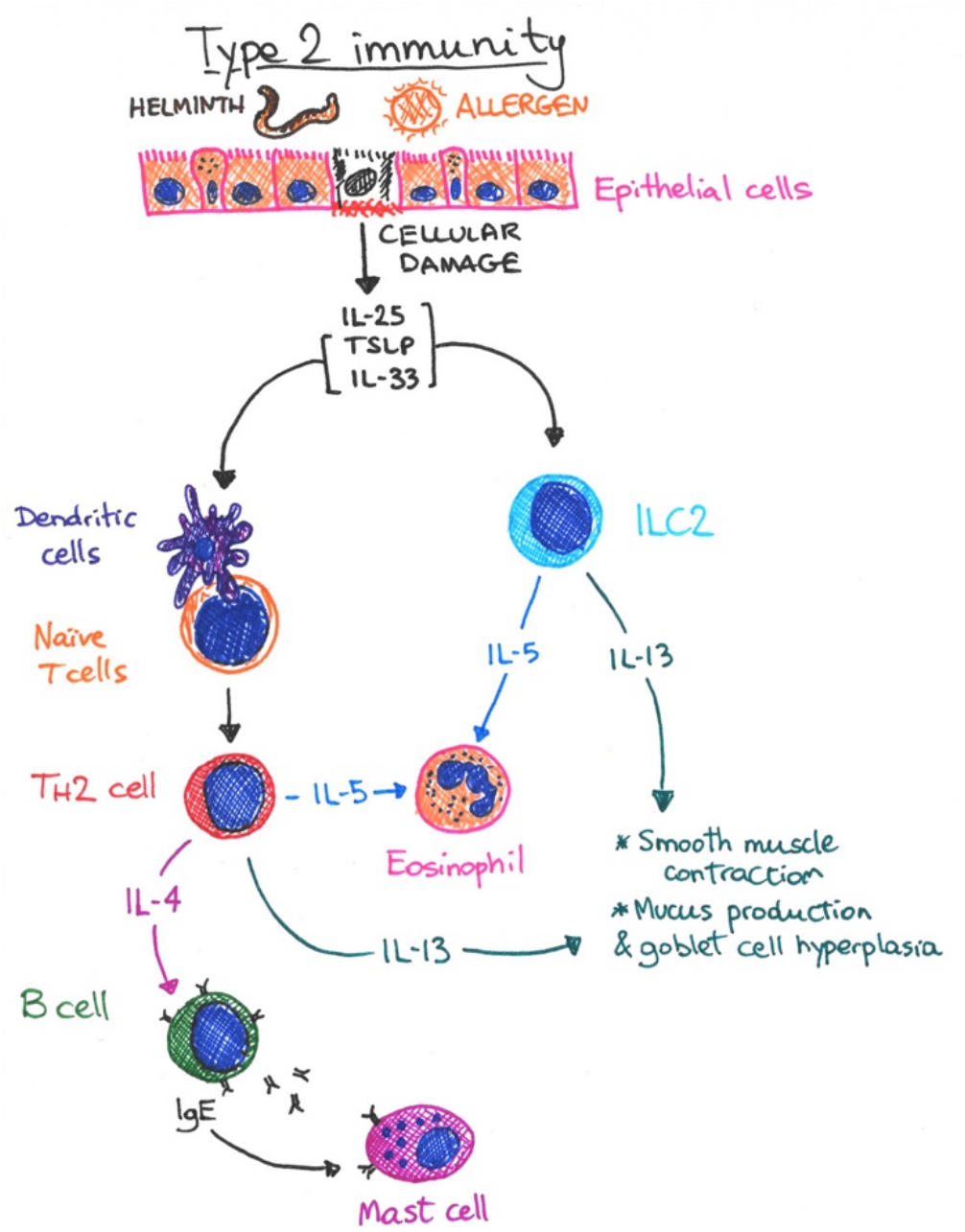


Figure 1.1 Type 2 immune responses are initiated by helminth infection and allergen.

Cellular damage in the airway or gut epithelium induces release of epithelial-derived cytokines such as IL-25, TSLP and IL-33. These cytokines act on several immune cells to start type 2 immune responses characterised by the production of IL-5, IL-13 and IgE, and the induction of mucus production, goblet cell hyperplasia and smooth muscle contraction.

1.4 Immune cells involved in type 2 immune responses

1.4.1 Epithelial cells

Epithelial cells (ECs) are the first barrier against the external environment and important contributors to the innate immune system. ECs can directly contribute to innate immunity to bacteria through the production of enzymes (such as peroxidases and lysozyme) and permeabilising peptides (e.g. cathelicidins and defensins) (Schleimer et al. 2007). ECs also express a broad range of Pattern Recognition Receptors (PRRs), such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs), to respond to damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). In response to signalling through these PRRs, epithelial cells produce and release chemokines, cytokines and antimicrobial peptides that attract and activate innate and adaptive immune cells (Hammad & Lambrecht 2015; Holgate 2009; Schleimer et al. 2007).

The airway and intestinal ECs act on the most proximal events in the initiation of type 2 immune responses. For instance, expression of TLR4 on airway ECs is essential for the responses against House dust mite (HDM) and LPS. Epithelial TLR4 is necessary to induce type 2 immune responses, such as production of IL-5 and IL-13, and recruitment of eosinophils. Treatment with TLR4 antagonist or absence of epithelial TLR4 expression reduces the asthmatic phenotype (Hammad et al. 2009). EC TLR4 signalling during HDM administration induces release of IL-1α, GM-CSF, IL-25, TSLP and IL-33, resulting in

recruitment of dendritic cells (DCs), and T_H2 induction (Hammad et al. 2009; Willart et al. 2012).

Gut ECs are critical in the detection and mediation of responses to parasitic antigens. For instance, the lyso-phosphatydilserine glycolypids and glycans from *Schistosoma mansoni* are recognised by CLRs (Ritter et al. 2010; Van der Kleij et al. 2002). Migrating parasites can provoke release of damage-induced IL-25, alarmins (such as IL-33), and other danger signals that can drive anti-helminthic type 2 immune responses (Sorobetea et al. 2018). During *T. muris* infection, absence of NF-κB specifically in epithelial cells prevents mounting of a protective immune response against the parasite (Zaph et al. 2007). Recently, a rare intestinal epithelial-like cell type has been identified and called: Tuft cells, which play an important role in the induction of IL-25-dependent type 2 immune responses.

Tuft cells expand during parasitic infection and are the major source for IL-25 in response to infection. They have chemosensory properties as isolated gut tuft cells highly express the G-protein gustducin and TRPM5, which have been associated with the taste sensory system (Howitt et al. 2016). Tuft cells can sense the metabolite succinate produced by bacteria and parasites such as *N. brasiliensis*, and, through calcium signalling, lead to the induction of type 2 immune responses (Von Moltke et al. 2016; Gerbe et al. 2016; Howitt et al. 2016; Schneider et al. 2018; Nadjsombati et al. 2018).

In the respiratory system, the existence of tuft cell-like chemosensory cells has been observed. In the nasal mucosa, chemosensory cells are responsible for the reactions in response to bacterial metabolite or irritant molecules, with these cells highly expressing

gustducin and TRPM5 (Tizzano et al. 2010). In the trachea and lungs, chemosensory tuft cells have been identified, and are known as brush cells (Gour & Lajoie 2016; Krasteva et al. 2011). However, their role in IL-25 secretion, and in allergies and asthma, has not yet being investigated. It is known that a subtype of asthma sufferers have an IL-25-high profile, with increased expression of IL-25 in the epithelium and increased levels in the blood (Cheng et al. 2014). IL-25 is also associated with asthma exacerbation, which is triggered by viral infections (Reid et al. 2005; Beale et al. 2014). These clinical observations may indicate a role for tuft cells in IL-25-high asthma.

1.4.1.1 The alarmin IL-33

Cytokines are known to be key to shape the immune responses, for instance tolerance to food in the gut and inhaled antigen in the airway is achieved with the secretion of TGFβ that acts on dendritic cells (DCs) to have a tolerogenic phenotype (Iliev et al. 2009; Wang et al. 2009). Conversely, in the initiation of immune responses, ECs can produce and release granulocyte macrophage stimulating factor (GM-CSF) which acts on DCs and macrophages, and is an important factor in allergic sensitisation inducing expression and release of IL-33 (Llop-Guevara et al. 2014).

In response to allergen, or during infection with parasites or viruses, ECs secrete IL-25, IL-33 and TSLP, cytokines that will act on a broad range of immune cells such as dendritic cells, type 2 innate lymphoid cells (ILC2s), basophils and mast cells (Kumar et al. 2014; Schmitz et al. 2005; Hammad & Lambrecht 2015). IL-33 has been mostly

studied in allergies, asthma and helminth infection. However, it could play an important role in other inflammatory conditions such as LPS-induced endotoxin shock, tissue repair and cancer (Kamijo et al. 2013; Serrels et al. 2017; Liew et al. 2016).

DAMPs have been recognised as inflammatory mediators of sterile inflammatory responses to injury and trauma for several years. They were believed to be stored inside the cells and passively released during necrosis, acting as alarm signals (alarmins). IL-33 is an alarmin as it is stored preformed in the nucleus and release upon cell necrosis.
IL-33 binds to its receptor ST2, which was discovered 20 years earlier than the cytokine and referred as an orphan receptor. IL-33 was identified in 2005 as a member of the IL-1 family (Schmitz et al. 2005). IL-33 was identified for its nuclear localisation and named nuclear factor from high endothelial venules (NF-HEV) (Kamijo et al. 2013; Schmitz et al. 2005; Martin & Martin 2016).

Full length IL-33 is constitutively expressed in the nuclei of epithelial cells at mucosal sites. In contrast with commonly secreted cytokines, IL-33 lacks a leader peptide, which is required for secretion. In addition IL-33 contains a nuclear localisation signal and DNA binding domain at its N terminus that results in IL-33 being trafficked to the nucleus and binding to chromatin (Travers et al. 2018; Cayrol & Girard 2018; Pichery et al. 2012). In addition to its N terminal chromatin binding domain, the IL-33 protein consists of a central domain and an interleukin-1-like domain at the C-terminus. In contrast to previous studies that suggested a possible role for IL-33 as a transcription factor due to its nuclear

localisation (Ali et al. 2011), recently, overexpression of IL-33 in an esophageal cell line was shown to not affect gene expression, ruling out a role as a transcription factor in this specific model (Travers et al. 2018). During apoptosis, IL-33 is processed and cleaved by caspase-3 and -7 which inactivate the cytokine. Cleavage occurs at a single conserved site in the interleukin-1-like domain as single amino acid mutation in the caspase cleavage site completely abrogates the activity of IL-33, indicating the important role of this domain for the cytokine activity (Lüthi et al. 2009; Cayrol & Girard 2018). As a DAMP, IL-33 is rapidly released in case of necrosis. In the absence of necrosis IL-33 is released in response to oxidative stress and ATP, however the specific mechanism involved in this release is still unclear and it might involve cell death or necrosis (Kouzaki et al. 2011; Uchida et al. 2017). Increasing the expression of antioxidant molecules in the airway epithelium reduced the release of IL-33 and reduced the asthmatic inflammatory phenotype (Uchida et al. 2017). In addition, released full-length IL-33 activity can be increased up to 10-fold by epithelial-derived calpain and allergen proteases such as *Alternaria*-derived cysteine proteases as well as cysteine- and serine- proteases produced by inflammatory cells (Scott et al. 2018; Lefrancais et al. 2012; Cayrol et al. 2018). Recently, IL-33 was shown to be released bound to histones and this association increased IL-33 activity if compared to full-length IL-33 alone (Travers et al. 2018). Histones have been considered as an alarm signal in sterile liver injury (Huang et al. 2011) and this newly-described synergistic activity of IL-33 and histones could give further insights into the inflammatory signals in this system. In sum, IL-33 is a cytokine that is stored pre-formed in the nuclei of epithelial cells and its released upon necrosis in complex with

histones or on its own. Once released, IL-33 can be activated by inflammatory proteases or inactivated by caspase, and it binds its receptor, ST2.

1.4.1.2 IL-33 receptor: ST2

After release IL-33 binds to ST2. ST2 was identified as member of the IL-1 receptor family due the presence of an intracellular domain called Toll/interleukin-1 receptor (TIR). ST2 is a typical class I receptor formed by three extracellular IgG-like domains, a transmembrane domain and an intracellular TIR domain (Sims et al. 1988), placing the receptor in two structurally defined families : triple IgG-domains that recognise β-trefoil class cytokines (i.e. IL-33, IL-1α/β); and cytosolic TIR domains involved in NF-κB signalling (Lingel et al. 2009). The affinity of IL-33 for ST2 is high and measured at dissociation constant (K_d) of 0.46 nM, and the formation of a 1:1 stochiometric complex. IL-33 engages ST2 in an extensive area of contact using 2 separate regions. Specifically, IL-33 region 1 is composed of strand β3 which interacts with ST2 domain 1 (D1) and domain 2 (D2), while IL-33 region 2 is the base of the β foil and interacts with ST2 domain 3 (D3) (Lingel et al. 2009). The electropositivity of ST2 D1 binds the IL-33 electronegative surface, initiating the specific recognition of IL-33 (Liu et al. 2013).

IL-33 binds to the extracellular domain of ST2 inducing a conformational change that leads to the recruitment of IL-1 receptor accessory protein (IL-1RAcP). The affinity of IL-1RAcP for the IL-33/ST2 complex has a K_d of 76 nM and a stoichiometry of 1:1:1. ST2 makes extensive contact with IL-1RAcP through its D3 region, and this stabilises the interaction. Interestingly, IL-33-induced ST2 conformational change is essential to

change the conformation of ST2 such that it can engage IL-1RAcP and form the IL-33R complex (Fig.1.2).

ST2 is expressed on several types of immune cells such as ILC2s, mast cells, T_H2 lymphocytes, basophils, eosinophils and M2-polarised macrophages (Martin & Martin 2016; Ball et al. 2018; Griesenauer & Paczesny 2017). IL-1RAcP is essential for the activation of the signal pathway cascade, the recruitment of MyD88 and the activation of NF-κB and MAP kinase (Griesenauer & Paczesny 2017), which lead to the production of pro-inflammatory cytokines, and GATA3 and Foxp3 expression (Schiering et al. 2014). While IL-1RAcP is promiscuously expressed in immune cells, ST2 expression dictates whether a cell will respond to IL-33 (Cayrol & Girard 2018). Ligation of ST2 by IL-33 induces internalisation of ST2 through activation of Focal Adhesion Kinase (FAK) and glycogen synthase kinase 3β (GSK3β). GSK3β interacts with ST2 at Ser446, inducing ST2 internalisation and degradation in the proteasome (Zhao et al. 2015; Zhao et al. 2012). ST2 exists in two isoforms, one membrane bound (ST2) and the other soluble (sST2), obtained through differential splicing (Iwahana et al. 1999). Soluble ST2 can be produced by activated T cells and mast cells (Lécart et al. 2002; Bandara et al. 2015), and it works as a decoy for IL-33 sequestering the cytokine and blocking signalling (Griesenauer & Paczesny 2017; Bandara et al. 2015).

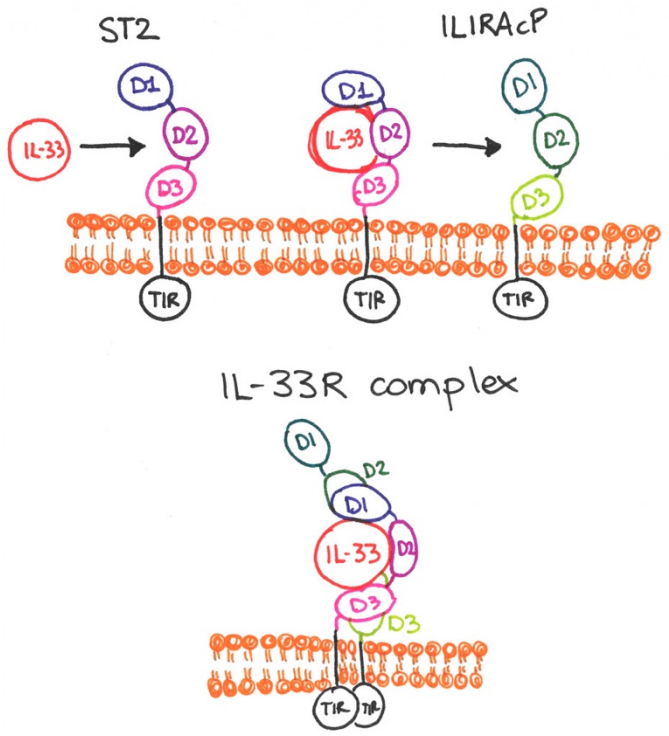


Figure 1.2 Formation of the IL-33 receptor complex.

The binding of IL-33 to ST2 occurs with all three domains of ST2. IL-33 binding induces a conformational change that induces the recruitment of IL1RAcP, which is required for IL-33-induced signalling. IL-1RAcP interacts with two of its three domains with ST2

1.4.1.3 Regulation of the IL-33 pathway

Activity and regulation of both IL-33 and ST2 exist at multiple levels to constrain the strong pro-inflammatory effect of the cytokine. As mentioned previously, one method of IL-33 regulation is through caspase cleavage during apoptosis (Cayrol & Girard 2018). Another mechanism is through IL-33 oxidation. The IL-1-like domain of IL-33 contains 4 free cysteines which are kept in a reduced state (not forming disulphide bonds) in the reducing environment of the nucleus. Release of the cytokine into the oxidative environment of the extracellular milieu induces a rapid oxidative process and formation of 2 disulphide bonds between the 4 free cysteines. This process induces a conformational change in IL-33 rendering it inactive and incapable of binding ST2. Oxidation occurs both *in vivo* and *in vitro*, and within 1h of IL-33 release almost 40% of the cytokine is inactivated (Cohen et al. 2015). The presence of a soluble form of both ST2 and IL-1RAcP inhibits IL-33 activity when it is released into the extracellular space, adding a further mechanism to block IL-33 responses (Ohto-Ozaki et al. 2010).

IL-33 signalling regulation also occurs at the level of ST2 signalling. ST2 can be negatively regulated by single immunoglobulin domain IL-1R-related molecule (SIGIRR, also known as TIR8), which binds to ST2 disrupting the dimerization with IL-1RAcP and stopping IL-33 signalling (Bulek et al. 2009; Liew et al. 2016). Specificity of IL-33 responses is achieved in some immune cells with ST2 interacting with other receptors: for instance IL-33 activation of mast cells requires interaction of ST2 and c-kit (Drube et al. 2010), and ST2-epidermal growth factor receptor (EGFR) interaction is

required to induce antigen-independent IL-13 production in T cells (Minutti et al. 2017; Monticelli et al. 2015; Molofsky, Savage, et al. 2015). The presence of multiple regulatory mechanisms implicates that IL-33-dependent exuberant immune responses need to be tightly regulated to avoid damaging inflammation.

1.4.1.4 IL-33 in asthma and helminth infection

The role of IL-33 in type 2 inflammation has been reported by several groups. Lethal multi-organ inflammation is induced by uncontrolled IL-33 release when the chromatin-binding domain is removed (Bessa et al. 2015). IL-33 is known to be a strong inducer of type 2 inflammation even in the absence of adaptive immunity: for instance intranasal administration of IL-33 induces IL-13-dependent goblet cells hyperplasia and airway hyperresponsiveness (AHR), even in $RAG^{-/-}$ mice that lack adaptive immunity (Kondo et al. 2008). Intraperitoneal administration of IL-33 induces accumulation of eosinophils, increased blood levels of IL-5 and IL-13, splenomegaly and mucus production in the lung (Ohto-Ozaki et al. 2010).

The involvement of IL-33 in asthma has been shown to be particularly important after the publication of several genome-wide association studies linking the *il33* and *il1rl1* genes to asthma susceptibility (Moffatt et al. 2010; Bønnelykke et al. 2013; Bønnelykke et al. 2014; Shrine et al. 2019). Clinical studies showed that IL-33 expression is increased in bronchial epithelial cells from patients with asthma compared to healthy controls (Prefontaine et al. 2009; Préfontaine et al. 2010), and IL-33 release is increased in asthma

sufferers compared to a control group, with IL-33 levels negatively correlating with lung function (Christianson et al. 2015).

Recently, IL-33 has been shown to accumulate physiologically in the developing lungs due to the mechanical damage induced by the first breath and the replacement of the aqueous environment with gas (Saluzzo et al. 2017; de Kleer et al. 2016). This release induces a type 2 environment during the alveolarization phase at days 7-14 of life in mice with accumulation of IL-33-responding immune cells, for example ILC2s, mast cells, eosinophils and T_H2 lymphocytes. Subsequently, mice at 14 days of age are more susceptible to allergic sensitisation that adult mice (de Kleer et al. 2016).

Increased IL-33 expression is observed in bronchiolar epithelium and smooth muscle from asthmatic patients (Préfontaine et al. 2010; Prefontaine et al. 2009). In addition, IL-33 has been correlated with disease severity: IL-33 detected in BAL of asthmatic patients positively correlates with levels of IL-13 and eosinophils, and negatively correlates with FEV_1 (Li et al. 2018). In paediatric patients, IL-33 has been correlated with airway remodelling and corticosteroid resistance (Saglani et al. 2013). Several different mouse models of asthma have demonstrated that IL-33 is required for driving the type 2 immune responses, and the cytokine can be detected in BAL within 15 min post allergen administration (Hammad et al. 2009; Snelgrove et al. 2014; McSorley et al. 2014; Halim et al. 2014; Scott et al. 2018).

The importance of the role of IL-33 during helminth infections comes mainly from mouse studies. ST2-deficient mice have a much slower expulsion of *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis,* both intestinal nematodes in mice (Coakley et al. 2017;

Hung et al. 2013), indicating that IL-33 signalling is required for optimal expulsion. Furthermore, recombinant IL-33 administration induces expulsion of *H. polygyrus* by recruitment of macrophages in the intestine (Yang et al. 2013). *N. brasiliensis* infection induces release of IL-33 and activation of IL-13-producing ILC2s which are required for parasite expulsion (Moro et al. 2010; Neill et al. 2010; Oliphant et al. 2014). In *Schistosoma mansoni* infection, ST2 signalling is required for the optimal development of T_H2 responses, formation of granulomas and recruitment of eosinophils (Townsend et al. 2000). IL-33 plays a key role during infection with *Trichuris muris*: administration of exogenous IL-33 induces parasite expulsion and IL-33 mRNA is upregulated at early stages of infection (Humphreys et al. 2008). Thus, IL-33 seems to be required for nematode expulsion. The importance of the IL-33 pathway is underlined by the fact that *H. polygyrus* targets the IL-33 pathway using several strategies that I will discuss in section 1.7.4.

1.4.2 Innate Lymphoid cells

Innate lymphoid cells are tissue-resident cells that originate in the bone marrow from common lymphoid progenitors (CPLs) (Diefenbach et al. 2014). Since 2008, it was believed that CPLs were able to generate only T and B lymphocytes – adaptive immune cells that express antigen receptors. However, with the isolation of lymphoid cells that did not express antigen receptor such as Natural killer cells (NK cells) and lymphoid tissue inducer (LTi), opened the identification of other subtypes of immune cells derived

from this lineage: these were named Innate Lymphoid cells (ILCs) (Spits et al. 2013) (Fig.1.3).

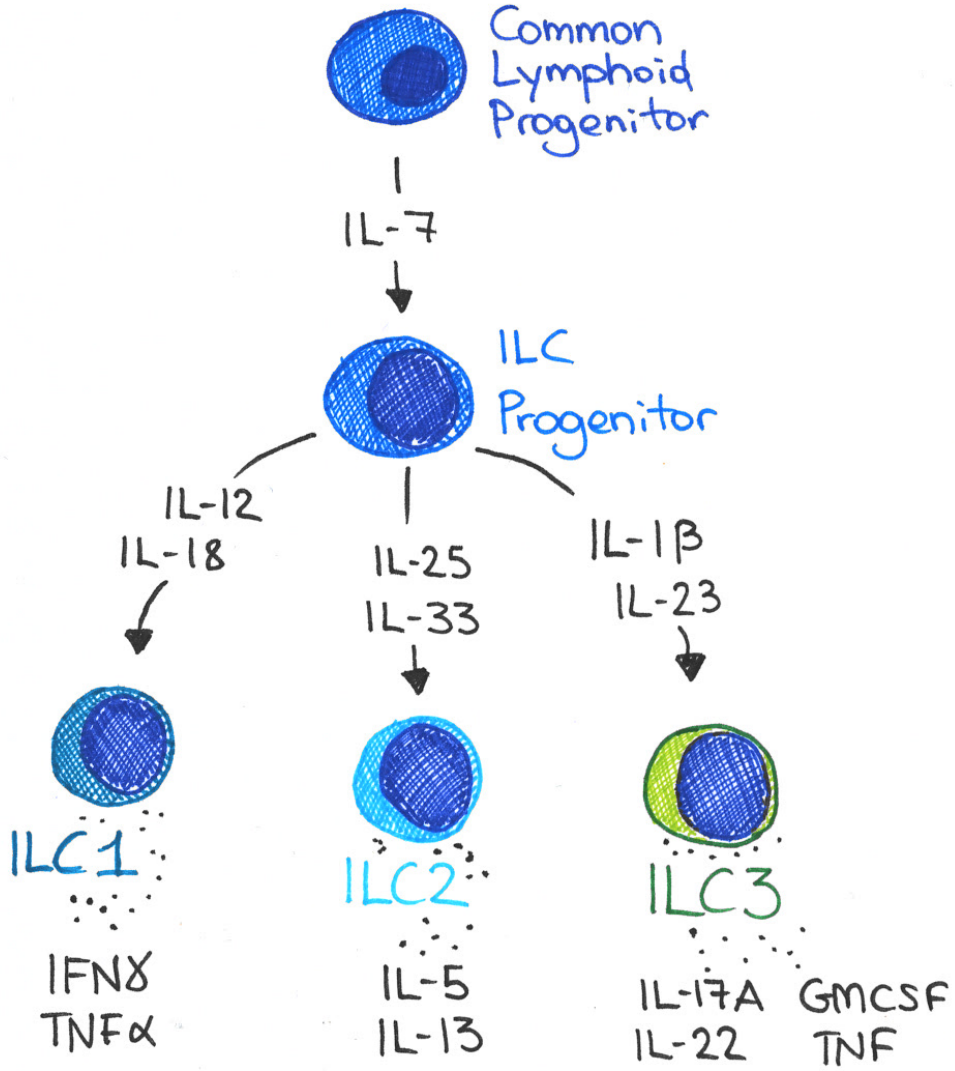


Figure 1.3 Innate Lymphoid Cells (ILCs) differentiation.

ILC progenitors differentiate from common lymphoid progenitor (CLP) under the production of IL-7. ILC subtypes originate

ILCs do not express recombination activating gene (RAG)-dependent rearranged antigen receptors, they lack phenotypical markers expressed by myeloid cells and dendritic cells, and they have a lymphoid morphology (Spits et al. 2013). They share a developmental process similar to T lymphocytes and they share phenotypic and functional similarities. In contrast to T lymphocytes, they respond rapidly to cytokines, stress signals, alarmins and microbial compound and they produce effector cytokines associated with helper T cells, and they are classified as ILCs type 1 (ILC1s) similar to T_H1 cells, type 2 (ILC2s) similar to T_H2 and type 3 (ILC3s) resembling T_H17 cells, and the already mentioned NK cells (Eberl et al. 2015). Further classification can be made between cytotoxic (NK cells) and non-cytotoxic ILCs (ILC1, ILC2 and ILC3) (Spits et al. 2013).

ILC1s are associated with the production of IFNγ and TNFα in response to intracellular bacteria (Fig.1.3). ILC2s respond to parasites and allergen by producing T_H2 cytokines such as IL-5, IL-13, IL-9 and they can produce amphiregulin to promote tissue repair (Klose & Artis 2016; Monticelli et al. 2011) (Fig.1.3 and 1.4). ILC3s produce IL-17A, IL-17F, IL-22, GM-CSF and TNF depending on the stimulation and they are believed to promote antibacterial immunity and chronic inflammation (Artis & Spits 2015) (Fig.1.3). ILCs do not express any classical lineage marker (i.e. CD3, CD4, CD5, CD8, CD19) and they do not express the T-cell receptor (TCR) (Halim 2016).

As a mirror of the T_H2 subset, and for their importance of type 2 immune responses in asthma and parasite infection, ILC2s will be the focus of this section (Fig.1.4).

ILC2s were first identified in the lung of *N. brasiliensis* infected mice and shown to be highly responsive to IL-33 and IL-25. These cytokines were essential for inducing IL-13-producing ILC2s and for worm expulsion (Neill et al. 2010). ILC2s require the transcription factors GATA3 and RORα and they are activated principally by IL-33 and IL-25, with further activation via TSLP, prostaglandins and neuromedin U (Moro et al. 2010; Neill et al. 2010; McKenzie et al. 2014; Klose & Artis 2016; Cardoso et al. 2017). Other cytokines that play a key role in ILC2 development and proliferation are IL-2 and IL-7. IL-7, together with IL-33, is required for type 2 cytokine production from ILC2s stimulated *in vitro* while a significant reduction of ILC2s is observed in IL-7-deficient mice (Moro et al. 2010; Moro et al. 2016).

The perinatal phase is essential to determine the ILC2s niche and IL-33 seems to play a key role for egression of ILC2 progenitors (ILC2Ps) from the bone marrow (de Kleer et al. 2016; Stier et al. 2018). ILC2s present a specific transcriptome depending on the tissue of residence, and tissue-derived signals drive ILC2s maturation: for example skin ILC2s development is independent from IL-25, TSLP and IL-33 (Ricardo-Gonzalez et al. 2018). ILC2s are major producers of IL-5, which induces eosinophilia, and IL-13, responsible for goblet cell hyperplasia, mucus production and airway hyperresponsiveness (AHR) (Diefenbach et al. 2014; Halim 2016). In addition, ILC2s can secrete amphiregulin to promote tissue repair (Monticelli et al. 2011; Klose & Artis 2016) and IL-9 that exacerbates type 2 inflammation, inducing mast cell activation, IgE production and goblet cell proliferation (Wilhelm et al. 2011; Klose & Artis 2016). ILC2s usually do not secrete significant quantities of IL-4, though isolated gut ILC2s can produce IL-4 in response to

leukotriene D_4 *in vitro*. Production of IL-4 from gut ILC2s is not observed if stimulated with IL-33 or IL-25 (Pelly et al. 2016). Interestingly, ILC2s express MHCII indicating a possible role for antigen presentation. ILC2s cannot process intact protein antigens as dendritic cells can, however they can present pre-processed antigen peptides through MHCII and they can activate $CD4^+$ T cells towards T_H2 both *in vitro* and *in vivo* (Mirchandani et al. 2014). MHCII expression is essential for helminth expulsion, and *in vitro* $MHCII^+$ ILC2s can activate T cells, albeit to a lower degree than DCs (Oliphant et al. 2014).

Recently, activation of ILC2 has been linked to neuronal regulation. ILC2 are closely associated with neurons in the lungs and they respond to neuronally-derived neuromedin U (NmU). NmU induces production of IL-5, IL-13 and AREG from cultured ILC2s in a MAPK-dependent and Ca^{2+}-dependent pathways (Cardoso et al. 2017). During *N. brasiliensis* infection NmU is induced in the lung at 2 dpi and in the gut at 6 dpi and NmU-KO mice have increased worm burden due to a lack of ILC2s responses (Cardoso et al. 2017). Hypothetically, the ILC2-neuron interaction might be important in the gut to induce contractility of the smooth muscle cells to induce worm expulsion and bronco-constriction in the airways during asthma. It is difficult to work on ILC2s in parasite-infected humans, therefore all our mechanistic data comes from mice. Nonetheless, human ILC2 could be activated in a similar way for example skin-penetrating parasites could induce release of TSLP or IL-33 and similarly parasites with a lung stage might induce release of IL-33 (Nausch & Mutapi 2018; Neill et al. 2010). Boyd and colleagues showed ILC2 and ILC3 expansion during filarial infections and the transcriptional

profiling suggests that these cells are ready for antigen sensing and ready to produce cytokines and chemokines (Boyd et al. 2014).

Several genome wide-association studies (GWAS) associated the risk of developing asthma with IL-33, IL-33 receptor, IL-13 and RORα, essential components of ILC2 responses (Moffatt et al. 2010; Bønnelykke et al. 2013), and a selection of these genes associated with asthma are shown in table 1.1.

Table 1.1 **Gene associated with risk of asthma development**

Asthma risk gene	**Product**	**Cellular expression**	**Asthma involvement**	**Reference**
IL-6R	IL-6 receptor	T and B lymphocytes	Induction of T_H17 Suppression of T_{REG}	(Westra et al. 2013)
FCER1G	IgE Fc receptor type 1 - γ	Eosinophils and mast cells	Mast cells degranulation and release of inflammatory mediators involved in allergic reaction	(Wu et al. 2010)
IL18RAP	IL-18 Receptor Accessory Protein	T_H1 and T_H2 NK cells Mast cells	Atopic asthma IL-18 signalling IL-4 and IL-13 production	(M. A. R. Ferreira et al. 2017)(Moffatt et al. 2010)

IL1RL1	ST2 – receptor for IL-33	T_H2, mast cells, ILC2, eosinophils and T_{REG}	Initiating type 2 immune responses	(Zhernakova et al. 2017; Bønnelykke et al. 2013)
TSLP	TSLP	Epithelial cells	Initiation of type 2 immune responses – DC polarisation	(Zhernakova et al. 2017; M. A. R. Ferreira et al. 2017)
ORMDL3	Orosomucoid like 3	Epithelial cells	Cellular stress and ureic acid production AHR and eosinophils recruitment	(M. A. R. Ferreira et al. 2017)
IL-33	IL-33	Epithelial cells	Released in case of necrosis Drives type 2 immune responses	(Moffatt et al. 2010)
RORα	RAR-related orphan receptor alpha	T_H2, ILC2s and T_{REG}	Transcription factor associated with ILC2s development	(Moffatt et al. 2010)
IL-2RB	IL-2 receptor subunit β	T lymphocytes, ILC2s, NK cells and T_{REG}	Differentiation and survival of immune cells	(Moffatt et al. 2010)
IL-13	IL-13	ILC2s, T_H2 and mast cells	Mucus production AHR	(Moffatt et al. 2010)

Stadhouders and colleagues analysed lung and mesenteric lymph node (MLN) ILC2 populations by RNAseq. ILC2s from the airways showed a more inflammatory phenotype with higher expression of ST2 and OX40, pro-inflammatory cytokines such as IL-9, IL-13 and IL-5, and chemokines such as CXCL3 (Stadhouders et al. 2018). MLN ILC2s present increased expression of genes involved in cell-cell interaction such as OX40L and MHCII (Stadhouders et al. 2018). These differences arise from a similar epigenome suggesting that ILC2s have a "flexible epigenome" that allow plasticity of ILC2s to respond and adapt to different stimulation in different compartments. Furthermore, it was demonstrated that ILC2s can become ILC1s under IL-12 stimulation, and this can be reversed with IL-4 (Bal et al. 2016). The switch between ILC2 to ILC1 is important during COPD and influenza virus infection as these conditions induce a reduction in the GATA-3 expression from ILC2s that transition to become ILC1s with a mixed T_H1-T_H2 phenotype (Bal et al. 2016; Ohne et al. 2016; Silver et al. 2016). All these data show the importance of ILC2 during parasitic infections and asthmatic responses, and the key role in inflammation, homeostasis and repair (Klose & Artis 2016).

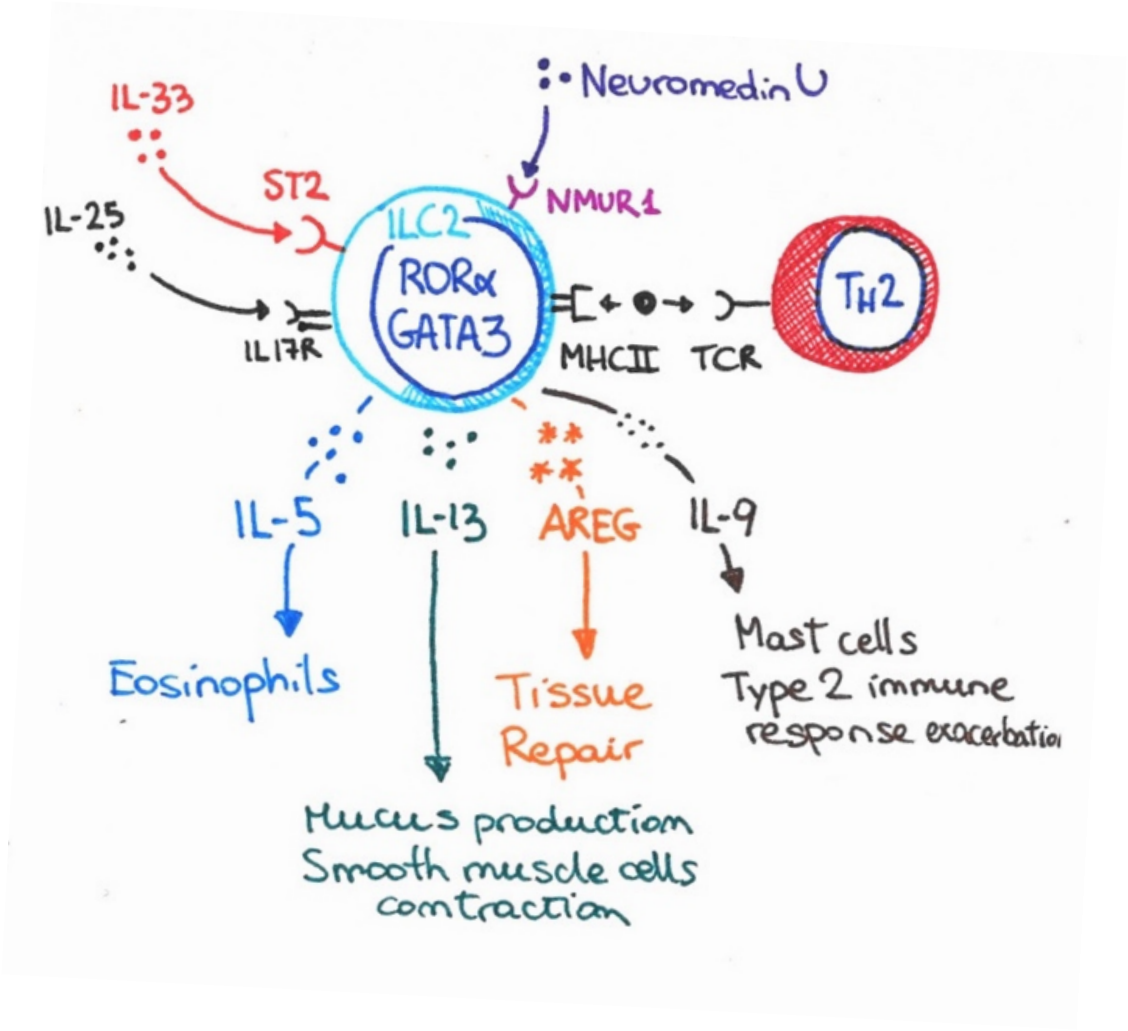


Figure 1.4 ILC2 transcription factor and activation.

Epithelial-derived cytokines such as IL-33 and IL-25, and the neuropeptide neuromedin U, can activate ILC2 to produce and release cytokines associated with type 2 immune responses e.g. IL-5, IL-13, IL-9; and cytokines associated with tissue repair and healing such as amphiregulin. ILC2s have been shown to present peptides through MHCII to naïve T cells, inducing T_H2 polarisation

1.4.3 Dendritic cells

Dendritic cells (DCs) play a key role for the initiation of type 2 immune responses. At the mucosal surfaces, DCs sense allergen and pathogens, they migrate to the lymph node where they activate $CD4^+$ T cells which in turn differentiate into T_H2, producing classical T_H2 cytokine such as IL-4, IL-5 and IL-13 (Lloyd & Snelgrove 2018).

DCs can be activated by allergen and parasite-derived products through different mechanisms, one of which is the presence of proteases in both allergen and parasite secretions (Lambrecht & Hammad 2010). For instance, the fungal allergen *Alternaria alternata* induces increased expression of surface molecules such as MHCII and co-stimulatory molecule such as CD40, CD80, CD86 and OX40 ligand (OX40L) involved in T_H2 polarisation (Kobayashi et al. 2009). *Alternaria*-activated DCs induce type 2 cytokine production *in vitro* and *in vivo* (Kobayashi et al. 2009). Activated DCs express CCR7, the receptor for the chemokines CCL19 and CCL21, which induce migration of DCs to the closest lymph node (Cook & MacDonald 2016) and in the lung $CD11c^+$ $MHCII^+$ have been shown to have a sentinel role, picking up antigen and presenting it to T cells in the lymph node (Vermaelen et al. 2001). DCs are a link between innate and adaptive immunity, and play a key role in allergen sensitisation process due to communication with epithelial cells. DCs can respond to the epithelial-derived cytokines IL-25, TSLP and IL-33, as well as reactive oxygen species, ATP and other DAMPs produced in response to allergens such as HDM. These signals can induce maturation of DCs that will polarise naïve $CD4^+$ T cells to T_H2 cells (Lambrecht & Hammad 2014; Cook & MacDonald 2016). For instance, TSLP is overexpressed in human asthmatic

airways suggesting a role in initiation of the disease (Ying et al. 2005). In addition, TSLP-activated DCs have increased expression of OX40L and ICOSL. OX40L has been shown to be essential activation of naïve T cells towards IL-4-, IL-5- and IL-13- producing T_H2 (Pattarini et al. 2017; Soumelis et al. 2002).

IL-33 can also activate DCs, inducing IL-6 production. IL-33-activated DCs had an increased expression of MHCII and they induced IL-5 and IL-13 production from $CD4^+$ T cells *in vitro* (Rank et al. 2009). In the context of HDM-induced allergic lung inflammation, DCs highly express ST2 and OX40L, which has been shown a strong costimulatory signal for T_H2 development (Pattarini et al. 2017; Plantinga et al. 2013; Gao et al. 2013).

Induction of type 2 immune responses are required during parasite infections and DCs plays a key role in this polarisation. In particular products secreted by parasites can drive the T_H2 polarisation observed *in vivo*. The excretory/secretory of the nematode *N. brasiliensis* (NES) can increase markers associated with T_H2 polarisation such as CD86 and OX40L in DCs cultured *in vitro* and this might explain the T_H2 responses observed *in vivo* (Balic et al. 2004; Lawrence et al. 1996). Another parasite associated with a strong induction of type 2 immunity is *Schistosoma mansoni*, and this strong polarisation is associated with eggs production (Pearce 2005). In particular, *S. mansoni* egg antigen (SEA) activated DCs to polarise naïve T cells towards T_H2 (MacDonald et al. 2001; Everts et al. 2009), however activation with SEA did not induce overexpression of OX40L, CD86 or other DCs co-stimulatory molecules (MacDonald et al. 2001). Among others, omega-1 has been identified as a single component of SEA identified for

conditioning DCs to polarise T_H2 cell (Everts et al. 2009; Everts et al. 2012). To underline the important role of DCs during *S. mansoni* infection, complete depletion of the DCs population dysregulates the induction of type 2 immune responses, resulting in an increased production of IFNγ (Phythian-Adams et al. 2010). These data are confirmed using other intestinal parasite such as *N. brasiliensis* and *H. polygyrus* as depletion of $CD11c^+$ DCs alters the T_H2 development without influencing innate type 2 immune responses (Smith et al. 2012). In conclusion, it is clear that DCs play an important role in influencing the adaptive immune responses in the gut and in the lungs, and these responses can be targeted by parasites such as *H. polygyrus* (Segura et al. 2007).

1.4.4 Eosinophils

Eosinophils are circulating granulocytes, described as cytotoxic cells and in some infection required for helminth expulsion (Jacobsen et al. 2012). They are produced in the bone marrow and they circulate in low levels in the blood stream in healthy individuals (1-3% of the leukocyte population), however they increase in asthmatic sufferers (6% of the leukocyte population) (Possa et al. 2013; Malm-Erjefält et al. 2005). IL-5 is a cytokine that drives eosinophilopoiesis, expansion and egression of eosinophils from the bone marrow (Possa et al. 2013). Recruitment of eosinophils in the tissue is driven by the production of eotaxin (CCL11), and expression in the vascular cells of vascular cell adhesion molecules-1 (VCAM-1) and P-selectin (Brightling 2011). For instance, increased eotaxin levels in human serum is observed both in severe asthmatic patients and during co-infection of intestinal helminth and *S. mansoni* (Geiger et al. 2013;

Lilly et al. 1999). During eosinophil maturation, driven by GATA1, eosinophils produce and store in their cytoplasmic granules major basic protein (MBP), peroxidase (EPO), cationic protein (ECP), neurotoxins and also cytokines and immune mediators, expanding the role of eosinophils in homeostasis and inflammation (Possa et al. 2013; Sonar et al. 2012; Roufosse 2018). The release of toxic granules from eosinophils induces tissue damage and leakage, mucus secretion and airway smooth muscle contraction (Liu et al. 2006). Eosinophils express receptors for multiple cytokines including IL-5, IL-13, IL-33 and TSLP, and chemokine receptors, particularly CCR3 which binds to CCL11 / eotaxin (McBrien & Menzies-Gow 2017).

Although there is large variability in eosinophil levels in asthmatic patients, eosinophilic infiltrates have been associated with asthma where they promote AHR and lung dysfunction (Wenzel 2006; Fahy 2015; Lambrecht & Hammad 2015). Eotaxin production by epithelial cells and IL-5 from ILC2 and T_H2 cells induces expansion and recruitment of eosinophils from the blood stream to the inflamed tissue (Sonar et al. 2012; Felton et al. 2014). Eosinophilia is observed in both acute and chronic mouse models of asthma using ovalbumin (Lloyd et al. 2018; Fernandez-Rodriguez et al. 2008), and the allergens papain (Halim et al. 2014; Kamijo et al. 2013), *Alternaria alternata* (Snelgrove et al. 2014; McSorley et al. 2014) and house-dust mite (HDM) (Hammad et al. 2009). These results support the findings in which alarmins such as IL-33 and IL-25 are essential to induce eosinophilia and type 2 immune responses (Morita et al. 2015; Stock et al. 2009). IL-33 not only induces eosinophil egression from the bone marrow through IL-5

production, but also seems to induce eosinophil survival in the tissue due to IL-5 and GM-CSF production (Willebrand & Voehringer 2016; Johnston & Bryce 2017).

Blood eosinophil levels correlate with disease severity and reduction in lung function (Bousquet et al. 1990). Eosinophil recruitment into the airways correlates with severity, and degranulation can be observed in the lung parenchyma and among epithelial cells (Bousquet et al. 1990). The release of EPO and MBP can be associated with the development of AHR, as EPO/MBP administration *in vivo* to primates and rats induces AHR. However the role of eosinophils in AHR development may be redundant due to other cells contributing to the asthmatic phenotype (Coyle et al. 1995; Gundel et al. 1991; McBrien & Menzies-Gow 2017; Grünig et al. 1998). Asthma severity correlates with reduced eosinophil apoptosis in sputum samples (Duncan et al. 2003) and therapies targeting IL-5 and eosinophils have been developed and showed promising results in those patients with eosinophilic asthma (Fahy 2015; Roufosse 2018).

In the gut during parasite infections eosinophils have been described to aid in worm expulsion (Huang & Appleton 2016). Eosinophils migrate towards different parasite species such as *C. elegans* and *N. brasiliensis* and this migration is driven by leukotrienes induced by the parasites (Patnode et al. 2014). *S. mansoni* eggs induce release of IL-5, resulting in eosinophilia, which is required for production of IL-4 in this model (Sabin et al. 1996), and IL-4 is known to be an important inducer of type 2 immune responses and IgE antibody production (Allen & Maizels 2011). Eosinophils promote repair and T_H2 responses through secretion of IL-4, and with the secretion of proteases they can directly target parasites (Buys et al. 1981; Goh et al. 2013; Huang & Appleton 2016). Therefore,

eosinophils are recruited at the site of parasitic infections and they characterise a subtype of asthmatic sufferers. Biological therapies targeting IL-5 reduced sputum and blood eosinophilia in asthmatic patients without affecting the allergen-induced later asthmatic responses, suggesting that eosinophils might not be a requisite for allergen challenge but it may play a role in reducing severity of asthma exacerbation (Leckie et al. 2000).

1.4.5 Mast cells

The skin, the gastrointestinal tract and the airways are enriched with mast cells (MCs). MCs derive from granulocyte/monocyte progenitors (GMPs) in the bone marrow, they circulate in the blood as mast cells progenitor (MCp) and they undergo complete differentiation in the tissue (Galli et al. 2005; Dahlin & Hallgren 2015). MCs are long-lived cells in the tissue, they undergo self-renewal, re-granulation and expansion, but MCp can be also recruited from the circulation to expand the MC population (Dahlin & Hallgren 2015). They mature under the influence of stem cell factor (SCF), IL-3 and other cytokines. The receptor for SCF, c-Kit, is required for MC maturation (Theoharides et al. 2007; Meurer et al. 2016; Gilfillan et al. 2011). The cytoplasm of MCs is filled with granules containing heparin, histamine, tryptase and TNF. Degranulation can be triggered by binding of IgE to the FcεR1 and MCs are common mediators of allergic inflammation (Joulia et al. 2015). MC degranulation releases large amounts of histamine, which induces bronchoconstriction, mucus production and oedema, mediating acute allergic responses, or anaphylaxis when systemic (Holgate 2000; Gilfillan et al. 2011). During parasitic infection, MCs have an essential role in induction of type 2 immune responses and so

contribute to worm expulsion (Hepworth et al. 2012; Reynolds et al. 2012). Recently, it was shown that mast cells can be activated by ATP released from necrotic cells during *H. polygyrus* infection. ATP induces release of IL-33 from MCs, which in turn activates ILC2s and contributes to the type 2 immune response (Shimokawa et al. 2017). Mast cells can recognise pathogens via surface-bound IgE, and secrete mediators that activate both the innate and adaptive immune system (Abraham & St. John 2010).

1.4.6 $CD4^+$ T lymphocytes

Different $CD4^+$ T cells subset have been identified to contribute to the immune-pathology of asthma. Naïve $CD4^+$ T cell polarisation occurs in the lymph nodes and, depending on specific stimulation and signals, several subset of helper T cells arise e.g. T_H1, T_H2, T_H9, T_H17 and T_{REG} (Ling & Luster 2016).

In this section I will focus the attention to the T_H2 subset, however due to the different asthmatic phenotypes other T_H- subsets have been involved in some asthmatic features, e.g. IL-9-producing T_H9 cells is involved in IgE class switching and mast cell activation, IL-17 produced by T_H17 cells might be involved in neutrophils recruitment and steroid-resistant asthma while T_{REG} and IL-10 could reduce allergic inflammation in the lungs (Lloyd & Hessel 2010).

$CD4^+$ T_H2 cells can produce IL-2, IL-4, IL-5 and IL-13 that contribute to the type 2 inflammation, and they are required for activation and IgE production from B cell (Muehling et al. 2017). A central cytokine in T_H2 development is IL-4 (Pelly et al. 2016).

In the absence of IL-4, $CD4^+$ T cells from *N. brasiliensis*-infected mice produced less type 2 cytokines (Kopf et al. 1993) while blocking IL-4 reduces protective immunity against *H. polygyrus* (Urban et al. 1991). T_H2 cells have been found to be elevated in asthmatic patients and they produce type 2 cytokines such as IL-4, IL-5 and IL-13. Production of IL-4 and IL-5 depends on the transcription factor GATA-3 and STAT-6, and both cytokines and transcription factors have been found elevated in bronchiolar byopsies from asthmatic patients (Taha et al. 2003; Lloyd & Hessel 2010). Disruption of the STAT-6 pathway in mice abrogates the activation of IL-4 signalling resulting in the loss of type 2 immune responses and IgE production (Shimoda et al. 1996). These results were supported by a study using *N. brasiliensis* infection in mice, in which splenic T cells from infected STAT-6-deficient mice have reduced production of IL-4, IL-5 and IL-10, while serum IgE was reduced in STAT-6-deficient mice compared to infected wild-type (Takeda et al. 1996). $CD4^+$ T cells are known to play a role together with ILC2s to induce protective immunity in the lungs against *N. brasiliensis*. In particular, $CD4^+$ T cells sustain the ILC2 population through production of IL-2, and IL-13 production by both ILC2s and T_H2 is required for M2 macrophage activation and worm killing (Bouchery et al. 2015). During helminth infections, $CD4^+$ T_H2 cells play an important role for protection against helminth infection and in case of secondary infection through the generation of a memory population. After *H. polygyrus* infection T_H2 memory cells persist in the lamina propria and in the peritoneal cavity, and they act as innate cells responding to cytokines such as IL-33 (Steinfelder et al. 2017).

As well as their beneficial effect in clearing parasitic infections, T_H2 cells also cause pathology in allergy, where $CD4^+$ T_H2 cells contribute to the immune-pathogenesis of asthma. Allergen-specific $CD4^+$ T cells respond quickly during allergen challenge, and in humans they have been described as a $CD161^{hi}$ sub-population of T_H2 which produce larger amounts of IL-5 and IL-9 compared to conventional T_H2 (Muehling et al. 2017; Fahy 2015; Wambre et al. 2017). At transcription levels the allergen-specific T_H2 have higher expression of the gene encoding for ST2, IL-25 receptor (IL-17RB), IL-5, IL-9 and genes involved in the arachidonic acid synthesis, all of which are involved in asthmatic responses (Wambre et al. 2017). $CD4^+$ T_H2 cells and ILC2s play similar roles in asthma pathogenesis, driving overlapping effector pathway depending on the availability of epithelial-derived cytokine and receptor expression (Lloyd & Hessel 2010). In addition, age of allergen exposure seems to be another factor to that can affect T cell responses. Administration of IL-33 or HDM to mice aged 3 days resulted in a large increase in IL-13^+ $CD4^+$ T_H2 cells, while adult mice showed a predominant increase in IL-13^+ ILC2s. In early life IL-13^+ $CD4^+$ T_H2 play an important role in the induction of AHR (Saglani et al. 2018). This is supported by the observations that in the developing lungs different immune cell populations have different dynamics, for instance accumulation of ILC2s is observed by day 7 and peak at 14 days post-birth while T cells peaks at day 1 post-birth, thus ILC2s may be more important in mice aged between 3 days and 21 days (de Kleer et al. 2016).

1.4.7 B lymphocytes, a focus on IgE

B lymphocytes are generated from haemopoietic stem cell (HSC) in the fetal liver and in the bone marrow during adulthood, circulating as pre-B cells and undergoing maturation to mature B cells in the spleen and lymph nodes (Lebien & Tedder 2008). B lymphocytes are known for the production of antibodies but they also contribute to an optimal lymphoid tissue development and they produce cytokines and signals that help the development the immune system (Lebien & Tedder 2008). B cells can specifically recognise antigen directly through expression of membrane-bound IgM and IgD. This first activation through antigen recognition is required to induce cross-talk with T lymphocytes. B cells present the antigen to T cells through MHCII, in response they stimulate heavy chain class switching through CD40L and production of specific cytokines will determine which antibody will be produced (Abbas et al. 2017). IgE production is frequently associated with parasite infections, allergen sensitisation and allergic responses. T_H2 cells induce IgE class switching through the production of IL-4, IL-13, IL-9 and CD40/CD40L interaction (Poulsen & Hummelshoj 2007; Geha et al. 2003).

Helminth infection induces a physiological type 2 immunity with production of IgE and induction of eosinophils to help with helminth clearance (Fitzsimmons et al. 2014; Svetić et al. 1993). It was shown that in the skin, IgE helps to block *N. brasiliensis* larvae in case of a second infection and this is mediated by basophils (Obata-Ninomiya et al. 2013). Studies in human infection with *S. mansoni* and *S. haematobium* also showed the protective role of IgE. Resistence to infection is associated with increased levels of IgE

against numerous parasite antigens. These IgE were shown to bind directly the parasite and they induced mast cell degranulation at the site of infection (Rihet et al. 1991; Hagan et al. 1991). In general IgE responses are believed to contribute to protection against helminth infections (Yazdanbakhsh et al. 2002). On the other hand, allergic asthma is an IgE-mediated disease and the release of IgE, which binds to FcεR1 expressed on mast cells, eosinophils and basophils, induces the release of pro-inflammatory mediators, driving type 2 immune responses and clinical symptoms (Oliveria et al. 2017). IgE-mediated allergic reactions occur rapidly and allergic (anaphylactic) reactions and asthma exacerbations can be fatal (Poulsen & Hummelshoj 2007). For this reason IgE production is negatively-regulated by cytokines such as IFNγ and IL-21 to control exuberant responses (Geha et al. 2003).

1.5 Respiratory Syncytial Virus (RSV)

Type 2 immune responses are physiologically elicited by parasite infections and they are necessary to have a "weep and sweep" effect against the worms. In contrast, type 2 immune responses mediate asthma via the mechanisms described above. Genetics play a role in the development of allergic immune responses and asthma, but an important role is played by environmental factors such as respiratory viral infections.

1.5.1 Respiratory Syncytial Virus structure and replication

RSV is single-stranded negative sense RNA virus, and is a member of the *Paramyxoviridae* family. Other members of this family include highly contagious viruses such as mumps and measles (Kiss et al. 2014). The RSV genome is formed by 10 open reading frames (ORFs) that encode for 11 structural and non-structural proteins (Huang et al. 1985) (Fig.1.5). The RSV envelope is formed by 3 proteins: the receptor attachment glycoprotein (G), the fusion protein (F), and the short hydrophobic protein (SH) (Kiss et al. 2014). As the names suggest, protein G and protein F are necessary for attachment to the cell membrane and fusion of the viral envelope and the cell membrane. Protein SH is less characterised but seems to form a pentameric ion channel and is associated with RSV pathogenesis (Gan et al. 2012; Whitehead et al. 1999). Other proteins are the non-structural protein 1 and 2 (NS1 and NS2), the nucleocapsid (N), the phosphoprotein (P), the large protein (L), which encodes the RNA polymerase, and the matrix protein (M) and M2, which has 2 open reading frame (Fig.1.6). The L gene is the last transcribed and

is often used as a measure of viral replication by qPCR, as it indicates a complete round of viral genome replication (Barik 1992; Braun et al. 2017; Lambert et al. 2014). Upon fusion of RSV to the cell membrane, the viral RNA is replicated in a positive-sense complimentary copy (antigenome), used as a template for synthesis of new genome copies. Transcription involves the generation of 10 mRNAs which are then translated into the viral proteins. The viral genome is then transported to the membrane of the infected cells and assembled together with the viral protein to form a new enveloped viral particle that will propagate the infection (Cowton et al. 2006).

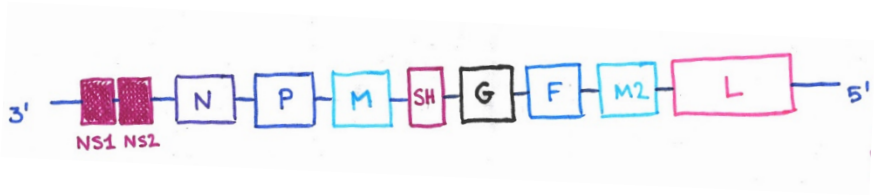


Figure 1.5 RSV genome

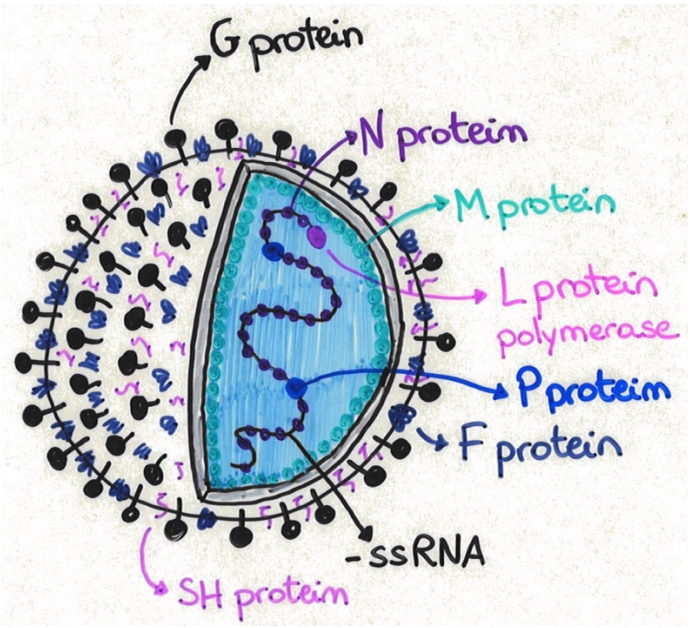


Figure 1.6 Respiratory Syncytial Virus structure

1.5.2 RSV epidemiology and disease

Isolated for the first time in 1956 in chimpanzees (Blount et al. 1956), RSV was detected in children with lower respiratory tract infection and was subsequently defined as a human pathogen (Lambert et al. 2014; Chanock et al. 1957). Shi et al. estimated that in 2015 around 33 million people worldwide suffered from RSV-dependent lower respiratory tract infection, with 3 million people hospitalised and 60,000 deaths in children younger than 5 years, with an overall mortality of 120,000 individuals, mainly in developing countries (Lambert et al. 2014; Shi et al. 2017). By the age of two, almost every child has experienced at least one RSV infection. The majority will develop mild symptoms, mainly restricted in the upper airways. A small portion will develop bronchiolitis, a lower respiratory tract infection characterised by inflammatory infiltrates, necrosis of epithelial cells and oedema (Lambert et al. 2014). These inflammatory processes lead to airway narrowing and impaired gas exchange, and in severe cases wheeze and hypoxia (Lambert et al. 2014; Tregoning & Schwarze 2010). Post-mortem characterisation of RSV pathology identified inflammatory infiltrates in the submucosa, dominated by T lymphocytes and alveolar macrophages with RSV infection extended from bronchial to alveolar airway epithelial cells (Johnson et al. 2007). Epithelial cells are believed to initiate inflammatory responses during RSV infection. BAL from children with RSV bronchiolitis have high levels of pro-inflammatory cytokines TNFα and IL-6, and chemokines CXCL10 and IL-8 (McNamara et al. 2005; McNamara et al. 2004). Neutrophils are recruited in the early stages of infection, with both beneficial and

pathogenic effects – neutrophils damage infected cells while simultaneously releasing proteases that damage the surrounding epithelium (McNamara et al. 2003).

No vaccine is available against RSV, and the only method to induce passive immunisation is palivizumab, a monoclonal antibody against RSV protein F. Palivizumab is only administrated to high risk infants (Blanken et al. 2013; Lambert et al. 2014), as it is prohibitively expensive, with a single dose costing around £5000 (Murray et al. 2014).

RSV infection can occur multiple times throughout life as people do not develop protective immune responses against the virus. In addition, poor understanding of RSV immunopathology, especially in determination of which responses are detrimental and which ones are protective lead to difficulties in developing efficient vaccination (Murray et al. 2014).

1.5.3 RSV and asthma

Numerous epidemiological studies have associated early-life severe RSV bronchiolitis with recurrent wheeze and development of asthma later in life (Henderson et al. 2005; Sigurs et al. 2005). Evidence for a causative role of RSV in the development of asthma comes from a study showing that children born 4 months before the winter peak of RSV transmission were more prone to develop early diagnosed asthma by the age of 5 (Wu et al. 2008). However, controversies still exist in defining a causative or correlative effect of RSV in subsequent asthma development due to possible shared genetic susceptibility between asthma and RSV infection. Several studies have been carried out using palivizumab as a prevention against RSV infection. Prevention of RSV infection reduced

reported wheeze up to 12 months after suspension of the treatment (Blanken et al. 2013). However, in another study in a 6 year follow-up, preventive RSV treatment did not affect asthma development or lung function (Scheltema et al. 2018).

Several considerations have to be taken into account when analysing these epidemiological studies. In particular, some studies consider wheeze episodes reported by parents and in this case it is essential to have a randomised design to avoid bias creation, while other focus on asthma diagnosed by a medical doctor. Another point is that assessing lung function in children younger than 6 years can be unreliable, creating further difficulties to standardise all these studies.

1.5.4 RSV and the IL-33 pathway

In recent years research has focussed on the role of RSV during childhood and in particular in type 2 immune responses associated with asthma. RSV replication occurs in the lung epithelium and it is hypothesised that extensive cellular damage induced the release of epithelial alarmins such as IL-33 (Kumar et al. 2014). In infants hospitalised with RSV bronchiolitis IL-33 is detectable in nasal aspirates, and IL-33 levels correlate with IL-13 (Saravia et al. 2015; García-García et al. 2017). Furthermore, SNPs in the *il1rl1* gene (encoding ST2) have been associated with the risk of RSV bronchiolitis while polymorphisms in the *il33* gene and *il1rl1* gene have been implicated in asthma development (Bønnelykke et al. 2013; Faber et al. 2012; Moffatt et al. 2010). In human challenges exist in studying the interaction between RSV and IL-33. Firstly because only

children hospitalised for severe RSV-bronchiolitis will be analysed and secondly it is challenging to determine at which day post infection children have been hospitalised. Therefore, detection of IL-33 in humans might be missed in most of the cases of RSV infection due to the fact that IL-33 is rapidly released and oxidised (Cohen et al. 2015).

Mouse models of RSV infection have been developed to help understand the mechanisms linking the IL-33 pathway and asthma. IL-33 has been detected in the airway of RSV infected mice, and anti-ST2 antibody treatment reduced the expression of type 2 cytokines and histopathology without affecting viral replication (Zeng et al. 2015). ILC2s and eosinophils accumulated in the lung of infected mice, and RSV-dependent IL-33 release induced ILC2s to produce IL-13 (Liu et al. 2015).

Other groups have carried out experiments using Pneumonia Virus of mice (PVM), a virus that is closely related to RSV and also causes pathology in the lower airways, but in contrast to RSV is a natural pathogen in mice (Rosenberg & Domachowske 2008). Co-administration of PVM and low dose of cockroach extract in neonatal mice induced the release of IL-33, which was responsible for sensitisation to the cockroach extract as blocking IL-33 prevented sensitisation. The PVM-cockroach allergen model is characterised by the development of type 2 immune responses and the onset and progression of asthma due to the IL-33 release (Lynch et al. 2016; Werder et al. 2018). Therefore, these studies suggest an important role for IL-33 in inducing sensitisation to allergen.

1.5.5 The neonatal model of RSV infection

RSV is a virus that infects human multiple times during the lifespan, however infants and the elderly are the two categories that have higher risk of RSV-dependent pathology. As RSV infection might be linked with the development of asthma, a neonatal mouse model of RSV infection is informative when studying pro-asthmatic responses. More than 15 years ago it was shown that age of first RSV infection can shape the responses at re-infection later in life. Especially, RSV infection in mice at 1 day of age induces T_H2 responses at re-infection 12 weeks after primary infection. T_H2 responses were not elicited if primary infection occurred in adulthood (Culley et al. 2002). In neonatal 5 day old mice, higher levels of IL-33 were observed 24 post-RSV infection compared to adults. Neonatal IL-33 release was associated with type 2 immune responses and asthmatic phenotype later in life upon re-infection. Type 2 immune responses at re-infection were prevented by administration of anti-IL-33 antibodies to neonates (Saravia et al. 2015). In adult mice, RSV reinfection did not induce a T_H2-skewed immune response, but intranasal administration of recombinant IL-33 during primary infection lead to the development of an asthmatic phenotype (Saravia et al. 2015). Therefore, IL-33 in early life infection might play a key role in the development of asthma later in life.

Recent studies on immune response dynamics in the developing lungs showed that IL-33 is released physiologically in the developing lung, providing a link between early life stimuli and increased induction of type 2 immune responses (de Kleer et al. 2016). In early life, DCs seems to play an important role. IL-33 was shown to activate DCs, increasing expression of OX40L and helping polarise naïve T cells to a T_H2 phenotype

(Murakami-Satsutani et al. 2014). IL-33 activates and recruits DCs *in vivo*, and in ST2 KO mice the absence of IL-33 signalling fails to activate DCs and to induce allergic responses (Besnard et al. 2011). The differences in DCs between neonatal and adult mice can be possibly explained from the different expression of IL-4Rα (Shrestha et al. 2017) as well as the increase of IL-33/ILC2s accumulation in neonatal lung (de Kleer et al. 2016).

DCs from neonatal mice express higher levels of IL-4Rα, which is linked with the suppression of maturation of DCs during RSV infection and T_H2-skewing of immune responses. Inducing DCs overexpression of IL-4Rα in adult mice during RSV infection reduces the presence of IFNγ$^+$ CD4$^+$ Th1 cells and increased IL-4$^+$ Th2 cells (Shrestha et al. 2017). As mentioned in the previous section, the role for IL-33 in inducing type 2 immune responses has also been analysed in PVM models in neonatal mice. PVM induces release of IL-33 at 10 dpi, and this release is enhanced if mice were co-exposed with cockroach allergen (CRE). CRE induces an ATP-dependent release of IL-33 at 3 dpi that reduces anti-viral immunity and predispose to the development of an asthmatic phenotype (Lynch et al. 2016). IL-33-release after CRE administration downregulates TLR7 expression on pDCs that induces a status of hypo-responsiveness with lower IFNα/β and higher IL-33 and a T_H2 skewed environment (Lynch et al. 2016). IL-33 and IFNs are able to counter-regulate each other, IFNγ has been shown to reduce ILC2s responsiveness to IL-33 *in vitro* and *in vivo*, and this is a mechanism to reduce type 2 immunopathology (Molofsky, Van Gool, et al. 2015; Duerr et al. 2016).

Therefore, in the developing lung the natural release of IL-33, accumulation of ILC2s and the specific pro-T_H2 DC phenotype leads to increase susceptibility to allergen. De Kleer and colleagues suggested that any increase in IL-33 levels, induced by allergen and possibly by RSV infection, cause sensitisation and development of asthma later in life.

1.6 Biological therapies in allergic asthma

Aberrant inflammatory responses are known to be responsible for the pathogenesis of asthma. However, the development of novel therapeutic has been challenging due to the heterogeneity of the disease. Around 5%-25% of asthma sufferers do not respond to traditional corticosteroids treatment and in the past decades several biological therapies have been developed and studied in clinical trials (McCracken et al. 2016; Global Asthma Network 2018). The attention of biological therapies has been focused on initiators of type 2 inflammation such as IL-33 and effector elements that are known to be responsible for the asthmatic pathogenesis such as IL-5, IL-4, IL-13 (and their receptor) and IgE. In this section I will give a brief overview on the current clinical trials and novel therapeutics in the field of asthma and allergies.

IgE has been targeted in biologic therapy against asthma and being one of the first clinically approved. Omalizumab is a monoclonal antibody against IgE that has been tested in phase III clinical trials for severe allergic asthma sufferers showing an reduction in incidence of exacerbations (Humbert et al. 2005; Djukanović et al. 2004; McCracken

et al. 2016). Omalizumab is clinically approved under the name of Xolaris and it is recommended for the treatment of allergic asthma and urticaria.

Dupilumab is a monoclonal antibody direct against IL-4Rα which is required for IL-4 and IL-13 signalling. In atopic dermatitis the drug is widely used due to its efficacy in reducing pruritus and symptoms of anxiety and depression with an evident quality of life improvement (Simpson et al. 2016). In severe asthma sufferers dupilumab has been observed to improve lung function when associated with corticosteroid treatment, reducing exacerbation cases and improving quality of life (Wenzel et al. 2016).

An effector cytokine widely associated with type 2 immune responses and eosinophilic inflammation in asthma is IL-5. Mepolizumab and reslizumab are anti-IL-5 therapies approved in the treatment of eosinophilic asthma. During clinical trials results were variable between the two compounds but in general they both showed a significant improvement in lung function, eosinophilia, improving asthma symptoms and quality of life (Pelaia et al. 2017; Castro et al. 2015; Castro et al. 2011). The only therapeutic drug targeting the IL-5R is benralizumab which has been shown to improve asthmatic symptoms in sufferers with no eosinophilic infiltrates as well as sufferers with eosinophilic asthma (Nair et al. 2017; FitzGerald et al. 2016; Castro et al. 2014). Analysing the data from 10 different clinical trials no differences in efficacy are observed using mepolizumab, reslizumab and benralizumab, and the conclusion is that therapies

targeting the IL-5 pathway have high efficacy in treatment of eosinophilic asthma (Cabon et al. 2017).

To target the initiation of asthmatic responses, several compounds are tested for targeting the IL-33 pathway. ANB020, an anti-IL-33 antibody, has been shown to be safe in patients and it is currently tested in asthma, peanut allergy and atopic dermatitis (Londei et al. 2017; AnaptysBio 2018). Another compound that target IL-33 is AMG282 used in patients with asthma and nasal polyps but no result have been published about safety and efficacy (NCT01928368).

GSK3772847 is another compound that targets ST2. It has been used in patients with moderate and severe asthma, but no results have been reported (NCT03207243), and in asthma sufferers with allergic fungal airway disease with the recruitment phase ongoing (NCT03393806).

Other clinical trials targeting epithelial-derived such as TSLP are underway, but no clinical trials for IL-25 have been reported. Anti-TSLP are being tested in asthma and COPD (NCT03423693, NCT02698501). Tezepelumab is an anti-TSLP mAb that showed improvement in uncontrolled asthma patients treated with β-agonist and inhaled corticosteroids, showing lower rates of asthma exacerbation compared to placebo (Corren et al. 2017).

In general, the heterogeneity of asthma makes challenging the development of a single treatment for asthmatic sufferers. Understanding the immune-pathophysiology of asthma

is a key factor for defining and stratifying asthmatic patients. Different subtypes of asthma have been identified depending on the presence of immune cells such as neutrophils or eosinophils, specific cytokines and resistance to corticosteroids treatmen. Key targets have been shown to drive disease and they can be targeted by novel therapeutics, showing improvement in the symptoms and quality of life of sufferers (Lawrence et al. 2018).

1.7 Hygiene hypothesis and parasite immunomodulation

1.7.1 Hygiene hypothesis

A rise in the prevalence of autoimmune diseases and allergic disorders has been observed in the last decades in industrialised countries and in the urbanised areas of developing countries (Bach 2002; Smits & Yazdanbakhsh 2007; Eder et al. 2006). This increase has been observed from the second half of the 20th century, for instance in 1964 19% of Australian children were reported to have diagnosed asthma or wheeze, while by 1990 such symptoms were present in 49% of children (Asher et al. 2006; Robertson et al. 1991). Changes in environmental factors, lower rate of infections and changes in the life style can influence our immune system (Lambrecht & Hammad 2017). For instance, reduced sanitation and no access to clean drinking water during childhood induce a spontaneous IL-10 production up to 8 years later of life (Figueiredo et al. 2009).

Environmental factor seems to play a key role, for instance, comparing two farming populations settled in the United States with a similar genetic background, the Amish and Hutterites, it was observed that the Amish had much lower levels of allergies and asthma. The Amish preserve a traditional farming society, where children are exposed to animals and to an environment rich in microbes, while the Hutterite have a modernised farming and children are exposed to much cleaner environments (Stein et al. 2016). This effect on asthma rates was hypothesised to be due to exposure to microbes or endotoxins influences the innate immune system, preventing hyperresponsiveness to allergen (Stein et al. 2016;

Braun-Fahrländer et al. 2002; Ege et al. 2011). Exposure to low dose of endotoxin (LPS) or farm dust (containing microbial products) abrogates the development of HDM-induced asthma, suppressing epithelial cytokines such as GM-CSF and IL-33 that recruit and activate DCs. The protective effects were lost if there is a loss in ubiquitin-modifying enzyme A20 in the lung epithelium (Schuijs et al. 2015), which has been shown to suppress TLR and IL-1 family signalling, and suppressing inflammasome activation (Jäättelä et al. 1996; Duong et al. 2015). These observations support the "hygiene hypothesis" developed in the 70s and formally defined in the late 80s, which stemmed from the detection of lower rates of eczema and allergies in children grown up in large families, due to the fact that these children were exposed to more recurrent infections (Strachan 1989). Early-life exposure to microorganisms and parasites is recognised to shape the immune responses in adulthood (Djuardi et al. 2011), and in the last decades several factors have been implicated in shaping our immune system e.g. mode of giving birth, breast feeding, number of siblings, infections, and contact with animals (Lambrecht & Hammad 2017).

1.7.2 Parasite and the hygiene hypothesis

A branch of the hygiene hypothesis involved the study of the immunomodulatory effect of parasites, due to the observation that in those area of the world were parasite infection are still common there is less incidence of allergies, asthma and auto-immune diseases.

A quarter of the world population is affected by parasitic infections (Jourdan et al. 2018). Helminths infections are common in developing countries, with soil-transmitted

nematodes (e.g. *Ascariasis lumbricoides, Trichuris trichiura* and hookworms such as *Ancylostoma duodenale*) being the most prevalent (Jourdan et al. 2018; Hotez et al. 2008). In industrialised countries many parasitic infections have been virtually eradicated in contrast to bacterial infection which have only been reduced (McSorley et al. 2013). A systematic review and meta-analysis identified the decreased risk of developing asthma in endemic hookworm infection areas (Leonardi-Bee et al. 2006). Thus, epidemiological observation suggested that parasites can modulate the immune system. This led to the proposal of using live parasitic infection to treat immune disorders, termed "helminth therapy". The first trials of helminth therapy were carried out to determine the safety of a porcine parasite, *Trichuris suis*, in patients suffering from Crohn's disease (CD) and ulcerative colitis (UC) (Summers et al. 2003). No adverse effects were detected during the infection and improvement in both CD and UC was observed (Summers et al. 2003; Summers et al. 2005). However, efficacy was not observed in the treatment of coeliac disease, allergic rhinitis and more recently Crohn's disease, even if immune responses were suppressed (Bager et al. 2010; McSorley et al. 2011; Daveson et al. 2011; Schölmerich et al. 2017). Using helminth therapy to treat asthma led to clinical trials with the hookworm *Necator americanus*. However these studies did not show improvement in lung function nor asthma amelioration (Blount et al. 2009; Feary et al. 2010).

Helminth therapy has been studied in auto-immune diseases such as multiple sclerosis (MS), following studies where in endemic helminth infection area helminth-infected MS patients showed the induction of a regulatory phenotype and reduced lesions (Correale et al. 2008). Clearing the infection using anti-helminthic drugs induced reappearance of the

symptoms suggesting that helminths were suppressing the exuberant immune responses (Correale & Farez 2007; Correale & Farez 2011). Recent studies using *T. suis* ova showed in general no clinical improvement in the treatment of MS, even if in the last trials results were variable (Voldsgaard et al. 2015; Fleming et al. 2017). Negative results from helminth therapy trials might depend on the fact that parasitic infections occur naturally in endemic areas. Infection can occur from a young age and recurrent infections are common, therefore in endemic areas, differently from a clinical setting where parasites are administered in pre-existing disease, parasites modulate the immune system for a longer period and before the initiation of the disease. In addition, other factors that can influence the outcome of the helminth therapy are when to administrate the helminth therapy after the disease started and the other challenging part is to decide the optimal dose of parasites.

1.7.3 Parasites immunomodulation and excretory/secretory products (ES)

Mouse model of parasitic infections have helped to understand their ability to modulate the host immune system. *S. mansoni* and *H. polygyrus* infection in mice reduces OVA-induced type 2 immune responses and airway hyperresponsiveness (Mangan et al. 2006; Smits et al. 2007; Wilson et al. 2005). Protection in the *S. mansoni* model was dependent on IL-10 production (Mangan et al. 2006). In a chronic model of *S. mansoni* infection, IL-10 was still necessary to induce suppression of OVA-induced type 2 inflammation, and B cells and T cells were involved in suppression (Smits et al. 2007). Moreover, isolated eggs from *S. mansoni* suppressed bystander OVA-specific T_H2 responses even if

a strong induction of type 2 immune responses was observed in response to the eggs. Eggs induced the generation of regulatory T cells and they impaired the recruitment of DCs, with reduced CCL2 levels in the BAL of egg-treated mice (Obieglo et al. 2018; Pacífico et al. 2009). Recently, there has been a focus on identifying a single component of *S. mansoni* eggs that replicates the immunomodulatory effect observed. Until now, a protein from *S. japonicum* called SjP40 has been shown to induce T_H1 immune responses suppressing allergic T_H2 inflammation (Ren et al. 2016), and recently the glycoprotein IPSE/alpha-1 from SEA was shown to induce IL-10 production from regulatory B cells, although the authors do not exclude the presence of other immunomodulatory molecules in SEA (Haeberlein et al. 2017).

Evidence that the immunomodulatory effects of parasites could be replicated by their excretory/secretory products have been observed in *N. brasiliensis* infection. *N. brasiliensis* infection is associated with reduction of allergen-dependent eosinophilia (Wohlleben et al. 2004) and reduced allergic type 2 immune responses were observed after *N. brasiliensis* ES (NES) administration (Trujillo-Vargas et al. 2007). Suppression by NES was independent from TLR2, TLR4, IFNγ and IL-10 and it was mediated by a nonprotein components of NES, as heat treatment or proteinase treatment did not affect NES effects (Trujillo-Vargas et al. 2007).

Similarly, infection with the nematode *H. polygyrus* suppressed allergen-induced T_H2 inflammation (Wilson et al. 2005) and this can be replicated by HES administration (McSorley et al. 2012). In the next section (1.7.3 *Heligmosomoides polygyrus*: life cycle and immunomodulation) I will analyse the immunomodulatory effects of *H. polygyrus*.

ES products have been characterised by mass spectrometry and together with a better knowledge of the parasites genomes, lead to the discovery of single proteins contained in ES with immunomodulatory effects (Maizels et al. 2018). For instance, ES-62 from the filarial nematode *Acanthocheilonema viteae* has been widely studied and it was shown that post-translational modification including phosphorylcholines moieties are responsible for the anti-inflammatory effects observed with ES-62 administration in a model of arthritis (Pineda et al. 2014; McInnes et al. 2003) and in a model of asthma (Rzepecka et al. 2013). This molecule targets several immune components, such as the BCR, TCR and TLRs, and suppresses aberrant MyD88 activation (Melendez et al. 2007; Ball et al. 2018; Pineda et al. 2014). Small molecule analogues have been created which mimic ES-62 phosphorylcholine moieties and some of them can replicate ES-62 effect *in vivo* and protect against collagen induced arthritis (Al-Riyami et al. 2013) and in models of asthma using cockroach extract and HDM (Janicova et al. 2016).

In addition, two proteins from the hookworm *N. americanus* and *Ancylostoma caninum*, anti-inflammatory protein 1 AIP-1 and AIP-2, have been identified and reduce inflammatory responses in model of colitis and allergic inflammation respectively. AIP-1 induces expansion of regulatory T cells in the gut mucosa due to increased production of IL-10 and TGFβ (I. B. Ferreira et al. 2017) while AIP-2 acts on $CD103^+$ DCs to induce regulatory T cells and reduce OVA-induced airway inflammation (Navarro et al. 2016). Therefore, parasite secretions have been shown to induce modulatory effect on the host immune system and these effects can be replicated by single molecules that can be identified and studied in clinically-relevant model of disease. An optimal model to use in

the lab is the strictly intestinal nematode *Heligmosomoides polygyrus* which ES has been shown to have immunomodulatory effect (McSorley et al. 2015; Johnston et al. 2015; Maizels et al. 2012), and it will will my focus on the next section.

1.7.4 *Heligmosomoides polygyrus*: life cycle and immunomodulation

Heligmosomoides polygyrus is a natural wild mouse parasite that has been successfully transferred to laboratory mice, and for years was called *Nematospiroides dubius* (Dobson & Owen 1977; Dobson & Tang 1991). It is an optimal model to study immunity and immune evasion. It is in the same Order (Strongylida) as the human hookworms (e.g. *Necator americanus* and *Ancylostoma duodenale*) and similarly to hookworms, it can establish a long-lasting chronic infection. In the laboratory setting, *H. polygyrus* L3 larvae are introduced by oral gavage in the mouse. Within 24h, larvae reaches the small intestine penetrating the submucosae (beneath the lamina propria) where they undergo two developmental molts. They subsequently emerge into the lumen as adult worms where they coil around the villi. Eggs are produced and released in the external environment through the faeces, eggs have to hatch and go through developing moults to generate infective L3 larvae, which can then spread the infection to other mice (Reynolds et al. 2012) (Fig.1.7). Adult worms from the intestine can be collected and used to produce HES (Reynolds et al. 2012; Johnston et al. 2015).

Live infection with *H. polygyrus* has shown protection in several model of allergic airway inflammation. Wilson et al. showed that infection with *H. polygyrus* suppresses OVA-dependent type 2 immune responses. Live infection induces reduction in inflammatory infiltrates in the lungs, especially eosinophils, mucus production and mast cell degranulation. Protection was observed if infection occurred at both sensitisation phase or challenge, and suppression was dependent on regulatory T cells. $CD4^+$ $CD25^+$ cells were transferred into OVA-sensitised mice and these cells were able to induce protection (Wilson et al. 2005). Kitagaki et al. showed suppression of pathology in an OVA-dependent model of asthma, although in contrast to the study by Wilson et al., they found this suppression was dependent on IL-10 (Kitagaki et al. 2006). In another model of allergen-induced asthma using the Der p1, an allergen found in HDM, *H. polygyrus* was still be able to suppress type 2 immune responses, and suppression could be transferred with $CD4^+$, $CD4^-$ and $CD19^+$ B cells isolated from the mesenteric lymph nodes of infected mice. In another model using HDM and *H. polygyrus*-infected mice, reduction in allergic responses associated with alteration of the gut microbiome with an increase production of short chain fatty acids (SCFAs). Transferred of the altered microbiota from infected mice was sufficient to induce protection against allergic asthma (Zaiss et al. 2015). *H. polygyrus* not only suppressed allergic responses but had immunomodulatory effects in an experimental model of autoimmune encephalomyelitis (Wilson et al. 2010), and, in a model of co-infection with RSV, *H. polygyrus* larvae induced protection against RSV pathology in an IFN-dependent and microbiota-dependent manner (McFarlane et al.

2017). Therefore, the enteric parasite *H. polygyrus* is able to modulate immune responses distally for example in the lungs and in the central nervous system.

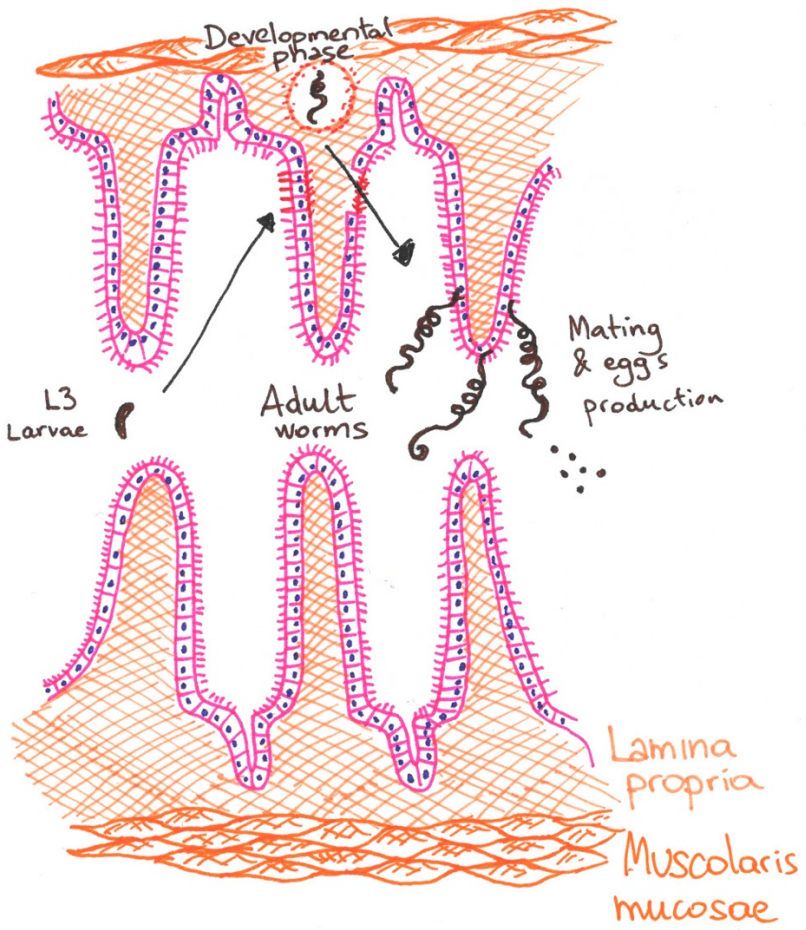


Figure 1.5 Heligmosomoides polygyrus life cycle

Infective L3 larvae are transmitted by feco-oral transmission in wild-mice and by oral gavages in the laboratory setting. Larvae migrate in the subserosa layer beneath the lamina propria of the duodenum and emerging in the intestinal lumen after approximately 8 days post infection.

1.7.5 *H. polygyrus* excretory/secretory products (HES) and its modulatory effects

Infection with *H. polygyrus* has been demonstrated to have broad effects in allergic airway inflammation. The immunomodulatory effects in allergic inflammation can be replicated with administration of HES (McSorley et al. 2012).

Adult worms can be collected from the gut of infected mice and cultivated *in vitro* to obtain HES (Johnston et al. 2015). HES is a complex mixture of molecules that contains around 400 identified proteins, with abundance of proteases, apyrases, acetylcholinesterase (AChE), and venom allergen-like (VAL) proteins, a conserved family identified in other parasites (Hewitson et al. 2011). Protein expression is different between larval ES and adult ES, underlining the different need of the parasite depending on the developmental stage and the location in the intestine (Hewitson et al. 2013). VAL proteins have been shown to exert some immunomodulatory effects such as suppressing basal immunity in plants, inhibition of cell adhesion and signalling pathway in B cells in mammals (Wilbers et al. 2018). However, in *H. polygyrus* no immunomodulation has been proved yet. Recently, HpVAL-4 protein has been show to bind to palmitate and sterol but more studies need to be carried out to determine possible immunomodulatory effect (Asojo et al. 2018). Another family of proteins that has been detected in HES and other parasites ES is the Sushi- or complement control protein (CCP) domain family (Hewitson et al. 2013). CCP domains are consensus sequences of around 60 amino acids containing 4 conserved cysteines residues linked by disulphide bonds and the presence of conserved tryptophan, leucine, glycine, proline and hydrophobic residues (Kirkitadze &

Barlow 2001; Soares et al. 2005). They were discovered in proteins from the complement system but subsequently found in neurotransmitters, interleukin receptors and other proteins outwith the complement system have been identified to be CCP domain proteins (Blein et al. 2004; Hewitson et al. 2013). Nematodes produce CCP domain-containing proteins and the CCP domain family underwent expansion in the *H. polygyrus* genome, with around 40 CCP domain proteins identified in *H. polygyrus* ES (Maizels et al. 2018; Hewitson et al. 2013). Different species of nematodes evolved independently, but evolution was driven by common environmental and host factors (Coghlan et al. 2019). Recently, the *H. polygyrus* genome was published, identifying around 27 thousand protein coding genes (Coghlan et al. 2019) (PRJEB1203). Sequencing the whole genome and knowing the protein produced by the parasite allows identification of further immunomodulatory molecules.

HES represent a major focus for immunological analyses due to its multiple immunomodulatory effects (Fig.1.8). HES induces expansion of $Foxp3^+$ T regulatory cells (Grainger et al. 2010), reduces activation of DCs (preventing subsequent stimulation of T cells) (Segura et al. 2007) and reduces airway inflammation by suppressing IL-33 responses (McSorley et al. 2012; McSorley et al. 2014). The importance of the IL-33 pathway is underlined by the fact that *H. polygyrus* targets this pathway using several strategies: reducing its expression through induction of IL-1β and targeting the expression of ST2 through miRNA from parasite-derived EV (Zaiss et al. 2013; Buck et al. 2014; Coakley et al. 2017). In addition these parasite-derived EV not only suppressed the

alternative activation of macrophages but also LPS-induced macrophage activation with suppression of IL-6, TNFα and inducible nitric oxide synthetase (iNOS) mRNA (Coakley et al. 2017). Therefore, the RNAs contained in the EV might play an important role in immunomodulation, however the authors do not exclude that another components of HES (such as protein or carbohydrates) can mediate the observed effects as HES depleted of EVs could still suppress responses (Coakley et al. 2017).

In a model of asthma inducing sensitisation to OVA with alum adjuvant intraperitoneally to mice, co-administration of HES suppressed OVA-dependent type 2 immune responses. HES reduced inflammation in the lungs, eosinophilia, airway hyperresponsiveness, and production of IL-4, IL-10, IL-13, IL-17A and IFN-γ. These effects were not dependent on the TGFβ activity of HES, and they depended on a heat-stable component of HES (McSorley et al. 2012). In addition, this suppressive effect was independent of MyD88 and TRIF: important downstream signalling adaptors of TLRs and the IL-1 receptor family (McSorley et al. 2015). It was shown that HES acted on the suppression of early type 2 immune responses and in the suppression of ILC2 activation (McSorley et al. 2015). However, using the alum as an adjuvant is not a clinically-relevant model to study asthma but rather study vaccination-like responses. In particular the observed unchanged inflammation after OVA-alum administration in MyD88-KO mice suggest that in contrast to the evidence from human asthma and other mouse models of asthma, these type 2 inflammatory responses are not dependent on IL-33 but on other pathways that HES suppresses.

Furthermore, in another model of asthma using a single dose of the fungal allergen *Alternaria* in combination with OVA mice can be sensitised against OVA and OVA-dependent type 2 immune responses can be elicited at challenge. Similarly, a single administration of HES at sensitisation suppressed eosinophilic infiltrates in the lung tissue and in the BAL but in this model the suppressive effect was associated with a heat-labile component of HES. HES reduced production of type 2 cytokine e.g. IL-5, IL-13 and IL-4 (McSorley et al. 2014).

IL-33 had a key role in this model, as ST2-deficient mice did not develop type 2 inflammation in response to *Alternaria* and administration of recombinant IL-33 abrogated HES suppressive effects (Snelgrove et al. 2014; Kouzaki et al. 2011). In this model HES suppressed the early IL-33 release in response to *Alternaria*, and early type 2 immune responses such as eosinophils 24h post-*Alternaria* administration and ILC2 activation (McSorley et al. 2014).

The several immunomodulatory effects associated with HES administration led to the identification of single molecules that can replicate the same effect. A single protein that has been identified from *H. polygyrus* genome is the cysteine protease inhibitor (HpCPI), which affects maturation of dendritic cells. This protein suppressed cysteine protease activity and when incubated with dendritic cells suppressed expression of MHCII (Sun et al. 2013). HpCPI reduced production of IL-6, IL-12 and TNFα from DCs stimulated with CpG, indicating that the recombinant protein affects DC responses, and this suppressive

effect can be observed in a T cell co-culture system in which HpCPI-treated DCs did not activate T cells (Sun et al. 2013).

In addition, as mentioned previously HES induced expansion of $Foxp3^+$ T_{REG} through the TGFβ pathway, and the transfer of T_{REG} from *H. polygyrus* infected animals induced protection in an asthma model (Wilson et al. 2005), while HES-induced T_{REG} suppressed T_H2 immune responses in a similar manner as TGFβ-induced T_{REG} (Grainger et al. 2010). Recently a parasite-derived TGFβ mimic named HpTGM was identified. This protein consists of 5 CCP domains and does not show homology with TGFβ (Johnston et al. 2017; Smyth et al. 2018). HpTGM binds directly to the TGFβ receptor, activating the signal transduction cascade associated downstream of the type I and type II TGFβ receptor. HpTGM is a promising molecule showing anti-inflammatory effects in a mouse model of skin allograft rejection (Johnston et al. 2017).

In conclusion, the advances in genomic and proteomic analysis lead to the understanding of how parasites interact with their host. Through their secretion parasites can release a broad range of molecules from protein to small RNAs that influences and modulate the host immune system. These molecules help to understand the mechanisms used by the parasites to survive within the host as well as giving the opportunity to use these molecules as therapeutic agents for human diseases.

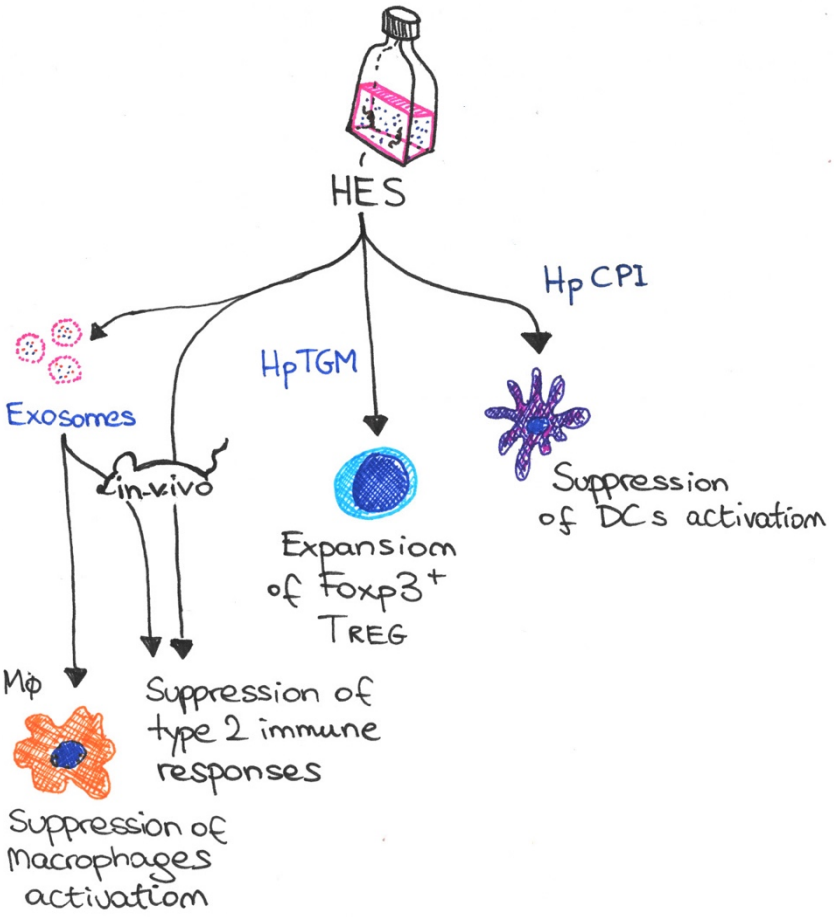


Figure 1.6 Administration of HES both *in vitro* and *in vivo* is associated with immune-modulatory effects

1.8 Hypothesis and aims

In the past years several groups have been studying how parasites modulate the host immune system, focusing on parasite ES products. Various proteins have been identified and to modulate the immune system. *H. polygyrus* is an intestinal nematode that has been used as an optimal laboratory model for chronic helminth infection. It modulates the immune system at different levels, and in particular it modulates the IL-33 pathway using different mechanisms.

H. polygyrus ES products called HES block IL-33 release and suppress ST2 (the IL-33 receptor) (Buck et al. 2014; Coakley et al. 2017; McSorley et al. 2014).

At the start of my project, a single protein called HpARI had been identified from HES which suppresses IL-33 release. Recombinant HpARI was produced in the lab and showed to suppress detection of IL-33 *in vitro* using freeze/thaw-induced IL-33 release. HpARI was tested *in vivo* and showed to suppress early IL-33 responses similarly to HES (Osbourn et al. 2017; McSorley et al. 2014). However, the mechanism was still unclear and it will be investigated with this project.

The hypotheses of this project are:

1) HpARI blocks IL-33 release by binding directly to the cytokine, preventing the cytokine from binding to its receptor.
2) IL-33 released during RSV infection is essential for inducing type 2 immune responses, and HpARI can suppress RSV-induced IL-33 release.
3) Another protein in HES suppresses ST2 expression.

The aims of this project are to:

1) Characterise the mechanistic effect of HpARI on IL-33 suppression. Binding studies will be performed to assess the ability of HpARI to bind murine IL-33 and human IL-33, and to block IL-33-ST2 interaction.
2) Develop an *in vitro* assay using bone marrow cells to study the induction of IL-33 dependent responses and the suppressive effect of parasite-derived proteins. In addition, this assay will test the specificity of the proteins against the IL-33 pathway.
3) Investigate whether HpARI can suppress IL-33-dependent RSV-induced immune responses in a neonatal mouse model of RSV infection and allergic sensitisation.
4) Analyse HES fractionation and HES mass spectrometry data to identify a novel protein which suppresses ST2. The protein will be recombinantly produced and analysed both *in vitro* and *in vivo*. The mechanism of action will be assessed.

Chapter 2

Materials and Methods

2.1 Mice and experimental model

BALB/cOlaHsd, C57BL/6JOlaHsd, IL-13-eGFP (C57BL/6 background (Neill et al. 2010)) and ST2-deficient mice (BALB/c background- kindly provided by Dr Andrew McKenzie, MRC Laboratory of Molecular Biology, Cambridge) were bred in house at the University of Edinburgh. For bone marrow collection males aged > 8 weeks were used.

For experimental procedures in adult mice, 6-10 weeks old female mice were used. For the neonatal model, breeding pairs were set up by technician in the animal facilities at the University of Edinburgh with a ratio of 1 male to 2 females. Pregnant mice were handled for 3 days a week prior to birth. After birth, dams were olfactorily conditioned with isofluorane, the anaesthetic used, and the ink used for tattooing neonates. Neonates were marked in the foot with tattooing ink as ears marking was not possible to perform because ears were too small at this age. Neonatal mice aged between 5 and 7 days were used in experimental procedure.

At the collection day, depending on the experiment BAL fluid 4x0.5 ml washes in cold PBS were collected for cytokine analysis and cellular analysis by flow cytometry. Half lung lobe was collected, homogenised and analysed by flow cytometry. The other half was used for RNA extraction and lung homogenate.

2.2 Culture media, cell lines and reagents

Complete culture media was supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% L-glutamine (all supplied by Gibco, ThermoFisher).

CMT-64 obtained from ECACC were cultured in complete DMEM (Gibco).

Hep2 cells were obtained from ATCC and cultured in complete RMPI (Gibco).

Expi293T obtained from Thermo Fisher Scientific were cultured as described by the manufacturer guidelines. Cells were grown in Expi293 Expression Medium (Gibco) at 37°C 8% CO_2 in constant shaking at 125 rpm and split when concentration was at 3-5x10^6 cells/ml.

Human nasal epithelial cells (hNECs) were obtained by brushing the medial aspect of the inferior turbinate of healthy volunteers under informed consent. Cells were collected in 1 ml BEGM media (Lonza) and plated immediately in BEGM. Cells were split when at 80% confluence, washed twice with HBSS (Gibco), trypsinised with trypsin/EDTA (Lonza) for 10 minutes at 37°C. Trypsin was neutralised using Trypsin Neutralising Solution (Lonza). Cells were used at passage 3 or 4.

General reagents:

FACS buffer

PBS 0.5% BSA 0.05% Sodium Azide

ELISA Block Buffer

PBS 1%BSA

ELISA Wash Buffer

1XTBS 0.05% Tween-20

Mouse ST2-Fc, mouse TRAP-Fc, human TRAP-Fc kindly provided by Suzanne Cohen (MedImmune, Cambridge, UK).

Alternaria alternata extract (Greer XPM1D3A25) was resuspended in PBS and concentration assessed by Pierce™ BCA assay (Thermo Fisher Scientific).

2.3 RSV, immunoplaque assay and *in vitro* RSV infection

Plaque-purified human RSV (Strain A2; ATCC, Manassas, Va) was grown in Hep-2 cells as previously described (Currie et al. 2013). The viral stock used in this study was produced and provided by the Schwarze lab (University of Edinburgh). Briefly, Hep2 cells were cultured and seeded at $5x10^6$ in a T175 flask and incubated overnight at 37°C 5% CO_2 in complete RPMI. The next day, cells were infected with human RSV A2 at a final concentration of 0.1 pfu/cell in a 3 ml volume of serum-free RPMI. Cells were incubated at 37°C 5% CO2 for 2 hours and rotated 90° every 15 minutes to be sure surface was covered equally. After 2 hours, 27 ml of complete RPMI were added to the culture and incubated until cytopatic effect was observed and 50% of the cells were floating (approximately around 5 days after). Cultures were then centifugated at 2000 rpm for 5 minutes, supernatants collected and aliquoted as viral stock. Tubes were snap frozen in liquid nitrogen.

Adult mice were infected with 50 μl of RSV stock (65000 PFU). Neonates were infected with 3250 PFU/ grams of body weight.

UV-RSV was generated by placing RSV stock on ice in the UV-Stratalinker 2400 (Stratagene, Agilent Technologies, Cheshire UK) and UV-irradiated for 15 minutes.

RSV titres were assessed diluting lung homogenates from infected mice in Hep-2 cells monolayers in 96-well flat bottom plate. Cells were washed with PBS 24h after infection and fixed with methanol 2% hydrogen peroxide (Sigma). Fixed cells were washed with PBS and incubated with biotin-conjugated goat anti-RSV antibody (Bio-rad, Watford, United Kingdom) diluted in PBS 1% BSA for 1 hour at RT. After, cells were washed three times with PBS. Cells were incubated with ExtrAvidin®-peroxidase (Sigma) diluted in PBS 1%BSA for 1 hour at RT. Infected cells were detected with 3-amino-9-ethylcarbazole, and infectious units were enumerated using light microscopy.

Human NECs obtained as described previously were infected with RSV (MOI 0.1) in the presence or absence of HpARI (1 ug/ml), when at around 60% confluence. Three day post infection NECs were washed with PBS and harvested using TRIzol. The study was approved by NHS Lothian.

2.4 HES/Exosomes preparation and fractionation

HES was prepared by Henry McSorley as described by Johnston et al. 2015. Exosomes were obtained from the Buck lab and prepared using ultracentrifugation as described by Buck et al. 2014 (Buck et al. 2014; Johnston et al. 2015). HES fractionation was performed by Henry McSorley as described in (Osbourn et al. 2017). In brief, Superdex 200 10/300 GL column was used for the size fractionation while MonoQ 5/50 GL column was used for the charge fractionation. A volume gradient from 20 mM TrisHCl pH 8 to a maximum of 30% 20 mM TrisHCl + 1 M NaCl pH 8 used as elution buffer and to obtain the charge fractionation.

2.5 Single lung cell suspension

A single lung cells suspension was obtained by digesting lung tissue from mice in 2 U/ml liberase TL (Roche, Burgess Hill, UK) and 80 U/ml DNase (Life technologies, Paisley, UK) shaking for 35 minutes at 37°C. Digested tissue was passed through a 70um strainer and red blood cell lysed with Red Blood Cells Lysis Buffer (Sigma). Live cells were counted using a haemocytometer and dead cells excluded using trypan blue.

2.6 *In vitro* HES fraction assay

Lung cells were resuspended at $5x10^5$ cells/well in a 96-well round bottom plate. Each HES fraction (1 µl of fraction in a 200 µl culture) were tested in the lung cells suspension for 24 h. HES, HpARI, exosomes and supernatants were added at the concentration

indicated in the respective graph and incubated at 37°C for 24 h. After 24 h supernatants were collected, cells were spin at 400 g for 5 minutes, washed and stained for flow cytometry.

2.7 Helminth-derived recombinant protein

HpARI was identified as described in (Osbourn et al. 2017). For ST2 suppression, candidate genes were selected comparing emPAI and ST2 suppression profile obtained by flow cytometry. The candidate Hp_I25642_IG17586_L548 was selected according to the suppression profile and the similar structure with HpARI, and for this reason was already being expressed in the lab as a part of another project to characterise the CCP domain protein family. Hp_I25642_IG17586_L548 was codon optimised for *Homo sapiens* and gene synthesised (GeneArt, Thermo Fisher) with AscI and NotI at the 5' and 3' respectively. The sequence was ligated into a pSecTAG2A expression vector (Thermo Fisher) using AscI and NotI-HF restriction enzymes (New England Biolabs).

JM109 cells were transformed with ligated construct and the plasmid was obtained using miniprep kit (Qiagen) according to manufacturer's instruction and Sanger sequenced using the service from Edinburgh Genomics.

Expi293 transfection system was used as described by the manufacturer's instruction and mainly performed by other members of the lab. In brief, $5x10^6$ cells/ml >95% viable were prepared into a 30 ml culture. Plasmid DNA (1 μg) was diluted in 3 ml Opti-MEM™ I reduced Serum Medium and incubated with ExpiFectamine™293 Reagent (ThermoFisher) for 20 minutes at room temperature, and added to the cells. The next day

transfection was enhanced using ExpiFectamine™ 293 Enhancer 1 and 2. Supernatants were collected 7 days after enhancement and purified using HisTrap excel column (GE Healthcare) and imidazole gradient to elute the bound protein. Fractions were assessed for protein content using Bradford assay and 5 μl of each fraction was run into a 4-12% Bis-Tris protein gel and stained with Coomassie blue. Fractions containing protein were pooled, dialysed into PBS, sterile filtered and assessed by absorbance at 280 nm to determine protein concentration. Concentration was corrected by the extinction coefficient calculate on Expasy protparam.

2.8 *In vitro* IL-33 release assay and oxidation assay

Confluent CMT-64 were plated at $5x10^5$ cells/well in a 96-well flat bottomed plate, frozen on dry ice and thawed at 37°C. Thawed plates were centrifuged at 400 g for 5 minutes and supernatants from all the wells collected and pooled together. In a new 96-well flat bottomed plate, 100 μl of thawed supernatants were added with or without 100 μl HpARI (2 μg/ml) and IL-33 levels assessed by ELISA.

2.9 *In vitro* bone marrow cultures

Tibias and femurs were obtained from euthanised mice in the animal facilities. Bones were placed in PBS and processed in the lab. Bones were placed in 70% ethanol for 5 minutes and washed with PBS. Bone marrow was flushed using a syringe with complete

RPMI and it was passed through a 70 μm strainer. Red blood cells were lysed using red blood cell lysis buffer (Sigma). Cells were resuspended in complete RPMI (Gibco). $1x10^6$ or $5x10^5$ cells were plated in a round bottom 96-well plate and co-cultured with IL-2, IL-7, IL-33 at 10 ng/ml (Biolegend), and our recombinant parasite-derived proteins at the concentration indicated in the experiment, at 37°C 5% CO2 for 24h, 3 days or 5 days as indicated in the figure legend. After incubation cells were washed and surface stained.

2.10 Flow cytometry staining

2.10.1 Flow cytometry surface staining

Lung cell suspension or bone marrow cells suspension were centrifuged at 400 g for 5 minutes after incubation. Supernatants were collected for further analysis or discarded. Cells were washed 3 times with PBS (200 μl/well for all washes). Cells were then incubated with 200 μl/well Fixable Blue Live/Dead (Thermo Fisher; 1:1000 in PBS) at 4°C for 20 minutes in the dark. Cells were spin down washed once with PBS and once with FACS buffer. Cells were blocked with anti-mouse CD16/32 antibody (Biolegend; 1:500 in FACS buffer) at 4°C for 10 minutes. Cells were washed twice with PBS and surface stained using antibodies indicated in table 2.1 at 4°C for 20 minutes. Cells were washed twice with FACS buffer and resuspended in 250 μl ready for analysis. Samples were aquired using 5L or 6L LSRFortessa (BD Biosciences) and analysed using FlowJo™ software (version 10).

2.10.2 Intracellular cytokine stain

To determine activation and to quantify type 2 immune responses, intracellular cytokine stain was performed to detect IL-5 and IL-13 by flow cytometry. Lung cells ($3x10^6$ cells/well) were stimulated with PMA (500 ng/ml), ionomycin (1 μg/ml) and brefeldin A(10 μg/ml) for 4h at 37°C 5%CO_2. Cells were surface stained as described previously. After surface stain, cells were incubated with 100 μl/well of IC Fixation buffer (eBioscience, UK) for 20 minutes at 4°C. Cells were washed twice with permeabilization buffer (eBioscience). Antibodies for intracellular cytokine stain were diluted in permeabilization buffer and cells were incubated at 4°C for 20 minutes. Subsequently cells were washed twice with FACS buffer and resuspended in 250 μl FACS buffer ready for flow cytometry.

2.10.3 BAL surface stain

Bronchoalveolar lavages were collected washing lungs with 4x0.5 ml in cold PBS placing an 18G x2" needle in the trachea. The first wash of 0.5 ml was used for cytokines measurement while the next 3x0.5 ml used for immune cells surface stain. Red blood cells were lysed and cells resuspended in FACS buffer. The surface stain protocol was followed starting from the blocking step.

Type 2 innate lymphoid cells (ILC2s) were identified in the live cell population as $CD45^+ICOS^+CD4^-Lineage^-$ (include CD11b, CD3, CD5, CD19,CD49b, GR1, TER-119).

CD25 geometric mean fluorescence intensity (MFI) and ST2 MFI were calculated within the ILC2 population. Eosinophils were identified as $CD45^{+}SiglecF^{+}CD11c^{-}$.

Table 2.1 **List of antibodies used for flow cytometry**

Antibodies (anti-mouse)	**Clone**	**Source**	**Dilution**	**Fluorochrome**
CD3	145-2C11	Biolegend	1:200 1:50	FITC Biotin
CD5	53-7.3	Biolegend	1:200	FITC
CD11b	M1/70	Biolegend	1:200 1:50	FITC Biotin
CD19	6D5	Biolegend	1:200	FITC
CD49b	DX5	eBioscience	1:200	FITC
GR1	RB6-8C5	Biolegend	1:200 1:50	FITC Biotin
TER-119	TER-119	Biolegend	1:50	Biotin
CD45R/B220	RA3-6B2	Biolegend	1:50	Biotin
CD45	30-F11	Biolegend	1:200	AF700
CD25	PC61	Biolegend	1:200	BV650
ICOS	15F9	eBioscience	1:100	PCP
ST2	RMST2-2	eBioscience	1:100	APC
CD4	RM4.5	Biolegend	1:200	PeDazzle

IL-5	TRFK5	Biolegend	1:200	PE
IL-13	eBio13A	eBioscience	1:200	PECy7
CD11c	N418	Biolegend	1:200	APC
Ly6C	HK1.4	Biolegend	1:200	PECY7
Ly6G	1A8	Biolegend	1:200	PerCP
SiglecF	ES22-10D8	Miltenyi	1:200	PE
Streptavidin		eBioscience	1:200	eF450

2.11 Enzyme-linked immunosorbent assay (ELISA)

Ready-SET-Go™ mouse IL-5, IL-13, IL-6 and IFNγ from eBioscience were used according to manufacturer's instructions. In brief, Nunc MaxiSorp™ plates (Thermo Fisher, UK) were coated with 50 µl/well of capture antibody diluted in 1X Coating buffer and incubated at 4°C overnight. The following day the plate was washed with ELISA Wash buffer three times and blocked with 150 µ/well of ELISA Blocking Buffer for 1h at RT. Block buffer was removed, plate washed three times with ELISA wash buffer and standard and samples (50 µl/well) were added to the plate. For bone marrow culture, supernatants were diluted 1:5 (3 days culture) or 1:10 (5 days culture) in block buffer. Samples and standards were incubated for 2h at RT. Plate was washed four/five times in ELISA wash buffer and incubated 1h at RT with 50 µl/well of detection antibody. After incubation, plate was washed four/five times and 50 µl/well of avidin-HRP (1:250 in block buffer) added to the plate for 30 minutes at RT. Plates were washed and 50 µl/well

of TMB substrate added to the plate and the reaction stopped with 50 µl/well of 2N H_2SO_4. Absorbance was read at 450 and background at 570 nm subtracted.

2.12 Solid-phase ELISA

Solid-phase ELISA was used to determine if constructs obtained from MedImmune were binding our parasite recombinant proteins.

Corning ™ Costar ™ 96-well EIA/RIA plate (Fisher Scientific, Thermo Fisher, UK) were coated overnight at 4°C with 1 µg/ml of HpBARI N-terminus or C-terminus tagged or HpARI diluted in 1X Coating buffer (eBioscience) (all at 50µl/well). Plate was washed three times with ELISA wash buffer and blocked with 150 µl/well ELISA block buffer for 1h at RT. Molar equivalents were used for mouse ST2-Fc, mouse and human TRAP-Fc and a 10-fold dilutions were performed starting at 11.1 nM. Constructs were diluted in ELISA block buffer, added to the protein-coated plate (50 µl/well) and incubated for 2h at room temperature. After the incubation, the plate was washed four times with ELISA wash buffer. Anti-human IgG HRP (Invitrogen) diluted 1:3000 in ELISA block buffer (50 µl/well) was incubated 1h at room temperature. Plate was washed four times and 1X TMB substrate was used (50 µl/well), stopping the enzymatic reaction with 2N H_2SO_4 (50 µl/well). Absorbance was read at 450.

2.13 Direct binding assay

Protein G dynabeads (Thermo Fisher) were coated with 5 μg anti-c-Myc (clone Myc.A7, Thermo fisher) or MOPC IgG1 isotype control antibody (in a volume of 200 μl). Mouse ST2-Fc, mouse TRAP-Fc and human IgG (MedImmune) were used at 20 mM (diluted in 100 μl of PBS 1%BSA). Conjugated beads were washed on a DynaMag-2 magnet with PBS 0.02% Tween 20. These were then used to immunoprecipitate human IL-33 and HpBARI. HpARI and HpBARI were used at 1 μg, while 100ng of IL-33 were used (diluted in 100 μl of PBS 1%BSA). Complexes were eluted using 30 μl of 50 mM glycine pH 2.8. Eluted proteins and unbound materials were ran on 4-12% SDS-PAGE gels (ThermoFisher) under non-reducing conditions using 1X MES buffer (Life technologies) for 35 minutes at 200V, and transferred to nitrocellulose membrane using 1X Transfer Buffer (Life Technologies) + 20% methanol (Scientific Laboratory Supply) for 80 minutes at 35V.

2.14 Western blot

Protein were transferred to nitrocellulose membrane and blocked with 5% BSA for 1h at room temperature. Membrane was washed 5 x 5 minutes with PBS containing 1% BSA and 0.05% Tween-20. Membranes were then incubated with primary antibody (e.g. anti-IL-33 or anti-c-myc) in constant shaking for 1h at room temperature or overnight at 4°C. Anti-mouse/human IL-33 (R&D System, goat polyclonal cat. AF3626/AF3625) were diluted 1:500 in PBS 1%BSA. Anti-c-myc (Thermo Fisher Scientific, mouse monoclonal

cat. MA1-21316) was diluted 1:2000 in PBS 1%BSA. After incubation, membrane was washed 5 x 5 minutes with PBS 1%BSA 0.05% Tween-20 and incubated with secondary antibody in constant shaking for 1h at room temperature. Secondary antibodies were diluted in PBS 1%BSA. Goat anti-mouse-HRP (Bio-Rad) diluted 1:3000 and donkey anti-goat (Thermo fisher Scientific) diluted 1:5000 were used, depending on primary antibody species. Membrane was washed 4 x 5 minutes with PBS 1%BSA 0.05% Tween-20 and one wash x 5 minutes with PBS. Membrane was incubated with WesternSure® Premium chemiluminescent substrate (LI-COR®) for 5 minutes at room temperature, and signal detected using LI-COR® C-DiGit™ Chemiluminescence Western Blot Scanner.

2.15 RNA extraction, reverse transcription and PCR

RNA was extracted from whole lung tissue or cells in culture.

From lung tissue, lung were placed in RNALater™ Stabilizing Solution (Thermo Fisher Scientific) and stored at -20°C. Lung tissues were transferred from RNALater™ to TRIzol (Thermo Fisher Scientific) and homogenised using 3mm stainless steel beads (Qiagen) in a TissueLyser II (Qiagen) set up at 25 Hz for 2 minutes.

Adherent nasal epithelial cells were washed 3 times with PBS and placed in 1 ml TRizol. RNA was extracted using the phenol/chloroform method. Complememtary DNA was made using Qiagen QuantiTect Reverse Transcription Kit (Qiagen) for RSV L-gene and IFNγ qPCR or High-Capacity cDNA Reverse Transcription Kit (Applied Biosustems by Thermo Fisher Scientific) for ST2 expression. Primers were diluted in TE buffer to a final

concentration of 0.025 nM/µl and probes to 0.005 nM/µl. Custom primers and probes were purchased from Jena Bioscience or Applied Biosystems. IFNγ and ST2 primer were purchased from Life technologies. PCR amplification for RSV L-gene was carried out in a 25 µl volume made up of custom 7 µl primer probe mix (300nM primers and 200nM probe), 12.5 µl TaqMan mastermix (Applied Biosystems); 1.75 µl H20; 1.25 µl 18S (Applied Biosystems); 2.5 µl DNA template.

For ST2 or IFNγ, PCR amplification was carried out in a 25 µl volume made up of 1.25 µl of pre-made primer probe mix, 12.5 µl TaqMan mastermix; 7.5 µl H20; 1.25 µl housekeeping primer ; 2.5 µl DNA template. Amplification was carried out using StepOne 48-well plate (Applied Biosystems). PCR data were analysed using the $2^{-\Delta\Delta CT}$ method. In brief, relative gene expression between different samples was calculated using the threshold cycles (CTs) generated by StepOne 48-well plate machine and software. ΔCT for each samples was calculated subtracting CT of the housekeeping gene (18s or RPL37) from the CT of the gene of interest. To obtain the relative gene expression between control group and treatment, ΔΔCTs were then obtained subtracting the average of the control group ΔCTs (e.g. PBS or RSV infected cells/mice) from the ΔCT of the sample. Subsequently, $2^{-\Delta\Delta CT}$ was calculated and plotted in a graph.

Table 2.2 **RSV L-gene custom primer sequence**

Gene	Probe (FAM-TAMRA 5'-3')
RSV-L	TTTGAACCTGTCTGAACATTCCCGGTT

2.16 Surface Plasmon Resonance (SPR)

SPR was performed at the Edinburgh Protein Production Facilities (EPPF) (University of Edinburgh) by the experience of Dr. Martin Wear. Measurements were performed using a BIAcore T200 instrument (GE Healthcare). Mouse ST2-Fc, mouse TRAP-Fc and human TRAP-Fc (10 nM) were immobilised on a Protein G sensor chip to 200 response unit (RU) for mouse ST2-Fc and to 400 RU for mouse and human TRAP-Fc. Five 3-fold dilution were made for HpBARI starting at 10 nM and run on the immobilised sensor or, a single 10 nM injection of HpBARI or IL-33 were performed, as described in figure legends.

2.17 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8. One way ANOVA followed by Bonferroni's multiple comparison post test or non-parametric t-test were used as indicated in each graph. Differences were considered to be significant at a P value <0.05 and represented by $*p<0.05$, $**p<0.005$, $***p<0.0005$, $****p<0.0001$.

Chapter 3

A helminth suppressor of the alarmin IL-33

3.1 Introduction

HES is a complex mixture of thousands of molecules excreted and secreted by *H. polygyrus* and a source of immunomodulatory molecules. HES administration replicates some of the effects of a live *H. polygyrus* infection (Hewitson et al. 2011; Segura et al. 2007; Grainger et al. 2010). Following these results, McSorley and colleagues decided to study responses in the airway epithelium using an allergen extract from *Alternaria alternata* (McSorley et al. 2014). Using this approach it is possible to sensitise mice against OVA and re-call type 2 immune responses at challenge (McSorley et al. 2014). Administration of HES during sensitisation abrogates the development of type 2 immune responses, reducing eosinophilia and T_H2 cytokine production. In particular, HES blocks IL-33 release and suppresses early type 2 immune responses such eosinophil infiltrates and ILC2s activation (McSorley et al. 2014).

Before the starting of my project, a parasite-derived protein HpARI (*Heligmosomoides polygyrus* Alarmin Release Inhibitor) was identified through HES fractionation and mass spectrometry analysis. A codon-optimised HpARI sequence was cloned into a pSecTAG2A vector and transfected into the HEK293T mammalian cell line using calcium-phosphate transfection, collecting supernatants containing expressed protein and

purifying by nickel-affinity cheomatography. The purified protein was tested both *in vitro* and *in vivo* and showed to suppress IL-33. HpARI was shown to bind to murine IL-33, but it was not known what form of IL-33 it could bind (reduced or oxidised) and whether its effect could be translated to human IL-33.

The main hypothesis is that HpARI binds directly to IL-33, preventing the cytokine from being detected and from activating pathways that lead to development of type 2 immune responses.

3.2 HpARI binds to IL-33

HpARI had been shown to bind murine IL-33 (Osbourn et al. 2017). Subsequently, we wanted to test if HpARI could bind in a similar way to human IL-33. Direct binding assay was performed: anti-c-Myc antibody was bound to protein G-coated beads. Myc-tagged HpARI was incubated with recombinant human IL-33 and analysed for interaction with the anti-myc tag conjugated beads. As revealed by an anti-human IL-33 western blot (Fig 3.1), HpARI bound to IL-33 and heat-treatment of HpARI ablated the ability to bind the cytokine. Heat-treatment was used to denature HpARI and to confirm that the IL-33 binding was due to the conformation of the protein. A weaker band could be detected in the unbound fraction when HpARI was used, confirming that HpARI was removing IL-33 from the solution by binding it.

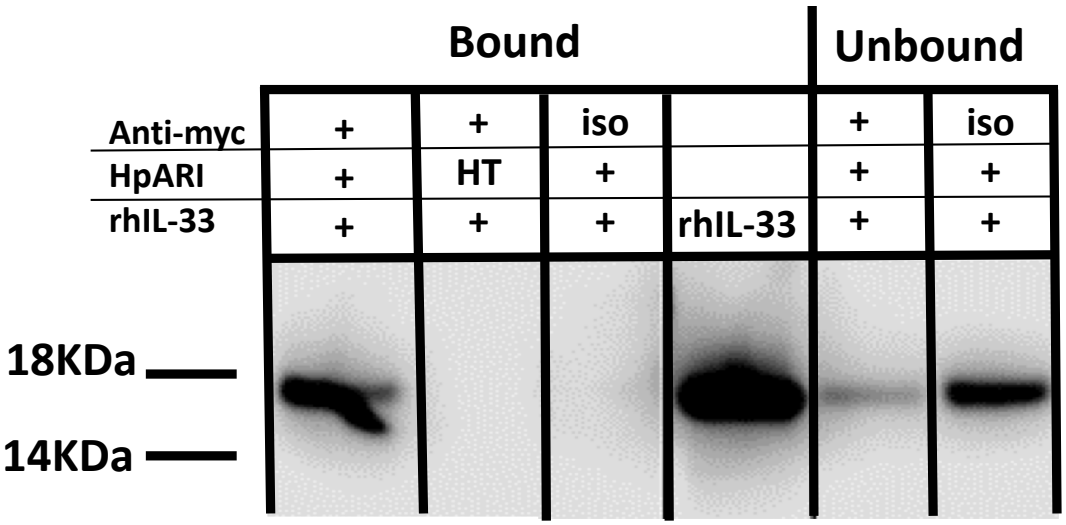


Figure 3.1 HpARI binds to human IL-33.

Protein G dynabeads were coated with anti-myc tag antibody or isotype control (iso). HpARI (1 µg) or heat-treated (HT) HpARI (90°C for 15 minutes) were incubated with human IL-33 (100 ng) to allow the formation of a complex. Subsequently, complexes were incubated with the coated beads and eluted using 50 mM glycine pH 2.8. Samples run in 4-12% Bis-Tris protein gel under reducing conditions. The line showing rhIL-33 is not a direct binding assay but it shows the recombinant protein used as a control. Western blot was probed using anti-human IL-33 antibody. Representative of 2 independent experiments. Published in (Osbourn et al. 2017)

3.3 HpARI does not bind inactive oxidised IL-33

To further study HpARI binding activity, a mouse lung epithelial carcinoma cell line CMT-64, which expresses high level of IL-33 in the nuclei (Osbourn et al. 2017), was used. Cells were exposed to freeze and thaw cycle to induce necrosis and release of IL-33. The hypothesis was that IL-33 released from necrotic CMT-64 can be detected by ELISA, and addition of HpARI prior to freeze/thaw will reduce IL-33 detection. As shown in Fig.3.2, CMT-64 cells exposed to freeze/thaw released IL-33 in the supernatants as detected by ELISA. If HpARI was added prior to freeze/thaw, lower level of IL-33 were detected by ELISA (Fig.3.2).

Furthermore, Cohen et al. showed that upon release, IL-33 is rapidly inactivated through the formation of two disulphide bonds that alter the structural conformation of the cytokine (Cohen et al. 2015). Thus, incubation of supernatants from necrotic CMT-64 at 37°C would induce IL-33 oxidation. However, the available commercial antibody against IL-33 cannot differentiate between reduced and oxidised form (Cohen et al. 2015).

It was hypothesised that HpARI can bind to reduced but not oxidised IL-33, if this is the case HpARI will suppress IL-33 detected by ELISA at earlier time point, when IL-33 is still mostly reduced, but not at later time points. After freeze/thaw supernatants were collected, pooled to achieve a uniform IL-33 concentration and 100 µl/well of thawed-CMT64 supernatant was plated in a 96-well plate.

To study IL-33 oxidation and HpARI binding, CMT-64 supernatants were incubated at 37°C for 0h, 1h, 2h and 4h, and compared to supernatants taken directly after thaw (0h). These time points were chosen according to the study by Cohen. et al in which they show

that after 1h almost 50% of IL-33 is in the oxidised form and more than the 90% by 4h (Cohen et al. 2015).

At each time point 100 μl of HpARI or proteinase K heat-treated HpARI (pK HpARI) were added to the supernatants and incubated for 1h at 37°C (Fig.3.3A). They were then collected and tested for IL-33 by ELISA. Proteinase K was used to proteolytically cleave HpARI and exclude any non-protein contaminant to have an effect in the binding. HpARI reduced IL-33 detection at 0h and 1h post IL-33 release but not after 2h or 4h. Proteinase K treatment of HpARI completely abrogated its effect (Fig 3.3B). These results were further confirmed by direct binding interaction assay and western blot in our group, showing HpARI specifically binding to reduced IL-33 (Osbourn et al. 2017). Therefore, HpARI discriminated between the two forms of IL-33, binding specifically to the active form and not to the oxidised cytokine.

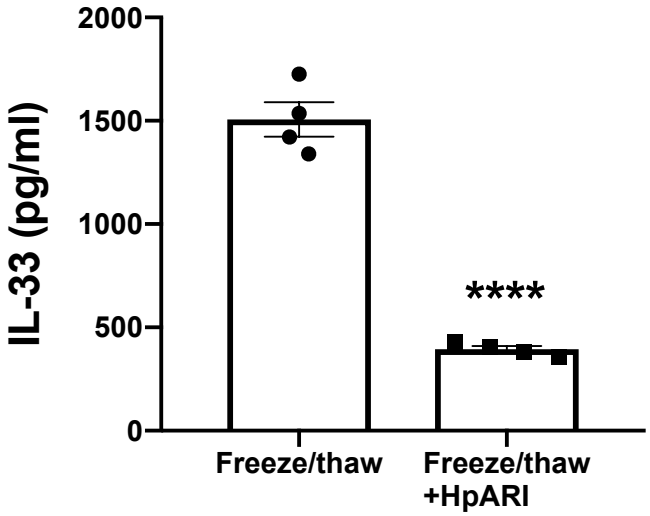


Figure 3.2 HpARI suppresses IL-33 in supernatants from necrotic CMT-64.

CMT-64 (5x105 cells/well) were plated in a 96 flat bottom well plate. HpARI (1 μg/ml) was added to the cells and the plate was frozen using dry ice. Thawed supernatants were collected and analysed by murine IL-33 ELISA kit. Bars representing mean ± SEM. Analysed with unpaired t test. ****<0.0001

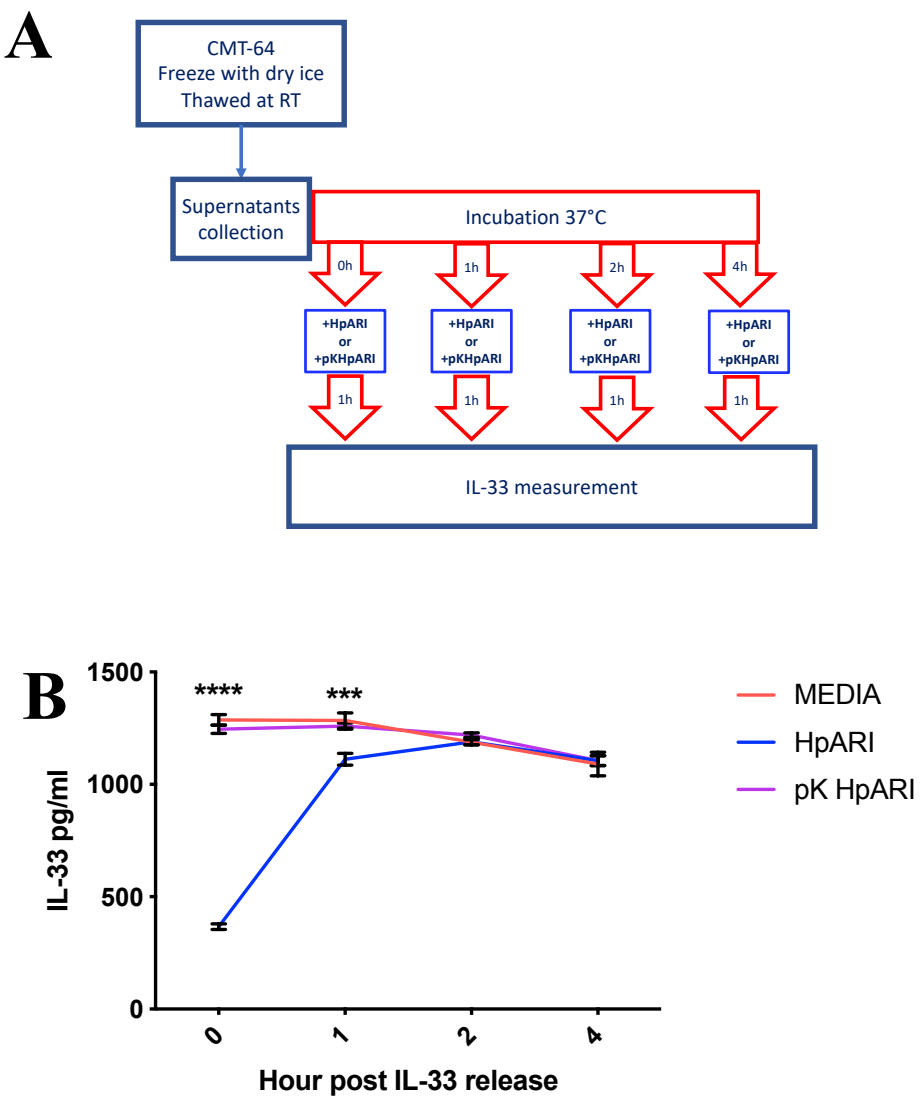


Figure 3.3 HpARI binds only active IL-33.

Experimental design showed in (A) and results in (B). CMT-64 cell cultures were frozen and thawed, supernatants were collected, pooled and incubated at 37°C. As indicted by the graph HpARI (1 μg/ml) or proteinase K treated HpARI (pK HpARI) (1 μg/ml) were added to the CMT-64 supernatants. Supernatants were incubated in medium alone, or with HpARI/pK HpARI for 1h at 37°C and analysed for mIL-33 concentration by ELISA. Point representing mean ± SEM. Representative graph of 3 independent experiments. N=4. Analysed by 2way ANOVA with Bonferroni multiple comparison test comparing MEDIA vs HpARI and HpARI vs pK HpARI. ***=<0.005; ****=<0.0001

3.4 HpARI blocks IL-33-ST2 interaction

ST2 is the cell-surface receptor for IL-33. We obtained two recombinant constructs from MedImmune: an Fc-tagged mouse ST2 (mST2-Fc) and a construct called mouse IL-33 TRAP (mTRAP-Fc) formed by the fusion of mouse ST2, mouse IL-1RAcP and an Fc-tag (Economides et al. 2003; Cohen et al. 2015). These constructs were used for binding studies, to test if HpARI could prevent IL-33 from binding to the IL-33R. The Fc-tag allowed binding of mST2-Fc or mTRAP-Fc to protein-G coated beads, and subsequent co-precipitation of IL-33 in the presence or absence of HpARI. Protein-G is a immunoglobulin derived from group G streptococci with high affinity for monoclonal and polyclonal antibodies, so optimal to conjugate beads or other surface with antibodies (Akerström et al. 1985). Human IgG antibody was used as a control to show that the binding is specific to the recombinant protein and not to the Fc-region. Fig 3.4 shows an anti-IL-33 western blot in which IL-33 was precipitated by bead-bound mST2-Fc and mTRAP-Fc. However, incubating IL-33 with HpARI completely abrogated the capability of the cytokine to bind both mST2 and mTRAP. Therefore, HpARI binds directly to IL-33 blocking binding of the cytokine to its receptor.

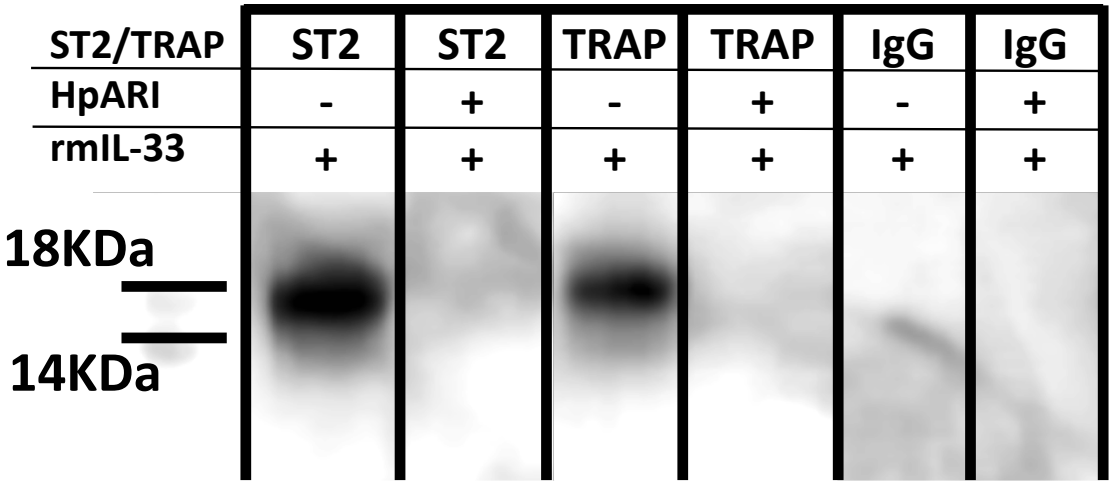


Figure 3.4 HpARI blocks IL-33 from binding its receptor.

Protein G dynabeads where coated with the same molar equivalent of mST2, mTRAP and human IgG (20 mM) as a control. IL-33 (100 ng) was incubated with HpARI 1 μg) for 20 min at RT and then the IL-33 ± HpARI was added to the coated beads. 50 mM glycine pH 2.8 was used to elute the samples. Samples were run on a 4-12% Bis-Tris protein gel, transferred to a nitrocellulose membrane and probed using anti-mouse IL-33 antibody. Representative of 2 independent experiments

3.5 Bone marrow assay to study IL-33-dependent responses and the effect of HpARI

The finding that HpARI binds IL-33 and blocks binding of IL-33 to ST2 in the direct binding assay suggests that HpARI might block IL-33-dependent responses. To further investigate if HpARI blocks IL-33 dependent responses, I set up an *in vitro* assay using whole bone marrow cells.

IL-33 signalling leads to development of type 2 immune responses with an increase in IL-5/IL-13 production (Halim 2016). The whole bone marrow preparation contains multiple cell populations, including type 2 innate lymphoid cells and their precursors. ILC2 can be activated *in vitro* to produce IL-5 and IL-13 when stimulated with IL-33 (Johansson et al. 2018; Brickshawana et al. 2011). Whole murine bone marrow (BM) was collected from wild-type C57BL/6 or IL-13eGFP transgenic mice, the latter of which express enhanced green fluorescence protein (eGFP) under the IL-13 gene (Neill et al. 2010). BM cells were incubated with IL-2 and IL-7 to induce ILC2 proliferation (Brickshawana et al. 2011; Neill et al. 2010), in the presence or absence of IL-33 and HpARI. Figure 3.5 shows the gating strategy used to identify the ILC2 population and the IL-13eGFP^{high} population in the bone marrow after 120 hours of culture. BM cultured in media without IL-2 and IL-7 contained less viable cells (Fig.3.6), and absence of ILC2 populations (Fig.3.7), especially at 120h (Fig.3.7B) compared with cells cultured in media supplemented with IL-2 and IL-7. IL-33 co-culture induced a decrease in viable cells and that might be due to cell activation and subsequent death. Further information

that can be obtained from this experiment is that co-culturing bone marrow cells with HpARI does not decrease cell viability, thus the protein does not induce cell death (Fig.3.6). Adding IL-33 induces expansion of the ILC2 population (Fig.3.7). Furthermore, it was investigated which cells are producing IL-13 (Fig.3.8). IL-33 induces an increase in the IL-13eGFP^{high} populations both at 72h (Fig.3.8A) and 120h (Fig.3.8B). The major population producing IL-13 at 72h seems to be an ICOS^{-} Lineage^{intermediate} population (Fig. 3.8A). I hypothesises that these could be mast cells or their precursors. Mast cells can be derived from the bone marrow and they can respond to IL-33 producing IL-13 (Ball et al. 2018; Meurer et al. 2016). However, by 120h the major population producing IL-13 were ILC2, identified as ICOS^{+}Lineage^{-} (Fig.3.8B). Following the gating strategy in Fig.3.5 ILC2s were gated and the percentage of ILC2 IL-13eGFP^{high} analysed. IL-33 induces an increase of IL-13eGFP^{+} cells (Fig.3.9), and blocking IL-33 with HpARI suppresses the response at 72h (Fig.3.9A) and 120h (Fig.3.9B). However, the increase of ILC2 IL-13eGFP^{high} was greater in the 120h culture. To further confirm the suppressive effect of HpARI, I focused on the IL-13eGFP^{high} from the whole live cells population, showing that IL-33 increased the percentage of 13eGFP^{high} and decrease of this population is observed with HpARI both at 72h and 120h (Fig.3.10).

To analyse cytokine secretion from whole bone marrow cultures and to test if HpARI suppresses IL-33-dependent responses, BM from C57BL/6 was cultured and stimulated with IL-33 +/- HpARI for 120h. Supernatants were collected and analysed for IL-5 and IL-13 by ELISA. IL-33 induced a significant increase in IL-5 and IL-13 production, while HpARI suppressed these responses (Fig.3.11). Furthermore, as showed in Fig.3.6 HpARI

did not reduce the number of viable cells, however, it might still reduce the general activation status of ILC2s, regardless of the stimulus used. To test this possibility, I stimulated bone marrow cells with IL-25. IL-25 is another alarmin that activates ILC2 and induce type 2 immune responses. Here I tested the release of IL-5 in response to IL-25, and the effect of HpARI, in whole bone marrow culture from wild-type and ST2 KO transgenic mice. Both IL-33 and IL-25 induces release of IL-5 in wild-type bone marrow culture while only IL-25 induces IL-5 in ST2 KO mice (Fig 3.12). HpARI only suppresses the IL-33-dependent response and not the IL-25-induced IL-5 production (Fig 3.12), confirming that HpARI is specific to IL-33 and it does not affect the activation status of ILC2 as they respond to another stimulation.

Therefore, HpARI blocks IL-33 from binding to ST2 as analysed by direct interaction and it suppresses only the IL-33-dependent responses in the bone marrow assay. In addition, the bone marrow assay is a useful method to test parasite-derived protein efficacy.

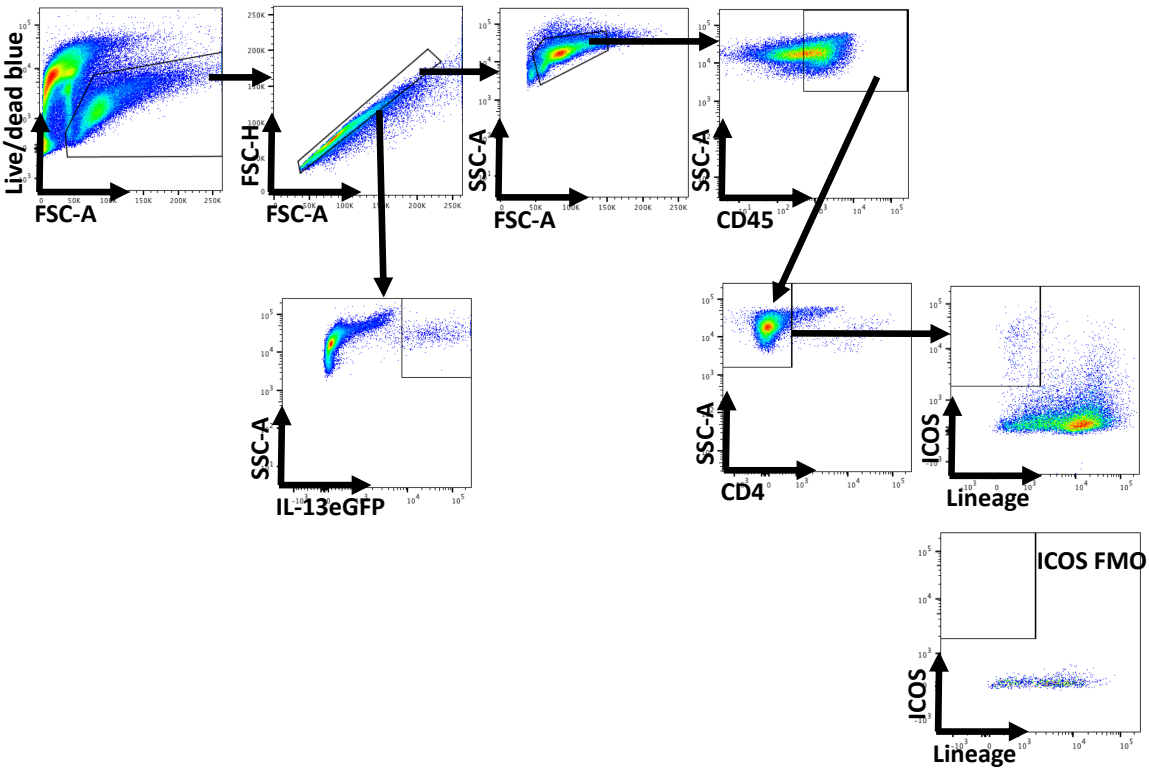


Figure 3.5 Gating strategy to identify the IL-13eGFPhigh population and the ILC2s population from 120h bone marrow cultures

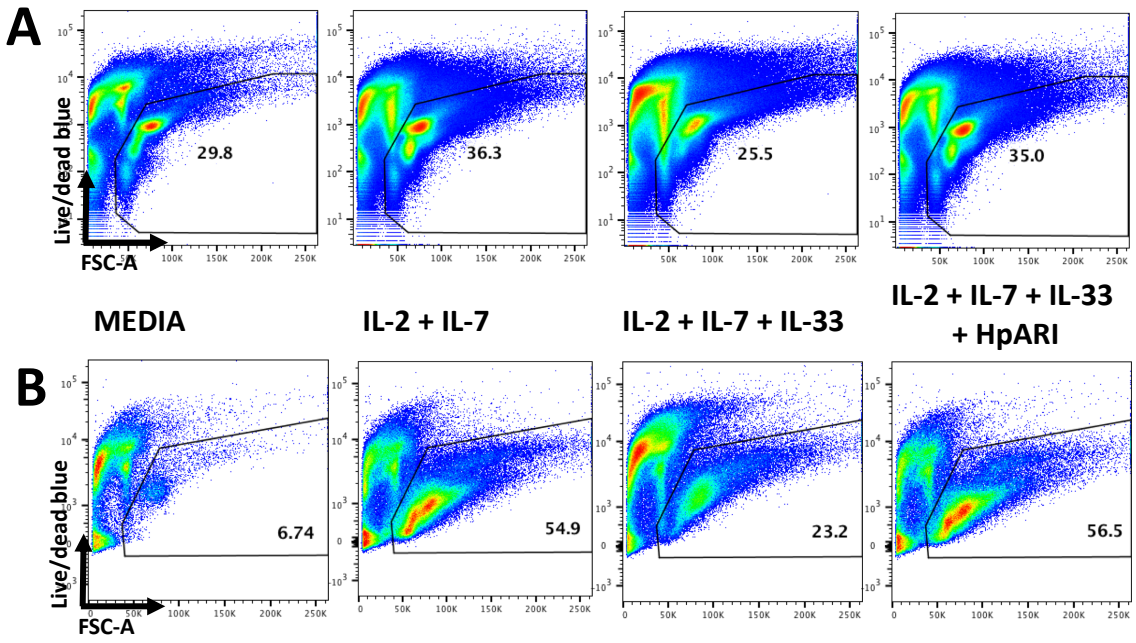


Figure 3.6 Live cells gate in 72h and 120h bone marrow culture.

IL-13eGFP bone marrow was harvested and cultured with media alone or supplemented with IL-2, IL-7 and IL-33 (all at 10 ng/ml) and HpARI (1 μg/ml) as indicated in the figure. After 72h (A) or 120h (B) incubation, cells were surface stained and analysed by flow cytometry. Representative FACS plot of 2 independent experiments (A) and one single experiment (B)

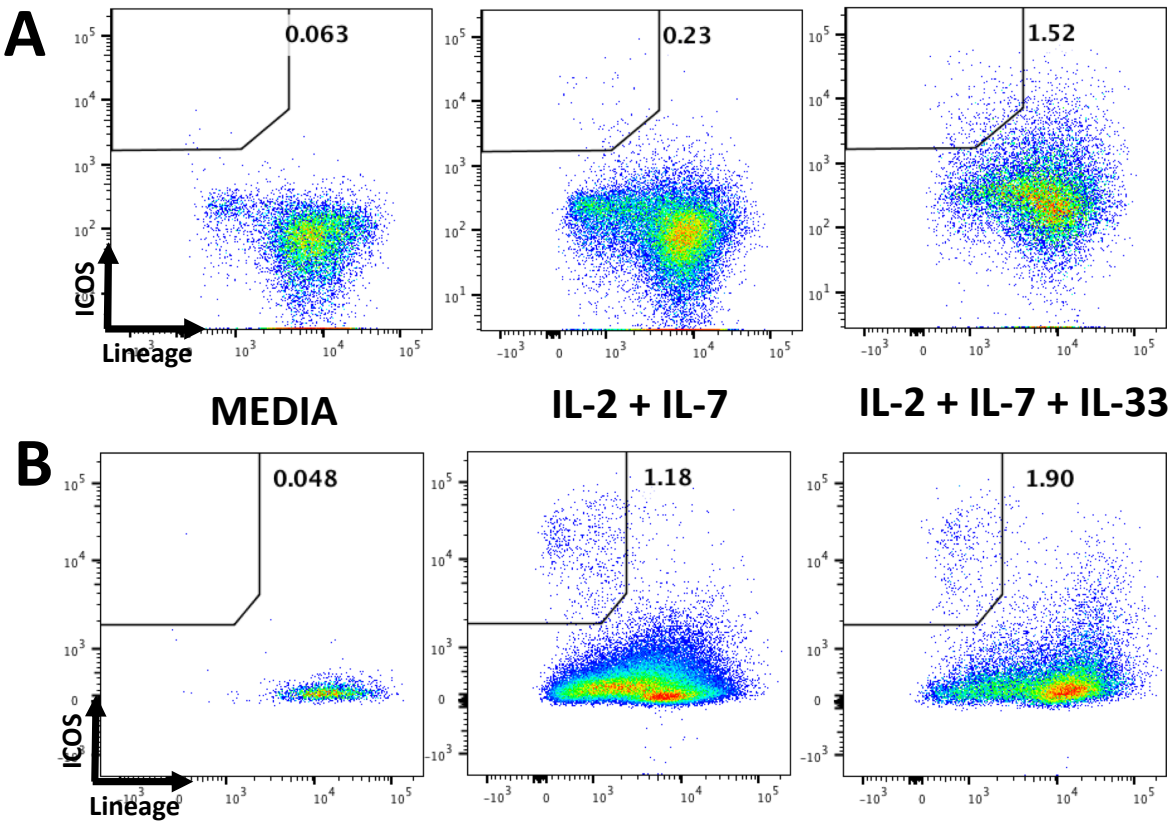


Figure 3.7 ILC2 activation in BM culture.

IL-13eGFP bone marrow was harvested and cultured with media alone or supplemented with IL-2, IL-7 and IL-33 (all at 10 ng/ml) as indicated in the figure. 72h culture (A) and 120h culture (B) were FACS stained and samples acquired by flow cytometry. ILC2s were identified as ICOS+ Lineage-. FACS plot showing one single experiment for B and a representative plot from 2 independent experiments for A

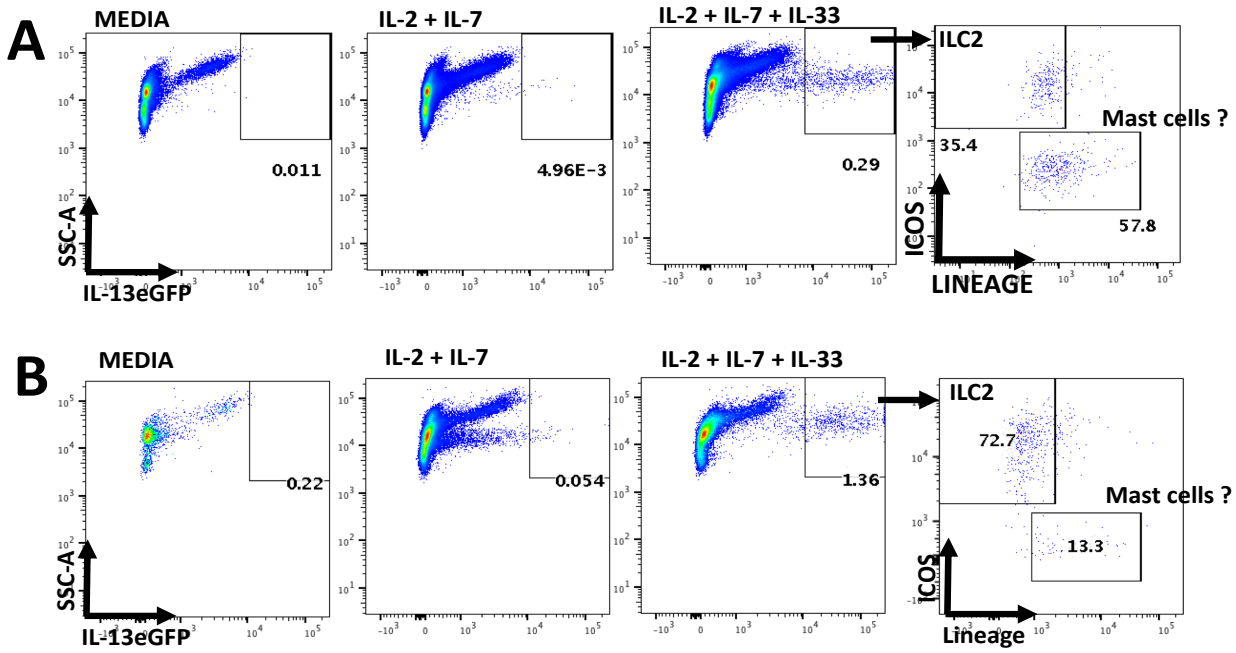


Figure 3.8 IL-13eGFP+ populations in cultured bone marrow cells.

Bone marrow cells were cultured with IL-2, IL-7, ± IL-33 (all cytokines at 10 ng/ml) and HpARI (1 µg/ml) for 72h (A) or 120h (B). Cells were then surface stained and analysed by flow cytometry. IL-13eGFP+ were gated from the live cells, singlets, CD45+ population. FACS plot from 72h representative of 2 independent experiments. FACS plot from 120h one single experiment. Showing percentages of cells.

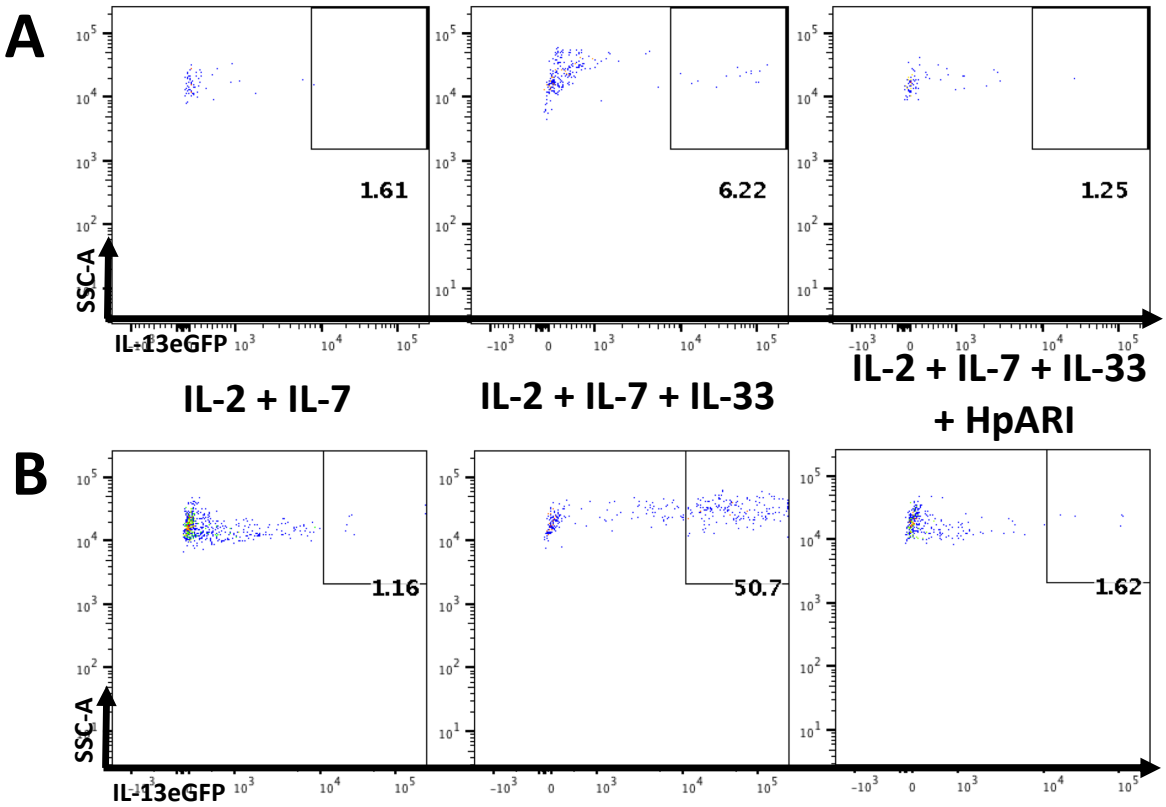


Figure 3.9 IL-13eGFPhigh ILC2s suppression with HpARI.

BM cells collected from IL-13eGFP mice were analysed by flow cytometry after 72h (A) and 120h (B) of culture. Cells were cultured with 10 ng/ml of IL-2 and IL-7, ± IL-33 and HpARI (1 µg/ml). Cells were surface stained and analysed. IL-13eGFPhigh ILC2 were gated considering around 1% of IL-13eGFPhigh in the IL-2 + IL-7 group. Representative FACS plot of 2 independent experiments for 72h time point. One single experiment for 120h time point

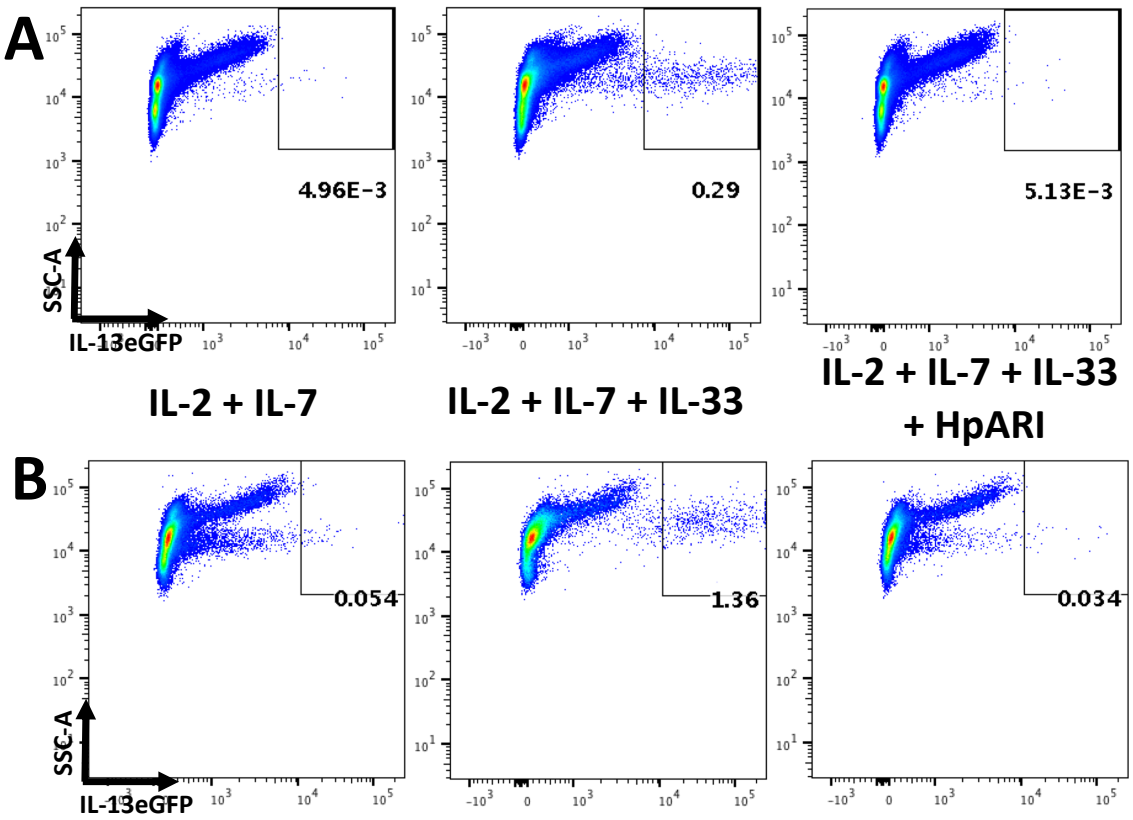


Figure 3.10 HpARI suppresses the total IL-13eGFPhigh population.

BM cells collected from IL-13eGFP mice was analysed by flow cytometry after 72h (A) and 120h (B) of culture. Cells were cultured with IL-2 and IL-7, ± IL-33 (all cytokines at 10 ng/ml) and HpARI (1 µg/ml). Cells were surface stained and analysed. IL-13eGFPhigh were considered from the live and singlets cells population. Representative FACS plot of 2 independent experiments for 72h time point. One single experiment for 120h time point

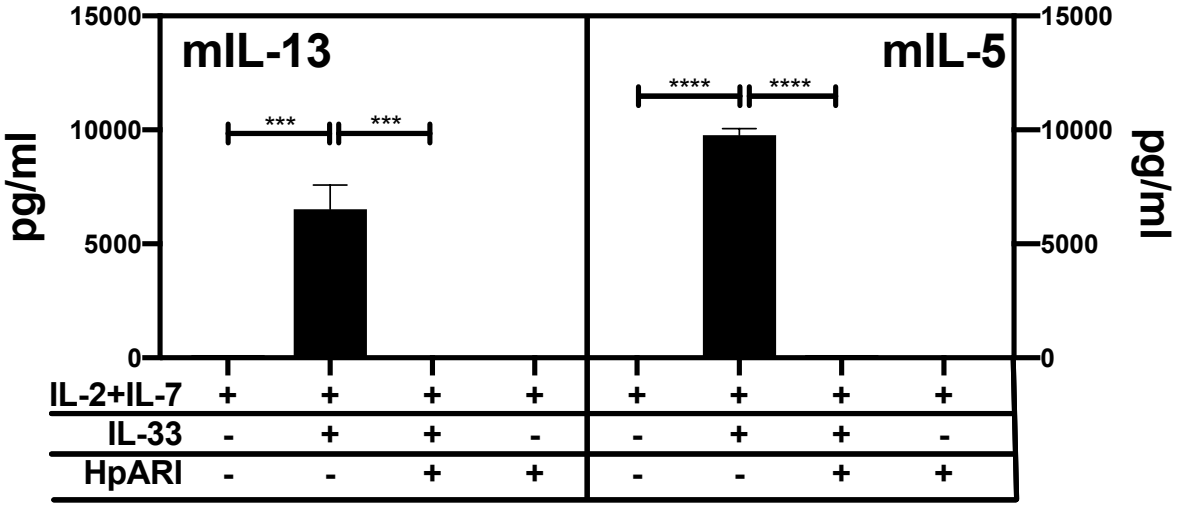


Figure 3.11 Release of IL-5 and IL-13 in response to IL-33 in the bone marrow assay

Whole C57BL/6 BM cells ($1x10^6$ cells/well) were cultured with IL-2, IL-7 in the absence or presence of recombinant IL-33 (all cytokines at 10 ng/ml) +/- HpARI (1 μg/ml) in a 96-well plate for 120h. Supernatants were collected and tested by ELISA. Bars representing mean and SEM. Results were analysed with One-way ANOVA with Bonferroni post-test. ***=0.005; ****=<0.0001. Representative graph of 4 independent experiments

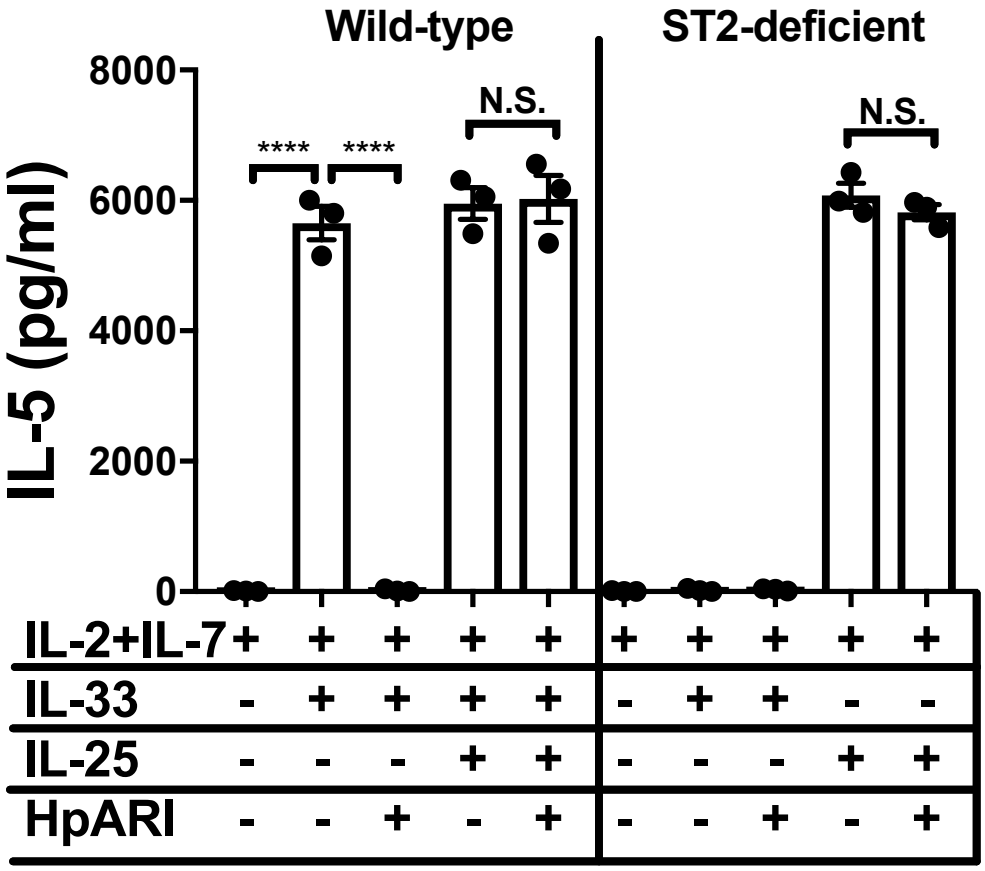


Figure 3.12 HpARI suppresses only IL-33-dependent responses

Whole BM cells ($1x10^6$ cells/well) from wild-type (BALB/c) and ST2-deficient mice (BALB/c background) were cultured with IL-2 and IL-7, plus IL-33 or IL-25 (all cytokines at 10 ng/ml) +/- HpARI (1 μg/ml) in a 96-well plate for 120h. Supernatants were collected and tested for IL-5 ELISA. Bars are mean and SEM. Data analysed with One-way ANOVA with Bonferroni multiple comparison post-test. ****<0.0001. Representative of 2 independent experiments

3.6 IL-33 titration in the bone marrow assay

The bone marrow assay is a useful tool to study IL-33 dependent responses and the effect of parasite-derived proteins before moving on to *in vivo* models. In this assay, bone marrow cells were treated with IL-2, IL-7 and IL-33 all at 10 ng/ml, this concentration was following a previous study by Duerr and colleagues, which had not determined an optimal concentration to stimulate bone marrow cells (Duerr et al. 2016). An IL-33 titration to test if maximal amounts of IL-5 and IL-13 were induced at 3 days post-stimulation with 10 ng/ml of IL-33. Due to the presence of different cell populations in the bone marrow culture, supernatants were also tested for IL-6 and IFNγ.

In BM culture, IFNγ may be produced by precursor of NK cells or γδ T cells (Liang et al. 2015) and IL-6 may be released by ILC2 and mast cells (Moro et al. 2010).

Maximal release of IL-5 (Fig.3.13A) and IL-13 (Fig.3.13B) is achieved at lower doses of IL-33 between 1 ng/ml and 0.5 ng/ml. IL-6 (Fig.3.13C) and IFNγ (Fig.3.13D) can be detected in the supernatants, and in contrast to IL-5 and IL-13 maximal release is observed at 10 ng/ml of IL-33. All data are presented in one graph (Fig.3.13E) as percentage of max value to appreciate those differences. Hence, lower dose could be used in the BM assay to test type 2 cytokine release.

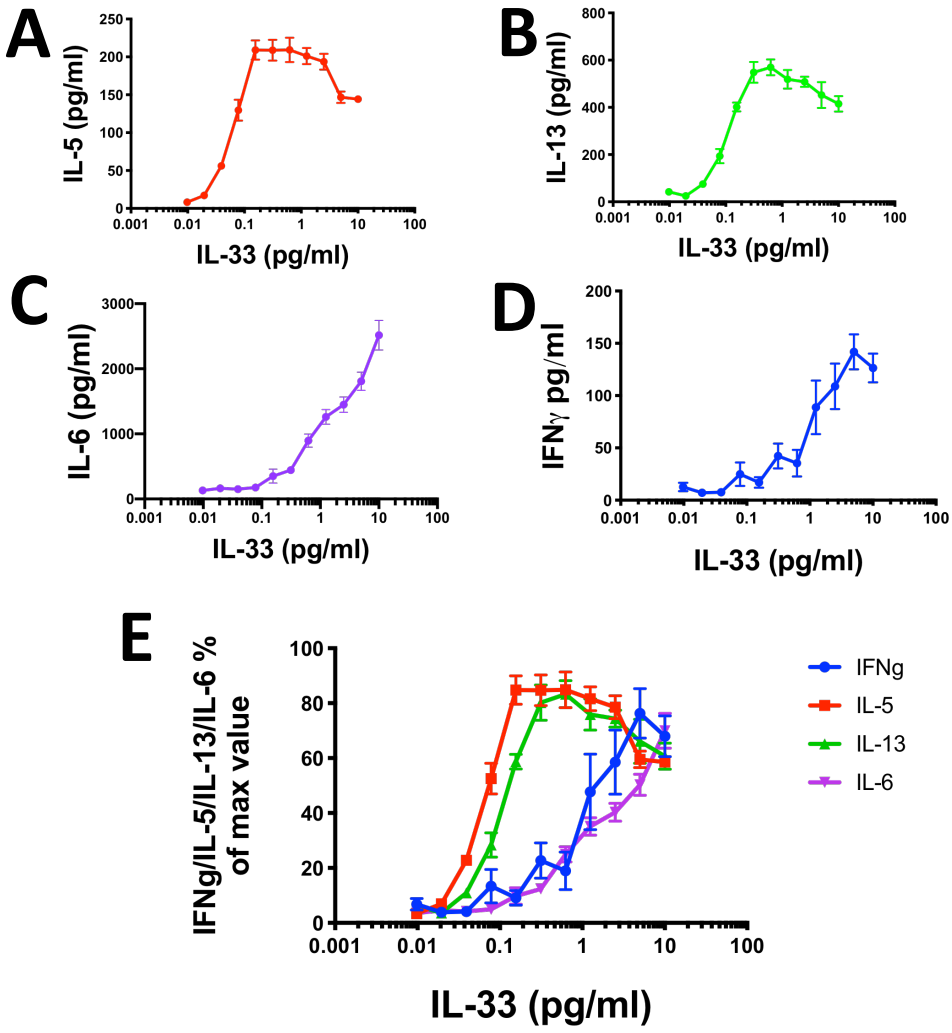


Figure 3.13 Maximum release of type 2 cytokine is observed at lower dose of IL-33 in the BM assay

C57BL6 BM was cultured with IL-2, IL-7 (10 ng/ml) and IL-33 (from 10 ng/ml to 0.01 ng/ml). Supernatants were collected at 72h and analysed for IL-5 (A), IL-13 (B), IL-6 (C), IFNγ (D) by ELISA. Merged data presented as % of max value (E). Points are mean ± SEM. N=6. Representative of 2 independent experiments

3.7 Suppression of ILC2 activation by HpARI *in vivo*

Allergens are commonly known to elicit asthma attacks. Fungal allergens like *Alternaria alternata* have proteolytic activity that can damage lung epithelial cells, inducing necrosis and release of IL-33, driving asthmatic inflammation (Snelgrove et al. 2014; McSorley et al. 2014). Previous experiments in this chapter show that HpARI is able to bind IL-33, preventing IL-33 from binding to ST2, and suppressing IL-33-dependent bone marrow responses *in vitro*. HpARI was tested *in vivo*, using *Alternaria alternata* as an allergen to induce release of IL-33 and activation of the ILC2 population using the same system that was used used to show suppression of IL-33-dependent responses by HES (McSorley et al. 2014). Mice were administered intranasally with *Alternaria* allergen, and lungs were harvested at 24 h after administration of the allergen. Lung cells were surface stained and intracellularly stained for IL-5 and IL-13. The gating strategy is shown in Fig.3.14.

Alternaria induces ILC2 activation, increasing the percentage (Fig 3.15A-C) and the cell counts (Fig. 3.15B-D) of ILC2 positive for IL-5 and IL-13. CD25 expression was measured as a marker of ILC2 activation, and *Alternaria* significantly increased the expression of CD25 on the surface of ILC2 (Fig. 3.15E). Blocking the IL-33 pathway using HpARI *in vivo* abrogated ILC2 activation, reducing expression of CD25, IL-5^+ and IL-13^+ ILC2s (Fig 3.15).

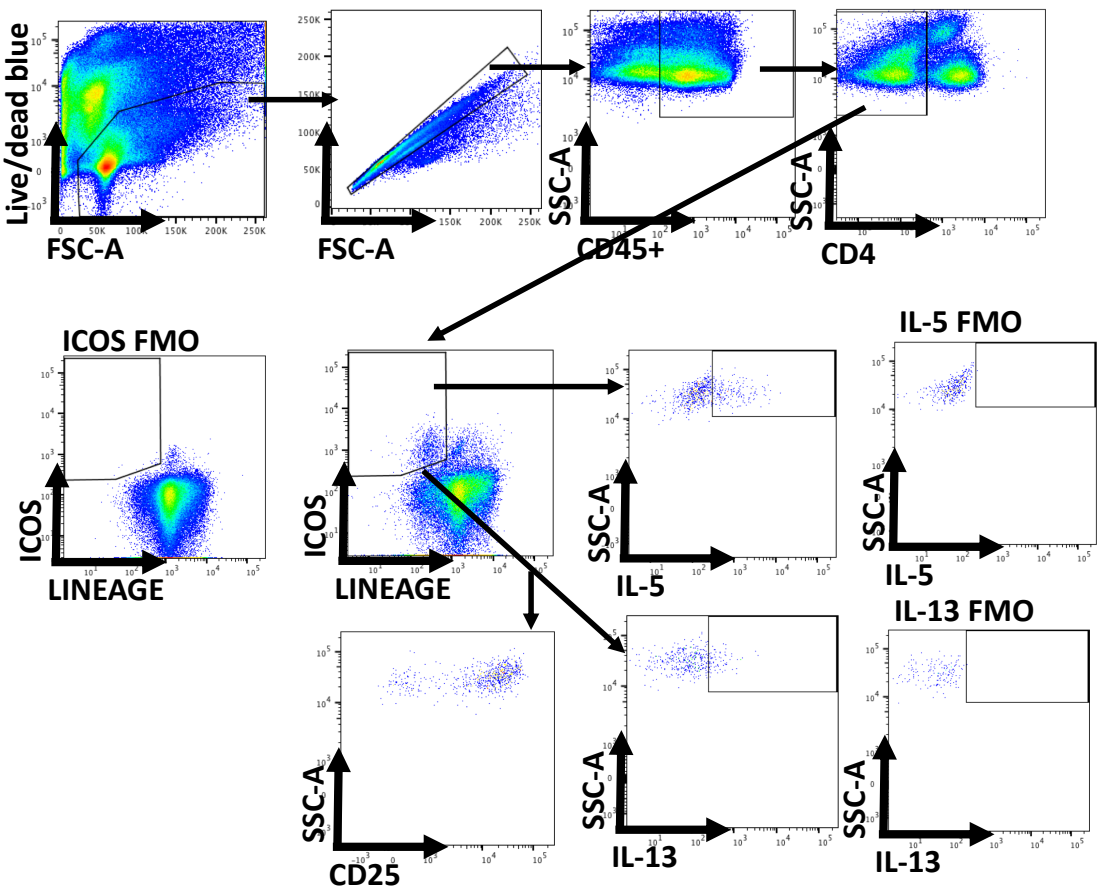


Figure 3.14 Lung cell suspension gating strategy with FMOs

Lung cells suspension was obtained as described in material and methods. Cells (3x106 cells/well) were then plated in a 96-well round bottom plate and incubated with PMA, ionomycin and brefeldin A for 4h. Next, cells were surface stained followed by intracellular staining. Stained cells were analysed by flow cytometry. Live cells then singlets were gated. ILC2s are identified as CD45+ CD4- ICOS+ Lineage-. IL-5+ cells, IL-13+ cells and CD25 MFI cells were measured and quantified

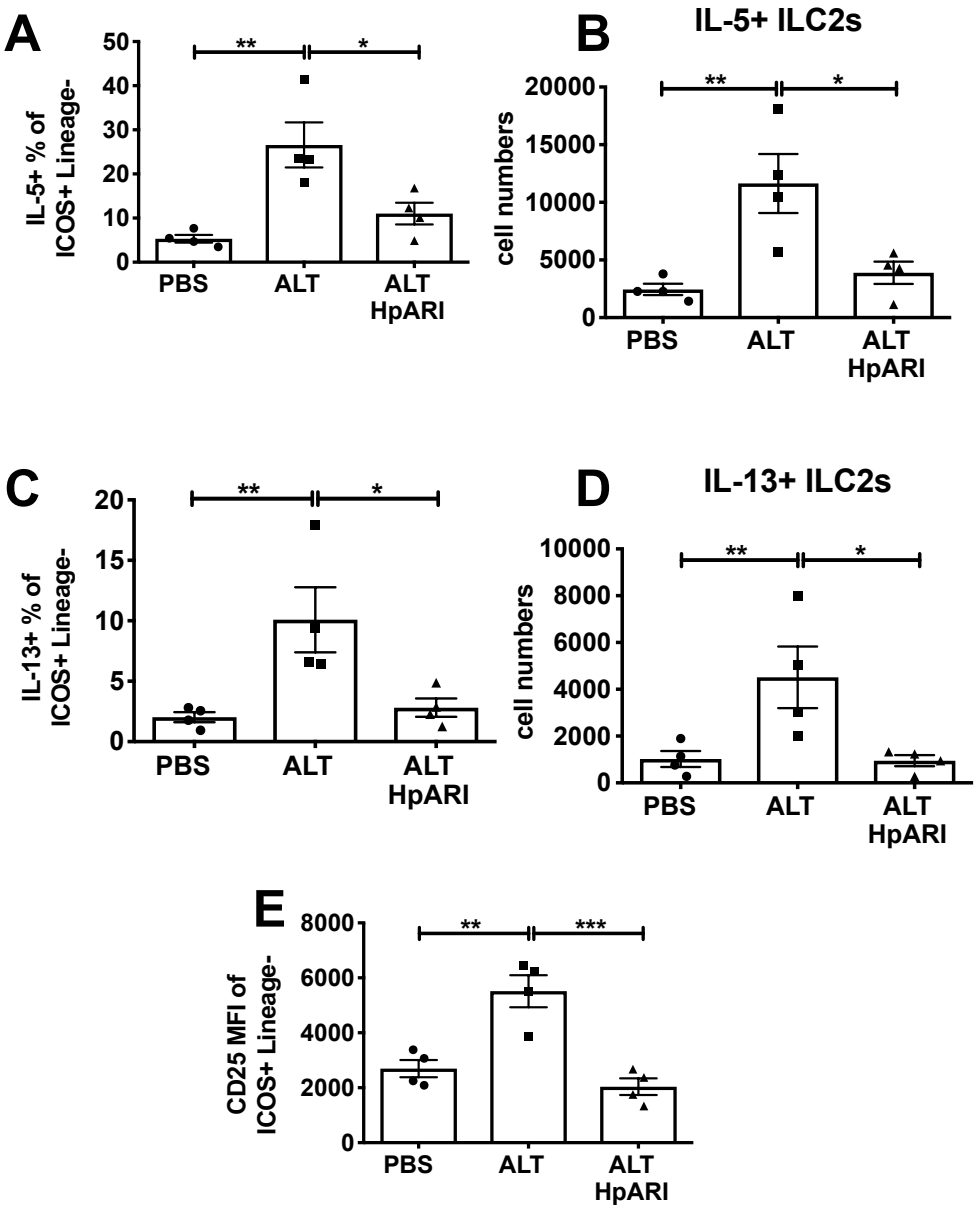


Figure 3.15 HpARI suppresses ILC2s activation induced by *Alternaria* allergen

Alternaria (25 μg) was administered intranasally with OVA protein (10 μg) and HpARI (10 μg). Lungs were collected at 24 h. Lung cells were stimulated with PMA, ionomycin and brefeldin A for 4h, intracellularly stained and analysed by flow cytometry. Bars represent mean and SEM. Data analysed with One-way ANOVA with Bonferroni multiple comparison post-test. *=<0.05, **=<0.005, ***=<0.001. One single experiment. Published in (Osbourn et al. 2017)

3.8 Discussion

Several immune modulatory effects have been associated with the excretory/secretory products of the helminth parasite *Heligmosomoides polygyrus* (HES) (Segura et al. 2007; Grainger et al. 2010; McSorley et al. 2014). Recent work showed that HES induction of T regulatory cells is performed by a mimic of host TGFβ named HpTGM (Grainger et al. 2010; Johnston et al. 2017). In recent years, researchers have been focusing on finding single immune modulatory molecules in parasite secretions with the purpose of identifying new therapeutical agents for allergic asthma (Navarro et al. 2016; Melendez et al. 2007; Johnston et al. 2017; Park et al. 2009; Ebner et al. 2014). HES administration is associated with suppression of IL-33 responses and it prevents inflammatory type 2 responses in a model of asthma (McSorley et al. 2014). Therefore, the focus of this project was to characterise a single protein identified in HES that could replicate the observed effect *in vivo.*

Here we investigate the mechanism of action of HpARI, a protein identified from HES and expressed in recombinant form in a mammalian cell line HEK293T. HpARI was discovered for its ability to block IL-33 release from necrotic epithelial cells. Mechanistically, HpARI binds directly to mouse and human IL-33, specifically blocking IL-33-dependent responses in the bone marrow assay *in vitro*, and ILC2 activation in response to *Alternaria* allergen *in vivo*. The HpARI binding affinity was confirmed using Surface Plasmon Resonance (SPR) by other members of the lab in collaboration with Dr Martin Wear. Binding affinity is measured by the K_d value.

Lower K_d values indicates stronger binding affinity, and in the case of IL-33 binding to ST2 the K_d is 0.46 nM (Lingel et al. 2009; Liu et al. 2013). The K_d of HpARI for murine IL-33 is 0.56 ± 0.1 nM, and 260 ± 13 nM for human IL-33, showing a strong affinity for murine IL-33 being similar to that of IL-33 to its receptor (Osbourn et al. 2017). Further experiments in the McSorley lab, measuring IL-33 by western blot led to the observation that HpARI not only suppresses detection of IL-33 by binding to the cytokine directly, but also inhibits release of IL-33. In addition HpARI localises in the nucleus of necrotic cells only by binding directly to DNA, as DNAse treatment ablates detection of HpARI. This was confirmed by a gel shift assay, in which if a protein binds to DNA this DNA will be bigger in size and will run more slowly in a DNA gel electrophoresis (Osbourn et al. 2017).

Subsequent experiments in the McSorley group, which I assisted with, showed that HpARI suppresses type 2 immune responses in a sensitisation and challenge model as described in (McSorley et al. 2014). Briefly, *Alternaria* allergen is able to induce sensitisation against a harmless protein: ovalbumin (OVA). After 14 days type 2 immune responses can be re-called using OVA in those mice that have been sensitised, while administering HpARI during sensitisation reduced antigen-specific type 2 immune responses and abrogated inflammatory changes in the lung, and lung resistance and compliance at challenge (Osbourn et al. 2017).

Helminths can establish chronic infection in the host, due to their ability to reduce type 2 immune responses that, if not controlled, will lead to parasite expulsion. *H. polygyrus* is an intestinal nematode of mice that can control at multiple levels the immune system through the secretion of HES products and exosomes (Segura et al. 2007; Johnston et al. 2017; McSorley et al. 2014; Buck et al. 2014; Coakley et al. 2017). The IL-33 pathway is targeted at different levels during *H. polygyrus* infection: for example HpARI binds and blocks IL-33; ST2 is suppressed at the transcriptional level by exosomes (Buck et al. 2014; Coakley et al. 2017); and an unidentified molecule induces IL-1β that subsequently downregulates IL-33 (Zaiss et al. 2013). The importance of IL-33 is demonstrated not only by the multiple ways the parasite uses to interfere with this pathway but experiments also showed increased infection burden in parasite-infected ST2-deficient mice (Coakley et al. 2017; Townsend et al. 2000). In particular, the *H. polygyrus* life cycle might induce multiple release of IL-33 due to epithelial cell damage. Larvae penetrate the intestinal submucosa 24h post-ingestion and after 10 days adult worms will migrate to the intestinal lumen (Maizels et al. 2012). This process can induce cell damage, and potentially release of IL-33, due to the fact that adult worms measure around 6-12 mm and they need to disrupt the epithelium to migrate into the lumen (Pritchett-Corning & Clifford 2012) . That suggest that the parasite needs to tightly regulate this pathway and this is an evolutionary adaptation to survive in the host. In particular, HpARI is produced both at the larval stage and by the adult worm (Hewitson et al. 2013) indicating the possibility of suppressing early anti-parasite immunity. To define the role of HpARI during live infection, mice could be vaccinated with HpARI prior to infection with H. polygyrus.

Protection could be assessed by numbers of eggs in the faeces and adult worm burden. Previous studies showed that vaccination with HES or exosomes provide protection against *H. polygyrus* (Hewitson et al. 2015; Coakley et al. 2017).

The main aim of this chapter is to understand the mechanism of action of HpARI and try to use HpARI as a model to develop novel therapeutic agents derived from a parasite. A parasite-derived molecule that has been developed toward the clinic is ES-62, a filarial protein. In humans, filariasis can be taken as an examples for immunomodulation. Filarial nematodes can survive in the host for several years, and this is believed to be due to the creation of a tolerance between the host and the parasites. Especially, the ES-62 protein from *Acanthocheilonema viteae* was shown to be extremely immune-modulatory affecting dendritic cells, LPS-responses, $CD4^+$ T cells, B lymphocytes (Harnett et al. 2004; Harnett et al. 2010), and reducing inflammation in a model of arthritis (McInnes et al. 2003; Doonan et al. 2018) and asthma (Rzepecka et al. 2013). ES-62 is conjugated with phosphorylcholine that sequestrates the adaptor MyD88 blocking TLR and IL-33 signalling (Pineda et al. 2014; Ball et al. 2018).

However, ES-62 is an immunogenic tetrameric protein not suitable to be used as a drug *per se*. The understanding of the molecular structure and its activity helped the development of small molecule analogues of phosphorylcholine that can be used as a therapeutic agents (Al-Riyami et al. 2013).

In light of this, HpARI is a smaller protein compared to ES-62, 26kDa versus 240 kDa respectively (Osbourn et al. 2017; Al-Riyami et al. 2013), but still immunogenic. Further experiments are planned to understand HpARI crystal structure and to understand at

which molecular site IL-33 and HpARI interact. It is known that the presence of 3 CCP domains is essential for the function of HpARI. CCP1 has been shown to bind DNA and the other 2 domains are required to selectively bind to active IL-33. These findings together with a structural characterisation could help to build a smaller and less immunogenic molecule that will resemble and mimic HpARI function. In particular, these CCP domains seems to undergo expansion in *H. polygyrus* (Maizels et al. 2018). CCP domain molecules have been associated with the complement system, cytokine receptors (i.e. IL-2) and neurotransmitters and many others. It is therefore interesting that in other living organism such as parasites, CCP domains have evolved to modulate the host immune system.

Several CCP domain molecules have been identified from HES, and two of them are: HpARI that blocks and suppresses IL-33-dependent responses, and is a promising molecule to be used against asthma initiation (Osbourn et al. 2017); and HpTGM which binds to TGF-β receptor but has no homology to TGF-β (Johnston et al. 2017), with 3 CCP domains out of 5 being required for its activity (Smyth et al. 2018). Taken together these findings indicate that CCP domain-containing proteins can be a target to identify immunomodulatory molecules from parasite secretions.

In conclusion, parasites have evolved to survive within the host and they modulate the host immune system during inflammatory initiation, adaptive responses and remodelling. Single molecules form parasite secretions can mimic the immunomodulatory effect of live infection (Harnett et al. 2010; Yazdanbakhsh & Matricardi 2004a; Lambrecht &

Hammad 2017), and in the case of HpARI used to target IL-33 and possibly asthma development. IL-33 is an emerging cytokine that plays a key role in asthma development by inducing a strong type 2 inflammation with activation of ILC2s (Cayrol & Girard 2018; Halim et al. 2014; Lloyd & Saglani 2015; Brickshawana et al. 2011). A role for IL-33 is supported by several GWAS studies (Bønnelykke et al. 2014; Moffatt et al. 2010; Bønnelykke et al. 2013). IL-33 can be detected in sputum and lung of patients with T_H2-asthma, together with activated ILC2s and IL-33 levels correlate with pathology (Kortekaas Krohn et al. 2018; Smith et al. 2016; Seys et al. 2013; Christianson et al. 2015). Consequently, IL-33 seems a promising target for asthma treatment and clinical trials are ongoing to test anti-IL-33 mAb in atopic dermatitis, asthma and COPD (AnaptysBio 2018; Londei et al. 2017). In particular mouse experiments suggest that blocking IL-33 reduces asthmatic responses during exacerbation (Werder et al. 2018).

Asthma exacerbations are commonly triggered by respiratory virus infections, with IL-33 released in the airway upon viral infection (Werder et al. 2018; Kumar et al. 2014; Jackson et al. 2014) . Lower respiratory tract infection, especially caused by Rhinovirus or Respiratory Syncytial Virus (RSV), during childhood increase the risk to allergen sensitisation and recurrent wheeze (Sigurs et al. 2000; Sigurs et al. 2005; Jackson et al. 2014; Korppi et al. 2004; Sigurs et al. 2010), with the mechanisms still poorly understood. Understanding the role for IL-33 in respiratory viral infection, and using HpARI to block IL-33, can offer new insight on the role of IL-33 for allergen sensitisation as well as a dampening role for anti-viral responses.

Chapter 4

Investigating the RSV-induced type 2 immune response and the effect of HpARI

4.1 Introduction

Respiratory Syncytial Virus (RSV) is the most common respiratory viral infection in children and leading cause of severe bronchiolitis (Smyth & Openshaw 2006; Lambert et al. 2014). Several epidemiological observations showed that RSV-induced severe bronchiolitis in children is a risk factor for the development of wheeze, allergic asthma and allergic rhinitis later in life (Korppi et al. 2004; Henderson et al. 2005; Sigurs et al. 1995; Sigurs et al. 2005; Sigurs et al. 2010). There is currently no vaccine against RSV and the only prophylactic treatment available is the humanized monoclonal antibody palivizumab for high-risk infants (Blanken et al. 2013; Olchanski et al. 2018; Mochizuki et al. 2017). Administration of palivizumab in healthy pre-term infants is associated with reduction in recurrent wheeze both during the first year of life and in a 6 year follow up (Blanken et al. 2013; Mochizuki et al. 2017). These studies suggest a possible causal link between RSV bronchiolitis during infancy and asthma development.

Lack of treatments and vaccination are in part due to a poor understanding RSV infection, especially when we consider detrimental and protective immune responses. In infants,

severe RSV infection is associated with airway obstruction, peribronchiolar inflammation, airway epithelium destruction and mucus production (Johnson et al. 2007). Damaged epithelium is known to release alarmins such as IL-33, which is a mediator for initiating type 2 immune responses and believed to be an important factor for allergen sensitisation and asthma development (Hammad & Lambrecht 2015; de Kleer et al. 2016).

Mucus production is a characteristic feature of type 2 immune inflammation driven by IL-13 production (Stier et al. 2016). Single nucleotide polymorphisms (SNPs) in the *IL-13* gene have been associated with severe RSV bronchiolitis during infancy, and these polymorphisms are associated with increased production of IL-13 (Forton et al. 2009). An emerging role for ILC2s has been shown in respiratory virus infection, and activation depending on pro-allergic epithelial cytokines release such as IL-33, IL-25 and TSLP (Monticelli et al. 2011; Hong et al. 2014; Chang et al. 2011; Stier et al. 2016). In particular, SNPs in the locus for the IL-33 receptor (*IL1RL1*) have been linked to asthma and allergic development (Bønnelykke et al. 2013) as well as disease severity of RSV infection (Faber et al. 2012), suggesting a key role for IL-33 in both asthma and RSV bronchiolitis.

To reproduce and study RSV infection, several groups have been using a neonatal mouse model of infection. Neonatal mice (<7 day old) mount a type 2 inflammation that is observed in human infants, and interestingly secondary RSV infection in later life lead to the development of IL-13-dependent AHR and mucus production. The induction of type 2 immunity in response to RSV infection in early life can drive the development of

asthma-like inflammation and phenotype, suggesting an important link between early life infection and subsequent asthma development later in life. Type 2 immune responses can be driven by alarmin cytokines like IL-33. Respiratory viruses replicate in the airway epithelial cells, inducing cell death during viral replication and consequent release of alarmins (Kumar et al. 2014). IL-33 has been shown to play a key role in a neonatal model of RSV infection. In neonatal mice, RSV induces release of IL-33 that is essential for the type 2 inflammation associated with subsequent RSV-reinfection and increased IL-33 levels are detected in nasal aspirates from human infants hospitalised for RSV infection (Saravia et al. 2015). In another study, using Pneumonia virus of mice (PVM) and cockroach extract, IL-33 is shown to play a pivotal role in the induction of asthma-like responses and decrease anti-viral immunity (Lynch et al. 2016).

PVM is a natural pathogen of mice, it is similar to RSV and as a natural pathogen, it induces pathology in mice at lower viral titre then RSV (Rosenberg & Domachowske 2008).

With this project I aim to study HpARI as a possible treatment for asthma. The risk of developing asthma and asthma exacerbation is associated with respiratory viral infections, especially severe RSV bronchiolitis. IL-33 seems a promising target, as it is released in the airway upon viral infection (Werder et al. 2018; Kumar et al. 2014; Jackson et al. 2014). More studies need to be carried out to understand the role of IL-33 in RSV bronchiolitis. Our aims are to investigate the role of IL-33 in a neonatal model of

RSV bronchiolitis, determining if HpARI has an effect and could subsequently be used as a treatment.

4.2 ILC2 activation 24h post RSV infection and the effect of HpARI

Saravia et al. showed a critical role for IL-33 during neonatal RSV-infection in mice. Early IL-33 release induces production of IL-13 in the neonatal airway with an increased accumulation of ILC2 (Saravia et al. 2015). In this study they infected neonatal mice aged 5 day or adult mice (4 weeks old) with RSV A2 at 2×10^5 50% tissue culture infectious dose (TCID50) per gram of body weight and they looked for ILC2s 1 day post-infection (dpi) and for lung histopathology, lung and BALF at 6 days post re-infection (Saravia et al. 2015).

Here we investigate the activation status of the ILC2 population, using flow cytometry to assess intracellular expression of IL-5 and IL-13. BALB/c timed matings were set up at least 20 days prior to the start of the experiment. Pregnant mice were carefully handled to let them become familiar with human presence and smell for 3 days one week before birth. After birth, dams were handled and underwent olfactory conditioning with isoflurane (anaesthetic used during the experimental procedure) and the ink used to mark the mice, for 3 days before the start of the experiment. Neonates were weighed and marked on the feet by tattooing, as ear marking was not an option due to the small size of neonatal ears. All these procedures were performed to reduce cannibalism, and none was

observed. A schematic representation of the procedure involved in the set-up of the experiments is shown in Figure 4.1 and in Figure 4.2 are shown some representative gating.

Neonatal mice, aged 7 days, were infected with RSV and culled 24h after infection. Active RSV infection induced an increase in lung IL-5^+/IL-13^+ ILC2s, while UV-inactivated RSV did not induce ILC2 activation (Fig.4.3). Co-administration of HpARI with RSV reduced subsequent ILC2 activation (Fig 4.3). ST2-deficient mice were also used to determine the role of IL-33 signalling on ILC2 activation and eosinophilic inflammation in the contest of RSV infection. Fig 4.3 shows IL-5^+/IL-13^+ ILC2 were significantly increased upon RSV infection, and this ILC2 activation was significantly reduced in the presence of HpARI. Infection of ST2-deficient mice did not result in an increase of IL-5^+/IL-13^+ ILC2s. This suggests that ILC2 activation in neonatal RSV infection depends on IL-33, and HpARI can be used to block this response and possibly reduce type 2 inflammation.

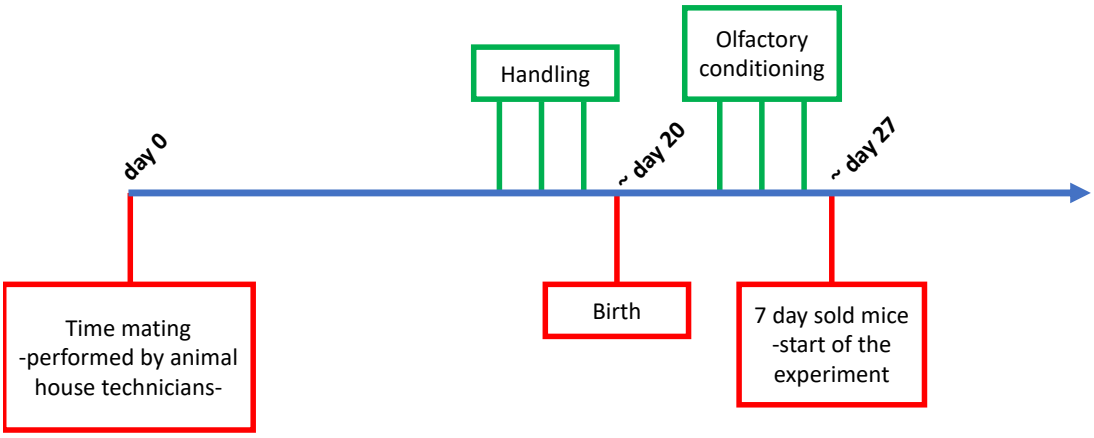


Figure 4.1 Representation of the dams' conditioning prior to start a neonate experiment

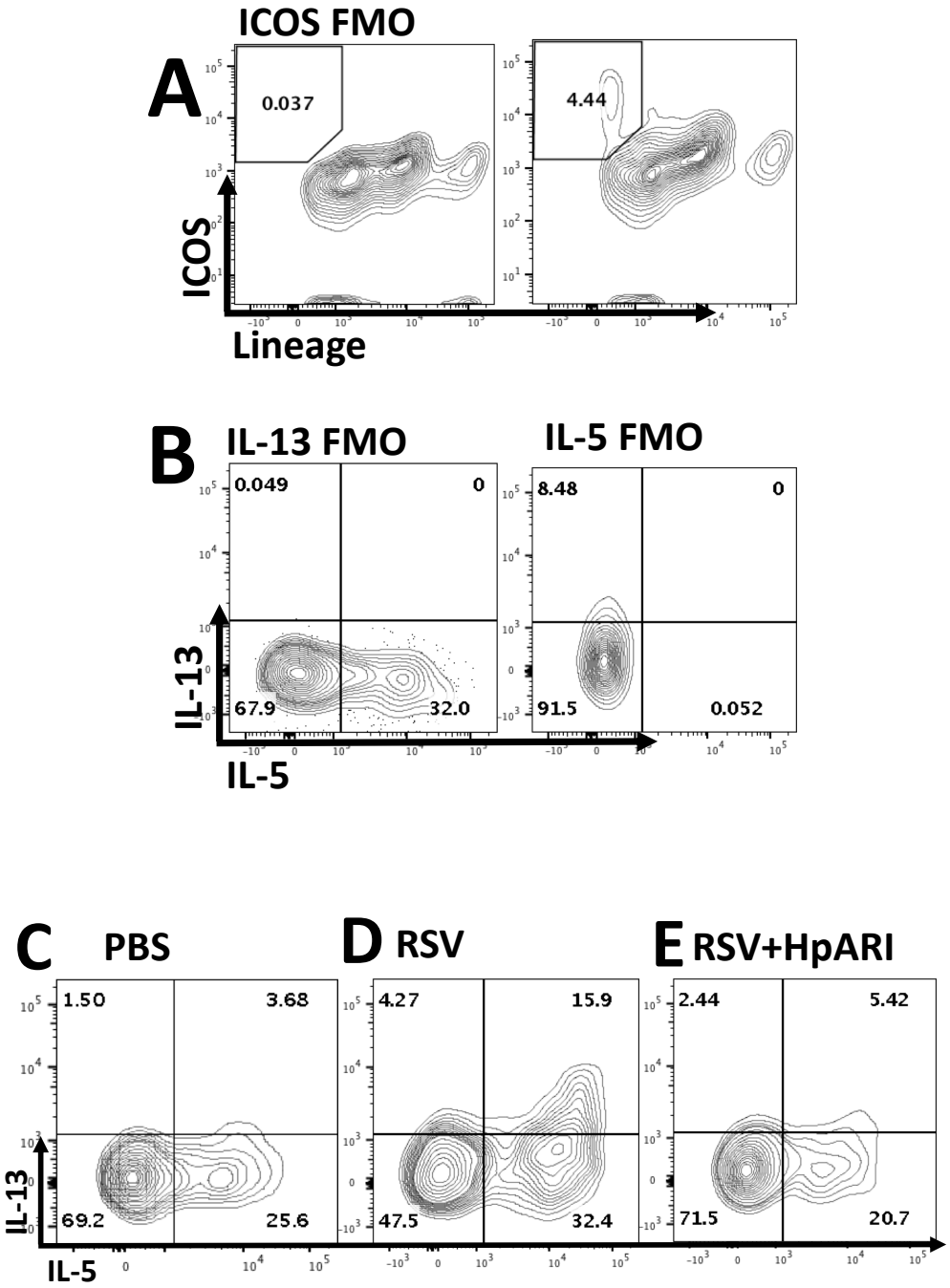


Figure 4.2 Representative FACS plot for the 24h RSV experiment

Lung cells were gated in live cell population, singlets, CD45+, CD4- and ILC2 identified as ICOS+Lineage-. Showing gate for ICOS+Lineage- population with ICOS FMO (A), IL-13 FMO and IL-5 FMO (B) and representative FACS plot from ICOS+Lineage- showing IL-5+IL-13+ for experiment in Fig.4.1A

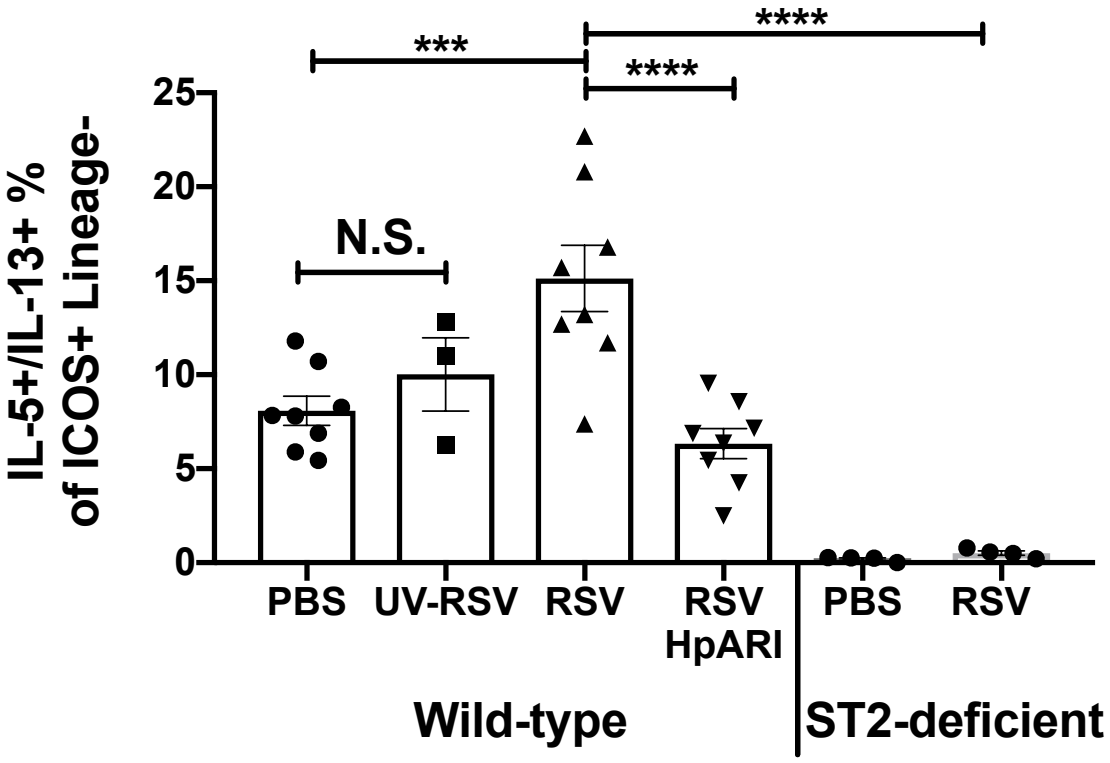


Figure 4.3 ILC2 activation 24h post-RSV infection of BALB/c neonates and ST2-deficient neonates

RSV (6.5x103 PFU/g) was administered intranasally to BALB/c neonates. Mice were sacrificed 24h post-infection and whole lung collected for intracellular cytokine stain. ILC2 were identified as CD45+ CD4- ICOS+ Lineage-. Bars showing mean ± SEM. N=3 to 8 from 2 independent experiments. Analysed with one-way ANOVA with Bonferroni's multiple comparison post-test. ***=<0.005, ****=<0.0001

4.3 Blocking IL-33 during primary infection reduces type 2 immune responses at re-infection

Re-infection in mice infected with RSV as neonates is associated with development of IL-33-dependent type 2 inflammation, which is not observed in adult mice (Saravia et al. 2015). In our model, BALB/c and ST2-deficient neonatal mice (day 5) were infected with RSV in the presence or absence of HpARI. Re-infection was performed 2 weeks after primary infection (Fig.4.4A). Immune responses were analysed 3 days post re-infection by collecting BALF, to analyse eosinophil numbers (gating strategy showed in Fig.4.4B), and lungs for intracellular cytokine staining of ILC2s. RSV re-infection seemed to increase eosinophil numbers in the BAL, and HpARI administered together with the virus at primary infection appeared to suppress this response. However, an outlier was identified which is shown in the graph, but has not been used to calculate the mean (Fig 4.5A – RSV-HpARI:RSV). Due to the large variance in the RSV-RSV group, statistical significance was not achieved. There was also a minor increase in eosinophil numbers at re-infection in ST2-deficient mice, which did not reach statistical significance. This suggests that eosinophilic responses are not completely IL-33-dependent (Fig 4.5A). When ILC2 activation was analysed, RSV re-infection induced a significant increase in IL-5^+/IL-13^+ ILC2s and blocking IL-33 with HpARI at primary infection decreased this response. ILC2 activation is IL-33 dependent in this model as no increase in IL-5^+/IL-13^+ ILC2s was observed in ST2 KO mice (Fig 4.5B).

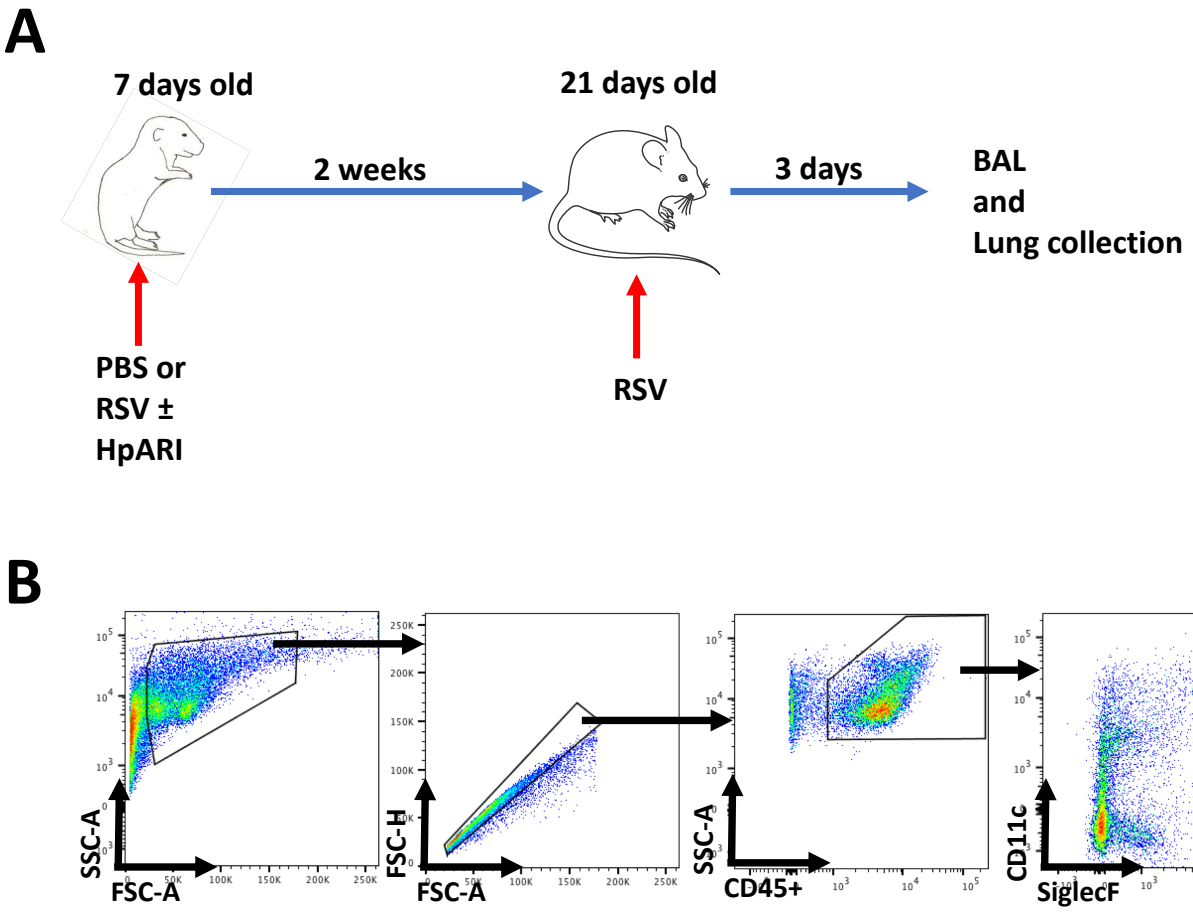


Figure 4.4 Experimental plan for the RSV re-infection experiment and eosinophil gating strategy

Experimental design for the re-infection experiment (A). Eosinophils were identified as $CD45^{+}SIglecF^{+}CD11c^{-}$ (B).

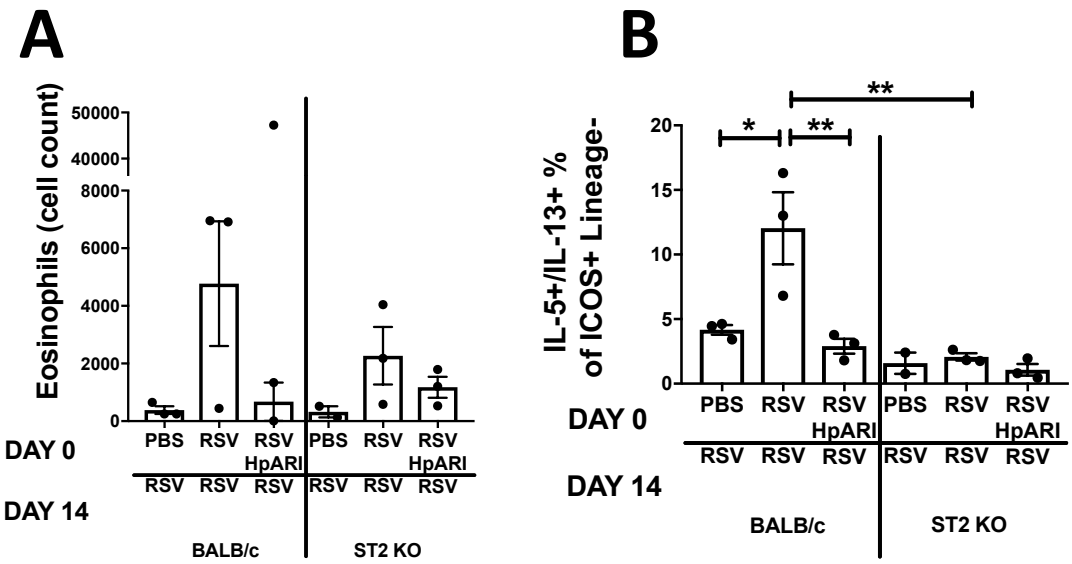


Figure 4.5 Eosinophils and ILC2 activation during RSV re-infection

Neonatal BALB/c and ST2 KO mice at 7 days of life were infected with RSV (6.5x103 PFU/g) (Day 0) and co-administered with HpARI (10 μg). 2 weeks after primary infection (Day 14) mice were re-infected with RSV and lungs collected 3 days after. BAL were collected and analysed for eosinophil infiltrates (A) and IL-5+/IL-13+ ILC2 (B). Bar graph representing mean ± SEM. N=3, one single experiment. Analysed with one-way ANOVA with Bonferroni's multiple comparison test. *=p<0.05, **=<0.005

4.4 Analysis of RSV L-gene and IFNγ expression by real time PCR

So far, I have shown that RSV infection in mice aged 7 days induces activation of ILC2s. This activation depended on IL-33 as ST2-deficient mice showed no ILC2 activation and HpARI suppressed this response. In addition, HpARI given only at primary infection reduced ILC2 activation at re-infection. Thus, HpARI can suppress ILC2 activation upon RSV infection presumably through blocking IL-33-mediated immune responses. However, another possibility is that HpARI reduces infectivity of RSV, inducing less cellular damage and less release of IL-33.

Next, to confirm that ILC2 suppression was due suppression of IL-33 and not anti-viral activity of HpARI, adult mice were infected with RSV and viral load was analysed by qPCR at 4 dpi. Two independent experiments were performed, lungs were collected, RNA extracted and converted to cDNA. To analyse viral replication, expression levels of the L-gene were tested. The L-gene is the last gene expressed during RSV infection and replication, and it has been used as a marker of active infection (Lambert et al. 2014). Merging together the results from the two experiments a decrease in L-gene transcription was observed with co-administration of HpARI during RSV infection (Fig.4.6A). Therefore, the reduction in type 2 immune responses might be caused by fewer viral particles as well as IL-33-blocking by HpARI. IL-33 has been associated with dampening anti-viral immunity in particular IFNs (Lynch et al. 2016; Werder et al. 2018). Thus, a decreased viral titre could be driven by increased IFNγ production due to lower level of

IL-33 and increased NK cells activity. I tested the IFNγ expression in this experiment (Fig.4.6B), and did not find any significant change following HpARI treatment. Thus, these results suggest that HpARI affect directly the viral particles and suppression of type 2 immune responses associated with viral infection can be explained with fewer infected cells and less IL-33 release.

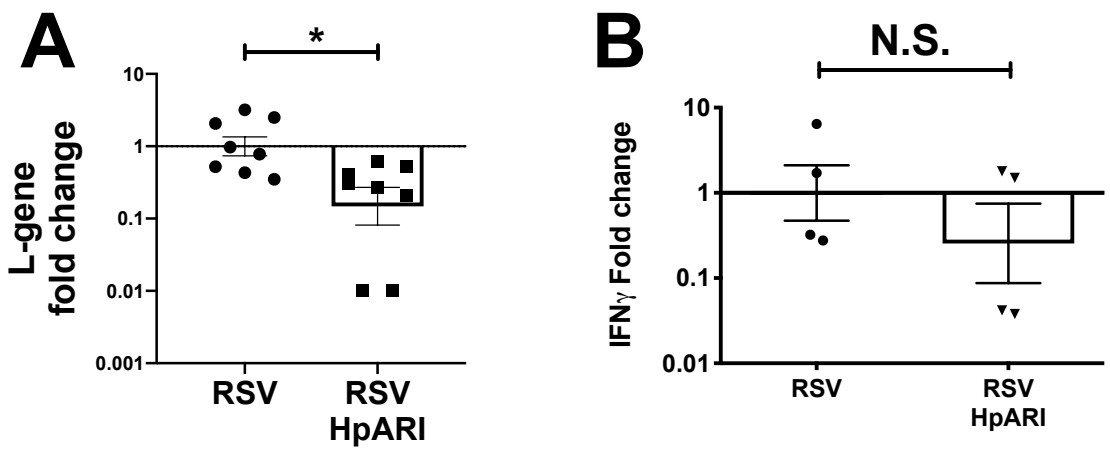


Figure 4.6 L-gene RSV and IFNγ expression in RSV infected mice and the effect of HpARI

HpARI (10 μg) was simultaneously intranasally administrated during RSV infection in adult BALB/c, and lungs collected 4 dpi. RNA was extracted and converted into cDNA as described in Materials and Methods. RSV L gene and IFNγ expression were analysed by qPCR. Gene expression was corrected for variation using 18S as the housekeeping gene, and normalised to the mean ΔCT of the RSV group. Bar graph showing fold change of expression ± SEM. Data pooled from two independent experiments, for a total N of 8 per group (A). Data from one single experiment, for a total N of 4 per group (B). Analysed with unpaired t-test. p=0.013

4.5 HpARI might affect RSV infectivity

To confirm the qPCR results and to assess viral replication, an *ex vivo* plaque assay was performed. The same experiment presented in the last chapter was repeated including more groups to better understand the suppressive effect of HpARI on RSV. HpARI was intranasally administered at several timepoints: 24h and 2h pre-infection to determine if the protein could have an effect on the epithelium and induce protection; simultaneously with RSV to check if it has a direct effect on the virus; and 2h post-infection to test the possibility of reducing viral spreading to the neighbouring cells. Importantly, HpARI can still block IL-33 even if administered 24h prior to stimulation (Osbourn et al. 2017).

As shown in Figure 4.7, at 4dpi a trend towards lower viral load was observed when HpARI was administered at the same time of the virus, and not with pre- or post-administration, however no statistical significance was observed.

A similar experiment was performed by Abbie Payne during an MSc project that I supervised. RSV and HpARI were co-administered to mice simultaneously, plaque assay was performed 4dpi and a similar trend towards viral titre suppression was observed. In Figure 4.8 the new experiment (performed by Abbie Payne) is shown together with the previous experiment shown in Fig.4.7 for the simultaneous administration only, and analysing these data with t-test statistical significance was achieved.

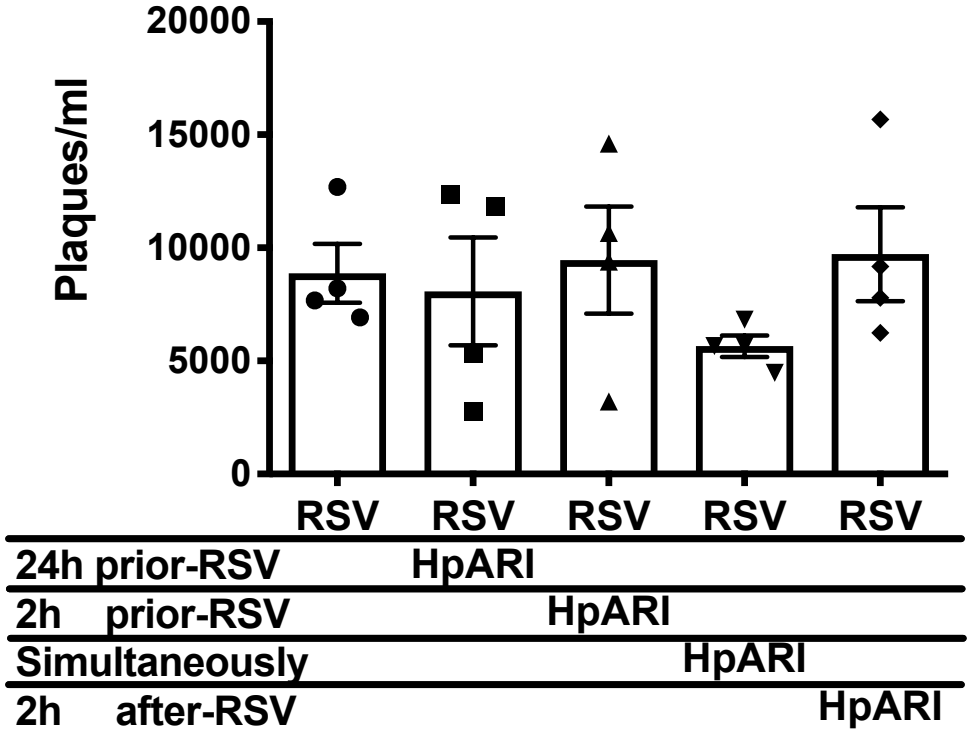


Figure 4.7 *Ex vivo* plaque assay – time course

Adult BALB/c mice were intranasally administered with HpARI (10 μg) 24h and 2h prior, simultaneously and 2h after RSV infection as indicated in the graph. 4 dpi mice were culled and lungs collected. Lung homogenate from each mouse was diluted and co-cultured with Hep2 cell line plated in a 96-well plate and a plaque assay was carried out. One single experiment, for a total of N of 4 per group. No statistical significance observed analysing the data with one-way ANOVA with Bonferroni's multiple comparison post-test.

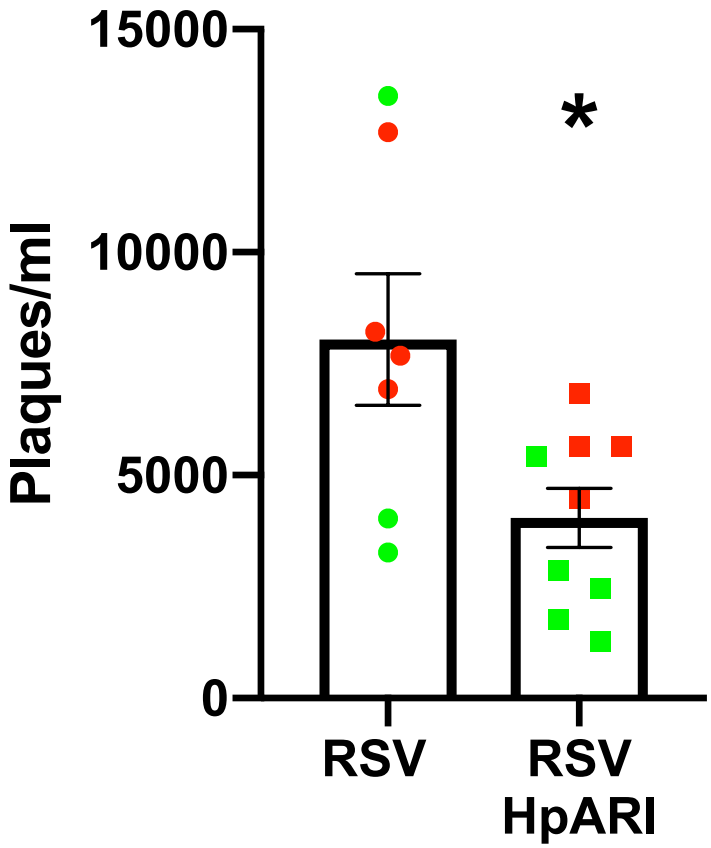


Figure 4.8 *Ex vivo* plaque assay

Adult BALB/c mice were intranasally co-administered with HpARI (10 µg) and RSV. 4 dpi mice were culled and lungs collected. Lung homogenate from each mouse was diluted and co-cultured with Hep2 cell line plated in a 96-well plate and a plaque assay was carried out. Showing 2 independent experiments, one already shown in Figure 4.7 (red symbols) and the experiment performed during MSc project (green symbols). Analysed with unpaired t-test. N=7-9. *=<0.05

4.6 Reduced viral replication in human nasal epithelial cells

RSV is a human virus, and not a natural pathogen in mice. I had the opportunity to perform some experiments using human nasal epithelial cells (hNECs) obtained from volunteers as described in Materials and Methods. hNECs were cultured for 4 days with RSV in the presence or absence of HpARI. Analysing viral load by qPCR showed a significant reduction in RSV L gene in this culture (Fig.4.9). Therefore, HpARI might have a directly antiviral effect. This experiment provided more evidence that the inhibitory effect observed in the RSV model may be explained by reducing RSV infectivity rather than IL-33 suppression.

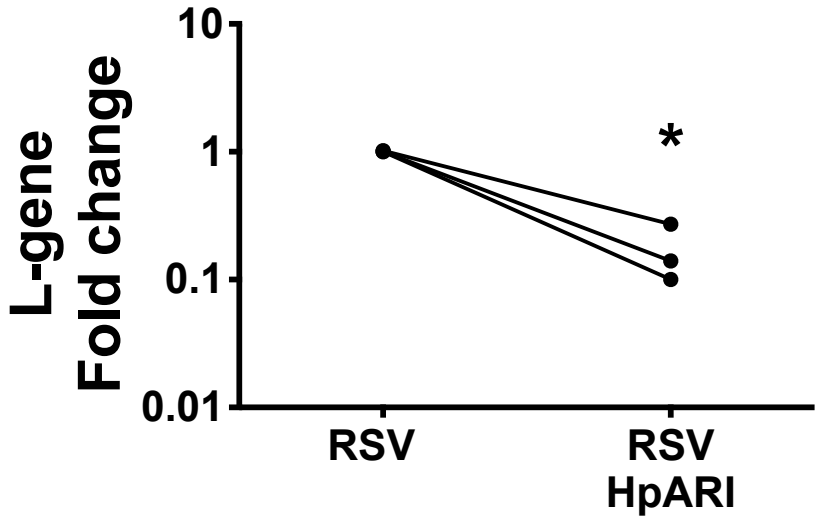


Figure 4.9 Antiviral effect of HpARI in human nasal epithelial cells (hNECs)

hNECs were obtained from volunteers as described in Materials and Methods. Cells were infected with RSV MOI 1 and simultaneously treated with HpARI (1 μg/ml). After 3 days RNA was extracted, cDNA converted and analysed by qPCR for L-gene expression. Gene expression was corrected for variation using 18S as the housekeeping gene, and normalised to the mean ΔCT of the RSV group. Bar graph showing fold change of expression. Data from 3 different biological replicates. Analysed with paired t test. p=0.023

4.7 Discussion

Asthma is a chronic airway disease characterised by reversible airflow obstruction with shortness of breath, wheeze and chest tightness. Respiratory viruses infection during childhood, especially RSV, have been associated with wheeze and asthma development later in life (Henderson et al. 2005; Sigurs et al. 2010). Studies in mice suggests that RSV infection could lead to a dysregulation of the response to bystander antigen and allergen (Schwarze et al. 1997; Siegle et al. 2010). The relationship between RSV infection and asthma development it is a subject of continuing discussion. In the past years, high interest has been focused on the epithelial-derived alarmin IL-33 as initiator of type 2 immune responses and it has been strongly associated with allergen sensitisation in the developing lung (de Kleer et al. 2016).

In this chapter, HpARI the *H. polygyrus*-derived IL-33 blocker was tested in a neonatal model of RSV infection. Setting up an RSV neonatal model was challenging due to unpredictable numbers of neonates, difficulties in breeding mice and tissue collection. For these reasons low numbers of mice were used, and low numbers of repeat experiments were carried out.

I have shown that RSV induces ILC2 activation 24h post RSV infection in a neonatal model and this response was significantly reduced with HpARI.

According to the study of Saravia and colleagues, IL-33 released during primary RSV infection induced type 2 immune responses at re-infection later in life (Saravia et al. 2015), therefore I tested HpARI in a re-infection model. IL-5^+ IL-13^+ ILC2 were

significantly increased at re-infection and IL-33 plays a pivotal role as ILC2s were not activated in ST2-deficient mice upon RSV re-infection. Eosinophil numbers in the BAL did not show statistical significance but a trend towards increased numbers was observed in the re-infection group. However, eosinophils numbers were lower than seen in other asthma models (e.g. between $5x10^5/1x10^6$ eosinophils compared to an average of 5000 eosinophils in our model) and possibly higher numbers might be observed at a later time point after re-infection e.g. 6 or 7 days after re-infection (Saravia et al. 2015; Schwarze et al. 1999, 2000; You et al. 2015). IL-33 has been suggested to be responsible for increasing sensitisation to allergen in the developing lung (de Kleer et al. 2016), raising the possibility to prevent allergic sensitisation by inhibition of IL-33 e.g. with HpARI. In addition, the key role of IL-33 during sensitisation has been shown in a respiratory viral model using pneumonia virus of mice (PVM). Co-exposure of mice to PVM and cockroach extract (CRE) induces an IL-33 dependent asthmatic phenotype with eosinophilia and mucus production and blocking IL-33 reverses this outcome. IL-33 blockade induces an increase in type I, type II and type III IFNs responses, restoring the anti-viral responses (Lynch et al. 2016; Werder et al. 2018). If on one side, during respiratory viral infection, IL-33 seems to be important in the initiation phase (Saravia et al. 2015; de Kleer et al. 2016), on the other side IL-33 could play a role during viral-induced exacerbation in experimental asthma model, in particular suppressing T_H1 development (Ravanetti et al. 2018; Werder et al. 2018). The role of RSV in exacerbation has not been studied yet in a laboratory setting, however blocking IL-33 with HpARI

might have a role during asthma exacerbation induced by viruses such as influenza or PVM (Ravanetti et al. 2018; Werder et al. 2018; Lynch et al. 2016).

Next, the viral load was measured after RSV infection by qPCR of the L-gene or *ex vivo* plaque assay. HpARI co-administration resulted in reduced RSV load, whether measured by qPCR or plaque assay, but this effect was only observed on co-administration and not if HpARI was administered before or after RSV. To test the effect of HpARI on RSV in a more relevant model I used primary human nasal cells obtained from healthy volunteers. Nasal cells were obtained from the inferior turbinate and cultured as described in Materials and Methods. Nasal cells were infected with MOI 0.1 and viral titre determined by qPCR 3 days post-infection. Co-administration with HpARI significantly reduced RSV-titre by almost 100-fold therefore HpARI might have direct effect on viral particles. I did not measure expression of type I or type III IFNs such as IFNβ or IFNλ in this experiment and I cannot exclude that HpARI induces other anti-viral responses. However in the *in vivo* experiment administration of HpARI 24h prior to RSV infection seemed not to affect the viral load, possibly indicating no effect of HpARI in IFNs production by epithelial cells. I believe that the anti-viral effect observed is dependent on the positively charged CCP1 domain of HpARI as similarly observed for the human cathelicidins LL-37 that through its cationic residues directly affects bacteria and disrupts viral membrane of Influenza A virus (Li et al. 2006; Tripathi et al. 2013; Currie et al. 2013; Sousa et al. 2017). Preliminary results from a MSc project in the lab which I supervised have shown that HpARI CCP1/2 reduced RSV infectivity in Hep2 cells but reduction was not

observed with HpARI CCP2/3. Therefore, HpARI could suppress RSV-induced ILC2s activation independently in two different ways. In neonate mice infected with RSV, HpARI reduces infectivity and consequently a reduced release of IL-33 induces decreased ILC2s responses. Released IL-33 could be still blocked by HpARI, further suppressing ILC2s activation.

More studies need to be carried out to understand the role of IL-33 in RSV bronchiolitis and the increased risk of developing allergies and asthma later in life. IL-33 seems to be a link between respiratory viral infections and asthma initiation in early life. In particular during the alveolar phase of the developing lung (between day 3 and day 21 post birth) an increase in ILC2, mast cells and eosinophils is observed in mice peaking at day 14 post birth (de Kleer et al. 2016), and this is driven by IL-33 which induce remodelling of the lungs. However, any further release of IL-33 induced by allergen, and possibly by viral infection, lead to sensitisation and to an asthmatic phenotype later in life (de Kleer et al. 2016; Saravia et al. 2015). Therefore in mice, the developing lung is already a TH2 polarised environment with physiological release of IL-33 and ILC2s infiltrates accumulating in the lungs. Epithelial cells damaged by RSV infection can release IL-33 and ILC2 activation, lowering the threshold to induce immunity to allergens during viral infection. In the neonatal model used in this chapter, lower eosinophil numbers were observed if compared with other asthma-like model. I suggest that to obtain increased type 2 immune responses neonatal mice might be infected at the peak of ILC2 and mast cells, thus using mice aged 14 days instead of 7 days as used here. In humans, Saravia

and colleagues reported an increased IL-33 and IL-13 release in nasal aspirates from infants hospitalised with severe RSV bronchiolitis. Levels of IL-33 increased in RSV-infected infants and IL-13 levels correlates with IL-33. With these results they showed a possible connection between severe RSV infection and the induction of a cytokine involved in type 2 immunity associated with asthmatic responses. However, it is important to appreciate that not all children with severe RSV bronchiolitis develop allergies or asthma later in life. These differences are still poorly understood, in particular genetic factor such as polymorphism in the IL1R (Faber et al. 2012) could be associated with the risk of asthma later in life as a consequence of severe infection. Also to be taken into consideration are potential differences in RSV clinical isolates, some of which have been shown to cause different immune responses (Moore et al. 2009; Stokes et al. 2011; You et al. 2006). Several factors might be responsible for these differences, as mentioned genetic factor, viral strain and recently the microbiome has been implicated in shaping the immune responses against RSV and the maturation of the gut microbiome is associated with the risk of asthma development (De Steenhuijsen Piters et al. 2016; Man et al. 2017; Stokholm et al. 2018). Nonetheless, developing new therapies for RSV or IL-33 might help the fight against allergies and asthma, while helping understand the basic biology of asthma induction.

Chapter 5

Helminth-derived suppressor of ST2

5.1 Introduction

Heligmosomoides polygyrus is a helminth parasite that establishes long-lasting infections in laboratory mice (Johnston et al. 2015). Infection occurs via the faecal-oral route when infective L3-stage larvae are ingested. Larvae reach the submucosa and they undergo maturation to L4-stage larvae and this period is associated with formation of granulomas. Adult worms start emerging from the gut wall around 8-10 days and emerge by day 14 into the lumen (Valanparambil et al. 2014; Johnston et al. 2015). Infection is restricted to the intestine but systemic effects are observed i.e. induction of both T_H2 and regulatory immune response (Maizels et al. 2012; Reynolds et al. 2012; McSorley et al. 2013). These effects can be mimicked by the *H. polygyrus* excretory/secretory products (HES) (Grainger et al. 2010; McSorley et al. 2014; McSorley et al. 2015), and dissected to single proteins produced by the parasite (Osbourn et al. 2017; Johnston et al. 2017; Smyth et al. 2018). HES administration is associated with decreased activation of dendritic cells (Segura et al. 2007), regulatory T cell induction (Grainger et al. 2010), interference with IL-33 (McSorley et al. 2014) and lower level of ST2 expression, the receptor for the alarmin IL-33 (Coakley et al. 2017; Buck et al. 2014). The IL-33 pathway, and ST2 in particular, is a promising target to develop treatments for asthma, as polymorphisms in

IL-33 and the ST2 coding genes are associated with asthma (Moffatt et al. 2010; Bønnelykke et al. 2013).

Parasitic E/S are a complex mixture of molecules including proteins, carbohydrates and lipids. Recently extra cellular vesicles (EV) (or "exosomes") have been identified in the E/S of various parasites. For example, EV-derived microRNAs from the parasite have been detected in the bloodstream of patients with *Schistosomiasis* (Meningher et al. 2017), as well as helminths such a *Fasciola hepatica* and *Echinostoma caproni* (Marcilla et al. 2012). EV have been hypothesised to play a role in communication and immunomodulation as they contain a subset of secreted proteins and miRNA (Buck et al. 2014; Deatheragea & Cooksona 2012; Filbey et al. 2014; Hewitson et al. 2011). Proteins and microRNAs within helminth EV have been shown to protect mice in experimental models of colitis: Intraperitoneal administration of *N. brasiliensis*-deried EV suppressed IL-6, IL-1β, IFNγ and IL-17a while inducing anti-inflammatory IL-10 in a mouse model of colitis (Eichenberger et al. 2018) and similar observations were made with administration of *F. hepatica* EV (Roig et al. 2018). Buck et al. showed that exosomes released by *H. polygyrus* are able to suppress Dusp1, and expression of ST2 on ILC2 and in an epithelial cell line, at the level of both surface ST2 protein, and ST2 gene transcription (Buck et al. 2014). However, depleting HES of the EV component still suppresses ST2 in M2 polarised macrophages both at surface protein levels and gene transcription (Coakley et al. 2017), indicating that non-EV mediators might also be responsible for ST2 suppression.

Prior to the start of this project, further data from the McSorley lab indicated a potent effect of HES on ST2 surface expression and that this suppression was not mediated by HpARI. Taken together with the results from Coakley and colleagues, HES, EV and non-EV components suppresses in two distinct ways: ST2 at surface protein expression and transcription levels.

In this chapter I will focus on the protein component of HES that are involved in ST2 suppression. Previous studies analysed HES, and different genes expression during larval stage and adult stage of *H. polygyrus* as well as the proteins content both in EV and non-vesicular component (Buck et al. 2014; Hewitson et al. 2013), giving information at which stage the protein is majorly produced by the parasite and where in the secretions each protein is most prevalent.

Our hypothesis was that a protein contained in HES is responsible for ST2 suppression. The aims for this part of the project are to identify the responsible molecule, through a process of screening HES fractions, and generate a recombinant protein to test *in vitro* and *in vivo*.

5.2 HES suppresses ST2 detection *in vitro*

As mentioned in the introduction, HES is a complex mixture of thousands of molecules, such as proteins, carbohydrates, lipids and EVs. HES can be obtained by cultivating adult *H. polygyrus* parasites *in vitro* as described in (Johnston et al. 2015). Buck and colleagues concentrated the EV components of HES from the non-vescicular supernatant component (EV-depleted HES) through ultracentrifugation (100,000 g) (Buck et al. 2014). EV were tested *in vivo* using the *Alternaria* model, showing suppression of type 2 immune responses and expression of ST2 on the surface of ILC2s. EV suppressed transcription of *ST2* and ST2 detection by flow cytometry in an epithelial cell line and in M2 polarised macrophages (Buck et al. 2014; Coakley et al. 2017).

To confirm these data, suppression of ST2 on lung cells by HES components was tested *in vitro*. Lungs were obtained from BALB/c mice, a single cell suspension was obtained and cells were cultured with a titration of HES, HES-derived exosomes (Exo) and exosomes-depleted HES (Sup). After 24h, cells were surfaced stained and expression of ST2 was measured in $ICOS^{+}Lineage^{-}CD4^{-}CD45^{+}$ ILC2s (lung flow cytometry gating shown in Figure 5.1). Lung cells cultured with media alone were used to determine the baseline levels of ST2 geometric Mean Fluorescence Intensity (MFI) on ILC2 (Fig.5.2A). Similarly, bone marrow cells from the same mice were cultured in media alone or HES, Exo and Sup for 24 h and analysed by flow cytometry (Fig.5.2B). ST2 was downregulated on ILC2s surface when lung and bone marrow cells are cultured with HES, exosomes or Sup. Lower concentration of HES preparation (0.01 μg/ml) do not affect ST2 detection. Thus, all HES preparations are able to suppress ST2 detection by flow cytometry in our

in vitro model and in addition, the suppression is similar among the preparations suggesting that there is a soluble element both in HES and HES-exosomes that carry out the suppression.

A timecourse was carried out to measure how long the suppressive effect of HES took to act.

After digestion, lung cells were incubated with HES for 30 minutes up to 24h. After the 37°C incubation cells were stained at 4°C and fixed with 2% paraformaldehyde. Fig. 5.3 shows that suppression occurs rapidly, after 30 minutes incubation, and that expression of ST2 increase overtime possibly due to the release of IL-33 from necrotic epithelial cells in these cultures (Osbourn et al. 2017). Hence, in our system ST2 expression is rapidly reduced by HES. I then hypothesised that this reduction is too rapid to involve a transcriptional effect and a reduction on the surface protein. Transcription regulation cannot be excluded at later time points, as shown in Buck et al (Buck et al. 2014; Coakley et al. 2017).

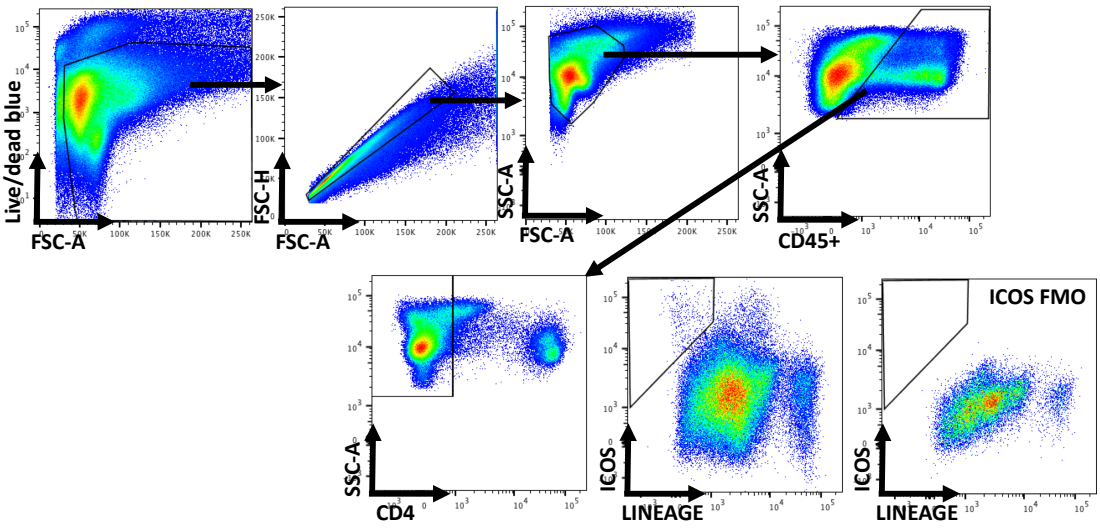


Figure 5.1 Lung gating strategy for measuring ST2 MFI in the ICOS+ Lineage- population

Gate made on live cells (UV450), Singlets, Lymphocytes, CD45+, CD4- and ST2 MFI was measured on the ICOS+ Lineage -

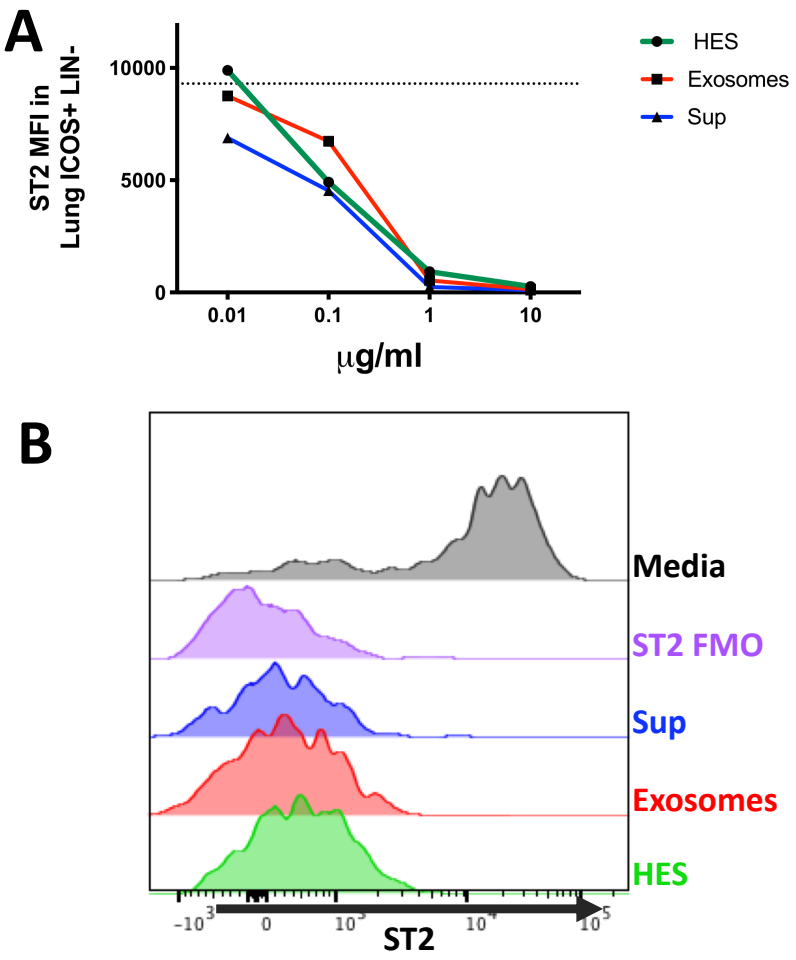


Figure 5.2 ST2 MFI in lung ICOS+ Lineage- populations

Lung and bone marrow cells (1x106 cells) were incubated with a titration of HES, HES-derived exosomes and exosomes-depleted HES (Sup) as indicated in the figures. Cells were harvested after 24h, surface stained and analysed by flow cytometry. ST2 MFI was measured as Geometric Mean of the ICOS+ Lineage- population (ILC2s). Graph showing ST2 MFI (A) and representative histogram for 1 µg/ml concentration (B). Dotted line representing the control expression of ST2 (A). One single experiment.

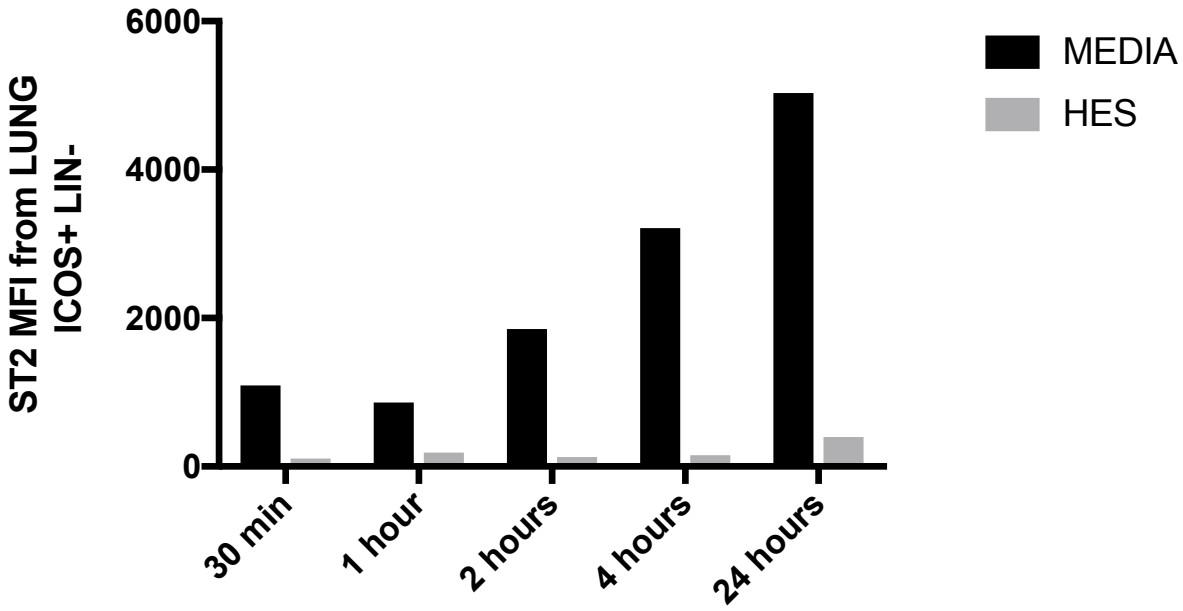


Figure 5.3 HES suppresses ST2 even at the earliest time points

WT mice lungs were harvested and digested to obtain a single cell suspension. After digestion, cells were incubated with total HES (1 μg/ml) as indicated in the graph. After incubation cells were surface stained, fixed and analysed by flow cytometry. One single experiment.

5.3 Identification of the ST2 suppressive protein

HES fractionation and mass spectrometry analysis were performed in the lab before I started the project as part of the identification of HpARI. The mass spectrometry data contains each protein in HES and the estimated abundance of each protein in the size and charge fractions. This proteomic data was compared to an in-house transcriptomic dataset from *H. polygyrus* (manuscript in preparation). Therefore, I tested each size and charge fraction for ST2 suppression by flow cytometry and the peak of ST2 suppression was identified from fraction 10 to fraction 13 for the size fractions and from fraction 45 to fraction 48 for the charge fractionation. Considering all the proteins that were in the ST2 suppression peak for the size fractionation (from fraction 10 to 13), 619 protein were identified, while for charge fraction (from 45 to 48) 578 proteins were identified. Matching the proteins shared between the 2 sets 113 proteins were identified. Among these 113 proteins, 20 of these contained a signal peptide. A signal peptide is a short amino acid sequence that indicates the possible secretion of the protein, and as we are interested in protein that have been secreted by the parasite, a signal peptide makes the protein a better candidate (Petersen et al. 2011). In the group of proteins that were expressing a signal peptide: 5 were identified as a apyrase, which have a highly conserved enzymatic function (Faria-Pinto et al. 2008) and unlikely to perform something novel as ST2 suppression; 2 were identified as VAL-family proteins that are protein expressed in almost all parasites secretions (Hewitson et al. 2013; Hewitson et al. 2011). Other proteins were identified as containing saponin B-like domain, thierodoxin, heat-shock protein and CCP domain proteins, which is an interesting family as two CCP domain-containing

proteins, HpARI and HpTGM, have been already identified as immunomodulators (Osbourn et al. 2017; Johnston et al. 2017) (Table 5.1). One candidate Hp_I25642_IG17586_L548, a CCP domain-containing protein, had already been gene synthesised in a parallel project in the lab to study the CCP-domain family and it was decided to be tested first. A schematic representation of the process is illustrated in Figure 5.4A. The ST2 suppression profile was compared to the exponentially modified protein abundance index (emPAI), which gives an estimation of the absolute protein content in complex mixture. In our case specifically, the emPAI profile estimates the protein content for the candidate Hp_I25642_IG17586_L548 in each charge and size fractions.

The emPAI profile of Hp_I25642_IG17586_L548 peaked at size fraction 11 while ST2 suppression peaked at fraction 10-13 (Fig 5.4B), and at charge fraction 46, with the suppression peak at fraction 42-44 (Fig 5.4C). As a reference, applying the same analysis system for Hp_I08175_IG02172_L1570 (HpARI) we can see that according to the size fractionation the emPAI value peaks at fraction 12, making it a good match with the ST2 suppression (Fig.5.4D). However, the peak for the charge fractionation is around fraction 24-25 making it a poor fit for the ST2 suppression (Fig.5.4E), and further confirming that Hp_I08175_IG02172_L1570 was not a good candidate.

Table 5.1 **Protein screening for ST2 suppressor candidate and annotation of protein superfamily identified with BLAST**

Genomic sequence	**Protein superfamily**
Hp_I30191_IG22135_L475	Unknown

Hp_I30075_IG22019_L477	Metallophosphoesterase
Hp_I28383_IG20327_L491	Unknown
Hp_I25828_IG17772_L540	Unknown
Hp_I25642_IG17586_L548	CCP domain
Hp_I25217_IG17161_L558	CCP domain
Hp_I21830_IG13774_L655	Unknown
Hp_I17392_IG09336_L916	Unknown
Hp_I15979_IG07923_L1089	Apyrase
Hp_I15931_IG07875_L1098	Apyrase
Hp_I12919_IG04863_L2064	Thioredoxin
Hp_I10525_IG03347_L606	Abhydrolase
Hp_I09769_IG02969_L1009	CCP domain
Hp_I08147_IG02158_L2251	Heat shock protein
Hp_I04668_IG00729_L1906	Apyrase
Hp_I04148_IG00569_L936	Saposin B-like domain
Hp_I01450_IG00104_L975	VAL protein
Hp_I04668_IG00729_L1906	Apyrase
Hpb-VAL7.1	VAL protein
Hp_I04667_IG00729_L1917	Apyrase

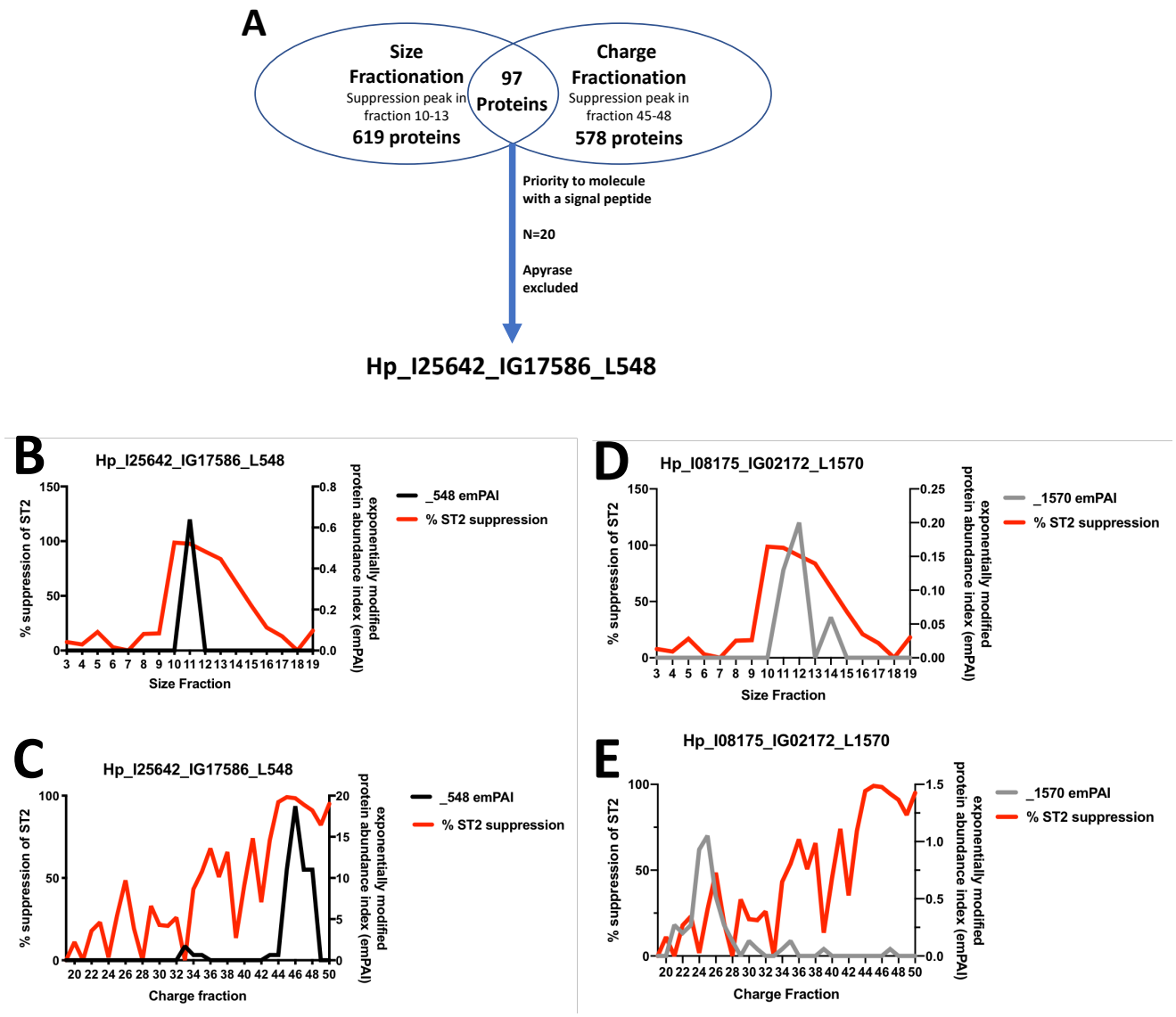


Figure 5.4 Identification of the ST2 suppressor protein

Candidate protein selection strategy (A). Size and charge fractions (1 µl per well in a 200 µl culture volume) were incubated with a single cell suspension from lungs (1x10^6 cells) obtained as described in Materials and Methods. Cells were collected 24h later, surface stained and analysed by flow cytometry. Suppression of ST2 was measured in the ILC2 population, comparing ST2 MFI decrease with the media alone group. Candidate Hp_I25642_IG17586_L548 emPAI profile and ST2 suppression profile in size (B) and charge (C) fractions. Hp_I08175_IG02172_L1570 emPAI profile and ST2 suppression profile in size (D) and charge (D) fractions.

5.4 Hp_I25642_IG17586_L548 is a CCP domain molecule

Hp_I25642_IG17586_L548 nucleotide sequence was identified through the available transcriptome data (manuscript in preparation). An identical transcript HPOL_0001228301-mRNA was identified using WormBase ParaSite, and that it has been annotated to be formed by 5 exons (546 nucleotide) that encodes for a 163 aa protein including a 18 aa signal peptide identified through SignaIP 4.1 (Petersen et al. 2011). Exons boundaries were mapped using WormBase ParaSite. Analysing the protein sequence by Expasy Prosite and EMBL-EBI InterPro predict a single CCP domain. However, when the amino acid sequence was aligned with other CCP domains, it appeared to have 2 CCP domains. In addition, as shown previously for HpARI and HpTGM, each CCP domain is encoded by 2 exons, confirming that Hp_I25642_IG17586_L548 has 2 CCP domains encoded by 2 exons each (Osbourn et al. 2017; Johnston et al. 2017; Smyth et al. 2018) (Fig.5.5). Furthermore, HpARI and Hp_I25642_IG17586_L548 CCP domains was aligned with two well-characterised CCP domain proteins: complement factor H CCP10 (Factor H CCP10) and complement receptor type 2 CCP2 (CR2 CCP2); as described in (Osbourn et al. 2017) (Fig. 5.6). Hp_I25642_IG17586_L548 CCP1 and CCP2 were aligned and it is possible to observe the 4 cysteines (C) in position 5, 69, 93 and 110, which define the CCP domain. At aa 99, another characteristic of a CCP domain is the presence of a tryptophan residue (W) that we can observe for Hp_I25642_IG17586_L548 CCP1 and CCP2, HpARI CCP2 and 3,

and CR2. A leucine (L) is observed in the same position for HpARI CCP1, but it is an atypical substitution that is observed in the sequence of complement factor H (Osbourn et al. 2017). Some other conserved traits can be observed in position 62 and 98 where a highly conserved glycine (G) residue is observed, and in position 66 with the presence of a valine (V) residue. Two atypical insertions can be found in HpARI CCP2 and CCP3 (from residue 7 to 33) that are not present in either Hp_I25642_IG17586_L548 CCP domains or the CR2 or Factor H CCP domains shown, as described in Osbourn *et al.* (Osbourn et al. 2017). However, Hp_I25642_IG17586_L548 CCP2 show an atypical insertion of 10 residues after the second cysteine which is not present in the other CCP domains analysed. This sequence analysis is presented to underline that Hp_I25642_IG17586_L548 shows typical characteristic of a CCP-domain protein with some divergences, similarly to HpARI, which also has atypical insertion in a different part of the sequence. These atypical insertion might have been developed by *H. polygyrus* to generate protein with activity or specific binding sites.

gtttaattacccaagtttgagatgcttcttctccaactacttcttgccgcgttcatcgct
M L L L Q L L L A A F I A
gaaggagcagatcaaagctgtaatgaagccccaaagggatactatgttcggctcaagtat
E G A D Q S C N E A P K G Y Y V R L K Y
agcggagataatcgcgtaagcggaaaatatcccagtggtacactagttgaagcatcctgt
S G D N R V S G K Y P S G T L V E A S C
acaaacggactacagatgatagaaggaagaatttttctcgatgcacaaacggaaagtgg
T N G L Q M I E G K N F S R C T N G K W
gtaccagggcttggccgctgcccatatcactgtccacttggcttcttcactggaagcaaa
V P G L G R C P Y H C P L G F F T G S K
taccaagtcgagccatatcctaacaaaggcaaaaaaaaaatggaatggagacctgatgga
Y Q V E P Y P N K G K K K M E W R P D G
tctaaggttttggcttattgcggatggaagagttccggtgcccaagagaggcgaatat
S K V L A Y C G W K S S G A Q E R G E Y
ggcgaatttcaagcgcgctcttacacctgtcgcgatggagactggctgacggacgacgga
G E F Q A R S Y T C R D G D W L T D D G
gaaccacacgatcaatgtattccagaaagttaggccgtttctctagttgtcaaataaagg
E P H D Q C I P E S -
tcattccg

Exons boundaries: Exon1 Exon2 Exon3 Exon4 Exon5
MLLLQ = Signal peptide
CNEAP= CCP-1 Hp_I25642_IG17586_L548
YTCRD= CCP-2 Hp_I25642_IG17586_L548
C = cysteine
W = tryptophan

Figure 5.5 Hp_I25642_IG17586_L548 genomic and amino acid sequence

The genomic sequence shows highlight of the 5 exons. The nucleotide sequence highlights the presence of 2 CCP-domains. The translated protein is showed in bold in the nucleotides sequence, the presence of 8 cysteine residues (C) is indicated with purple letters and the tryptophan (W) residues in red.

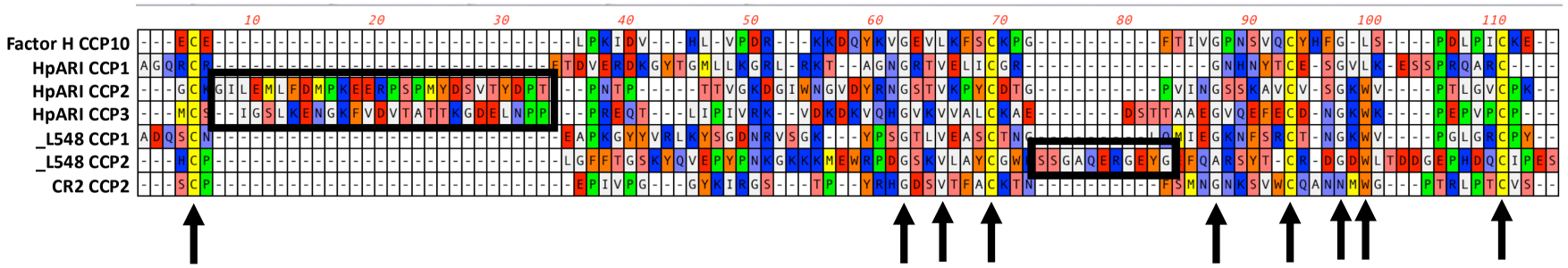


Figure 5.6 Sequences alignment of HpARI and Hp_I25642_IG17586_L548 CCP domains

Sequences were obtained from the translation of the genomic sequence. Factor H CCP10 and Complement receptor type 2 (CR2) CCP2 were aligned with HpARI CCP-domain as described in (Osbourn et al. 2017) and subsequently Hp_I25642_IG17586_L548 was aligned. Sequences have been split after position 51 and following aa showed below. Some characteristic features of CCP-domain molecules are highlighted in. Black box showing atypical insertions between cysteine 1 and 2 in HpARI CCP1 and CCP2, and between cysteine 2 and 3 in _L548 CCP2. Arrows indicate conserved cysteines, valines, glycine and leucine/tryptophan sites.

5.5 Cloning and expressing Hp_I25642_IG17586_L548

I identified the *Hp_I25642_IG17586_L548* gene that encodes a possible candidate for ST2 suppression. To start producing the recombinant protein, a sequence optimised for mammalian expression was generated. The sequence was codon optimised to increase expression in a mammalian cell line with Invitrogen GeneArt Gene Synthesis and 2 restriction sites were added to the sequence: AscI at the 5' and NotI at the 3' (Fig.5.7), to allow sticky-end cloning into an expression vector. Gene codon optimisation consists in choosing the most common tRNAs for the species where the protein will be produced, adding these tRNAs at the codons of the sequence.

Figure 5.8A shows an alignment of the genomic sequence of Hp_I25642_IG17586_L548 directly from the parasites genome versus the sequence obtained after codon optimisation, to show the divergences in the nucleotide sequence. However, even if the two nucleotide sequences have some divergences in figure 5.8B the alignment of the translated amino acid sequence showed that they both generate the same protein and they align after the end of the signal peptide of _548. The codon optimised sequence was gene synthesised in a pMA-RQ plasmid and subsequently restriction digested, purified and ligated into a pSecTAG2A vector that includes a myc-tag and a polyhistidine tag (6-His) at the C-terminus of the protein (Fig.5.9). The 6-His tag allows purification of the protein from

transfected cell supernatants by nickel affinity chromatography using HisTrap excel columns and eluting bound proteins using an imidazole gradient.

Figure 5.9 shows the amino acid sequence cloned into the pSecTAG2A vector highlighting enzyme restriction sites. JM109 competent cells were transformed with the pSecTAG2A vector containing Hp_I25642_IG17586_L548 sequence. As described in Materials and Methods bacterial midiprep was carried out and the plasmid transfected using Expi293T system. Supernatant containing protein were collected and purified with a HisTRAP excel column. Purified protein was run in a 4-12% Bis-Tris protein gel and the gel stained with Coomassie blue (Fig.5.10). Hp_I25642_IG17586_L548 is 163 aa long protein, with a predicted molecular weight of 18.1 kDa and a theoretical pI of 7.50. The recombinant protein generated, including tags, is a 209 aa long protein, and has a predicted molecular weight of 23 kDa, however in the protein gel the size of the protein appears to be around 30 kDa. Using NetNGlyc 1.0 and NetOGlyc 4.0, no N-glycosylation or O-glycosylation sites respectively are predicted for the protein, thus the differences between the predicted molecular weight and the protein gel might be dependent on other post-translational modification than N- or O- glycosylation. Moreover, these are prediction algorithms only and protein glycosylation might still occur.

Codon optimised Hp_I25642_IG17586_L548 sequence with AscI, NotI and TEV sites:

GGCGCGCCTCTTGTAACGAGGCCCCCAAGGGCTACTACGTGCGGCTGAAGTACAG
CGGCGACAACCGGGTGTCCGGCAAGTACCCTTCTGGCACCCTGGTGGAAGCCAGC
TGCACCAACGGCCTGCAGATGATCGAGGGCAAGAACTTCAGCAGATGCACCAATG
GCAAATGGGTGCCCGGCCTGGGCAGATGCCCTTACCATTGCCCCCTGGGCTTTTT
CACCGGCAGCAAGTACCAGGTGGAACCCTACCCCAACAAGGGCAAAAAGAAAATG
GAATGGCGGCCTGACGGCTCCAAGGTGCTGGCCTACTGTGGCTGGAAGTCCTCTG
GCGCCCAGGAAAGAGGCGAGTACGGCGAGTTTCAGGCCAGAAGCTACACCTGTCG
GGACGGCGACTGGCTGACCGATGATGGCGAACCCCACGACCAGTGCATCCCCGAG
AGCGAGAACCTGTACTTCCAGTCTGCGGCCGC

Figure 5.7 Hp_I25642_IG17586_L548 codon optimised sequence

Invitrogen GeneArt Gene Synthesis was used to generate a codon optimised sequence. AscI and NotI restriction enzyme sites were added to the sequence and they are highlighted in red and green respectively.

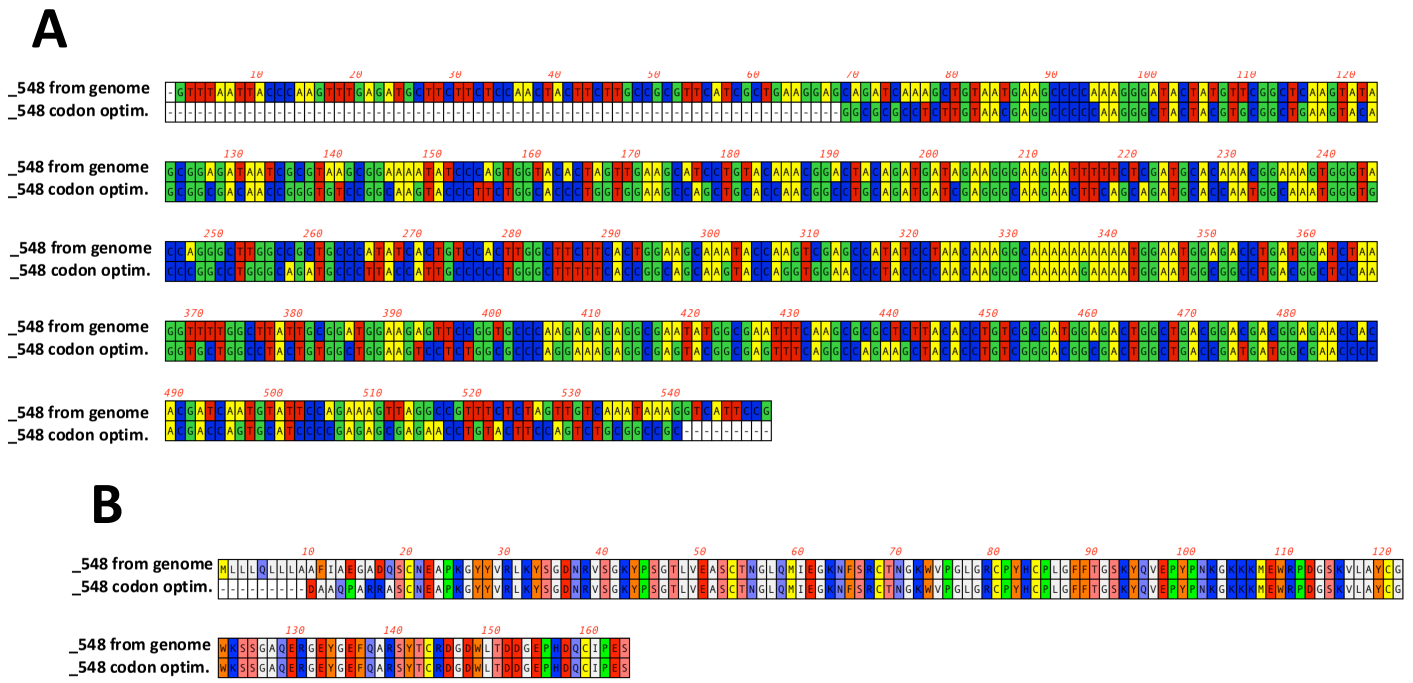
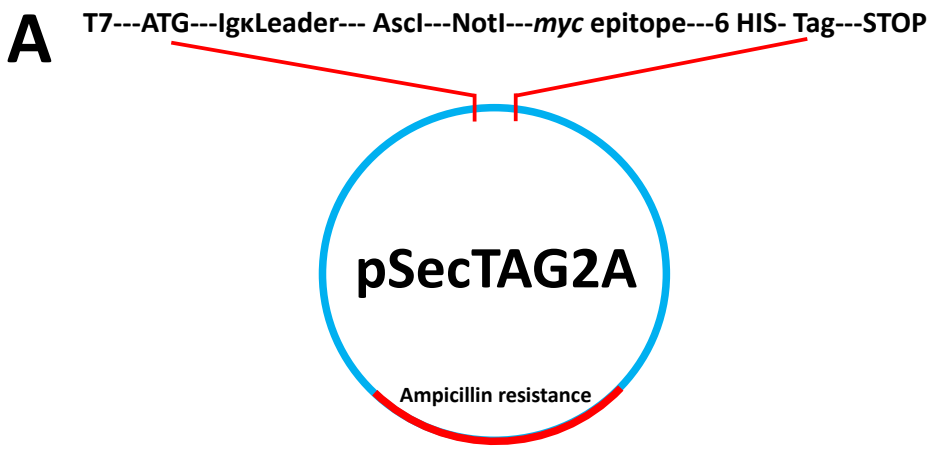


Figure 5.8 Alignment of the _548 genomic sequence from *H. polygyrus* versus the codon optimised sequence

Genomic sequence alignment (A) and protein sequence alignment (B).



B pSectAG2A leader sequence, Other pSectag2A sequence, AscI sequence, Hp_I25642_IG17586_L548 , TEV site, NotI sequence, myc epitope, His tag

-METDTLLLWVLLLWVPGSTGDAAQPARRASCNEAPKGYYVRLKYSGDNRVSGKYPSGTLVEASCTNGLQ
MIEGKNFSRCTNGKWVPGLGRCPYHCPLGFFTGSKYQVEPYPNKGKKKMEWRPDGSKVLAYCGWKSSGA
QERGEYGEFQARSYTCRDGDWLTDDGEPHDQCIPESENLYFQSAAARGGPEQKLISEEDLNSAVDHHHHHH-

Figure 5.9 Schematic representation of the cloning strategy with sequences

Representation of pSecTAG2A vector used to insert Hp_I25642_IG17586_L548 optimised genomic sequence (A). Amino acid sequence of Hp_I25642_IG17586_L548 cloned in the pSecTAG2A vector highlighting leader sequence (black), AscI sequence (green), Hp_I25642_IG17586_L548 (blue), TEV cleavage site (brown), NotI sequence (orange), myc-tag (purple) and 6-His (light blue).

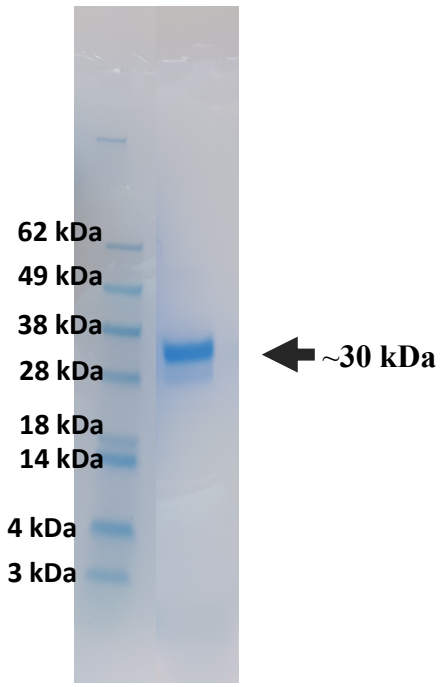


Figure 5.10 Coomassie blue gel of purified C-terminus tagged Hp_I25642_IG17586_L548

Purified protein (1 μg) was run into a 4-12% Bis-Tris protein gel and subsequently the gel was stained with Coomassie Brilliant Blue for 1h. Lanes in the gel have been removed

5.7 The C-terminus tag affects the functionality of the molecule

After purification, the protein was tested in a bone marrow assay. A titration was performed to detect the optimal concentration to use the protein. Compared to HES, the recombinant protein suppressed IL-5 and IL-13 at a very high concentration of 100 μg/ml, while HES suppressed at 1 μg/ml (Fig.5.11A-B). As shown by Osbourn and colleagues, recombinant HpARI suppresses IL-33 at lower concentrations compared to HES, and this is what should be observed as we are purifying a protein that it is contained in HES (Osbourn et al. 2017). In addition, ST2 suppression seems not to correlate with cytokine production in response to IL-33 (Fig.5.11C).

Hp_I25642_IG17586_L548 seems to reduce expression of ST2 at 100 μg/ml and 10 μg/ml if compared with cell cultured with IL-33 (Fig.5.11C). However, HES suppresses ST2 better even below baseline level (IL-2+IL-7 group). The fractionation screening identified Hp_I25642_IG17586_L548 as an optimal candidate to be the ST2 suppressor. The purified protein seems to be less active than the endogenous HES protein. I believe it is the correct protein as using HpARI or other control protein did not shown the same effect (Data not shown).

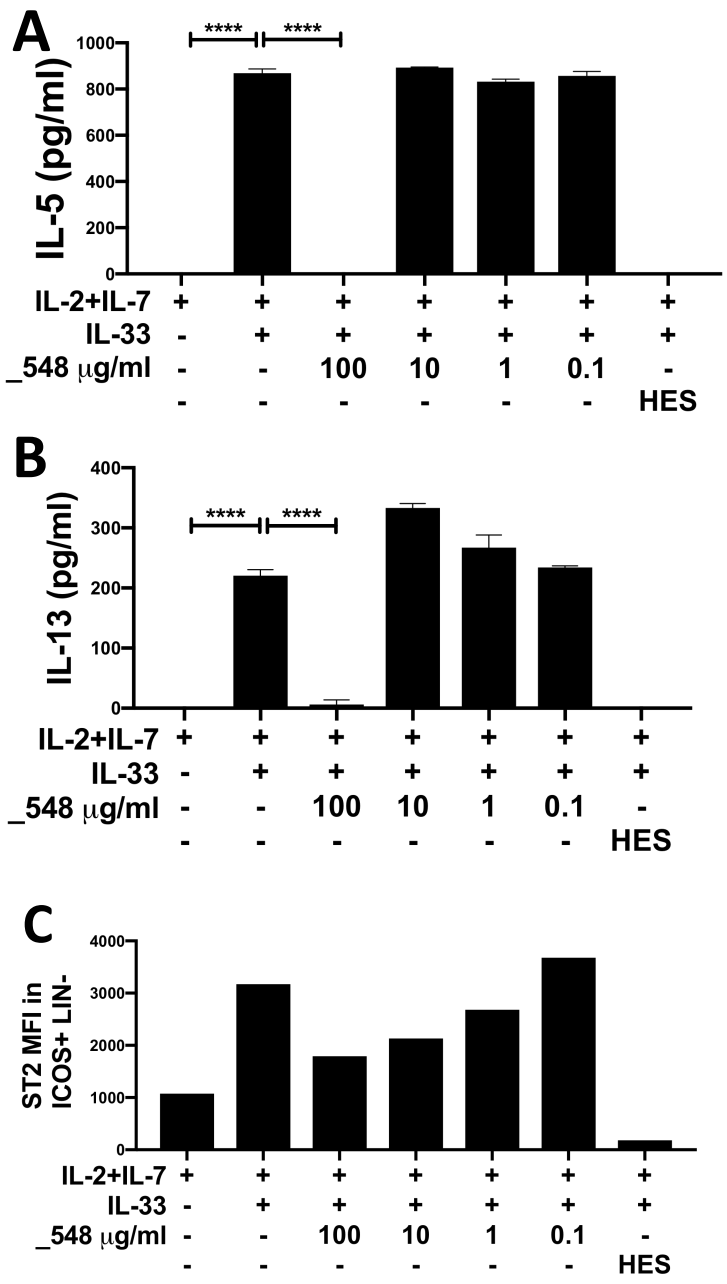


Figure 5.11 Hp_I25642_IG17586_L548 effects in the BM culture

BM cells ($5x10^5$ cells/well) were incubated with IL-2, IL-7, IL-33 (10 ng/ml) and _548 titration as indicated in the graph. Supernatants were collected at 72h and analysed for IL-5 (A) and IL-13 (B). Cells from 3 wells were surface stained and analysed by flow cytometry for ST2 expression (C).

5.8 N-terminus tagged Hp_I25642_IG17586_L548 suppresses IL-33 dependent responses

This difference between HES and purified protein suggests that the purified _548 protein has lost activity compared to the endogenous form of _548 contained in HES. I hypothesised that the C-terminal tags might have an effect on protein functionality. Through PCR and using a specific reverse primer containing a stop codon after the TEV cleavage site, a construct was made to express a protein without C-terminal tags. In addition, a new sequence was made to have a N-terminus tagged protein. Codon optimisation was performed, the new sequences cloned and transfected in Expi293T mammalian cell line. Amino acid sequence alignment is shown in Figure 5.12A. In Figure 5.12B is shown the sequence as it was cloned in the pSecTAG2A vector. The untagged protein has a predicted molecular weight of 17.9 kDa while the N-terminus tagged protein has a predicted molecular weight of 18.2 kDa.

Proteins were then purified as shown in the Coomassie blue stained protein gel (Fig.5.13). In Table 5.2 are reported the predicted molecular weight (MW) and the approximate MW from the protein gel.

Table 5.2 **Predicted molecular weight of tagged and untagged protein (using the ExPASy protparam tool) and approximate molecular weight in Coomassie blue gel.**

	Predicted MW	MW protein gel (approx.)
C-terminus _548	23.4 kDa	30 kDa
N-terminus _548	18.2 kDa	28 kDa
Untagged _548	17.9 kDa	26-27 kDa

Subsequently, purified C-terminus and N-terminus tagged protein, and untagged protein supernatants were tested in bone marrow cells stimulated with IL-33 and measuring IL-5, IL-13 and IL-6 by ELISA. Purified N-terminus and untagged proteins suppress IL-33 responses in a similar manner. Suppression of IL-5 production (Fig.5.14A) and suppression of IL-13 (Fig.5.14B) are observed up to 0.1 μg/ml of purified protein and 0.1 μl of supernatants from transfected cells with the untagged _548 protein. As a control, empty-vector-transfected supernatants had no effect (data not shown). Production of IL-6 (Fig.5.14C) was observed with IL-33 and suppression was observed with N-terminus and untagged protein up to 0.01 μg/ml or 0.01 μl of protein respectively. C-terminus tagged protein do not suppresses any of the IL-33 dependent responses in this assay, at any concentration up to 10 ug/ml (Fig.5.14). Therefore, the C-terminus tags affected the functionality of the protein while the N-terminus tags allow to purify and retain protein activity.

A

METDTLLLWVLLLWVPGSTGDAAQPARRASCNEAPKGYYVRL
METDTLLLWVLLLWVPGSTGDAAQPARRASCNEAPKGYYVRL
HHHHHHEQKLISEEDLMETDTLLLWVLLLWVPGSTGDAAQPARRASCNEAPKGYYVRL

KYSGDNRVSGKYPSGTLVEASCTNGLQMIEGKNFSRCTNGKWVPGLGRCPYHCPLGFF
KYSGDNRVSGKYPSGTLVEASCTNGLQMIEGKNFSRCTNGKWVPGLGRCPYHCPLGFF
KYSGDNRVSGKYPSGTLVEASCTNGLQMIEGKNFSRCTNGKWVPGLGRCPYHCPLGFF

TGSKYQVEPYPNKGKKKMEWRPDGSKVLAYCGWKSSGAQERGEYGEFQARSYTCRDGD
TGSKYQVEPYPNKGKKKMEWRPDGSKVLAYCGWKSSGAQERGEYGEFQARSYTCRDGD
TGSKYQVEPYPNKGKKKMEWRPDGSKVLAYCGWKSSGAQERGEYGEFQARSYTCRDGD

WLTDDGEPHDQCIPES-
WLTDDGEPHDQCIPESENLYFQSAAARGGPEQKLISEEDLNSAVDHHHHHH-
WLTDDGEPHDQCIPES-

B

pSecTAG2A leader sequence, Other pSectag2A sequence, AscI sequence, His tag, myc epitope,Hp_I25642_IG17586_L548

METDTLLLWVLLLWVPGSTGDAAQPARRAHHHHHHEQKLISEEDLSCNEAPKGYYVRLK
YSGDNRVSGKYPSGTLVEASCTNGLQMIEGKNFSRCTNGKWVPGLGRCPYHCPLGFFTG
SKYQVEPYPNKGKKKMEWRPDGSKVLAYCGWKSSGAQERGEYGEFQARSYTCRDGDWLT
DDGEPHDQCIPES-

Figure 5.12 Sequence alignment of different tagged version of Hp_I25642_IG17586_L548

Untagged (red), C-terminus tag (black) and N-terminus tag (purple) Hp_I25642_IG17586_L548 alignment. Underlined is the _548 sequence. Highlighted in green is the TEV cleavage site, in yellow the myc tag and in light blue the polyhistidine tag. Final aa sequence cloned in pSecTAG2A (B).

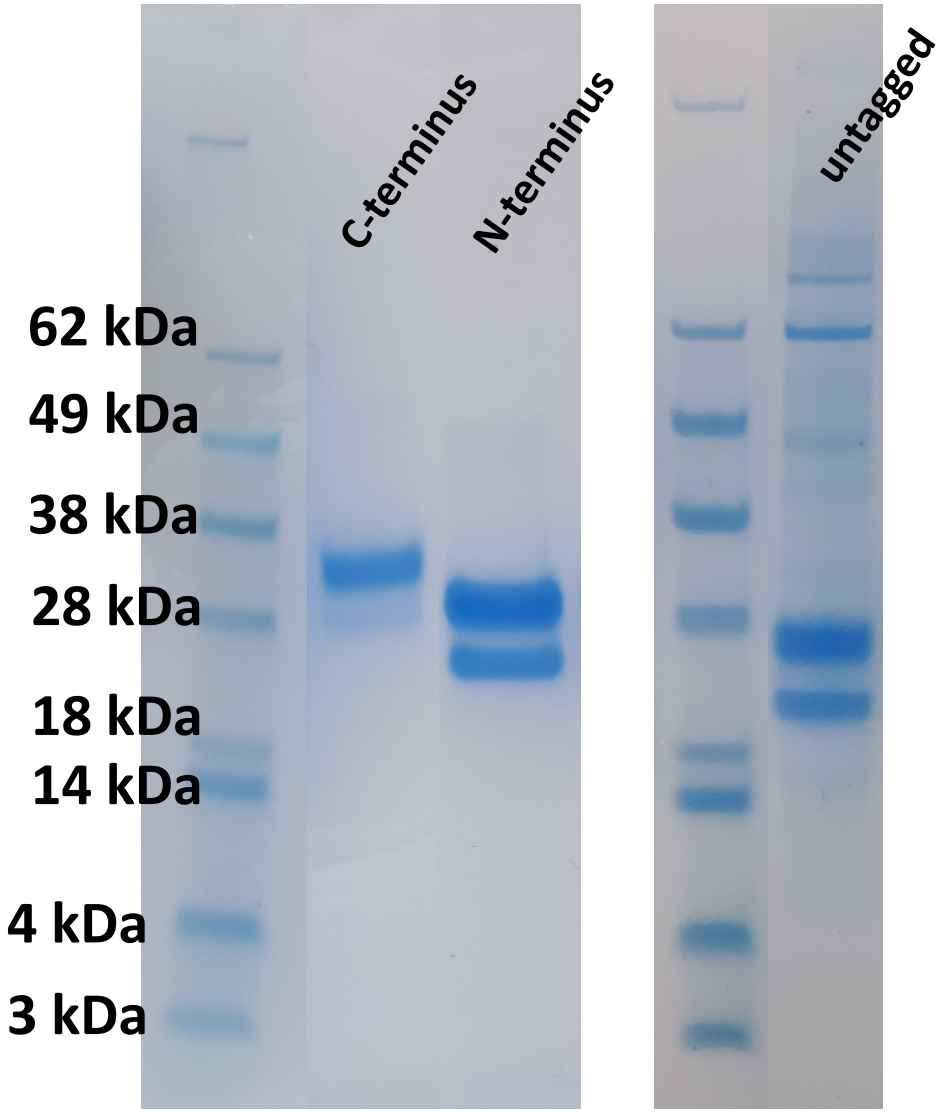


Figure 5.13 Coomassie blue-stained SDS-PAGE gel of purified _548 protein and untagged _548-transfected HEK293 supernatants

Tagged _548 protein (1 μg) and untagged _548 protein (5 μl of unpurified supernatant) were run into a 4-12% Bis-Tris protein gel electrophoresis under reducing conditions. Gel was stained with commassie blue. Lanes were removed for clarity.

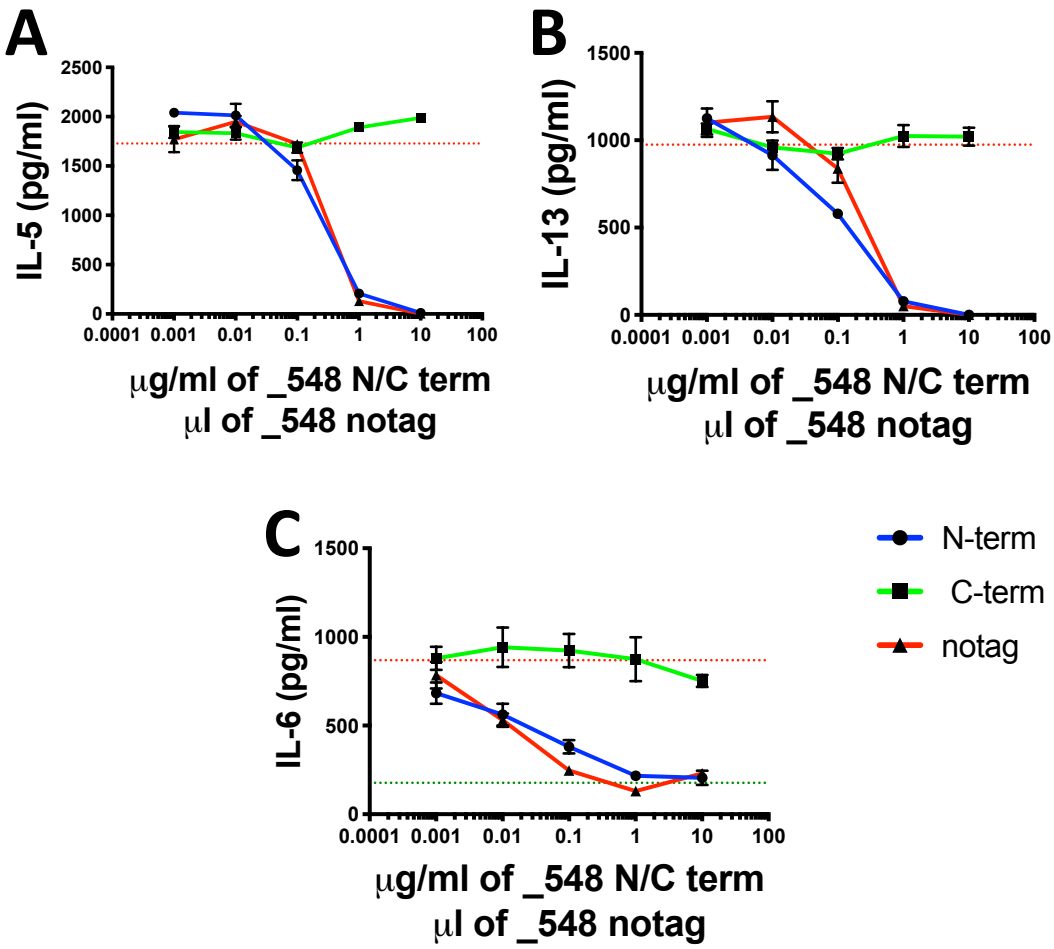


Figure 5.14 N-terminus tagged Hp_I25642_IG17586_L548 suppresses IL-33 dependent responses

Bone marrow cells ($5x10^5$ cells/well) were cultured with a titration of untagged _548-transfected HEK293 supernatants ("_548 notag") and a titration of purified C-terminus and N-terminus tagged proteins for 72h. Supernatants were collected and tested for IL-5 (A), IL-13 (B) and IL-6 (C) by ELISA. Symbols are mean ± SEM of three replicate cultures. Representative of >3 repeats.

5.9 Discussion

Suppression of ST2 is associated with HES administration both *in vitro* and *in vivo* (Buck et al. 2014; Coakley et al. 2017). EV contained in HES are able to suppress ST2 transcription and ST2 receptor protein on the cell surface, but when HES was depleted of its EV component ST2 suppression at both transcription level and membrane receptor was still observed (Coakley et al. 2017), suggesting that a soluble element was still able to suppress ST2. In addition, blocking IL-33 with the newly discovered parasite protein HpARI during *Alternaria* allergen stimulation *in vivo* did not suppress ST2 expression, as was shown in previous results prior my project start. Taken together all this data suggests the presence of a protein, different from HpARI, that suppresses ST2.

In the *in vitro* model used in this section, a single lung cell suspension or bone marrow cells were cultured with a titration of HES, EV and EV-depleted HES (kindly provided by Dr. Amy Buck) and suppression of ST2 detection was observed with all HES components, replicating what already showed by Coakley and colleagues using a different system.

Here we identified the *Hp_I25642_IG17586_L548* gene that encodes a protein that suppresses ST2. The gene was identified by screening the available HES fractions for ST2 suppression and matching these results with HES mass spectrometry analysis and genome sequencing. Among the proteins that correlated with suppression of ST2 and expressed a signal peptide *Hp_I25642_IG17586_L548* was tested first as was already being expressed in the lab for another project involving the CCP domain proteins family.

A C-terminus tagged protein was purified and proved that suppressed IL-5 and IL-13 in bone marrow cultures, and ST2 expression on ILC2s, but it was less effective than expected. It was shown that the tag modified the efficacy of the protein as 1 μg/ml HES suppressed IL-33-dependent responses in the BM assay compared to 100 μg/ml of purified protein. An untagged version and an N-terminus tagged version were generated and both were able to suppress production of IL-5, IL-13 and IL-6 in response to IL-33 at lower concentration compared to the C-terminus tagged protein, indicating that the N-terminus tagged protein is more effective. Taking this observation, the C-terminus of the protein might contain the key element for the protein functionality and in particular, blockade of IL-33 responses. Adding a tag in this section might induce a conformational change or steric hinderance of an active site that is detrimental for the protein activity.

From a mechanistic point of view, and from the results showing HES suppressing ST2 detection after 30 minutes incubation, it was hypothesised that the protein binds directly to ST2 and I will discuss this in the next chapter. As I have now shown that the Hp_I25642_IG17586_L548 protein is the active constituent that blocks ST2 detection and prevents responses to the alarmin cytokine IL-33, the protein was renamed as ***Heligmosomoides polygyrus*** **Binds Alarmin Receptor and Inhibits** or **HpBARI**.

HpBARI was considered as an optimal candidate for two reasons: suppression peak matched the emPAI profile; and EMBL-EBI InterPro identified the protein as a CCP domain-containing protein. From the translated sequence some conserved CCP domain

features can be observed for example: the 4 cysteines that form a CCP module and the presence of leucine, tryptophan, glycine and valine residues. The alignment of the sequence to other CCP domain-containing proteins highlighted the presence of 2 CCP modules.

HpARI CCP domains have some conserved characteristics but they show some atypical divergences that are not observed in other CCP proteins (Osbourn et al. 2017). In particular, two insertion of ≈ 20 aa between cysteine 1 and cysteine 2 in CCP2 and CCP3. Other experiments in the McSorley lab showed that these atypical insertions might be essential for HpARI binding activity to IL-33, as deleting them ablates HpARI's activity (unpublished data). An atypical insertion of around 10 aa is observed in HpBARI CCP2 that is not observed in either CR2 CCP2 and factor H CCP10. The presence of this atypical insertion might be the reason why both Expasy Prosite and EMBL-EBI InterPro do not predict the second CCP domain in Hp_I25642_IG17586_L548.

The presence of this atypical insertion in HpBARI CCP2 is of high interest to be considered in future studies for analysing the mechanism of action of the protein. In particular, next steps will be to clone and express the single HpBARI CCP domains and determine if both are required for its activity. Furthermore, it is possible to modify or remove the atypical insertion from HpBARI sequence and determine if this is essential for the protein function as showed for HpARI. Analogously to HpARI and HpTGM, HpBARI is a CCP domain protein. HpARI and HpTGM CCP domains are essential for the protein function (Smyth et al. 2018; Osbourn et al. 2017). We hypothesise that CCP domains represent an adaptable scaffold used by numerous parasites, especially *H.*

polygyrus, to achieve immune modulation (Maizels et al. 2018). HpBARI CCP domains need to be consider for its functionality and in particular if each of them execute a different function.

In summary, a novel protein that suppresses ST2 detection by flow cytometry and blocks IL-33-dependent responses was identified from HES. The protein was named HpBARI, as it is hypothesised that it binds to the alarmin receptor ST2, and inhibits the responses. It shows typical characteristics of a CCP domain protein indicating with more evidence that *H. polygyrus* uses these modules as an evolutionary conserved system to modulate the host immune system. The HpBARI gene sequence show no close homologues with other parasites. In addition, analysing the proteomic data from the Buck and colleagues, HpBARI is predominantly detected in the non-EV fraction of HES (Buck et al. 2014). This indicates that suppression of ST2 transcription by EV is unlikely to be mediated by HpBARI, but potentially by the miRNAs detected in the EV. Further investigation need to be carried out to understand the mechanism of action of HpBARI. *In vitro* HpBARI shows suppression of IL-33-dependent responses, therefore I hypothesised that HpBARI may suppress in IL-33-dependent mouse models of asthma *in vivo*. Furthermore, due to the rapid suppression of ST2, I further hypothesised that the HpBARI mechanism of action is through direct binding to ST2. These hypotheses will be tested in the next chapter.

Chapter 6

Dissecting the mechanisms of action of HpBARI

6.1 Introduction

In the previous chapter a new transcript Hp_I25642_IG17586_L548 was identified from the excretory/secretory products of *H. polygyrus*, and it was re-named HpBARI. *In vitro*, HpBARI suppresses the production of IL-5, IL-13 and IL-6 in bone marrow culture in response to IL-33. The HpBARI protein consist of 2 atypical CCP domain and it suppresses detection of ST2 by flow cytometry. I hypothesised that suppression of ST2 induces blockade of IL-33-dependent responses *in vivo*. I will use the *Alternaria* model to induce rapid release of IL-33 and I will detect eosinophils in both lung tissue and BALF. In addition, I will analyse ILC2 activation in lung tissue, and ST2 transcription levels. After I will test my second hypothesis that HpBARI suppresses ST2 detection by binding directly to ST2.

To test the binding I will carry out two assays: direct binding assay using ST2-conjugated beads and solid-phase ELISA, coating the ELISA plate with the protein of interest and using ST2 to determine binding activity. I hypothesise that the binding of HpBARI to ST2 is sterically interfering with the detection of ST2 by flow cytometry antibodies and that explains why HpBARI reduced detection of ST2. Furthermore, I hypothesise that this binding might interfere with the IL-33 binding site on ST2 and subsequently, the cytokine cannot bind and induce type 2 immune responses.

6.2 HpBARI *in vivo* suppresses ST2 and type 2 immune responses

HpBARI N-terminus tagged has been shown to suppress IL-33-dependent responses in whole bone marrow cell cultures. Next, HpBARI was tested *in vivo* using the *Alternaria* model used for HpARI (Osbourn et al. 2017). Type 2 immune responses were assessed 24h after *Alternaria* allergen administration. As expected *Alternaria* administration induces an increase in BAL (Fig.6.1A) and lung eosinophils (Fig.6.1B), and HpBARI significantly suppressed this response.

I then checked IL-13$^+$ ILC2 by intracellular cytokine stain (ICS). *Alternaria* induced an increase percentage of IL-13$^+$ ILC2 (Fig.6.1C), and while HpBARI co-administration appeared to suppress this, it did not quite reach statistical significance (p=0.0566). IL-5$^+$ ILC2 were also analysed by ICS, while an increase was observed with *Alternaria* administration only a small trend toward suppression was observed with HpBARI (Fig.6.1D). CD25 MFI was measured in the ILC2 population as a measure of cellular activation (Bartemes et al. 2011), showing significant increase with *Alternaria* and CD25 MFI reduction with HpBARI co-administration (Fig.6.1E). When the BAL fluids were analysed for IL-5 levels, *Alternaria* significantly increased the IL-5 levels and HpBARI suppressed this response (Fig.6.1F). IL-13 ELISA was performed but IL-13 levels were below the detection limits (data not shown).

The levels of ST2 detected on ILC2 was significantly suppressed with HpBARI when *Alternaria* was co-administered, compared to PBS control levels (Fig.6.2A-B-C).

Furthermore, as total HES and its EV content is associated with reduced ST2 transcription, ST2 expression was examined in this experiment. ST2 transcription was significantly increase with administration of *Alternaria*, and this increase was suppressed by blocking ST2 and IL-33 responses (Fig.6.2D). However no further suppression below baseline (PBS group) was observed. Therefore, taken together all these data indicate that HpBARI reduces type 2 immune responses *in vivo,* suppressing ST2 on ILC2s surface and this suppression is specific to the protein level and not to the transcription of ST2.

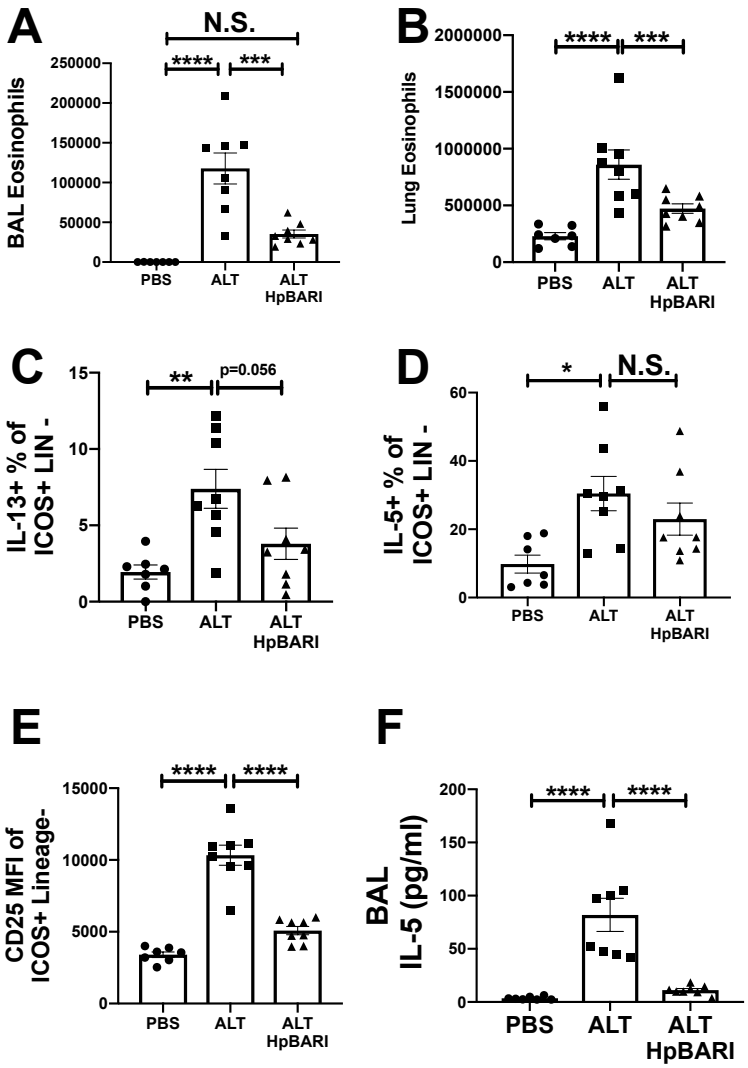


Figure 6.1 HpBARI effects in the *in vivo Alternaria* model.

Alternaria allergen (10 μg) was co-administered with HpBARI N-terminus tagged (10 μg). BALs were collected and analysed for eosinophil numbers (identified as CD45+ SiglecF+ CD11c-) (A). Lungs were collected and a single lung cell suspension was obtained as described in Materials and Methods. Cells were surface stained and number of eosinophils determined (B), ST2 MFI of ILC2s (C) and CD25 MFI (D). Intracellular cytokine stains was performed in a single lung cell suspension stimulated with PMA, ionomycin and brefeldin A and IL-13+ ILC2s determined (E). BAL fluids were analysed for IL-5 by ELISA (F). Bar graphs are mean ± SEM. Data pooled from two independent experiment for a total of N number of 7-8. Analysed by one-way ANOVA with multiple comparison test with Bonferroni's post-test **=p<0.005, ***=p<0.001, ****=p<0.0001

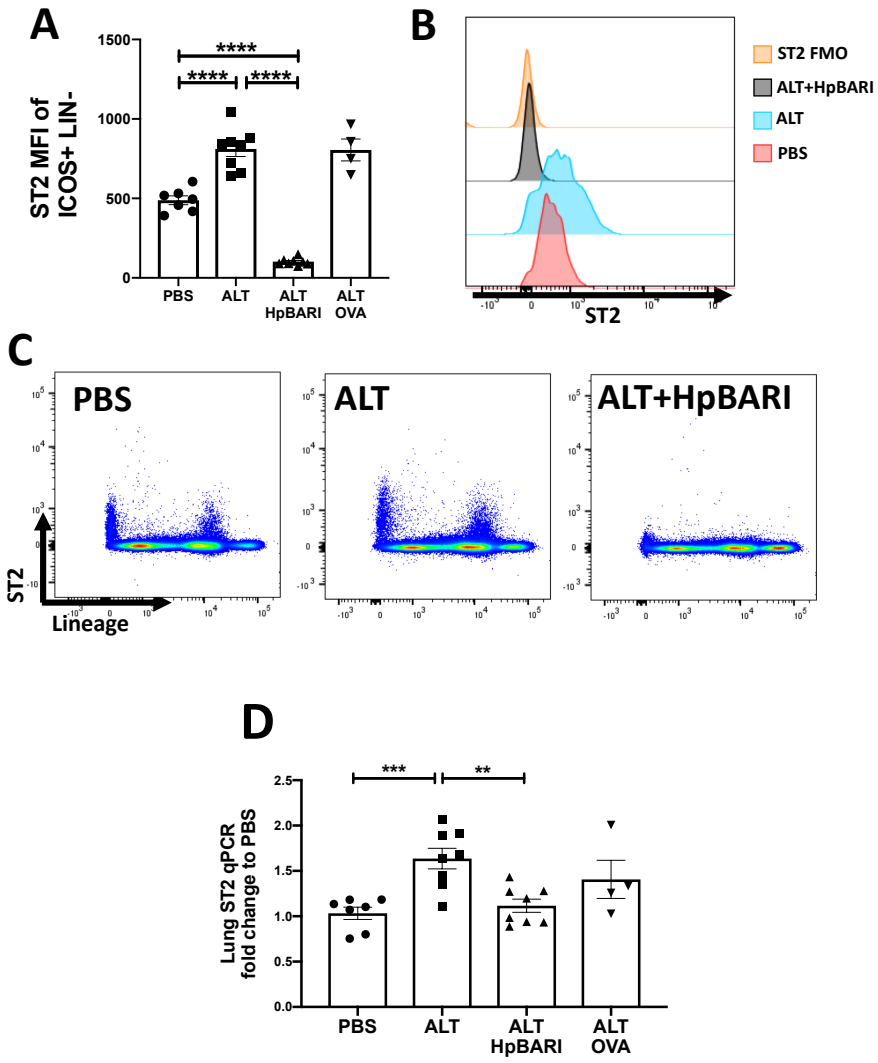


Figure 6.2 HpBARI suppressed ST2 detection by flow cytometry but did not change ST2 transcription in the lung

Alternaria allergen (10 μg) was co-administered with HpBARI N-terminus tagged (10 μg). Analysed tissues were from the same experiment in Fig.6.1. Lungs were collected and a single lung cell suspension was obtained as described in Materials and Methods. Cells were surface stained and ST2 MFI quantified (A). Representative histogram (B) and FACS plot (C) for ST2 suppression. RNA was extracted and converted into cDNA as described in Materials and Methods. TaqMan qPCR was performed to analyse transcription of the il1rl gene (ST2) (D). Data pooled from two independent experiment (A and D) for a total N of 7-8, except for ALT-OVA group which is one single experiment N=4. Analysed with one-way ANOVA using Bonferroni's multiple comparison post-test. **=p<0.005, ***=p<0.001, ****=p<0.0001.

6.3 Solid phase ELISA confirms that HpBARI binds to ST2

HpBARI suppresses detection of ST2 by flow cytometry *in vivo*. This suppressive effect is associated with reduced eosinophils and ILC2s activation. The observation that HES suppresses ST2 detection after 30 minutes incubation (Fig.5.3) suggests that the protein acts rapidly and it might be a direct binding interaction. To test the hypothesis that HpBARI was binding to ST2, N-terminus and C-terminus tagged purified protein were used to coat an EIA/RIA plate to perform solid-phase ELISA, with HpARI-coated wells used as a control.

I hypothesised that HpBARI binds to ST2 so if I add to the wells the mST2-Fc or mTRAP-Fc construct, if my hypothesis is true, I should be able to detect the Fc tag in the ST2 or TRAP using an anti-human IgG HRP subsequently. When the ELISA plate was coated with N-terminus tagged HpBARI, an increase in optical density was observed with mST2-Fc and mTRAP-Fc (Fig.6.3A) that was not observed when the plate was coated with HpBARI C-terminus tag (Fig.6.3B) and with HpARI (Fig.6.3C). However no increase in optical density was observed with the human TRAP construct. Therefore, mST2-Fc and mTRAP-Fc can bind to HpBARI N-terminus tagged protein and this binding property is lost with the C-terminus tag. In addition, this binding cannot be replicated using a human construct suggesting that HpBARI specifically binds to the murine receptor.

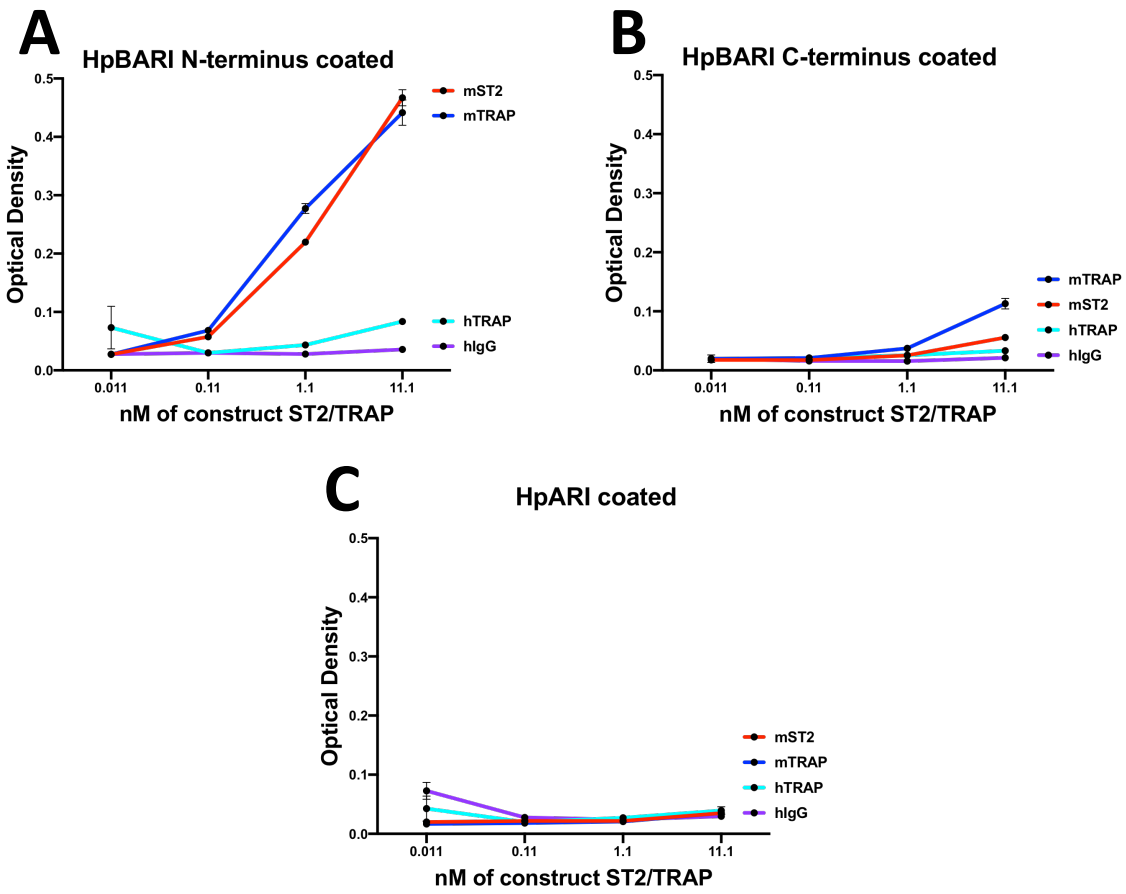


Figure 6.3 mST2 and mTRAP binds to N-terminus tagged HpBARI

An ELISA plate was coated with 1 µg/µl of purified N-terminus (A), C-terminus tagged HpBARI (B) and HpARI (C). A titration of the same molarity of mST2-Fc, mTRAP-Fc and hTRAP-Fc was used as indicated in the graph. This was based on the top concentration of mST2-Fc of 1 µg/ml = 11.1 nM. Anti-human IgG was used as a control. Points represent mean ± SEM from three replicate wells. Representative of 3 independent experiments

6.4 HpBARI binds to ST2 and blocks binding of IL-33 to its receptor

To further test the hypothesis that HpBARI binds to ST2, thus inhibiting IL-33-ST2 interactions, analysis of direct interactions was performed using mST2-Fc obtained from MedImmune. Protein G dynabeads were conjugated with mST2-Fc, HpBARI was allowed to bind to ST2-conjugated beads and IL-33 incubated subsequently, to see if it could bind to its receptor (Fig.6.4).

After IL-33 incubation, samples eluted from the beads were collected and anti-myc and anti-mouse IL-33 western blots performed. HpBARI was detected by western blot when the beads were conjugated with mST2-Fc (Fig.6.5) confirming that HpBARI N-terminus was bound to ST2, and the binding was specific to the construct and not to the Fc region as IgG-conjugated beads could not interact with HpBARI (Data not shown). IL-33 could be detected only in the absence of N-terminus tagged HpBARI. HpBARI C-terminus tagged did not show binding to ST2, and IL-33 was able to bind its receptor (Fig.6.4)

Therefore, HpBARI binds directly to ST2 and this interaction prevents binding of IL-33 to ST2.

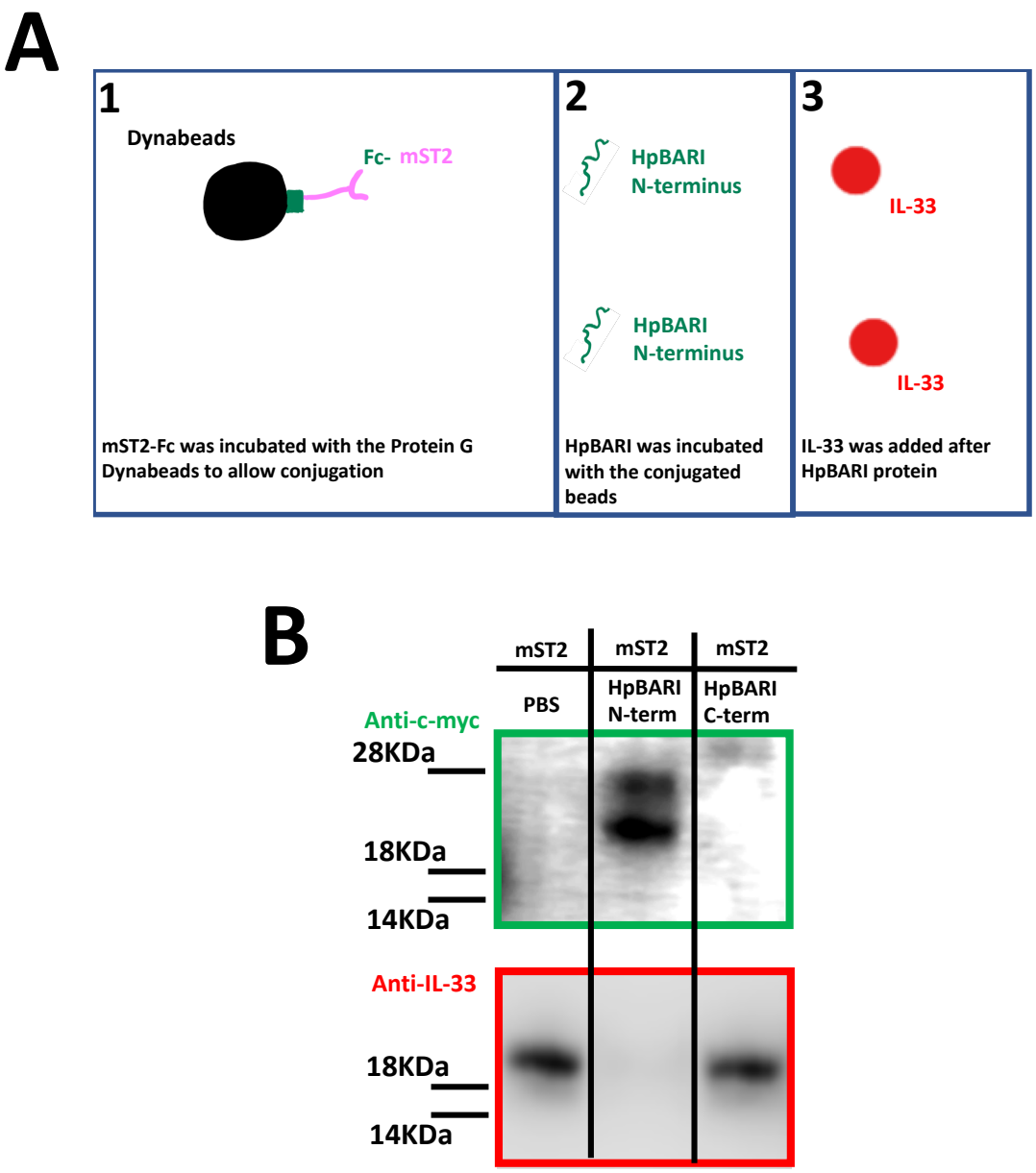


Figure 6.4 HpBARI binds directly to ST2 and blocks IL-33 to bind its receptor.

Graphic representation of the direct binding assay (A). Protein G dynabeads were conjugated with mST2-Fc (1 µg) obtained from MedImmune (1). HpBARI N-terminus tag protein was allowed to bind the conjugated beads (2). Subsequently, IL-33 (100 ng) was added (3). Samples eluted from the binding assay were run in a 4-12% Bis-Tris protein gel and western blot was performed. Top membrane was revealed with anti-c-myc antibody to detect tagged HpBARI. Bottom membrane was incubated with anti-mIL-33 to determine if IL-33 was still able to bind ST2. Representative of 2 independent experiments

6.5 Surface Plasmon Resonance

The solid-phase ELISA result and the direct binding assay result suggested that the N-terminus BARI can bind ST2 and block IL-33-ST2 interaction. To investigate this further and to quantify the affinity of this interaction, surface plasmon resonance (SPR) was performed. Surface plasmon resonance is a bioanalytical technique that allows quantification of a binding interaction using a gold-coated chip. It is not the purpose of this section to explain the bio-physics of SPR but in brief, SPR measures the refractive index of light at a specific angle that hit the chip. Any proteins that bind to the chip will generate a variation of the refractive index, which can be measured and displayed as a sensogram. The sensogram provides real time information about the binding and the specificity of the interaction. Mouse ST2-Fc, mTRAP-Fc and hTRAP-Fc were coated onto a protein G chip via their Fc tags. The binding of HpBARI N-terminus was measured subsequently. HpBARI was run onto the coated chip at five 3-fold dilution starting at 10 nM. In Fig.6.5 the sensogram shows that HpBARI N-terminus tagged bound mST2-Fc and mTRAP-Fc but not hTRAP-Fc. The sensogram is a representative of 3 repeats and the analysis was performed with the help of Dr. Martin Wear (Edinburgh Protein Production Facility). Modelling the binding curve on a 1:1 stoichiometry interaction the mean K_d for BARI binding to ST2 is 0.34 ± 0.25 nM, with a complex half life time of 760s, that means it takes around 12 minutes for half of the HpBARI molecules to dissociate from ST2. The mean K_d for BARI binding to mTRAP is 0.2 ± 0.16 nM and a complex half life time of 1150 s. The K_d for HpBARI binding to hTRAP was ≥ 100 nM, indicating that no binding was observed. Furthermore, the binding profile of IL-33 to

mTRAP-Fc was determined by SPR showing that IL-33 is binding to the construct mTRAP as expected, with high affinity K_d 0.1 ± 0.4 nM (Fig.6.6A). Following the result showing that HpBARI blocked IL-33/ST2 interaction in the binding assay, this blocking activity was tested using SPR. Mouse TRAP-Fc (10 nM) was coated onto the SPR chip, a single injection of murine IL-33 (10 nM) was carried out and showing binding to the mTRAP (Fig.6.6B – black line). A single injection of HpBARI N-terminus (10 nM) was performed into a different channel of th coated chip showing binding to the construct (Fig.6.6B - red line). After equilibrium was reached between HpBARI and mTRAP, a single injection of IL-33 (10 nM) was performed showing no binding (Fig.6.6B). Therefore, we confirmed using SPR what we observed in the direct binding assay, HpBARI binds to ST2 blocking IL-33/ST2 interaction.

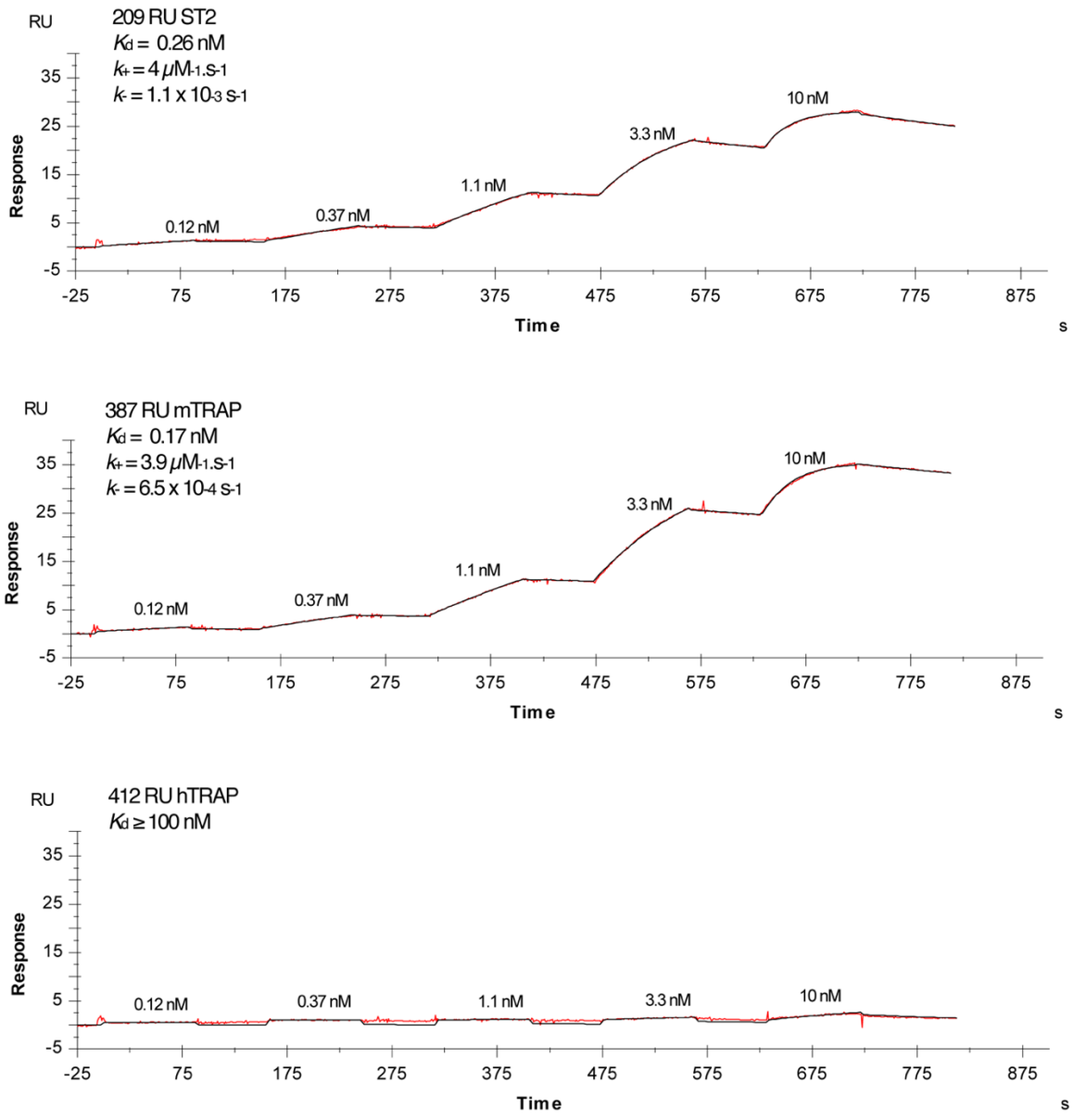


Figure 6.5 Surface plasmon resonance using Biacore T200 to determine HpBARI-ST2 binding

Protein G chip was coated with 10 nM of mST2, mTRAP and hTRAP. As indicated in the graph, dilution of HpBARI N-terminus were run through the coated chip. Reference corrected single kinetic titration SPR binding curves (red), and a globally fitted 1:1 kinetic binding model (black). Representative of three single runs. Analysis performed by Martin Wear (University of Edinburgh)

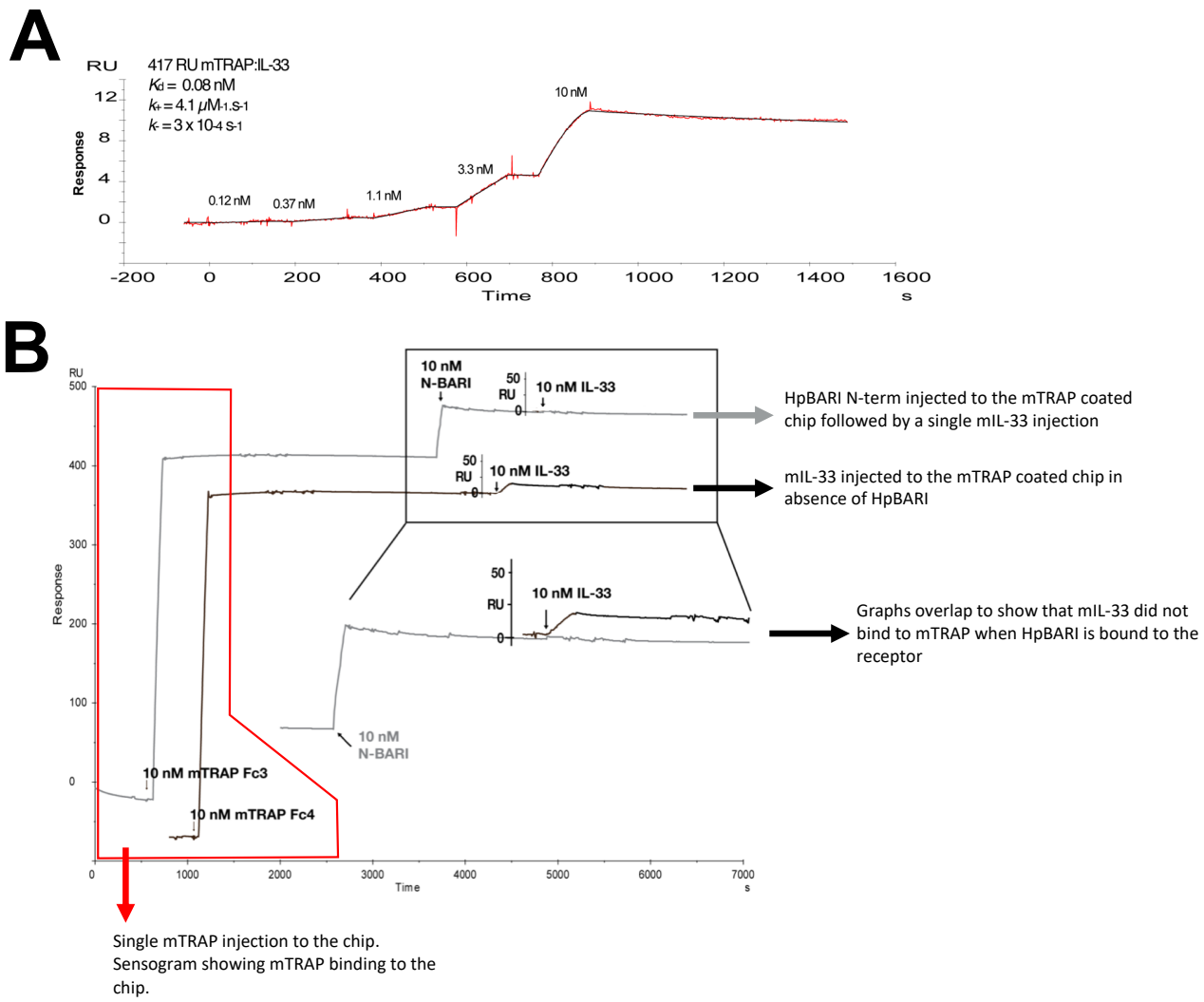


Figure 6.6 Using SPR to determine IL-33 binding profile to ST2

Protein G chip was coated with 10 nM mTRAP and, once stability of the interaction was reached, IL-33 was run at different concentration as indicate in the graph (A). In panel B, mouse TRAP was coated to the chip (binding indicated in the red box by the grey and black line). Injection to the chip are represented by small black arrow with indication of construct injected and concentration. Interaction between mTRAP and HpBARI was assessed performing a single injection of HpBARI (10 nM) to a mTRAP-coated chip (as indicated by the grey line) and, when equilibrium was reached, a single injection of mIL-33 (10 nM) was performed. As a control of IL-33 binding, a single mIL-33 injection (10 nM) was performed in a mTRAP-coated chip (black line). Analysis performed by Martin Wear (University of Edinburgh).

6.6 Discussion

Here I showed that HpBARI was capable of suppressing IL-33-mediated responses *in vivo*, suppressing ST2 at the level of protein expression, but not ST2 transcript. Using a combination of solid-phase ELISA, direct binding assay and SPR, I further showed that HpBARI binds directly to ST2, inhibiting the interaction of IL-33 with its receptor. Following the discoveries of the *H. polygyrus*-derived immunomodulatory proteins HpARI and HpTGM (Osbourn et al. 2017; Johnston et al. 2017), both of which consist of a series of CCP domains, here we identified HpBARI - a novel double CCP domain molecule that suppresses IL-33 responses *in vitro* and *in vivo*. I showed that the insertion of tags at the C-terminus region prevented the protein from binding to ST2 as detected in the solid-phase ELISA and western blot of the direct interaction assay. As the orientation of CCP domains and the flexibility of intramodular junction are required for their functionality (Henderson et al. 2001; Barlow & Campbell 1994), I hypothesised that adding tags at the C-terminus could modify the tertiary structure and sterically hinder the binding site. In collaboration with Dr Martin Wear, SPR was performed to confirm these data. The binding affinity of HpBARI N-terminus tagged for mST2 and mTRAP was measured and the affinity of the interactions quantified at a K_d of 0.34 ± 0.25 nM and 0.2 ± 0.16 nM respectively. HpBARI binds strongly this construct considering that the affinity for mouse IL-33 to mTRAP showed a K_d of 0.1 nM in our experiment while the published affinity of IL-33 to ST2 has been published and being around 0.5 and 0.7 nM (Lingel et al. 2009; Günther et al. 2017). Therefore, HpBARI binds with high affinity to ST2 and it might compete with IL-33. However, from the *in vivo* data, HpBARI did not

completely suppress eosinophilia and ILC2 activation, suggesting that IL-33 might still bind ST2 quicker. This led to partial activation of ILC2s, while any further release of IL-33 did not activate ILC2 as HpBARI bound to ST2 blocked any IL-33-dependent response. Therefore, I hypothesise that HpBARI should be administered prior to *Alternaria* to achieve a better suppression of type 2 immune responses.

Furthermore, I showed that no binding was observed using the human TRAP-Fc. Mouse and human ST2 are 67% identical in the amino acid sequence (Tominaga et al. 1992), and HpBARI is a protein produced by a murine nematode, thus it is perhaps not surprising that the protein is specific for mouse ST2. As we showed in Osbourn et al., HpARI is a recombinant protein that binds to both mouse and human IL-33, however the affinity of binding to mouse IL-33 is higher than that to human IL-33 (Osbourn et al. 2017). So far, it was demonstrated that HpBARI binds directly to mouse ST2, preventing the interaction between IL-33 and its receptor, and our data indicate that it does not bind the human target. We could speculate that the binding site might be in one of the region that diverge from human and mouse ST2, and possibly a nearby region to the IL-33 binding site as HpBARI block IL-33-ST2 interaction. Interaction at the molecular level should be characterised performing structural studies, for example obtaining a structure of the HpBARI-ST2 complex. I observed a difference in IL-33-suppressive activity between the C-terminus tagged and N-terminus tagged protein. C-terminus tagged HpBARI cannot suppress IL-33-dependent responses in the bone marrow assay and no binding interaction with ST2 was detected by solid-phase ELISA. Adding a tag at the C-terminus of HpBARI might compromise the folding of the protein and disrupting, or hiding, the binding site of

the protein reducing its stability, inducing a rapid dissociation from the receptor. Thus, IL-33 has high affinity for ST2 and once HpBARI has dissociated from the receptor, IL-33 can still bind ST2 and allowing IL-33-dependent immune cells activation.

In addition to HpARI and *H. polygyrus* EV, HpBARI identification add a new element produced by *H. polygyrus* to target the IL-33 pathway and the initiation of type 2 immune responses. This corroborates previous studies where they showed that EV can suppress ST2 and alternative macrophage activation (Coakley et al. 2017). In the study by Coakley and colleague, ST2 suppression was observed both at the level of mRNA (measured by qPCR) and at surface receptor expression (measured by flow cytometry) both with EV and with EV-depleted HES (called supernatants by Coakley and colleagues), and in a previous study proteomic characterisation of EV and supernatants indicates that HpBARI is more abundant in HES supernatants (Buck et al. 2014). Therefore, HpBARI interferes with ST2 protein expressed on the surface of immune cells while miRNAs interfere with ST2 transcription.

Expression levels of ST2 during the *Alternaria in vivo* experiment were checked. *Alternaria* induced increase of ST2 transcription and, blocking ST2 with HpBARI did suppress *Alternaria*-dependent ST2 transcription but no differences were observed between PBS group and HpBARI, indicating that HpBARI did not interfere with ST2 transcription levels.

The identification of HpBARI is an addition to the strategies that *H. polygyrus* uses to target the IL-33 pathway indicating that this pathway is essential for type 2 immune

responses induction that lead to the parasite expulsion. The importance of the IL-33 pathway during *H. polygyrus* infection has been shown in ST2-deficient mice, which are more susceptible to infection (Coakley et al. 2017).

As mentioned before, *H. polygyrus* has evolved using CCP domain-containing protein to modulate the host immune system (Maizels et al. 2018) and it will be interesting in the future to determine if human-specific parasites can use a similar strategy to evade immune responses.

Targeting the IL-33 pathway has been shown to reduce allergen sensitisation in early life and reducing type 2 immune responses (de Kleer et al. 2016; Lynch et al. 2016; Werder et al. 2018). Biological therapies targeting IL-33 or ST2 are currently under investigation in clinical trials for the treatment of asthma and dermatitis. Experiments using human ST2 indicate that HpBARI does not target the human IL-33 receptor, making this protein not exploitable to target human disease. Nonetheless, it is a tool to manipulate ST2 and the IL-33 pathway in mice and with further investigation at the structural and interaction levels it might offer a strategy to design novel therapeutics.

Chapter 7

Final discussion and future work

In the past decades an increase in cases of allergies, asthma and auto-immune disease has been observed in industrialised countries (Lambrecht & Hammad 2017). The origin of asthma is multi-factorial and the heterogeneity of the disease has made it a challenge to develop new therapeutics. New biological therapies, which target immuno-mediators involved in the pathogenesis of asthma, are currently showing encouraging results in clinical trials, and allow specific targeting of asthma endotypes. This project stems from epidemiological studies in which a lower incidence in allergic disease and asthma is observed in areas of the world where helminth infections are still common (Leonardi-Bee et al. 2006; Yazdanbakhsh & Matricardi 2004b).

In the past years various clinical trials have been carried out to test the efficacy of live helminth infection on inflammatory disease such as IBD, Crohn's disease, coeliac disease, asthma and multiple sclerosis. Parasites are known to modulate the host immune system at different levels through the secretion of soluble molecules, which among other effects, target type 2 immune responses (Harnett & Harnett 2017; Maizels et al. 2018). Type 2 immune responses are required for fighting parasitic infection, but aberrant type 2 immune responses have been associated with asthmatic immune responses (Lloyd & Snelgrove 2018). Helminth-derived immunomodulators offers a novel approach for the design of future therapies as demonstrated with viruses, fungi and bacteria in the past

(Kaparakis-Liaskos & Ferrero 2015; Felix & Savvides 2017). Viruses have been shown to produce an array of cytokine and chemokine homologues (e.g. homologues of IL-10), numerous cytokines-like receptor (e.g. TNF decoy receptor) and proteins that bind cytokines and receptors (Felix & Savvides 2017). For instance, viral-derived CC-chemokine inhibitor (vCCI) a protein derived from pox virus was shown to bind specifically to human and rodent CC-chemokines, inhibiting allergic inflammatory responses in the lungs (Dabbagh et al. 2000). CCP domain proteins have been identified in viruses, for instance vaccinia virus complement control protein (VCP) (Henderson et al. 2001). VCP is a 4 CCP domain protein that has been shown to inhibit complement proteins as well as playing a role in pathogenesis by blocking antibody-mediated neutralisation which is dependent on complement proteins (Agrawal et al. 2017). In the last decade the field of immunomodulation by parasite secretion has grown and led to the identification of an array of single molecules that can suppress inflammatory responses and relevant for this project, aberrant allergic type 2 immune responses (Maizels et al. 2018).

The IL-33 pathway is emerging as a key initiator of type 2 immune responses. IL-33 is an important mediator of the allergic immune responses and a promising clinical target. Clinical and genetic studies in human and experimental model in mice have demonstrated the important role for the IL-33 pathway in allergic responses. Initiation and exacerbation of asthma has been linked to respiratory viral infection, especially severe RSV bronchiolitis in early life have been correlated with wheeze and asthma diagnosis later in

life (Edwards et al. 2012; Sigurs et al. 2010). In neonatal mice IL-33 has been shown to be required for the development of type 2 asthmatic immune responses during RSV re-infection (Saravia et al. 2015). The cytokine might be an important target for prophylactic intervention in high risk infants to develop asthma, and for therapeutic treatment of asthma in adults. Several clinical trials are being carried out to test the efficacy of anti-IL-33 treatment in diseases such as COPD, acute respiratory syndrome and asthma (NCT03546907, NCT03469934, NCT02492204), but no results have been published yet. IL-33 is not only confined in the lung environment, but it plays a role in driving pathology in other organs such as the gut (e.g. IBD), the skin (e.g. atopic dermatitis) and it might be involved in metabolic disease, graft-versus-host disease (GVHD) and fibrosis (Griesenauer & Paczesny 2017; Scott et al. 2016). On the other hand IL-33 has a protective role during sepsis (Alves-Filho et al. 2010) and a role in tissue repair in several tissues such as lung, gut and CNS (Molofsky, Savage, et al. 2015). The IL-33/ILC2 axis has been demonstrated to be essential for fat metabolism, as IL-33-activated ILC2s have been shown to promote metabolic homeostasis and regulation of adipose tissue (Brestoff et al. 2015), as well as promoting amphiregulin production, tissue repair and homeostasis after influenza infection (Monticelli et al. 2011). Therefore suppression of the IL-33 pathway need to be careful planned to avoid deleterious side effects.

In this project the excretory/secretory products of the intestinal murine nematode *Heligmosomoides polygyrus* (HES) were studied for their suppressive effects on the IL-33 pathway (McSorley et al. 2012; McSorley et al. 2014; Buck et al. 2014; Coakley et al.

2017), a cytokine involved in the initiation of type 2 immune responses and asthma (Johansson & McSorley 2019).

I studied two HES-derived proteins: *H. polygyrus* Alarmin Release Inhibitor (HpARI) was already identified prior to my project while *H. polygyrus* Binds Alarmin Receptor and Inhibits (HpBARI), was identified *de novo* from HES. Both proteins interfere with the IL-33 pathway. I was able to show that HpARI binds to the reduced (active) form of IL-33, suppressing IL-33/ST2 interaction and blocking IL-33-dependent responses *in vitro* using a bone marrow assay and *in vivo* using a model of allergen-induced asthma. HpBARI binds to ST2, blocking IL-33-ST2 interactions and suppressing responses *in vitro* and *in vivo*. Both HpARI and HpBARI are CCP domain-containing proteins, with 3 CCP and 2 CCP domains spanning the length of HpARI and HpBARI respectively. Further experiments by other members of the McSorley lab showed that HpARI binds DNA via its positively-charged CCP1 domain, as expressing a mutated HpARI CCP2/3 (which lacks CCP1) did not show any DNA binding. Furthermore, HpARI CCP2/3 mediates the binding to IL-33 (Osbourn et al. 2017): a summary of the mechanism of action of HpARI is a shown in Fig.7.1. Although truncation studies of HpBARI have not yet been carried out, the fact that the C-terminal tagged HpBARI protein had reduced activity may give clues as to the structure and function of HpBARI. In particular, during the generation and purification of HpBARI the C-terminus tagged protein showed a decreased activity compared to the N-terminus tagged protein. Thus, the C-terminus might contain a key site that induces suppression of ST2 and IL-33 responses. Previous studies showed that the receptor ST2 is formed by 3 domains, and all of them interact

directly with IL-33 (Lingel et al. 2009). HpBARI binds to ST2 and blocks ST2/IL-33 interaction suggesting that HpBARI might bind one or multiple ST2 domains, blocking the cytokine to bind its receptor (Fig.7.2). Future plans will include the expression and purification of the single HpBARI CCP domains and determine which CCP domain (or both) is required for suppression of ST2 and the IL-33-dependent responses. I speculate that the HpBARI CCP2, which contains an atypical insertion of 10 aa, might be required to block the responses to IL-33 and as mentioned before, adding a myc and a 6-HIS tag at the end of this region might induce the steric hinderance resulting in low affinity to ST2. Furthermore, X-ray crystallography would help to better characterise the interaction between HpARI/IL-33 and HpBARI/ST2.

The purpose of this project was to identify immunomodulatory molecules from HES, with an ultimate aim of developing novel therapeutic agents against asthma and allergic diseases. The development of new tools to experimentally manipulate the IL-33 pathway might offer the possibility to better understand the role of IL-33 in disease and homeostasis. Understanding the interactions between our parasite-derived proteins and their targets will define at which site the binding occurs, and together with the knowledge about the CCP domains could lead to the design of smaller and less immunogenic molecules that can be further developed for human treatments. Targeting the immunogenicity is an essential requirement to prevent the formation of anti-drug-antibodies (ADAs) that will make the drug ineffective and in rare case induce auto-immune reactions (Tovey & Lallemand 2011). While HpARI binds to both murine and human IL-33, HpBARI binds only to murine (and not human) ST2, making the use of

HpBARI more challenging as a human therapeutic. Nonetheless, one strategy that could be used is to determine the HpBARI/ST2 binding region through determining the structure of the HpBARI-ST2 complex. Knowing the interaction at molecular level could lead to engineering a protein, mutating the amino acids involved in the HpBARI/ST2 interaction and generating a mutant protein that could bind human ST2 with high affinity.

Another strategy that could help with engineering a protein that binds to human ST2 is to investigate the presence of homologues of HpBARI, as similarly done with HpTGM (Smyth et al. 2018; Johnston et al. 2017). A family of nine related HpTGM molecules secreted by *H. polygyrus* was identified showing that only two of these homologues were as active as HpTGM, while for the others the function is still unclear (Smyth et al. 2018). Similarly, homologues of HpARI have been identified showing different affinities to mouse and human IL-33 (Manuscript in preparation).

HpBARI homologues have been identified during the screening for an ST2 suppressor candidate and these will be investigated further in the future.

H. polygyrus seems to have developed the CCP domain as a structural scaffold to generate an array of different proteins that act on the host immune system, in particular by blocking IL-33 and the induction of type 2 immune responses, and by induction of Foxp3 T_{REG} to induce an anti-inflammatory environment. Furthermore, other CCP proteins might have been developed by *H. polygyrus* to target other pathways that are involved in ILC2 activation and type 2 immune responses, for examples IL-25, neuromedinU and TSLP (Cardoso et al. 2017; Zaiss et al. 2013; Ricardo-Gonzalez et al. 2018). CCP domains are

present in other parasites and interestingly, in human parasites (Maizels et al. 2018), indicating that immunomodulatory molecules could be identified from ES products of human parasites. Cultivation and obtaining ES in the lab from human helminths has been challenging, and achieved for some species e.g. *Schistosoma japonicum, Schistosoma mansoni* or *Brugia malayi* (Falcone et al. 1995; Ye et al. 2013; Frahm et al. 2019) However, parasite genomic data has been constantly expanded over recent years, with new genomes added regularly into the WormBase ParaSite database and recently the addition of numerous genomes from helminth species (Coghlan et al. 2019). This database can be used to identify similar genomic sequences and proteins secreted by different human and murine parasites, and this was used to identify homologues proteins of HpARI and HpBARI. In addition, the role of parasite-derived immunomodulators could be studied in the context of vaccination. With over one fourth of the human population infected by parasites, no vaccine has been developed for human use yet. Mouse models have suggested that vaccination against, for example, ES products or EV induces protection against infection (Coakley et al. 2017; Hewitson et al. 2013). Future works could involve the production of monoclonal antibodies against HpARI or HpBARI and test mAb during *H. polygyrus* live infection, measuring egg count and worm burden to determine severity of infection.

In conclusion, using HpARI and HpBARI to modulate the IL-33 pathway in the context of pathological responses offers an important tool to better understand the role of IL-33 and ST2 in disease development. HpARI and HpBARI are further evidence that *H.*

polygyrus secretes a pool of proteins with immunomodulatory effects, and the IL-33 pathway seems to be essential for parasite expulsion as it is targeted at multiple levels. In particular, both proteins are members of the CCP domain family, and so it appears that this family has been exploited by *H. polygyrus* as a scaffold to generate a range of immunomodulatory proteins. This is an exciting area as genomic data from parasites has been expanding in the last years, enabling the identification of similar or novel immunomodulatory molecules in human parasites in the future. Furthermore, understanding at molecular levels HpARI/IL-33 and HpBARI/ST2 interaction can help to design single peptides or smaller molecules that block the IL-33 pathway, retaining the biological function, being less immunogenic. This may lead to further development of such compounds as possible therapeutic in different condition where the IL-33 pathway plays a key role.

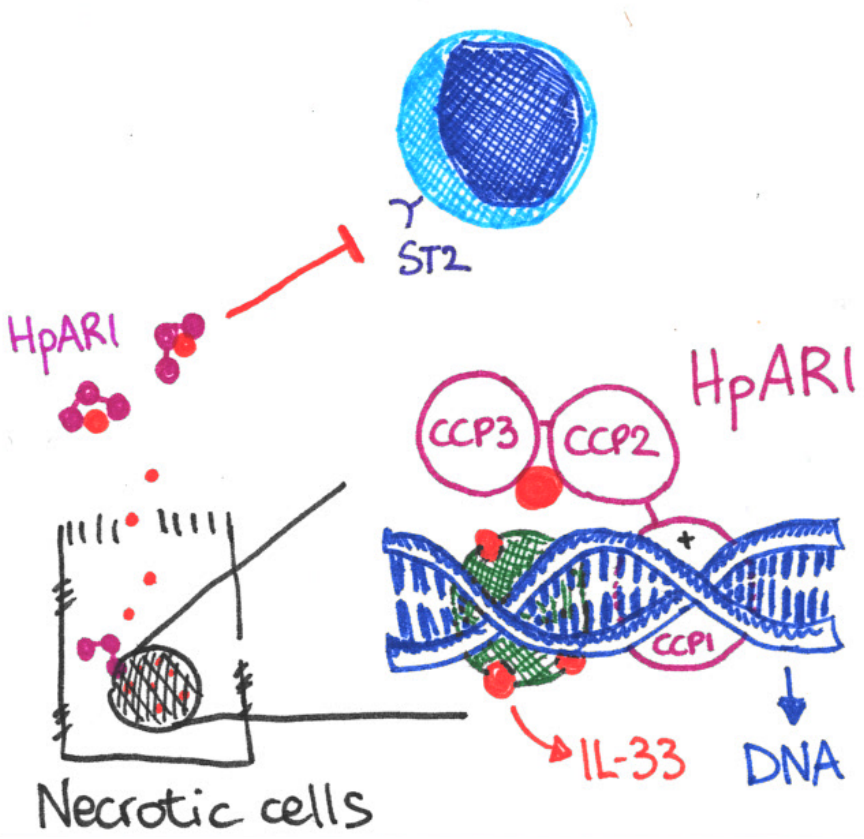


Figure 7.1 HpARI mechanism of action

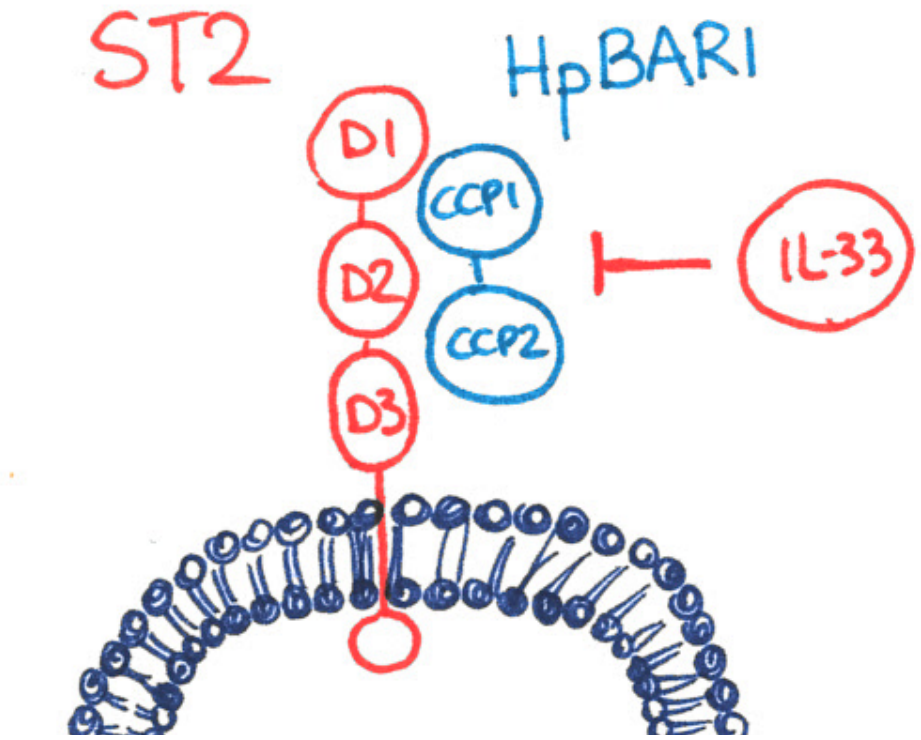


Figure 7.2 HpBARI mechanism of action.

HpBARI binds directly to ST2 blocking IL-33-ST2 interaction. This figure is not representative of the interaction between ST2 domains and HpBARI CCP domains as the real interaction between HpBARI CCP domains and ST2 domains is still unknown.

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Appendix

HpARI Protein Secreted by a Helminth Parasite Suppresses Interleukin-33

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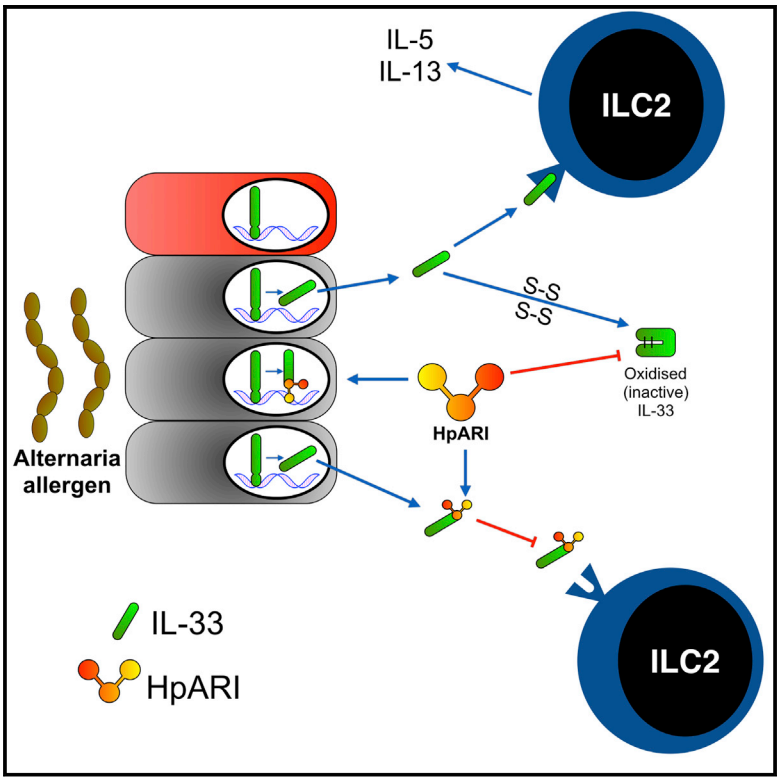
Article

Immunity

HpARI Protein Secreted by a Helminth Parasite Suppresses Interleukin-33

Graphical Abstract



Highlights

- HpARI is a suppressor of IL-33 release and consequent allergic sensitization
- HpARI binds active IL-33 and nuclear DNA, tethering IL-33 within necrotic cells
- HpARI is active against both human and murine IL-33

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In Brief

Osbourn et al identified HpARI, a protein secreted by a helminth parasite that is capable of suppressing allergic responses. HpARI binds to IL-33 (a critical inducer of allergy) and nuclear DNA, preventing the release of IL-33 from necrotic epithelial cells.

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HpARI Protein Secreted by a Helminth Parasite Suppresses Interleukin-33

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SUMMARY

Infection by helminth parasites is associated with amelioration of allergic reactivity, but mechanistic insights into this association are lacking. Products secreted by the mouse parasite *Heligmosomoides polygyrus* suppress type 2 (allergic) immune responses through interference in the interleukin-33 (IL-33) pathway. Here, we identified *H. polygyrus* Alarmin Release Inhibitor (HpARI), an IL-33-suppressive 26-kDa protein, containing three predicted complement control protein (CCP) modules. *In vivo*, recombinant HpARI abrogated IL-33, group 2 innate lymphoid cell (ILC2) and eosinophilic responses to *Alternaria* allergen administration, and diminished eosinophilic responses to *Nippostrongylus brasiliensis*, increasing parasite burden. HpARI bound directly to both mouse and human IL-33 (in the cytokine's activated state) and also to nuclear DNA via its N-terminal CCP module pair (CCP1/2), tethering active IL-33 within necrotic cells, preventing its release, and forestalling initiation of type 2 allergic responses. Thus, HpARI employs a novel molecular strategy to suppress type 2 immunity in both infection and allergy.

INTRODUCTION

Infection with helminth parasites negatively correlates with prevalence of allergic disease, and parasitic infection is associated with immunosuppression (Maizels and McSorley, 2016). Many researchers, ourselves included, have demonstrated that helminths release immunomodulatory proteins to control anti-parasite immune responses and maintain their persistence in the host (Maizels and McSorley, 2016). We previously showed that the excretory–secretory products of the mouse intestinal parasite *Heligmosomoides polygyrus* (HES) suppress allergic responses in mouse models of asthma (Buck et al., 2014; McSorley et al., 2015; McSorley et al., 2014; McSorley et al., 2012). HES administration blocks the interleukin-33 (IL-33) response to inhaled *Alternaria* (fungal) allergen (McSorley et al., 2014) leading to reduced type 2 innate lymphoid cell (ILC2) responses and abrogating lung pathology.

IL33 and its receptor (*IL1RL1*) are both among the 10 genes most strongly linked to allergic sensitization (Bønnelykke et al., 2013) and asthma (Bønnelykke et al., 2014; Moffatt et al., 2010) in genome-wide association studies. IL-33 concentration is increased in the lungs of severe asthmatics (Castanhinha et al., 2015; Saglani et al., 2013), correlating negatively with lung function (Christianson et al., 2015). Respiratory viral infections are implicated in both initiation and exacerbation of asthma, an effect that is also associated with IL-33 release (Jackson et al., 2014; Saravia et al., 2015).

The IL-33 receptor (ST2, IL1RL1, IL-33R) is expressed by a wide range of cells, notably T cells, macrophages, endothelial cells, epithelial cells, and ILC2 (Cayrol and Girard, 2014). Through these interactions, IL-33 drives type 2 immune responses in a range of diseases including asthma, atopic dermatitis, food allergy, COPD, eosinophilic inflammatory bowel disease, eosinophilic esophagitis, and age-related macular degeneration (De Salvo et al., 2016; Liew et al., 2016; Simon et al., 2015; Tordesillas et al., 2014). IL-33 is a member of the

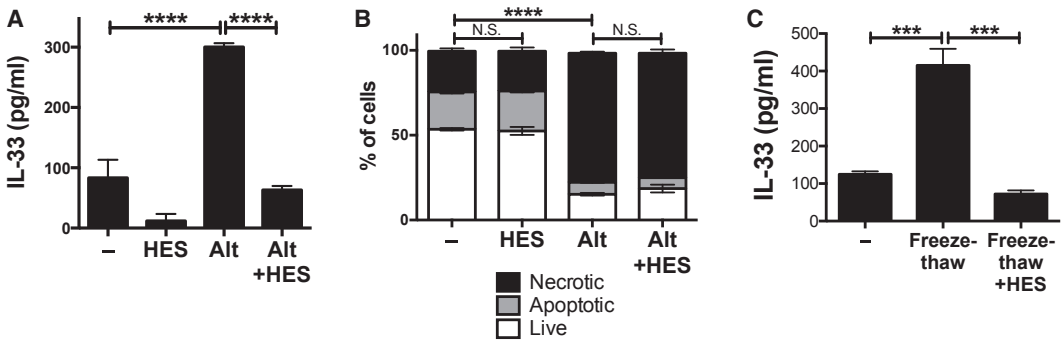

Figure 1. HES Suppression of IL-33

(A) IL-33 levels (ELISA) in supernatants of naive murine lung cells (1×10^5 per well), cultured for 1 hr with *Alternaria* (Alt) allergen (200 μg/ml) and HES (10 μg/ml).
(B) Propidium iodide (PI) and annexin V (AnnV) staining of cells from (A) was used to assess apoptosis (PI–AnnV+) versus necrosis (PI+AnnV+).
(C) IL-33 levels (ELISA) in supernatants of naive murine lung cells, freeze-thawed in the presence of HES.
All data shows SEM of 2–3 replicates, and are representative of 2–3 repeat experiments. Error bars show SEM.

IL-1 family of cytokines. It is stored preformed in the nucleus bound to heterochromatin, and its dominant function is as an alarmin cytokine. Active IL-33 is released from the nucleus under conditions of necrosis, while during apoptosis active caspases cleave IL-33 within its receptor-binding domain, abolishing activity (Lefrançais and Cayrol, 2012). Although the full-length, 30 kDa form of IL-33 is functional, the activity of IL-33 is increased 10-fold through cleavage between the DNA-binding and receptor-binding domains by proteases such as calpain-2 (Hristova et al., 2016), neutrophil elastase, cathepsin G (Lefrançais et al., 2012), and mast cell tryptase (Lefrançais et al., 2014) releasing 18–21 kDa mature forms. Active IL-33 is released in a reduced form, which under physiological conditions rapidly oxidizes, forming new disulfide bonds and changing conformation, rendering it unable to bind to the IL-33R beyond a short temporal and spatial range (Cohen et al., 2015).

Here, we identified *H. polygyrus* Alarmin Release Inhibitor (HpARI), a HES-derived recombinant protein that can replicate the IL-33-suppressive effects of total HES. HpARI bound directly to active murine or human IL-33 and nuclear DNA. This dual binding blocked the interaction of IL-33 with its receptor, and tethered IL-33 within necrotic cells, preventing its release, and blocking allergic response initiation. Thus, HpARI prevents initiation of parasite-toxic IL-33-mediated type 2 immune responses and suppresses the development of allergic airway inflammation.

RESULTS

In Vitro Suppression of IL-33 by HES

Previous studies established that HES ablates detectable IL-33 in the bronchoalveolar milieu after *Alternaria* allergen administration, suppressing downstream allergic responses (McSorley et al., 2014). To further investigate the IL-33-suppressive activity of HES, we developed an *in vitro* assay for IL-33 release: a single cell suspension of naïve total murine lung cells cultured for 1 hr in the presence of *Alternaria* allergen and HES. In this assay, HES markedly reduced the amount of IL-33 in culture supernatants, as detected by ELISA (Figure 1A).

IL-33 is released from lung epithelial cells under conditions of necrosis, whereas activated caspases cleave IL-33 within the IL-1-like cytokine domain, inactivating IL-33 under conditions of apoptosis (Lefrançais and Cayrol, 2012). We therefore hypothesized that HES could be activating caspase and/or apoptosis pathways. Propidium iodide and annexin V staining showed that cells incubated with *Alternaria* allergen were highly necrotic and that this was unaffected by the presence of HES (Figure 1B). Necrosis induced by freeze-thaw treatment of lung cells also resulted in substantial IL-33 release, which again was abrogated by treatment of cells with HES immediately prior to freezing (Figure 1C). Therefore we conclude that HES suppression of IL-33 does not depend on activation of the apoptosis pathway, but instead acts on pre-formed IL-33 released from necrotic cells.

Identification and Characterization of HpARI Protein

A process of fractionation, screening, and proteomic analysis of HES was used to identify candidate IL-33-suppressive proteins. Gel filtration and anion exchange FPLC were used to fractionate HES by size and charge, respectively. IL-33 suppressive activity peaked around size fraction 11 (Figure 2A) and charge fraction 25 (Figure 2B). Each size and charge fraction was subjected to trypsin digestion followed by liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS), and the exponentially modified protein abundance index (emPAI) value for each HES protein in every fraction was calculated, and compared to the profile of IL-33 suppression.

By size fractionation, 220 proteins were found with emPAI values which peaked around size fraction 11 (peak value in fractions 10–12), while 371 proteins were found with emPAIs which peaked around charge fraction 25 (peak value in fractions 23–27), 54 of which were shared between the two fractionation techniques. Proteins were prioritized wherein more than one peptide was detected in size fraction 11 and charge fraction 25, resulting in a short-list of 25 candidate proteins (Table S1). The emPAI values for each of these 25 candidates for all size and charge fractions was then manually compared to the IL-33 suppression profile (Figure S1A), and 4 candidates were selected for initial screening (Figure S1B).

The 4 candidate IL-33 suppressive genes were transfected into HEK293T cells for expression, and screened for suppression

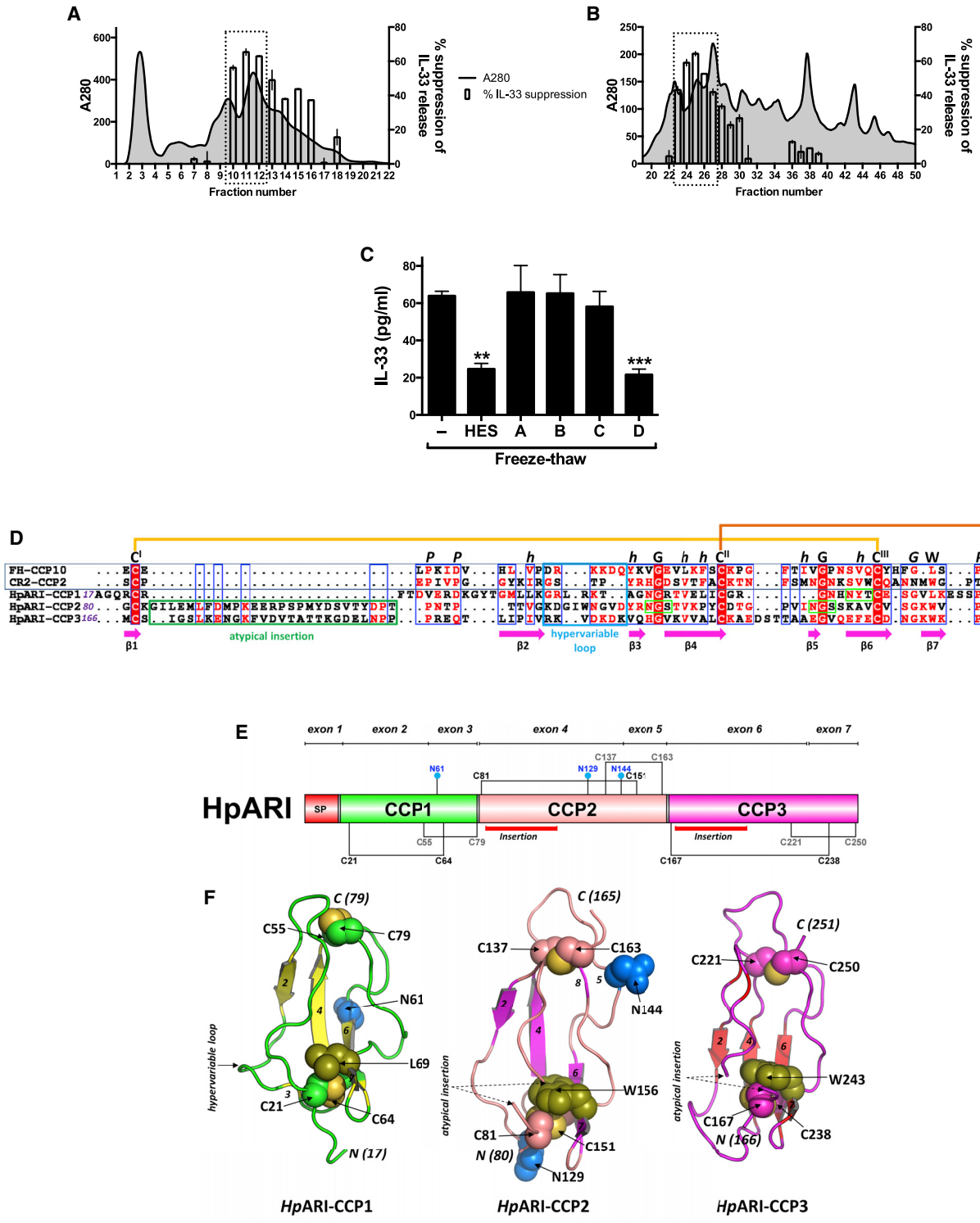

Figure 2. Identification and Bioinformatic Characterization of HpARI Sequence and Structure

(A) IL-33 suppression by HES size fractions.

(B) IL-33 suppression by HES charge fractions.

Data in (A) and (B) are percentage suppression of the IL-33 signal compared to *Alternaria*-only control. Dotted rectangles indicate peaks used for selection of candidates.

(C) IL-33 levels (ELISA) in supernatants of naïve murine lung cells, freeze-thawed in the presence of supernatants of HEK293T cells transfected with four candidate genes. Mean and SEM are shown of three replicate wells, representative of three repeat experiments.

(legend continued on next page)

of the IL-33 signal *in vitro*. Of the 4 candidates, only the transcript named Hp_I08176_IG02172_L1157 in our in-house sequencing (candidate "D" in Figure 2C) significantly suppressed IL-33; this protein was consequently renamed as *H. polygyrus* Alarmin Release Inhibitor (HpARI). Subsequently, an identical transcript was found at WormBase Parasite: HPBE_0000813301.

The *HpARI* gene is made up of 7 exons, encoding a 251-aa protein including a 16-aa signal peptide motif (Figure S2A), with a deduced mature molecular weight of 26 kDa. The mature protein contains three predicted Complement Control Protein (CCP)-like modules (also known as Short Consensus Repeats (SCRs) or sushi-domains, PFAM00084) (Figure 2D). CCP1–3 all contain features of a CCP module such as the four consensus Cysteine residues (Cys^{I} to Cys^{IV}, consistent with formation of disulfide bonds in a Cys^{I}-Cys^{III} and Cys^{II}-Cys^{IV} pattern), the Trp/Leu residue between Cys^{III} and Cys^{IV} and other structurally important residues typical of a CCP module (Figures 2D and 2E and STAR Methods) (Kirkitadze and Barlow, 2001; Soares et al., 2005). Compared to archetypal CCP modules (Soares and Barlow, 2005), all three are atypical in part with divergent sequence features, including an absence of conserved Proline residues after Cys^{I} in CCP1, and atypical insertions of ~20 amino acid residues between Cys^{I} and Cys^{II} in CCP 2 and CCP3, which are unique compared to previously identified CCP domains. Each CCP module is encoded by two exons with the second exon boundary in each case falling between adjacent predicted CCP modules (i.e. between Cys^{IV} of one module and Cys^{I} of the next) lending further support to the discerned domain boundaries (Figure 2E and Figure S2A).

The three predicted HpARI CCP module sequences were modelled individually based upon their top ranked CCP module template structures. Each CCP module 3-D model is characterized by a β-sheet framework, held together by two disulfide bridges. Other key structural features such as the location of the buried Trp/Leu, hypervariable loop, and potential N-glycosylation sites are indicated along with the relative positions of the novel insertions in CCP2 and CCP3, which could not be modelled on conventional experimentally determined CCP module structures (Figure 2F).

In Vitro and In Vivo IL-33 Suppression by HpARI

Recombinant mature 6-His and Myc-tagged HpARI protein was purified by metal chelating chromatography (Figure S2B), and tested for IL-33 suppression *in vitro*. HpARI was active at <10 ng/ml, while HES required an approximately 50-fold higher concentration for a similar effect (Figure 3A). The IL-33-suppressive activity of HpARI in response to *Alternaria* culture or freeze-thaw was ablated on heat-treatment, as with HES (Figures S3A and S3B).

HpARI also effectively suppressed IL-33 detected in bronchoalveolar lavage (BAL) fluids in response to *Alternaria* allergen *in vivo* (Figure 3B). Again this effect replicated that of HES (McSorley et al., 2014) and suppression was ablated when HpARI was proteolytically cleaved and heat-treated, ruling out a role for non-protein contaminants. In addition, the IL-33-suppressive effects of HpARI could pre-condition airway tissues, substantially reducing the IL-33 response to *Alternaria* allergen 24 hr later, with a degree of protection in some animals even after 72 hr (Figure 3C). Thus, HpARI appears to be a critical IL-33-suppressive factor in HES.

Suppression of In Vivo Type 2 Responses by HpARI

Alternaria exposure induces a rapid T cell-independent eosinophilia within 24 hr of administration. This response is driven by ILC2 cytokine release, and is critically dependent on IL-33 (Bartemes et al., 2012). Recombinant HpARI co-administration with *Alternaria* allergen abrogated BAL eosinophilia (Figure 3D) and lung ILC2 IL-5 (Figure 3E) and IL-13 production (Figure 3F), 24 hr later, again replicating the effects observed with total HES. IL-13-eGFP reporter mice were used to assess ILC2 cytokine responses in the absence of PMA and Ionomycin stimulation, confirming profound suppression of IL-13 reporter expression in $ICOS^{+}CD90.2^{+}IL\text{-}33R^{+}CD127^{+}CD45^{+}lineage^{-}$ ILC2s by HpARI (Figures S3C–S3E).

HpARI was administered in a T cell-dependent model of asthma, in which OVA protein is first co-administered with *Alternaria*, and antigen-specific type 2 responses recalled 2 weeks later by challenge with OVA protein alone (McSorley et al., 2014). Again HpARI replicated the suppressive effects of HES on BAL eosinophilia and lung ILC2 responses (Figures 4A–4C). Furthermore, this suppression led to significantly abrogated lung resistance and compliance at challenge (Figures 4D and 4E), as well as reduced inflammation and mucus production assessed by histological staining (Figure 4F–4H).

Finally, the role of HpARI in parasite infections was addressed using *Nippostrongylus brasiliensis* infection, a parasite which (unlike *H. polygyrus*) migrates through the lung and leads to early IL-33-dependent type 2 responses (Hung et al., 2013). Similarly to the phenotype seen in an IL-33-deficient mouse, HpARI administration did not affect worm burden at early timepoints, but increased numbers of adult parasites found in the intestinal lumen at day 6 (Figures 4I and 4J). This suppression of parasite rejection was associated with reduced BAL eosinophilia, reaching significance at day 6 (Figure 4K). Thus, HpARI abrogates parasite- or allergen-induced IL-33-dependent type 2 immune responses, abrogating parasite ejection and suppressing allergic pathology.

HpARI Binding to IL-33

We hypothesized that HpARI could act by binding directly to IL-33. To investigate this, we incubated Myc-tagged HpARI with murine lung cell homogenates, and immunoprecipitated with anti-c-Myc antibody bound to protein G-coated beads.

(D) Alignment of HpARI CCP-like modules with complement receptor type 2 CCP2 (CR2-CCP2) and complement factor H CCP10 (FH-CCP10). The putative disulfide bonding pattern (C^{I}-C^{III}; C^{II}-C^{IV}), conserved tryptophan (W) and structurally-important proline (P), glycine (G), and hydrophobic amino acid residues (h), characteristic of a CCP-module are indicated. Atypical insertions in CCP2/3 (green box), the hypervariable loop (cyan box), and beta-strands (pink arrows) are indicated, based on known CCP secondary structure of CR2-CCP2, as well as three potential N-linked glycosylation sites (light green box).
(E) HpARI domain schematic, with putative disulfide bonding pattern and location of insertions indicated.
(F) Structural models of the three HpARI CCP-like modules.
Error bars show SEM.

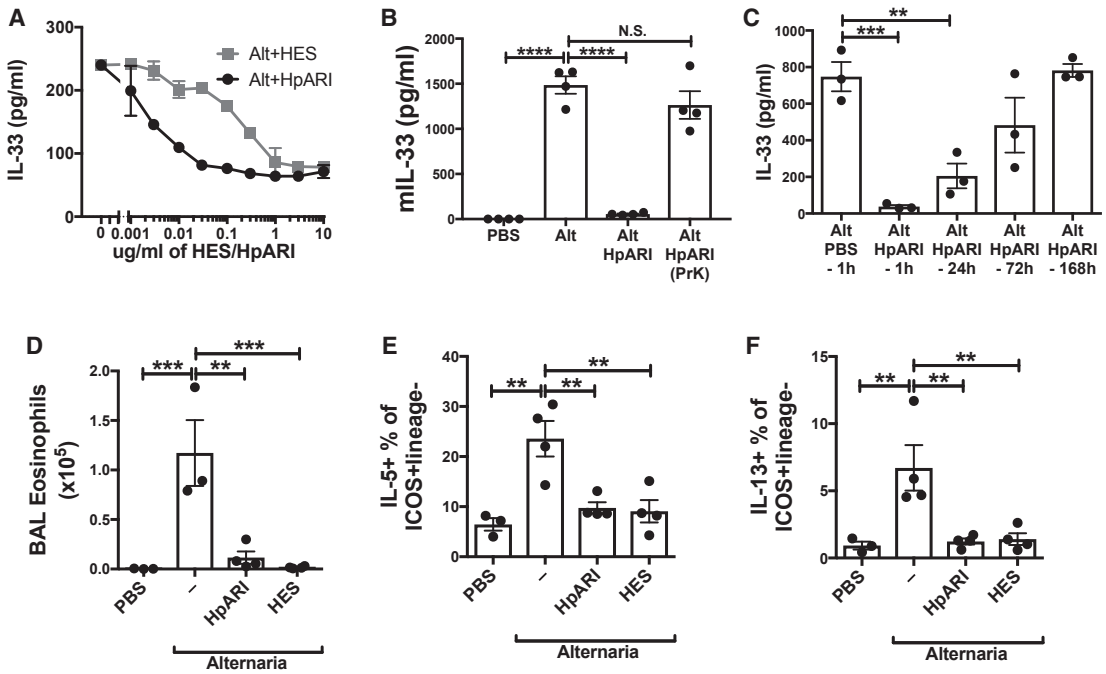

Figure 3. HpARI Suppresses Responses to *Alternaria* Allergen

(A) IL-33 levels (ELISA) in supernatants of naive mouse lung cells, cultured for 1 hr in the presence of *Alternaria* (200 μg/ml) and HES or HpARI.

(B) IL-33 levels (ELISA) in BAL 1 hr after *Alternaria* allergen administration with HpARI (5 μg) or proteinase K-degraded and heat-treated HpARI ("HpARI (prK)").

(C) IL-33 levels (ELISA) in BAL 1 hr after *Alternaria* allergen administration, with HpARI (5 μg) administered 1, 24, 72, or 168 hr prior to *Alternaria* allergen.

(D) BAL eosinophil numbers 24 hr after *Alternaria* allergen, HpARI, and HES administration.

(E) Lung ILC2 IL-5 staining from mice in (D).

(F) Lung ILC2 IL-13 staining from mice in (D).

All data representative of 2–3 repeat experiments, each with 3–4 replicates/mice per group. Error bars show SEM.

HpARI immunoprecipitated a clear band at ~18 kDa in Myc-tagged complexes eluted from anti-c-Myc-coated, but not isotype control-coated beads, as revealed by anti-IL-33 western blotting (Figure 5A). Unbound material (supernatants from co-immunoprecipitation) showed undetectable or very faint bands for IL-33 under these conditions, reflecting the manner in which immunoprecipitation concentrates ligand sufficiently for detection. No band could be detected for full-length IL-33 (30 kDa) in these experiments (data not shown).

Despite human and murine IL-33 sharing only 52% amino acid identity, we found that human IL-33 also co-immunoprecipitates with HpARI after incubation with human lung homogenates, seen as an ~18 kDa band corresponding to mature human IL-33 (Figure 5B). In this case, unbound human IL-33 could be detected in supernatants from co-immunoprecipitation or control conditions, also at ~18 kDa.

To biochemically characterize the binding of human and mouse IL-33 with HpARI, we assessed the interactions between these proteins by surface plasmon resonance (SPR) (Figures 5C and 5D). The equilibrium dissociation constant (KD) of HpARI for murine IL-33 is 0.56 ± 0.1 nM, and 260 ± 13 nM for human IL-33.

Oxidation of IL-33

Recently, it was shown that IL-33 is released in an active reduced form, which is quickly oxidized (<4 hr after release) and inactivated under physiological conditions (Cohen et al., 2015). Commercially-available IL-33 ELISA kits do not differentiate between the reduced and oxidized forms. Therefore we decided to investigate whether HpARI preferentially bound to reduced or oxidized IL-33.

To attain a source of oxidized and reduced IL-33, we subjected lung cells to freeze and thaw-mediated necrosis, harvested IL-33-containing supernatants immediately post-thaw, and incubated these at 37°C for 1–4 hr to oxidize IL-33 (Cohen et al., 2015). When HpARI was added to supernatants directly post-thaw, or up to 2 hr later, it was able to significantly reduce the IL-33 signal as measured by ELISA, whereas by 4 hr post-thaw, no effect of HpARI could be seen (Figure 5E and Figure S4A). Therefore we hypothesized that HpARI binds only to active (reduced) IL-33.

HpARI co-immunoprecipitation experiments were then repeated with either untreated recombinant murine IL-33 (rmIL-33) or rmIL-33 which had been oxidized by incubation for 24 hr at 37°C in tissue culture medium. Eluted complexes were run on non-reducing SDS-PAGE gels to distinguish reduced and oxidized IL-33 by their differential migration under non-reducing conditions, the more compact oxidized form migrating more rapidly (Cohen et al., 2015). A strong bias for binding of HpARI to the reduced form could be seen, with unbound supernatants containing the oxidized form, while no unbound reduced IL-33 could be detected (Figure 5F).

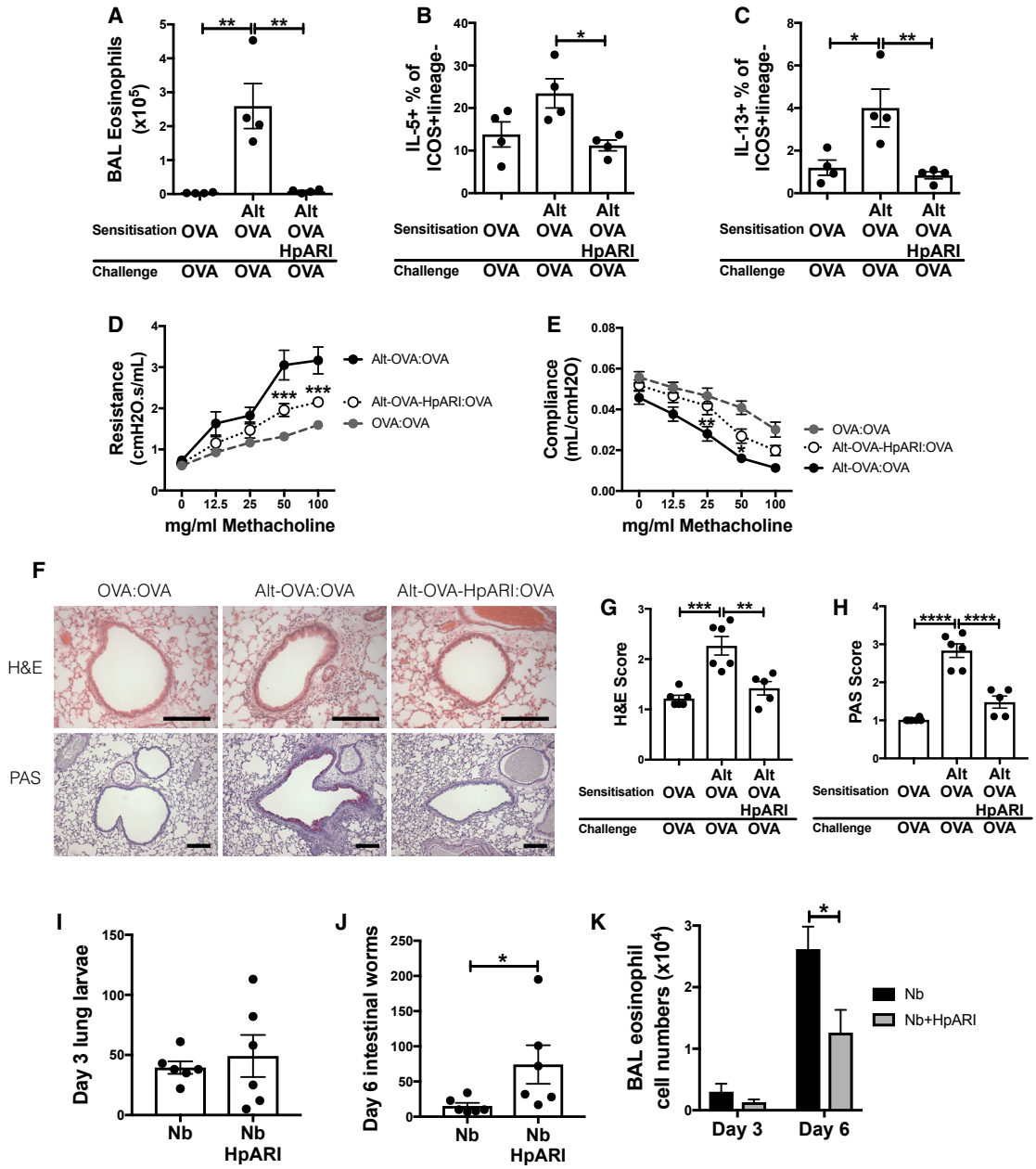

Figure 4. HpARI Suppresses Responses to *Alternaria* Allergen

(A) Day 17 BAL eosinophil numbers after *Alternaria* allergen, OVA protein, and HpARI administration on day 0 (sensitization), and OVA protein alone on days 14, 15, and 16 (challenge).

(B) Lung ILC2 IL-5 production from mice in (A).

(C) Lung ILC2 IL-13 production from mice in (A).

(D) Lung resistance in methacholine challenge from mice treated as in (A).

(E) Lung compliance in methacholine challenge from mice treated as in (A).

(F) H&E- (top panels) and PAS-stained (bottom panels) lung sections from mice treated as in (A). Scale bars indicate 100 μm.

(G) H&E scoring of sections from mice treated as in (A).

(H) PAS scoring of sections from mice treated as in (A).

Alternaria model data representative of 2–3 repeat experiments, each with 4–6 mice per group.

(I) Mice were subcutaneously infected with *N. brasiliensis*, and HpARI administered intranasally on days 0, 1, and 2 of infection. Lung larvae were counted 3 days after infection.

(J) Day 6 intestinal *N. brasiliensis* worms from mice treated as in (I).

(K) Day 3 and day 6 BAL eosinophil numbers from mice treated as in (I).

Error bars show SEM.

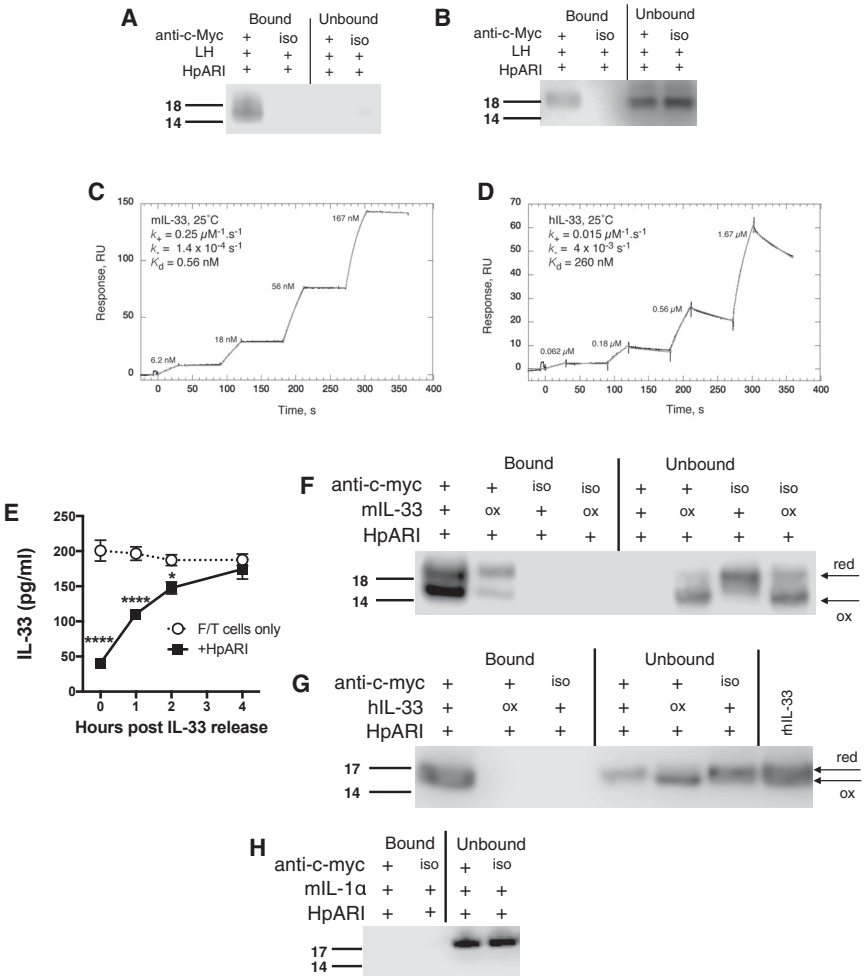

Figure 5. HpARI Binds Active Murine and Human IL-33

(A) Murine IL-33 western blot (non-reducing) of HpARI immunoprecipitation of mouse lung homogenates, using anti-c-Myc antibody, or MOPC isotype control (iso).

(B) Human IL-33 western blot (non-reducing) of HpARI immunoprecipitation of human lung homogenates, as in (A).

(C) Characterization of the interaction of mouse IL-33 (mIL-33) with HpARI by surface plasmon resonance (SPR - BIAcore T200). Reference corrected single kinetic titration SPR binding curves (black), and a globally fitted 1:1 kinetic binding model (grey).

(D) Characterization by SPR of the interaction of human IL-33 (hIL-33) with HpARI, as in (C).

(E) IL-33 levels (ELISA) in supernatants of freeze-thawed murine lung cells, incubated at 37°C for 0, 1, 2, or 4 hr, before addition of 1 μg/ml HpARI, and a further incubation for 1 hr at 37°C.

(F) Untreated or oxidized recombinant murine IL-33 immunoprecipitated with HpARI as in (A).

(G) Untreated or oxidized recombinant human IL-33 immunoprecipitated with HpARI as in (B).

(H) Immunoprecipitation experiments repeated with recombinant murine IL-1α, and probed with anti-murine IL-1α.

Arrows indicate specific IL-33 or IL-1α bands, and IL-33 reduced ("red") or oxidized ("ox") bands. All data are representative of at least two independent repeats. Error bars show SEM.

Co-immunoprecipitation was repeated with recombinant human IL-33 (rhIL-33), either untreated or oxidized under the same conditions as applied to murine IL-33. Similarly to murine IL-33, rhIL-33 could only be bound by HpARI in its reduced, active form, with oxidation of IL-33 abolishing its ability to be co-precipitated (Figure 5G).

Finally, we ensured that the binding of HpARI is specific to IL-33, by binding studies with the closely-related IL-1 family cytokine IL-1α. No binding of HpARI to IL-1α could be detected, either by co-immunoprecipitation (Figure 5H) or by SPR (Figure S4B). Thus, HpARI specifically and with high affinity, binds to the active, reduced form of IL-33.

HpARI Prevents Binding of Active IL-33 to the IL-33 Receptor

To investigate whether HpARI binding IL-33 consequently affected downstream responses to IL-33, we investigated the binding of IL-33 to its receptor ST2. Recombinant mIL-33 was incubated alone or with HpARI, then immunoprecipitation was carried out using an ST2-Fc fusion protein bound to protein G-coated magnetic beads. The presence of HpARI completely blocked immunoprecipitation of rmIL-33 by ST2-Fc (Figure 6A), implying that HpARI prevents IL-33 from binding to its receptor.

Furthermore, when rmIL-33 was administered intranasally to mice, IL-33-mediated ILC2 activation (measured by IL-5 and IL-13 production) was effectively ablated by HpARI co-administration (Figures 6B and 6C). Thus HpARI, through binding to IL-33, can prevent the activation of ILC2s through ST2 ligation.

HpARI Inhibits Release of IL-33

As HpARI directly binds IL-33, it could also interfere with detection of the cytokine by ELISA through masking epitopes bound by assay antibodies. This could affect our early screening results, (Figures 1, 2, and 3) as these are largely dependent on ELISA to measure concentrations of IL-33. To investigate the possibility of undetectable HpARI-bound IL-33 in BAL supernatants, we measured IL-33 by both ELISA and western blot, as the latter reduces, denatures and dissociates protein complexes. Mice were treated with *Alternaria* allergen and BAL taken 15 min later (at which timepoint the majority of IL-33 released is active and reduced [Cohen et al., 2015]), HpARI coadministration ablated the IL-33 signal by ELISA (Figure 6D), and significantly inhibited (but did not ablate) the IL-33 signal by western blot (Figure 6E), implying that although HpARI binding interferes with IL-33 detection by ELISA, IL-33 release is indeed diminished with HpARI administration. In contrast, HpARI could not affect the release of HMGB1, another nuclear-localised alarmin cytokine released on necrosis, (Figure S4C), demonstrating that the effects of HpARI are specific to IL-33.

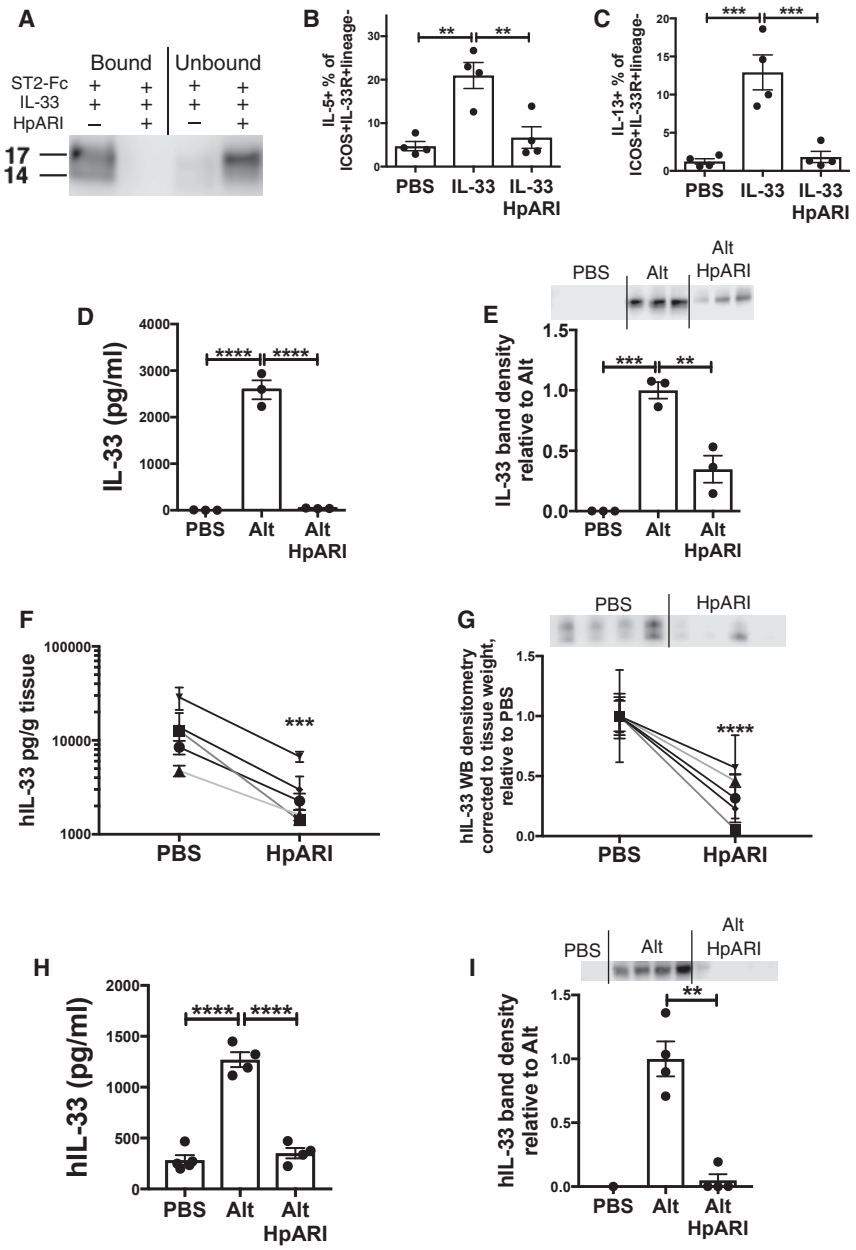

Figure 6. HpARI Blocks IL-33-ST2 Interactions and Inhibits IL-33 Release

(A) IL-33 western blot (non-reducing) of ST2-Fc fusion protein immunoprecipitation of recombinant murine IL-33 in the presence or absence of HpARI.

(B) Lung ILC2 IL-5 production 24 hr after intranasal administration of recombinant murine IL-33 (200 ng/mouse) with 5 μg HpARI.

(C) Lung ILC2 IL-5 production from mice described in (B).

(D) Murine IL-33 levels (ELISA) in BAL 15 min after *Alternaria* allergen and HpARI were intranasally administered.

(E) Murine IL-33 western blot (~20 kDa band and densitometry analysis) of BAL from mice described in (D).

(F) Human IL-33 levels (ELISA) in supernatants of human lung explants cultured for 1 hr with HpARI.

(G) Human IL-33 western blot (~20 kDa band and densitometry analysis) of supernatants from human lung explants cultures described in (F).

(H) Human IL-33 levels (ELISA) in BAL fluid of human IL-33-transgenic mice, 30 min after *Alternaria* allergen and HpARI intranasal administration.

(I) Human IL-33 western blot (~20 kDa band and densitometry analysis) of BAL from human IL-33-transgenic mice described in (H).

Mouse data (A–E, H–I) representative of 2–4 repeat experiments, each with 3–4 mice per group. Human data (C and D) shows 5 independent subjects. Error bars show SEM.

To translate these results to human biology, we cultured human lung explants for 1 hr with HpARI, a system and timepoint in which lung explants spontaneously release reduced (active) human IL-33 (Cohen et al., 2015). Similarly to the murine system, a reduction in IL-33 signal was seen with HpARI coadministration, as measured by both ELISA and western blot (Figures 6F and 6G). Furthermore, HpARI was administered with *Alternaria* to human IL-33 transgenic mice (Cohen et al., 2015), where it again suppressed human IL-33 release into the BAL (Figures 6H and 6I). Thus, HpARI reduces the release of both mouse and human IL-33.

Immunofluorescent Localization of HpARI

To further investigate the mechanism of action of HpARI, we utilized the CMT-64 mouse lung epithelial carcinoma cell line, which we found stores high amounts of IL-33 in the nucleus (Figure S5A). Similarly to lung cells cultured *in vitro*, IL-33 is released from freeze-thawed CMT-64 cells, and this response is suppressed by HpARI (Figure S5B). We then produced an HpARI_mCherry fusion protein, allowing fluorescent localization of HpARI binding, while retaining IL-33-suppressive activity (Figure S5C).

Although we found no HpARI_mCherry staining of live CMT-64 cells, binding was evident in freeze-thaw treated necrotic cells (Figure 7A), where it bound in the nucleus (Figure 7B). Surprisingly, we found HpARI_mCherry binds the nucleus independently of IL-33 expression, as similar staining could be seen in HEK293 cells (Figure 7C), from which no IL-33 could be detected (data not shown). As binding of HpARI in the nucleus of CMT-64 or HEK293 cells was ablated by addition of DNAse I (Figure 7C), we hypothesized that HpARI binds directly to DNA in the nucleus of necrotic epithelial cells.

In vivo, DNAse co-administration with *Alternaria* allergen abrogated HpARI suppression of IL-33 as measured by western blot, but not by ELISA, in the latter case presumably due to steric hindrance of ELISA antibodies on released HpARI-bound IL-33 (Figures 7D and 7E). We conclude that dual binding of DNA and IL-33 by HpARI results in retention of IL-33 within the necrotic cell nucleus, conferring a tethering function

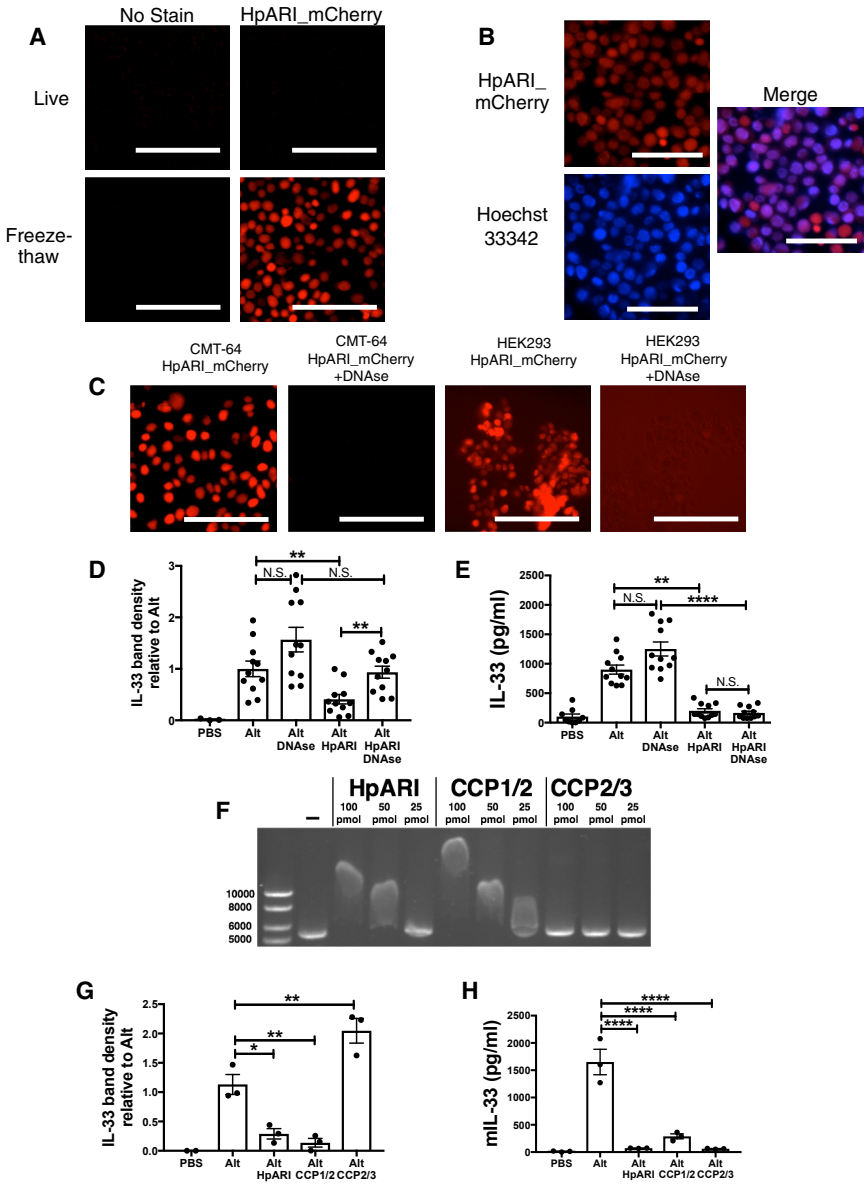

Figure 7. HpARI Binds Nuclear DNA, Tethering IL-33 within Necrotic Cells

(A) Live (top panels) or freeze-thawed (bottom panels) CMT-64 cells were incubated for 1 hr at 37°C with 5 μg/ml HpARI_mCherry.

(B) HpARI_mCherry-stained freeze-thawed CMT-64 cells, with Hoechst 33342 nuclear co-stain.

(C) Freeze-thawed CMT-64 or HEK293T cells were stained with HpARI_mCherry with 100 U/ml DNAse I.

(D) Murine IL-33 western blot densitometry of BAL taken 15 min after *Alternaria* allergen, HpARI and DNAse (100 U) intranasal administration.

(E) Murine IL-33 levels (ELISA) IL-33 in BAL fluid from mice described in (D)

(F) Gel shift assay of linearised plasmid DNA, incubated with 100, 50 or 25 pmol of HpARI, CCP1/2 or CCP2/3 truncated proteins.

(G) Murine IL-33 western blot densitometry of BAL taken 15 min after *Alternaria* allergen, HpARI or CCP1/2 or CCP2/3 HpARI truncated proteins intranasal administration.

(H) Murine IL-33 levels (ELISA) in BAL from mice described in (G).

All data representative of at least 2 repeat experiments. Data in (D) and (E) shows mean and SEM of 3 pooled experiments, data log-transformed for statistical analysis to equalize variances. Scale bars = 100 μm. Error bars show SEM.

on HpARI in addition to its ability to block IL-33 in the fluid phase.

Binding of DNA by HpARI was confirmed using a gel shift assay, in which addition of HpARI retarded the migration of linear plasmid DNA through an agarose gel in a concentration-dependent manner (Figure 7F), and by immunoprecipitation of plasmid DNA by HpARI (Figure S5D). We hypothesised that HpARI could bind DNA through electrostatic interactions, as shown for other CCP module-containing proteins (Sjöberg et al., 2007; Trouw et al., 2005). When the isoelectric point (pI) of each of the three CCP domains of HpARI were calculated, CCP2 and CCP3 were found to be relatively acidic (pI 6.32 and 5.34 respectively), while CCP1 was strongly basic (pI 9.79). Indeed, an electrostatic surface representation of our 3-D model of CCP1 (Figure S5E), reveals clusters of solvent-exposed positively charged residues that could serve as a binding site for oppositely-charged (acidic) DNA. We produced truncated versions of HpARI, either encoding CCP1/2 or CCP2/3. As predicted, we found that only the CCP1/2 truncation caused a shift in DNA migration (Figure 7F), supporting a role for CCP1 in binding to DNA.

In vivo, only the CCP1/2 HpARI truncation could inhibit the release of IL-33 as measured by western blot, while CCP2/3 actually increased total quantities of IL-33 detected in the BAL (Figure 7G). Both constructs suppressed IL-33 detection by ELISA (Figure 7H), indicating they could both bind IL-33 and inhibit binding of ELISA antibodies. Therefore we propose that CCP2/3 does not inhibit IL-33 release but instead binds it in solution, prevent it from being degraded or taken up via its receptor. This data supports a model by which HpARI binds to IL-33 through its CCP2 domain, and to DNA through its CCP1 domain, tethering IL-33 within the necrotic cell nucleus.

DISCUSSION

IL-33 has emerged as a critical initiator of allergic responses in diseases such as asthma, sparking an array of type 2 reactions in innate lymphoid cells, eosinophils, macrophages, and T cells (Liew et al., 2016). Through screening of the secreted products of a helminth parasite we identified HpARI, a CCP module-containing protein that inhibits IL-33 release. Recombinant HpARI is non-cell permeable, and can only gain access to the nucleus of necrotic cells, where it binds directly to IL-33 and nuclear DNA, tethering IL-33 within necrotic cells and preventing binding to

the IL-33R, thereby suppressing ILC2 responses and eosinophilia in the lung after *Alternaria* administration.

The primary mechanistic effect of HpARI is to bind IL-33: remarkably, this extends from murine to human IL-33. Although the affinity of HpARI for human IL-33 is lower than that of mouse IL-33, this binding is sufficient to prevent human IL-33 release, with a reduced IL-33 signal in human lung explant supernatants when cultured with HpARI, and reduced human IL-33 release in the lungs of human IL-33 transgenic mice. In the mouse, HpARI proved to be highly suppressive *in vivo*, recapitulating and exceeding the effects of total parasite secretions (HES), and able to inhibit IL-33 release even when administered 24 hr prior to allergen challenge.

Although it is clear that IL-33 is released at high levels during tissue injury and necrosis, it is presently unclear how IL-33 is secreted during homeostasis (Liew et al., 2016). We showed that HpARI was not able to penetrate intact cells thus, in the absence of cell membrane damage, HpARI would be unable to mediate the nuclear retention of IL-33. HpARI's unique mechanism of action and specificity provide an interesting tool to investigate the role of IL-33 as an alarmin—preventing the release of IL-33 from necrotic cells while leaving other responses (necrosis, HMGB1 or IL-1α release) unaffected. Recently, IL-33 production and release by activated mast cells in response to extracellular ATP release was demonstrated in *H. polygyrus* infection (Shimokawa et al., 2017), and extracellular ATP has previously been shown to induce IL-33 release in response to *Alternaria* administration (Kouzaki et al., 2011). These findings might explain the lack of total ablation of IL-33 release with HpARI administration, as some cytokine might be actively secreted by live mast cells, against which the tethering function of HpARI would be inactive, without exposed DNA in a necrotic, lysed cell. In this context, the role of *H. polygyrus* secreted apyrases (Hewitson et al., 2011)—enzymes which degrade extracellular ATP—might have a further role.

Binding to nuclear DNA allows HpARI to hold active IL-33 within the necrotic cell, and ablates allergic sensitization. Although the affinity for DNA was not determined in this study, evidence from gel shift and co-immunoprecipitation assays, as well as ablation of necrotic nuclear localization and IL-33 tethering function on DNAse treatment, strongly supports binding of HpARI to DNA. Truncated HpARI lacking CCP1 has no activity in the gel shift assay and lacks IL-33 tethering functionality, and molecular modeling of CCP1 revealed 2 exposed basic patches as putative DNA binding sites. Of note, the mammalian CCP domain-containing proteins C4b-binding protein (C4BP) (Trouw et al., 2005) and complement factor H (Leffler et al., 2010), also bind DNA through basic CCP modules. The importance of IL-33 localization to the nucleus has been shown in transgenic mice lacking the nuclear localization domain of IL-33, which develop lethal eosinophil-dominant multi-organ inflammation (Bessa et al., 2014), and in human endothelial cells, where extracellular IL-33 leads to inflammatory responses, while nuclear IL-33 does not (Gautier et al., 2016).

Three predicted CCP modules span the length of mature HpARI. CCP module-containing proteins are present in different phyla including chordates and nematodes, with notable expansion and diversification in parasitic species such as *H. polygyrus* (Hewitson et al., 2013). The functions of CCP modules are diverse, underlining the versatility of this structural scaffold that has evolved to serve many purposes (Kirkitadze and Barlow, 2001; Soares and Barlow, 2005; Soares et al., 2005). Of note, no non-host CCP module-containing protein has previously been shown to have immunomodulatory function outside of the complement system, and hence the co-option of this module by a parasite to block a mammalian immunological pathway is remarkable.

The suppression of the IL-33 pathway by *H. polygyrus* at the level of the IL-33 cytokine (mediated by HpARI) and the IL-33 receptor (mediated by secreted exosomes [Buck et al., 2014]) indicates that this pathway might be critical to persistence of the parasite. Indeed administration of exogenous IL-33 induces expulsion of *H. polygyrus* (Yang et al., 2013), while IL-33R-deficient mice are slow to expel this parasite even when immunized with a vaccine that induces sterile immunity in wild-type mice (Coakley et al., 2017). Similarly, in many helminth infections IL-33 administration can drive immunity, while deficiency of IL-33 or the IL-33 receptor leads to increased parasite load (Maizels and McSorley, 2016). Hence, the ability of *H. polygyrus* to pre-empt the IL-33 alarmin system is likely to be a pivotal evolutionary adaptation to allow establishment in the mammalian host.

HpARI administration suppressed the eosinophilic response to *N. brasiliensis* infection, leading to reduced ejection of adult parasites from the intestinal lumen, similarly to the phenotype seen in IL-33-deficient animals (Hung et al., 2013). Thus HpARI is capable of suppressing early innate anti-parasite immunity, a role we hypothesize it to play in the early stages of *H. polygyrus* infection where IL-33 is critical for resistance (Coakley et al., 2017).

During an *H. polygyrus* infection, larvae penetrate the gut wall, undergo two molts in the subserosal membrane, and emerge back into the lumen of the gut as adults (Maizels et al., 2012). As the parasite penetrates the intestinal wall, it damages epithelial cells which could result in the release of pre-formed IL-33 and induction of a parasite-toxic type 2 immune response. HpARI is secreted by the parasite larvae and adult (Hewitson et al., 2013) and so is well positioned to ablate this IL-33 response.

Recently, IL-33 was implicated in activation of intestinal Foxp3$^+$ regulatory T (Treg) cells (Schiering et al., 2014) raising the possibility that HpARI could interfere with Treg cell-mediated suppression. However, in mouse models of asthma, IL-33 signaling to IL-33R$^+$Foxp3$^+$ Treg cells results in their expression of Th2 cytokines, and abrogation of suppressive ability (Chen et al., 2017). Thus, in asthmatic responses at least, IL-33 appears to have an inflammatory, rather than suppressive effect.

In conclusion, we have identified a CCP module-containing protein with the unique ability to selectively bind to IL-33 and DNA within necrotic epithelial cells. This activity potently suppresses the release and the biological activity of IL-33, resulting in suppression of type 2 responses to allergen challenge. IL-33 is a critical mediator in allergic disease and an important clinical target. HpARI could be a potent agent for prevention of IL-33-mediated pathology, as well as a new tool for manipulation of IL-33 release, leading to better understanding of the IL-33 pathway.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one tables and can be found with this article online at https://doi.org/10.1016/j.immuni.2017.09.015.

AUTHOR CONTRIBUTIONS

M.O., F.V., E.S.C., I.C.S., W.F.G., M.T., A.M.K., D.J.S., H.H., A.G., C.E., and H.J.M. carried out the experiments. D.C.S., A.M.K., T.B., M.W., and A.C.I. carried out bioinformatic and structural analyses. A.M.K., K.J.F., J.P.H., and H.J.M. designed and carried out fractionation experiments. W.A.W. provided human tissue. E.S.C., I.C.S., S.V., A.L.A., J.S., R.M.M., and H.J.M. designed the experiments. M.O., D.C.S., E.S.C., I.C.S., R.M.M., and H.J.M. wrote the manuscript.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD3 (clone 145-2C11)	Biolegend	100306
Anti-mouse CD4 (clone RM4.5)	Biolegend	100566
Anti-mouse CD5 (clone 53-7.3)	Biolegend	100606
Anti-mouse CD11b (clone M1/70)	Biolegend	101224
Anti-mouse CD11c (clone N418)	Biolegend	117312
Anti-mouse CD19 (clone 6D5)	Biolegend	11506
Anti-mouse CD25 (clone PC61)	Biolegend	102038
Anti-mouse CD45 (clone 30-F11)	Biolegend	103128
Anti-mouse CD49b (clone DX5)	eBioscience	11-5971-85
Anti-mouse CD127 (clone A7R34)	Biolegend	135013
Anti-mouse ICOS (clone 15F9	eBioscience	46-9940-82
Anti-mouse GR1 (clone RB6-8C5)	Biolegend	108406
Anti-mouse IL-5 (clone TRFK5	Biolegend	504304
Anti-mouse IL-13 (clone eBio13A)	eBioscience	25-7133-82
Anti-mouse Ly6G (clone 1A8)	Biolegend	127616
Anti-mouse SiglecF (clone ES22-10D8)	Miltenyi	130-102-274
Anti-mouse ST2 (clone RMST2-2)	eBioscience	17-9335-82
Anti-mouse TER119 (clone TER-119)	Biolegend	116220
Anti-HMGB-1 rabbit polyclonal	Abcam	Ab18256
Anti-c-myc (clone Myc.A7)	Thermo Fisher Scientific	MA1-21316
Anti-human IL-33 goat polyclonal	R&D Systems	AF3625
Anti-mouse IL-33 goat polyclonal	R&D Systems	AF3626
Anti-mouse IL-1α	R&D Systems	AF-400-NA
IgG1 isotype control antibody (clone MOPC-21)	Produced in-house	N/A
Bacterial and Virus Strains		
Heligmosomoides polygyrus	(Johnston et al, 2015)	N/A
Nippostrongylus brasiliensis	(Lawrence et al, 1996)	N/A
Biological Samples		
Human lung tissue	Lothian NRS Bioresource	15/ES/0094
Chemicals, Peptides, and Recombinant Proteins		
Recombinant mouse IL-1α	Biolegend	575002
Recombinant mouse IL-33	Biolegend	580506
Recombinant human IL-33	Biolegend	581806
ST2-Fc	Biolegend	557904
Dynabeads Protein G	Thermo Fisher Scientific	10004D
Proteinase K	Sigma	557904
DNAse (protease-free)	Sigma	4536282001
Liberase TL	Sigma	05401020001
Methylcholine chloride	Sigma	A2251
Hoescht 33342	Thermo Fisher Scientific	H3570
Critical Commercial Assays		
Mouse IL-33 Duoset ELISA	R&D systems	DY3626
Human IL-33 Duoset ELISA	R&D systems	DY3625B

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Annexin V Apoptosis Detection Kit	eBioscience	88-8005-72
Limulus Amoebocyte Lysate assay	Lonza	QCL-1000
Experimental Models: Cell Lines		
HEK293T	ATCC	CRL-3216
CMT-64	ECACC	10032301
Experimental Models: Organisms/Strains		
Mouse: IL-13-eGFP (C57BL/6J)	(Neill et al., 2010)	N/A
Mouse: hIL-33$^{+/+}$ / mIL-33$^{-/-}$ (humanized IL-33) (BALB/c)	(Cohen et al., 2015)	N/A
Recombinant DNA		
pSecTAG2A plasmid	Thermo Fisher Scientific	V90020
Software and Algorithms		
ClustalX	(Thompson et al., 1997)	www.clustal.org
Mascot v2.4	Matrix Science	www.matrixscience.com
SMART	(Letunic et al., 2015)	smart.embl-heidelberg.de/
HHpred	(Söding, 2005)	toolkit.tuebingen.mpg.de/#/tools/hhpred
Modeller v9.12	(Sali and Blundell, 1993)	salilab.org/modeller/
APBS	(Baker et al, 2001)	www.poissonboltzmann.org/
ESPript v3	(Robert and Gouet, 2014)	espript.ibcp.fr/
PyMOL	Schrödinger, LLC	www.pymol.org
PROSITE	(de Castro et al., 2006)	prosite.expasy.org/
Protein Data Bank	(Berman et al., 2000)	www.rcsb.org/pdb
Wormbase ParaSite	(Howe et al., 2016)	parasite.wormbase.org/
FlowJo v9.1	Flowjo, LLC	www.flowjo.com/
Prism v7	Graphpad Software	www.graphpad.com/scientific-software/prism/
BIAcore T200 software v2.01	GE Healthcare	N/A
Other		
Superdex 200 10/300 GL	GE Healthcare	17517501
MonoQ 5/50 GL	GE Healthcare	17-5166-01
Series S Sensor Chip NTA	GE Healthcare	BR-1005-32

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and request for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Henry McSorley (henry.mcsorley@ed.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

BALB/cOlaHsd, C57BL/6JOlaHsd, IL-13-eGFP (C57BL/6 background) (Neill et al., 2010) and ST2-deficient (BALB/c background, kindly provided by Dr Andrew McKenzie, MRC Laboratory of Molecular Biology, Cambridge) mice, male or female (single sex within an experiment), 6-10 weeks old, were bred in-house at the University of Edinburgh. hIL-33$^{+/+}$, mIL-33$^{-/-}$ (humanised IL-33) transgenic mice (BALB/c background) (Cohen et al., 2015) were bred in-house at the Babraham Institute, Cambridge. All mice were accommodated, and procedures performed under UK Home Office licenses with institutional oversight performed by qualified veterinarians.

Human Tissue Samples

Non-cancerous adjacent tissue from lung cancer patients was collected by Lothian NRS Bioresource, and cultured as previously described (Cohen et al., 2015). The study was approved by Lothian NRS Bioresource (15/ES/0094) and tissue was donated with the informed consent of patients.

METHOD DETAILS

Parasite lifecycles, Infection, and HES Preparation

The life cycle of *H. polygyrus bakeri* was maintained, and HES products prepared, as previously described (Johnston et al., 2015). The life cycle of *N. brasiliensis* was maintained in Sprague-Dawley rats as previously described (Lawrence et al., 1996), and infective L3 larvae were prepared from 1-3 week rat fecal cultures. BALB/c mice were subcutaneously infected with 500 L3 *N. brasiliensis* larvae. At day 3 post-infection, larvae were counted in the bronchoalveolar lavage and in lung tissue, by dicing lungs and placing them in a cheese-cloth bag in a 50 ml tube containing PBS at 37°C for at least 3 h. Day 3 lung counts reflect a sum of the BAL and lung larval counts for each animal. At day 6 intestinal worms were recovered from intestinal tissue using an adapted Baermann apparatus.

Reagents

Alternaria alternata extract (Greer XPM1D3A25) was resuspended in PBS, filter sterilized and concentration assessed by BCA assay (Pierce). CMT-64 cells (ECACC 10032301) and HEK293T cells (ATCC CRL-3216) were maintained by serial passage in DMEM medium containing 10% fetal bovine serum, 2 mM L-glutamine and 1 μg ml^{-1} penicillin/streptomycin. Human and murine IL-33 and murine IL-1α were purchased from BioLegend.

In Vitro IL-33 Release Assay

HES, candidate proteins or HpARI were cultured with total murine lung cells prepared by Liberase/DNAse digestion of naïve mouse lungs or CMT-64 cells for 1 h at 37°C, 5% CO_2, with *Alternaria* allergen (200 μg ml^{-1}), or were frozen on dry ice, and thawed at 37°C.

Preparation of Murine Lung Single Cell Suspension

Single-cell suspensions of naïve murine lung tissue were prepared by digesting in 2 U ml^{-1} liberase TL (Roche, Burgess Hill, UK) and 80 U ml^{-1} DNase (Life Technologies, Paisley, UK) at 37°C with agitation for 35 min. Digested tissue was macerated through a 70 μm cell strainer (BD Biosciences), treated with red blood cells lysis buffer (Sigma), and live cells counted on a haemocytometer, excluding dead cells by trypan blue staining.

Cytokine Measurement

R&D Systems Duoset kits were used to measure human and murine IL-33 by ELISA, while western blotting was carried out using polyclonal goat anti-mouse IL-33, goat anti-human IL-33 or goat anti-mouse IL-1α (R&D Systems) with a rabbit anti-goat IgG HRP secondary antibody (Thermo Fisher), and detected using WesternSure Premium reagent (Licor).

Fractionation and Mass Spectrometry

HES was separated into 1 ml fractions by size exclusion chromatography using a Superdex 200 10/300 GL column, or by anion exchange chromatography using a MonoQ 5/50 GL column (GE Healthcare) in a 40 column volume gradient from 20 mM TrisHCl pH 8 (start buffer) to a maximum of 30% 20 mM TrisHCl + 1 M NaCl pH 8 (elution buffer). All fractions were trypsinized and analyzed by LC MS/MS on an on-line system consisting of a capillary-pump Agilent 1200 HPLC system (Agilent, UK) coupled to an Orbitrap XL mass spectrometer (Thermo Scientific) as previously described (Hewitson et al., 2011; Hewitson et al., 2013). LC MS/MS data was analyzed using Mascot (v2.4, Matrix Science) and searched against an improved in-house BLASTx annotated database obtained by 454 sequencing of *H. polygyrus* adults, with additional full length *H. polygyrus* sequences from NCBI, WormBase ParaSite (Howe et al., 2016) and our own Sanger sequencing (Harcus Y. et al, manuscript in preparation). Peptides identified were ranked by Mascot protein score, with a minimum cutoff score of 20, with a significance threshold of $p<0.05$. Protein abundance was estimated by emPAI (exponentially modified protein abundance index).

Protein Expression and Purification

Candidate genes were selected by comparison of emPAI and IL-33-suppression profiles in all fractions (Figures S1 and S2). Candidate genes A-D (Figure 2A, respectively Hp_I10793_IG03481_L623, Hp_I15874_IG07818_L1106, Hp_I46029_IG37973_L313 and Hp_I08176_IG02172_L1157 transcripts) were codon optimised for *Homo sapiens* and gene synthesised (GeneArt, Thermo Fisher) with 5' AscI and 3' NotI restriction enzyme sites. CCP1/2 (amino acids 17-165) and CCP2/3 (amino acids 80-251) constructs were created using PCR of codon-optimised HpARI, and primers which added a NotI site 3' of the CCP2 module (5'GCGGCCGCCTTGGGGCACACGCCCAG3', primes reverse of LGVCPK amino acid sequence, for CCP1/2 construct), or an AscI site 5' of the CCP2 module (5' 5'GGCGCGCCGGCTGCAAGGGCATCCTG3', primes GCKGIL amino acid sequence, for CCP2/3 construct), combined with vector-specific T7 (5' of insert) and BGH (3' of insert) primers. The HpARI_mCherry fusion protein was created by cloning in a codon-optimised gene-synthesised mCherry sequence (ANO45948.1) at the C-terminus of the HpARI protein, using an mCherry 5' NotI site and a 3' ApaI site. These constructs were sub-cloned into the pSecTAG2A expression vector (Thermo Fisher), using AscI, NotI-HF and Apa-1 restriction enzyme digestion (New England Biolabs), followed by T4 DNA ligation (Thermo Fisher).

JM109 cells were transformed with ligated constructs and plasmids were midiprepped using the PureLink HiPure midiprep kit (Thermo Fisher) according to manufacturer's instructions, and Sanger sequenced. Plasmid constructs were transfected into HEK293T cells using the calcium phosphate technique (Jordan et al., 1996), using 15 μg plasmid DNA per 100 mm tissue culture

dish of HEK293T cells at 20% confluency. Stable cell lines were maintained using Zeocin (Thermo Fisher) selection in DMEM medium containing 10% fetal bovine serum, 2 mM L-glutamine and 1 μg ml^{-1} Penicillin/Streptomycin.

Resulting expressed proteins secreted to the medium contained C-terminal myc and 6-His tags. For large scale expression of constructs, transfected cells were transferred to 293 SFM II media (Thermo Fisher) and protein purified from supernatant by nickel affinity chromatography using HiTrap chelating HP columns (GE Healthcare), eluting bound proteins using an imidazole gradient. Fractions containing pure expressed protein were pooled, dialysed into PBS, sterile filtered and concentration assessed by absorbance at 280 nm, corrected by calculated extinction coefficient.

Purified HpARI had an endotoxin content of below 0.5 U LPS per μg protein, as measured by the Limulus Amoebocyte Lysate assay (Lonza).

Bioinformatics Characterization and Modeling

Domain identification and assignment were undertaken using a combination of SMART (Letunic et al., 2015), an HHPred search against the pdb70 database (accessed March 2016) (Berman et al., 2000; Söding, 2005), and refined manually based upon positioning of the four Cysteine residues that typify CCP module sequences (Soares et al., 2005). PROSITE (de Castro et al., 2006) was used for short motif searches. ESPript v3 (Robert and Gouet, 2014) was used for alignment figure preparation.

The three predicted HpARI CCP module sequences were modeled based upon their top ranked CCP module template structure 'hits' as suggested by HHPred. HpARI-CCP1 was modeled based upon CR2-CCP2 (PDB ID: 1LY2) (Prota et al., 2002) (after a manual switch of Leu^{69} with Trp^{69} to help identify this CCP module using HHPred; note Leu/Trp substitutions exist in other experimentally-determined CCP module structures such as complement Factor H CCP10 and CCP20 (Makou et al., 2012; Morgan et al., 2012); HpARI-CCP2 on CSMD1-CCP3 (PDB ID: 2EHF) (RIKEN Structural Genomics/Proteomics Initiative); HpARI-CCP3 on GABABR1α-CCP2 (PDB ID: 1SRZ) (Blein et al., 2004). The target-template alignment in each case was based upon the initial HHPred alignment, then extended to include the first Cysteine residue in each domain, realigned using ClustalX (Thompson et al., 1997), and finally subjected to manual editing to optimally position known consensus residues, secondary structure elements and gaps (Soares et al., 2005). Note, an alternative alignment for the atypical insertion in CCP3 is possible where it can be accommodated after the hypervariable loop (not shown). A total of 100 models for each CCP module were built using Modeller v9.12 (Sali and Blundell, 1993), and the model with the lowest DOPE (Shen and Sali, 2006) energy score selected as the representative model in each case and evaluated for valid stereochemistry (Lovell et al., 2003). Electrostatic surface potential was calculated using APBS (Baker et al, 2001). PyMOL (http://www.pymol.org/; Schrödinger, LLC.) was used for visualization, and figure preparation.

Alternaria Models

Alternaria models, lung cell preparation, flow cytometry and lung histology were carried out as previously described (McSorley et al., 2014). *Alternaria* allergen (25 μg) was administered intranasally with 20 μg OVA protein (Sigma) and HpARI (10 μg). In some experiments, the OVA-specific response was recalled by daily intranasal administration of 20 μg OVA protein on days 14, 15 and 16. Mice were culled 15 min, 1 h, 24 h or 17 days after the initial administration, as indicated. Bronchoalveloar lavage was collected (4 lavages with 0.5 ml ice-cold PBS), followed by lung dissection for tissue digestion and single cell preparation (see below), or lungs were inflated with 10% neutral buffered formalin for histology. Formalin-fixed lungs were transferred into 70% ethanol 24 h after collection, paraffin, embedded and sectioned (5 μm), prior to staining with haemotoxylin and eosin (H&E) or Periodic Acid Schiff (PAS). H&E and PAS-stained sections were scored blindly according to the following criteria: H&E stain at 200X magnification on an increasing severity score of 1–4 in both the peri-vascular and peri-bronchiolar compartments (1 = <5, 2 = 5-20, 3 = 20-100, 4 = >100 cells), giving an average overall score of 5-10 fields of view per section. PAS stained sections were scored at 100X magnification, on percentage of mucous-positive epithelial cells (1 = <1%, 2 = 1-20%, 3 = 20-50%, 4 = 50-100%), of 5-10 fields of view per section.

Measurement of Airway Hyperresponsiveness

A Flexivent system (Scireq, Montreal, Canada) was used to measure dynamic resistance and compliance. Mice were anaesthetised with intraperitoneal ketamine 200 mg/kg and pentobarbitone (50 mg/kg), tracheotomised and mechanically ventillated. Lung resistance and compliance were measured in response to nebulised methacholine (Sigma).

Immunoprecipitation

Protein G dynabeads (Thermo Fisher) were coated with 5 μg anti-c-Myc (clone Myc.A7, Thermo Fisher), MOPC (IgG1 isotype control antibody) or ST2-Fc fusion protein (Biolegend), and washed on a DynaMag-2 magnet with PBS containing 0.02% Tween 20. These were then used to immunoprecipitate HpARI-IL-33 complexes, following manufacturer's instructions.

Where human or mouse lung homogenates were used, these were prepared by homogenizing (Tissuelyser II, QIAGEN) one lung lobe (mouse) in 1 ml PBS, or 400 mg human lung tissue in 1 ml PBS. Lung homogenates (100 ul) or 100 ng human or murine recombinant IL-33 (Biolegend) were then mixed with 1 μg HpARI in PBS containing 100 ug/ml OVA protein, and incubated for 30 min at 37°C. Complexes were then added to coated dynabeads, incubated for 10 min at room temperature, and unbound material collected. Bound material on beads was washed 3 times in PBS+0.02% PBS on a DynaMag-2 magnet, before transferring to a fresh tube and eluting bound complexes using 50 mM glycine pH 2.8 (non-denaturing), before neutralising in 1M Tris buffer, pH 8. Eluted proteins and unbound supernatants were ran on 4-12% SDS-PAGE gels (Thermo Fisher) under non-reducing conditions, and transferred to nitrocellulose membranes for western blotting.

Surface Plasmon Resonance (SPR)

SPR measurements were performed using a BIAcore T200 instrument (GE Healthcare). Ni^{2+}-nitrilotriacetic acid (NTA) sensor chips were purchased from GE Healthcare. HpARI was immobilised on an NTA sensor surface to 400 RU, which gave essential zero baseline drift over the time course of the experiments performed: the apparent k- for the His-tagged HpARI Ni^{2+}-NTA surfaces was significantly slower than the complex being studied $\sim 5 \times 10^{-5}$ s^{-1} for HpARI-Ni-NTA *vs* $\sim 14\text{-}400 \times 10^{-5}$ s^{-1} for HpARI-IL-33, therefore short cycle (400-600 s total run times) single kinetic analysis could be reliably performed. Following Ni^{2+} priming (30 sec injection of 500 μM $NiCl_2$ at 5 $\mu l \cdot min^{-1}$) 50 nM HpARI, in 10 mM NaH_2PO_4, pH 7.5; 150 mM NaCl; 50 μM EDTA; 0.05% surfactant P20, was captured *via* the 6-His tag by injection for 15 seconds, at 30 $\mu l \cdot min^{-1}$. Surface regeneration between cycles and/or experiments was performed by dissociating any immobilised His-tagged protein or complex by a 90 s injection of 350 mM EDTA, in 10 mM NaH_2PO_4, pH 7.5; 150 mM NaCl; 0.05% surfactant P20 followed by a 30 s injection of 50 mM NaOH at the same flow rate.

SPR kinetic titration binding experiments were performed at 25°C. Three-fold dilution series of mIL-33 (6.2 nM to 167 nM) or hIL-33 (0.062 μM to 1.67 μM), were injected over the sensor surface, in 10 mM NaH_2PO_4, pH 7.5; 150 mM NaCl; 50 μM EDTA; 0.05% surfactant P20, at 30 $\mu l.min^{-1}$ for 30 s followed by a 60 s dissociation phase. The same concentration series of mIL-33/hIL-33 were ran over Ni^{2+}-charged NTA surfaces, and showed no evidence of non-specific interaction of mIL-33/hIL-33 interacting with these surfaces. All experiments were performed on Ni^{2+}-charged surfaces following non-specific binding assessment and were double referenced using similar blank surface responses for run-noise corrections. The on- (k_+) and off-rate (k_-) constants and the equilibrium dissociation constant (KD) were calculated by global fitting all three surfaces simultaneously to a 1:1 interaction model, with mass transport considerations, to the double reference corrected sensorgrams, using analysis software (v.2.01, GE Healthcare) provided with the BIAcore T200 instrument.

Both interactions were extremely well fit by a simple 1:1 interaction model (Chi^2 values of 0.457 and 0.395, mIL-33 and hIL-33 respectively), with RUmax values close to the theoretical maximum expected for a 1:1 stoichiometric interaction with high specific activity (~ 180 RU; 173 RU and 169.3 RU, mIL-33 and hIL-33 respectively) and showed no evidence of mass transport issues.

Human Lung Explant Culture

Approximately 5 g of lung tissue was washed 3 times in PBS and ~0.5 mm^2 tissue explants prepared using sterilized scissors. Explants were incubated in 400 μl PBS+0.1% BSA +/- 10 μg/ml HpARI in wells of a 48-well tissue culture plate (Costar) for 1 h, at 37°C, 5% CO_2. Each condition was performed with 8 replicates for IL-33 measurement by ELISA, and pairs of supernatants were pooled (to make 4 replicates) for IL-33 western blot. After culture, tissue pieces were weighed, and IL-33 levels calculated relative to tissue weight.

Gel Shift Assay

Linearized Not-HF-cut pSecTAG2A plasmid (10 ng) was mixed with HpARI, CCP1/2 and CCP2/3 proteins, in 10 mM TrisCl, 1 mM EDTA, and incubated for 30 min at 37°C. Complexes were ran on a 0.7% agarose gel and imaged with Gelred (Biotium).

QUANTIFICATION AND STATISTICAL ANALYSIS

All data was analyzed using Prism (Graphpad Software Inc.). Where two groups were compared, Student's t-test was used, where there were 3 or more groups, one-way ANOVA with a Bonferroni's post test was used, and for comparing groups at multiple timepoints two-way ANOVA with a Sidak's post test was used. **** = $p<0.0001$, *** = $p<0.001$, ** = $p<0.01$, * = $p<0.05$, N.S. = Not Significant ($p>0.05$).

DATA AND SOFTWARE AVAILABILITY

The accession number for the HpARI transcript sequence as reported in this paper is Wormbase Parasite: HPBE_0000813301.