# Properties and function of *Heligmosomoides polygyrus* secreted apyrases

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A thesis submitted for the degree of Doctor of Philosophy (PhD)

## Declaration of Originality

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## Funding

This PhD was funded via a scholarship by Association Philippe Jabre (APJ) – Beirut, Lebanon. I also received a travel award from Imperial College London to attend conferences during the time of my PhD. I would like to thank all of these bodies for their financial support.

## Abstract

Helminth parasite secreted molecules have been shown to modulate the host immune system to the extent of alleviating symptoms of immune disorders such as allergy and autoimmunity. Apyrases secreted by helminth parasites have potential immunoregulatory functions. They constitute a family of nucleotide-metabolising enzymes which can disturb purinergic signalling pathways of immune cells via hydrolysing inflammatory ATP released following tissue damage. In this thesis, the biochemical properties of the five apyrases secreted by the intestinal nematode *Heligmosomoides polygyrus* were elucidated via heterologous expression in the yeast *Pichia pastoris.* Results showed that the enzymes belonged to a group of calcium-dependent apyrases with a broad optimum pH and a broad substrate specificity, catalysing the hydrolysis of both nucleoside tri- and diphosphates. In an attempt to understand if any immune modulation was displayed by apyrases, *in vivo* studies were performed. Apyrase-1 and -3 were expressed in *Trypanosoma musculi*, a suitable *in vivo* vehicle for the expression of genes encoding secreted proteins of nematode parasites. Among the results shown, the transgenes grew faster compared to control trypanosomes, and splenocytes from mice infected with *T. musculi* expressing Apy-3 produced higher levels of IL-5 and IL-13. Both immunological and physiological factors appear to be responsible for these changes, suggesting that apyrases might modulate the immune response in addition to influencing the availability of extracellular purines for salvage by parasites. The effect of *H. polygyrus* secreted apyrases on type 2 immunity was also examined in this thesis during an acute model of allergic inflammation and during nematode infection. Intranasal administration of recombinant Apy-1 and Apy-3 did not seem to have an effect in regulating immunological responses, at least in the models tested. Further work is required to probe the precise function of apyrases secreted by parasitic nematodes and the possible immune modulatory effects exerted by these enzymes.

## Acknowledgments

I would like to express my acknowledgements to my supervisors Niki Gounaris and Murray Selkirk and to the Selkirk-Gounaris Lab who have assisted me in the accomplishment of this PhD thesis and whom I have had the great pleasure to work with. I am indebted to Niki and Murray who have provided me with professional and personal guidance in this research project. Thank you for being very supportive and patient and for your assistance at all levels in spite of your academic and professional commitments. You have shown me what a good researcher should be and have taught me more than I could ever give you credit for here.

I also would like to acknowledge the most amazing colleagues I have been working with: Corinna Schnoeller, Rachel Vaux and Luke Roberts who have been actively involved in this thesis work. With your great support, help, guidance, and friendship you helped me to become who I am today. I owe you so much! Acknowledgements also go to the rest of the lab in particular Jana Hagen. During my PhD, I had the opportunity to work within Rick Maizels lab at University of Glasgow. A huge thanks goes to everyone, particularly to Danielle Smyth who provided me with lots of advice and help for which I am very grateful. Additionally, I would like to thank everyone in the Southall Lab, including Tony Southall, Alicia Estacio Gomez, Amira Hassan, Colin McClure, Gabriel Aughey and Emma Walmsley for their incredible support, kindness and friendship.

A huge thanks has to go to my friends away from work: Joelle Jerjess, Christina Howayek, Stephanie Harb, Tracy Matta, and the sisters of Religious Mary Immaculate especially Sister Carmen. Through your loyal friendship and love, you have supported me and encouraged me in my darkest moments and helped me to accomplish this thesis.

An endless gratitude goes to my family who provided me with unending encouragement, who always believed in me and supported me in whatever I undertake in my life and who have always been there for me. My parents Atiye and Tony and my brothers Hany and George, your love and support mean a lot to me. Thank you from the bottom of my heart!

Last but not least, I am thankful to God, Saint Charbel and Saint Rita for walking with me during my years of study and life, and for giving me the strength to hold on every time I gave up. Thank you!

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# List of Abbreviations

| Abbreviation | Full term |
|---|---|
| AAM | Alternatively activated macrophage |
| ACK | Ammonium chloride potassium lysis buffer |
| ACR | Apyrase conserved region |
| ADA | Adenosine deaminase |
| ADP | Adenosine diphosphate |
| ADK | Adenosine kinase |
| ALT | Allergen extract from *Alternaria alternata* |
| AMP | Adenosine 5´-monophosphate |
| APC | Antigen presenting cell |
| ApE | A plasmid Editor |
| Apy | Apyrase |
| Apy-1a | Recombinant enzymatically active Apy-1 |
| Apy-1i | Recombinant enzymatically inactive Apy-1 |
| AR | Adenosine receptor |
| Arg1 | Arginase 1 |
| ATP | Adenosine triphosphate |
| B2m | Beta-2 microglobulin |
| BAL | Bronchoalveolar lavage |
| BALF | Bronchoalveolar lavage fluid |
| BiP | Binding protein |
| BMGY | Buffered complex glycerol medium |
| BMMY | Buffered complex methanol medium |
| BSA | Bovine serum albumin |
| cDNA | Complementary DNA |
| CDP | Cytidine diphosphate |
| Chi3l3 | Chitinase-like 3/YM1 |
| CNS | Central nervous system |
| CNT | Concentrative nucleoside transporter |

| | |
|---|---|
| COPD | Chronic obstructive pulmonary disease |
| Cx | Connexin |
| DAMP | Danger-associate molecular pattern |
| DC | Dendritic cell |
| DEAE | Diethylaminoethyl |
| $dH_2O$ | Distilled water |
| DIDS | 4,40-diisothiocyanatostilbene 2,20-disulfonic acid |
| DMEM | Dulbecco's Modified Eagle's Minimal Essential Medium |
| DNA | Deoxyribonucleic acid |
| dsRNA | Double-stranded RNA |
| DTT | Dithiothreitol |
| ecto-NTPDase or ENTPDase | Ecto-nucleoside triphosphate diphosphohydrolase |
| EDTA | Ethylene diamine tetraacetic acid |
| EGTA | Ethylene glycol tetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ENT | Equilibrative nucleoside transporter |
| epg | Eggs per gram |
| ER | Endoplasmic reticulum |
| ES | Excretory-secretory |
| FACS | Fluorescence activated cell sorting |
| FCS | Foetal calf serum |
| Foxp3 | Forkhead box P3 |
| FSC | Forward scatter |
| gDNA | Genomic DNA |
| GDP | Guanosine diphosphate |
| GFP | Green fluorescent protein |
| GMP | Guanosine monophosphate |
| GTP | Guanosine triphosphate |
| HDL | High-density lipoprotein |
| HpARI | *H. polygyrus* Alarmin Release Inhibitor |
| HEPES | 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid |

| | |
|---|---|
| HMBS | Hydroxy-methylbilane synthase |
| HPRT | Hypoxanthine guanine phosphoribosyl transferase |
| Hp-TGM | *H. polygyrus* TGFβ mimic |
| HRP | Horseradish peroxidase |
| HSP | Heat shock proteins |
| IBD | Inflammatory bowel disease |
| ICOS | Inducible co-stimulator |
| IFNγ | Interferon γ |
| Ig | Immunoglobulin |
| IG | Intergenic |
| IL | Interleukin |
| ILC | Innate lymphoid cell |
| ILC2 | Type 2 innate lymphoid cell |
| iILC2 | Inflammatory ILC2 |
| IMP | Inosine monophosphate |
| i.n. | Intranasally |
| iNOS | Inducible nitric oxide synthase |
| kDa | Kilodalton |
| kDNA | Kinetoplast DNA |
| KLRG1 | Killer-cell lectin like receptor G1 |
| L | Larvae |
| LB | Luria Broth |
| LPS | Lipopolysaccharide |
| Luc | Luciferase |
| mApy | Mutant apyrase |
| MFI | Geometric mean fluorescence intensity |
| MHC | Major histocompatibility complex |
| MIF | Macrophage inhibitory factor |
| Mrc1 | Mannose receptor C-type-1 |
| mRNA | Messenger RNA |
| MS | Multiple sclerosis |

| | |
|---|---|
| MWCO | Molecular weight cut-off |
| NADH | Nicotinamide adenine dinucleotide (NAD) + hydrogen (H) |
| NFW | Nuclease-free water |
| NIF | Neutrophil inhibitory factor |
| Ni-NTA | Nickel nitrilotriacetic acid |
| nILC2 | Natural ILC2 |
| NK | Natural killer |
| NO | Nitric oxide |
| NOS2 | nitric oxide synthase-2 |
| NSPs | Novel secreted proteins |
| PAMP | Pathogen-associated molecular pattern |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PFR | Paraflagellar rod |
| p.i. | Post-infection |
| $P_i$ | Inorganic phosphate |
| PKA | protein Kinase A |
| PMA | Phorbol myristate acetate |
| PSG | Phosphate saline glucose |
| PVDF | Polyvinylidene fluoride |
| Px | Pannexin |
| qPCR | Quantitative PCR |
| RA | Rheumatoid arthritis |
| RELMα | Resistin-like molecule-alpha |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| ROS | Reactive oxygen species |
| RPMI 1640 medium | Roswell park memorial institute 1640 |
| RT | Reverse transcriptase |
| RT-PCR | Reverse transcription PCR |
| s.c. | Sub-cutaneous |

| | |
|---|---|
| SDS-PAGE | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis |
| siRNA | Small interfering RNA |
| SL RNA | spliced leader RNA |
| SOCS1 | Suppressor of cytokine signaling-1 |
| SSC | Sideways scatter |
| SSU rRNA | Small subunit of ribosomal RNA |
| ST2 | Suppression of tumorigenicity 2 |
| TBS-T | Tris-buffered saline-Tween |
| TCR | T cell receptor |
| TGFβ | Transforming growth factor β |
| Th | T helper |
| Tm | *Trypanosoma musculi* |
| TNFα | Tumour necrosis factor α |
| Treg | Regulatory T cell |
| TLR | Toll-like receptor |
| TSLP | Thymic stromal lymphopoeitin |
| UTP | Uridine triphosphate |
| UDP | Uridine diphosphate |
| VAL | Venom-allergen-like |
| WT | Wild type |
| YPD | Yeast extract peptone dextrose |
| YPDS | Yeast extract peptone dextrose sorbitol |
| 5´-NT | 5´-nucleotidase |

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# CHAPTER 1

# General Introduction

## 1.1 Helminth parasites

### Helminth parasite infections

Almost one third of the global human and livestock population are infected with one or more helminth species occurring mostly in the poor areas of the developing world (Hotez *et al.*, 2008; WHO, 2011; Savioli, 2012). Helminths have a significant impact on economy and health. They impose widespread agricultural losses and a considerable disease burden associated with diverse pathologies ranging from malnutrition to impaired growth and anaemia resulting in severe morbidity (Hotez *et al.*, 2004, 2008; Brooker, Hotez and Bundy, 2008; Sanchez *et al.*, 2013; Gyorkos and Gilbert, 2014; Jourdan *et al.*, 2017; Sotillo *et al.*, 2017; Wright *et al.*, 2018).

Helminths, derived from the Greek word "helmins" meaning worms, are large multicellular eukaryotic invertebrates that were either parasitic or free-living throughout evolutionary history. The two major phyla that have received the greatest amount of interest in research over the past decade are: Nematoda and Platyhelminths. Nematodes also known as roundworms, include the intestinal worms or soil-transmitted helminths (STH) (i.e. *Trichuris trichiura*, *Necator americanus* and *Ancylostoma duodenale*), and the filarial worms that cause lymphatic filariasis (LF) and onchocerciasis (Faust, Russell and Jung, 1970; Castro, 1996). Molecular phylogenetic analysis arranged this phylum into five major clades (Clade I, II, III, IV and V) (Blaxter *et al.*, 1998; Blaxter and Koutsovoulos, 2015), all of which include about 25,000 described species (Zhang, 2013). Platyhelminths or flatworms include the trematodes (flukes) such as schistosome species that cause schistosomiasis, and

cestodes (tapeworms) like *Taenia* and *Echinococcus* (Faust, Russell and Jung, 1970; Castro, 1996). The Acanthocephala (thorny-headed worms) which have an evolutionary relationship in-between cestodes and nematodes, and the Annelida (segmented worms) are another two phyla which are also thought to belong to helminths.

Parasitic helminths reside in their hosts for substantial periods of time, secreting a range of molecules that facilitate their migratory paths to reach the final destination (Sotillo *et al.*, 2017). They have adapted to colonise diverse niches such as the gastrointestinal tract, bloodstream, lymphatic system, liver, lungs or subcutaneous tissues. The developmental process of these organisms comprises egg, larval and adult stages, however their life cycles and routes of entry vary. For instance, the infection of the hookworms *Necator americanus* or *Nippostrongylus brasiliensis* is acquired through penetration of the skin by third stage larvae (L3) which then migrate through the bloodstream to the lungs. Adult worms develop in the small intestine after the L4 larvae are coughed up and swallowed. The eggs released in the faeces, hatch and develop into infective larvae (Ogilvie and Jones, 1971; Brooker, Bethony and Hotez, 2004; Brooker and Bundy, 2013). Other gastrointestinal worms, such as *Heligmosomoides polygyrus,* are transmitted orally by ingesting infective larvae (Camberis, Le Gros and Urban, 2003).

**Immune response against parasitic helminths**

The host's immune response generated against all helminth infections comprise both innate and adaptive immunity regardless of the various colonisation sites of helminth species

(Maizels and Yazdanbakhsh, 2003; Anthony *et al.*, 2007; Saenz, Noti and Artis, 2010; Harris and Loke, 2017). This immune response is predominantly characterised by a type 2 arm with T helper 2 (Th2) cells and innate lymphoid type 2 cells (ILC2s) as key players. It is generally associated with an elevated secretion of several protective cytokines resulting in the activation of specialised effector cells such as mast cells, eosinophils and basophils, as well as the expansion and activation of alternatively activated macrophages (Figure 1.1A) (Hagan *et al.*, 1991; Finkelman *et al.*, 2004; Jackson *et al.*, 2004; Fallon *et al.*, 2006; Turner *et al.*, 2008; Sallusto and Lanzavecchia, 2009; Moro *et al.*, 2010; Neill *et al.*, 2010a; Price *et al.*, 2010; Yasuda *et al.*, 2012).

During helminth infections, epithelial cells and other cells provoke the release of alarmin cytokines IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) (Owyang *et al.*, 2006; Zaph *et al.*, 2007; Saenz, Taylor and Artis, 2008; Massacand *et al.*, 2009; Taylor *et al.*, 2009; Saenz, Noti and Artis, 2010; Hepworth *et al.*, 2012; Allen and Sutherland, 2014; Cayrol and Girard, 2014; Shimokawa *et al.*, 2017). IL-33, a member of the IL-1 family (Schmitz *et al.*, 2005; Arend, Palmer and Gabay, 2008), binds to its receptor ST2 (suppression of tumorigenicity 2) resulting in the activation of a wide range of immune cells and inducing the production of an array of type 2 cytokines including IL-4, IL-5 and IL-13 by Th2 cells, basophils, mast cells and ILC2s (Schmitz *et al.*, 2005; Ho *et al.*, 2007; Humphreys *et al.*, 2008; Kondo *et al.*, 2008; Voehringer, 2009; Liew, 2012; Walker and McKenzie, 2013; Hung *et al.*, 2013; Scalfone *et al.*, 2013; Cayrol and Girard, 2014; Liew, Girard and Turnquist, 2016). Th2 cytokines produced by ILC2s and other cells trigger the expulsion of most helminths and mediate resistance to re-infection (Maizels, Hewitson and Smith, 2012; Medzhitov, Schneider and Soares, 2012; Grencis, 2015).

Expansion of Th2 cells induces eosinophilia via IL-5 (Yasuda *et al.*, 2012), and promotes B cell isotype switching to immunoglobulin (Ig)E and IgG1 (mice) or IgG4 (humans) by IL-4 and IL-13 (Harris and Gause, 2011). The latter can also increase smooth muscle contractility, epithelial cell electrolyte secretion and mucus production, resulting in the clearance of most intestinal parasites (Urban *et al.*, 1998; Akiho *et al.*, 2002; Finkelman *et al.*, 2004; Cliffe *et al.*, 2005; Hasnain *et al.*, 2011, 2013). Through the activation of FcεR receptor, IgE triggers the degranulation of basophils, eosinophils and mast cells, causing the release of IL-4, IL-13, TGFβ, and inflammatory mediators like histamine and leukotrienes (Stone, Prussin and Metcalfe, 2010; Warrington *et al.*, 2011).

When macrophages are exposed to IL-4, IL-13 and IL-33 (Mantovani, Sica and Locati, 2005; Jackson-Jones *et al.*, 2016), they are polarised towards an alternatively activated macrophage phenotype (AAM or M2) (Chen *et al.*, 2012; Du *et al.*, 2014) involved in wound healing and helminth killing (Flores *et al.*, 1994; Herbert *et al.*, 2004; Kreider *et al.*, 2007; Koh and DiPietro, 2011; Mantovani *et al.*, 2013). AAMs release the chitinase-like protein Ym1 that elicits the recruitment of neutrophils and the production of IL-17 from γδT cells (Sutherland *et al.*, 2014), together with resistin-like molecule (Relmα) and programmed death ligand 2 (PD-L2) which regulate Th2 responses (Herbert *et al.*, 2009; Pesce *et al.*, 2009; Huber *et al.*, 2010; van der Werf *et al.*, 2013). It was also shown that IL-33 induces ILC2s to produce IL-9 which in turns stimulates mast cells to secrete IL-2, leading to further ILC2 expansion (Mohapatra *et al.*, 2016; Moretti *et al.*, 2017).

Furthermore, recent studies investigated the role of tuft cells following helminth infections and showed that they are important cells in driving mucosal type 2 responses and

worm expulsion. Tuft cells secrete IL-25, a member of the IL-17 family, which activates IL-13 production by ILC2s, further promoting the differentiation of tuft and goblet cells and amplifying a positive-feedback loop of type-2-cell-mediated responses (Gerbe, Legraverend and Jay, 2012; Gerbe *et al.*, 2016; Von Moltke *et al.*, 2016). On the other hand, TSLP, an IL-7-like cytokine (Park *et al.*, 2000), was shown to induce a Th2 cytokine response via the activation of B cells and dendritic cells (DCs) (Leonard, 2002) and the differentiation of CD4 Th2 cells (Zaph *et al.*, 2007; Massacand *et al.*, 2009; Taylor *et al.*, 2009; Ziegler and Artis, 2010; Kim *et al.*, 2013).

## 1.2 Therapeutic benefits of helminth products

### The Yin-Yang of helminth infections

In Western society, the reduced exposure to certain infectious agents (such as helminths) during early age and therefore the early stages of immune development, was noticed to be associated with an expansion of allergic and autoimmune diseases; whereas in the presence of helminth infections, especially in developing countries, a lower incidence of immunopathological disorders was observed. This inverse relationship, termed 'Hygiene Hypothesis', was supported by a large number of studies and is believed to be caused by molecules secreted by helminths (Greenwood, 1968; Strachan, 1989; Wills-Karp, Santeliz and Karp, 2001; Yazdanbakhsh, Kremsner and Van Ree, 2002; Bloomfield *et al.*, 2006; Zandman-Goddard and Shoenfeld, 2009; Hewitson, Grainger and Maizels, 2009; Okada *et*

*al.*, 2010; Mustonen *et al.*, 2013; Parker and Ollerton, 2013; Prokopakis *et al.*, 2013; Maizels, Mcsorley and Smyth, 2014; Helmby, 2015; Villeneuve *et al.*, 2018; Haspeslagh *et al.*, 2018).

Helminths and vertebrates have co-evolved over millions of years, with parasites developing complex mechanisms to suppress the host immune response from the early stages of infection. They promote long-term survival enabling protective pathways to maintain their feeding, life cycle completion, and successful reproduction. It happens that both the host and the parasite benefit from this stealthy strategy: first it allows the parasite to evade the host immune system preventing it from being killed or expelled; and in doing so it also inhibits responses to unrelated antigens in allergy and autoimmunity (Allen and Maizels, 2011; Smallwood *et al.*, 2017; Maruszewska-Cheruiyot, Donskow-Lysoniewska and Doligalska, 2018). The questions raised by this process are: how do helminths evade the host immune system, what mechanisms do they employ to limit inflammation, and do parasitic worms provide new insights into drug-based therapies protecting against inflammation and autoimmune diseases?

Using live pathogens as clinical treatments is probably not feasible on a large scale and has several disadvantages, thus an alternative to helminth therapy is the identification and characterisation of individual molecules in the complex mixture of secreted products and that might be applied to treat inflammatory diseases. A substantial amount of human epidemiological studies coupled with mouse models of pathological disorders examined the effects of different helminth infections. They confirmed that helminth parasites secrete a wide range of molecules which can act as immunomodulators and hold potential for future human therapy in the prevention or suppression of immune-mediated diseases such as

allergy, autoimmunity and colitis (Erb, 2009; Hewitson, Grainger and Maizels, 2009; Baqueiro *et al.*, 2010; Harnett and Harnett, 2010; Allen and Maizels, 2011; Brenna *et al.*, 2013; Viehmann Milam *et al.*, 2014; Harnett, 2014; Johnston *et al.*, 2014; Shepherd *et al.*, 2015; Bashi *et al.*, 2015; Navarro *et al.*, 2016; Nascimento Santos *et al.*, 2017; Caraballo, 2018).

**Immunoregulatory effects**

Given that helminth excretory/secretory (ES) products have been shown to modulate the host immune system in a protective way, it is essential to investigate which molecules are immunoregulators. A number of studies have identified these proteins using various techniques including genomics, transcriptomics and mass spectrometry, and characterised them by expressing their active forms in suitable expression systems such as bacteria (*Escherichia coli*), yeast (*Pichia pastoris*), protozoa (*Trypanosoma musculi*), insect cells and mammalian cells (Frenzel, Hust and Schirrmann, 2013).

For instance, one of the extensively studied helminth molecules is the glycoprotein ES-62 derived from the filarial nematode *Acanthocheilonema viteae* (Harnett, Harnett and Byron, 2003), which showed modulatory effects in a range of inflammatory models including arthritis (Harnett, Melendez and Harnett, 2010; Pineda *et al.*, 2012; Al-Riyami *et al.*, 2013; Rzepecka *et al.*, 2013; Harnett, Harnett and Pineda, 2014; Coltherd *et al.*, 2016; Janicova *et al.*, 2016; Lumb *et al.*, 2017). Additionally, the recombinant cystatin, a cysteine protease inhibitor, and secreted cathepsins which belong to the cysteine protease family, have been

reported to suppress allergic airway inflammation and colitis (Carmona *et al.*, 1993; Stack *et al.*, 2005; Donnelly *et al.*, 2010; Smooker *et al.*, 2010; Jang *et al.*, 2011; Kang *et al.*, 2011; Sun *et al.*, 2013; Daniłowicz-Luebert *et al.*, 2013; Ji *et al.*, 2014; Ziegler *et al.*, 2015; Coronado *et al.*, 2016; Wang *et al.*, 2016; Coronado *et al.*, 2017; Venugopal *et al.*, 2017). Interestingly, helminth parasites also secrete molecules that can interrupt immune cell signalling pathways, such as purinergic and cholinergic signalling disrupted by apyrase and acetylcholinesterase respectively (Ogilvie *et al.*, 1973; Nisbet *et al.*, 2011; Darby *et al.*, 2015). Other helminth-derived molecules have been studied and was shown to alter intestinal and airway inflammation, like the *S. mansoni* egg-derived glycoprotein ω1, macrophage migration inhibitory factor (MIF), heat shock proteins (HSP), neutrophil inhibitory factor (NIF), and many more reviewed in depth previously (Khan and Fallon, 2013; Maizels and McSorley, 2016; Harnett and Harnett, 2017; Nascimento Santos *et al.*, 2017; Kahl, Brattig and Liebau, 2018; Schwartz, Hams and Fallon, 2018). Parasitic helminths are able to secrete not only proteins but also glycans, small-molecules and metabolites, short-chain fatty acids, as well as exosomes (extracellular vesicles) containing immunomodulatory microRNAs (Buck *et al.*, 2014; Shepherd *et al.*, 2015; Siles-Lucas *et al.*, 2015; Cai, Gobert and McManus, 2016; Entwistle and Wilson, 2017).

These products exert their effect in different ways modifying or suppressing Th2 immune response induced to clear helminth infections (Correale, Farez and Razzitte, 2008; Taylor *et al.*, 2009; Amu *et al.*, 2010; Hussaarts *et al.*, 2011; Khan *et al.*, 2015; Yang, Seoh and Jang, 2017). The control of Th2 responses by helminth parasites is mediated by several mechanisms including a reduction in IgE, IL-4 and IL-5 levels as well as eosinophilia, and an inhibition of the pro-inflammatory cytokines IL-2, interferon (IFN)-γ, and IL-17 (Schnoeller *et*

*al.*, 2008; Kim *et al.*, 2010; Ji *et al.*, 2014). Furthermore, some molecules are able to stimulate the generation of regulatory T cells (Tregs) (Layland *et al.*, 2013), resulting in the production of anti-inflammatory cytokines, particularly IL-10 and transforming growth factor (TGF)-β (Figure 1.1B) (Shiny *et al.*, 2011; Jang and Nair, 2013; Finlay, Walsh and Mills, 2014). Studies have also shown that *H. polygyrus* secreted products can block the release of alarmin cytokine IL-33 from epithelial cells in response to fungal allergen (McSorley *et al.*, 2014). Other helminths including *Nippostrongylus brasiliensis* are able to inhibit IL-12 production from dendritic cells suppressing proinflammatory Th1 immune responses (Balic *et al.*, 2004; Segura *et al.*, 2007; Massacand *et al.*, 2009; Donnelly *et al.*, 2010). These diverse regulatory pathways enforced by parasites or their products not only allow helminths to establish chronic infections, but also promote a wound repair function limiting host tissue damage (Bashi *et al.*, 2015; Maizels and McSorley, 2016). Collectively, helminth secreted products may lead to the discovery and generation of new drugs treating immunopathologies, thus improving life span.

**Immune-related disorders versus parasitic helminths**

When functioning properly, the immune system protects humans from pathogens (bacteria, virus or parasite) and other invaders, maintaining a state of homeostasis through a cascade of reactions, the so-called immune response. However, in the presence of immune dysregulation, uncontrolled inflammation could lead to an increased susceptibility to chronic pathologies causing inflammatory and autoimmune diseases. Interestingly, and as mentioned earlier, helminths induce a strong type 2 immune response. However, through

their regulatory mechanisms, parasites have developed the ability to target Th2- and Th1/Th17-driven inflammation, thus constraining the symptoms associated with allergy and autoimmunity (Wilson *et al.*, 2005; Mangan *et al.*, 2006; Schnoeller *et al.*, 2008; Kobayashi *et al.*, 2009; Amu *et al.*, 2010; Correale and Farez, 2011; Cosnes *et al.*, 2011; Kondrashova *et al.*, 2013; Finlay, Walsh and Mills, 2014; Lambrecht and Hammad, 2017; Schwartz, Hams and Fallon, 2018).

Asthma (airway inflammatory disease) and allergic rhinitis (hay fever) are characterised by a Th2 response associated with high levels of IL-4, IL-5, IL-13 and IgE, in addition to infiltration of eosinophils (Figure 1.1C) (Poole and Rosenwasser, 2005; Kim, Dekruyff and Umetsu, 2010). The protective effects against allergy evoked by some parasitic helminths are mediated by a modified Th2 type response, suppressing the secretion of pro-inflammatory cytokines and switching of IgE to IgG4, leading to high levels of IL-10 and TGFβ, as well as Tregs (Hussain, Poindexter and Ottesen, 1992; Akdis *et al.*, 1998; Van Den Biggelaar *et al.*, 2000; Bashir *et al.*, 2002; Francis, Till and Durham, 2003; Mangan *et al.*, 2004; Fallon and Mangan, 2007; Mo *et al.*, 2008; Adjobimey and Hoerauf, 2010; Navarro *et al.*, 2016; Titz *et al.*, 2017; Logan *et al.*, 2018; Midttun *et al.*, 2018). Another activator of allergic responses is the alarmin cytokine IL-33, which was shown to be blocked by parasitic secreted products (Neukirch *et al.*, 1999; Agarwal, 2011; Kouzaki *et al.*, 2011; Cayrol and Girard, 2014; McSorley *et al.*, 2014; Snelgrove *et al.*, 2014; Christianson *et al.*, 2015; De Salvo *et al.*, 2016; Liew, Girard and Turnquist, 2016).

In autoimmune diseases, the immune system recognises normal body parts as foreign, damaging its own healthy tissues and causing diseases such as coeliac disease,

multiple sclerosis (MS), rheumatoid arthritis (RA), type-1 diabetes and inflammatory bowel disease (IBD - including ulcerative colitis and Crohn's disease). As in allergic disorders, evidence suggested that helminths and their products can ameliorate autoimmune pathologies in humans and animal models (Rook, 2012) via multiple mechanisms depending on the disease and on the worm. Parasites are able to downregulate Th1 or Th17 pathways mediated during autoimmune diseases (Bettelli, Oukka and Kuchroo, 2007; Zaccone *et al.*, 2010; Du *et al.*, 2011; Chen *et al.*, 2014; Lund *et al.*, 2014). Several studies have examined the administration of whole products and extracts from the rat tapeworm *Hymenolepis diminuta*, the hookworms *Ancylostoma ceylanicum* and *Ancylostoma caninum*, the nematode *Trichinella spiralis*, the filarial nematode *Litomosoides sigmodontis*, and schistosome worms on a range of colitis and MS mouse models. They reported a reduction in Th1 markers such as TNFα and IFNγ, an increase in regulatory markers (IL-10 and TGFβ), and activation of Tregs, along with an increase in AAM markers (Elliott *et al.*, 2003, 2008; Walsh *et al.*, 2009; Hübner, Thomas Stocker and Mitre, 2009; Motomura *et al.*, 2009; Wilson *et al.*, 2010; Johnston *et al.*, 2010; Cançado *et al.*, 2011; Du *et al.*, 2011; Hubner *et al.*, 2012; Ferreira *et al.*, 2013; Heylen *et al.*, 2014).

Although relatively limited, clinical trials were also performed using helminth infections to treat immune diseases. Crohn's disease and ulcerative colitis patients infected with the pig whipworm *Trichuris suis*, which is naturally expelled within 6 weeks, reported improvements in disease outcome, however these results are inconclusive due to lack of efficacy in some studies (Summers *et al.*, 2003, 2005; Shi *et al.*, 2011; Graepel *et al.*, 2013; Sandborn *et al.*, 2013; Weinstock and Elliott, 2013; Fleming and Weinstock, 2015; Helmby, 2015; Mckay, 2015).

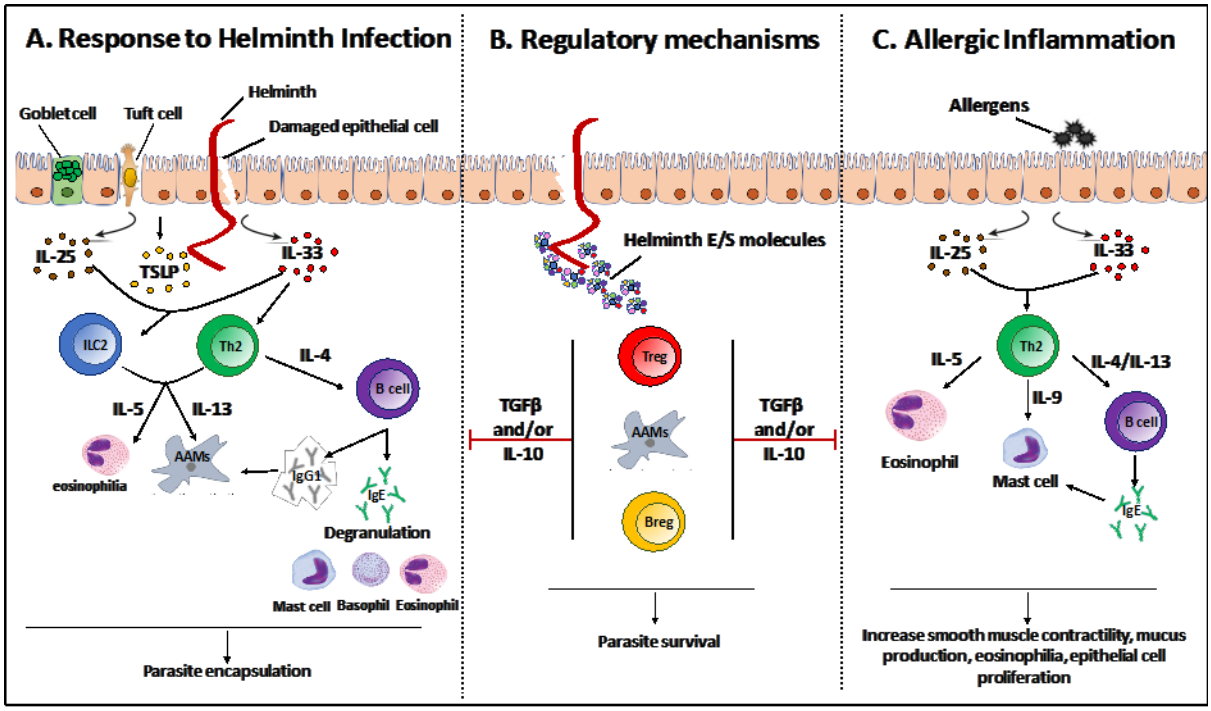


**Figure 1.1: Type 2 immune response and helminth regulatory mechanisms**

**(A) Response to helminth infection.** IL-25, IL-33 and TSLP released by epithelial cells during helminth infections, drive type-2 responses stimulating ILC2s and Th2 cells to produce several protective cytokines including IL-4, IL-5 and IL-13. This results in B cell isotype switching to IgE and IgG1, the activation of specialised effector cells such as mast cells, eosinophils and basophils, the increase of eosinophilia, as well as the expansion and activation of alternatively activated macrophages (AAMs) which can initiate wound healing and tissue repair. The cytokines released trigger the parasite expulsion.

**(B) Helminth-induced regulatory mechanisms.** Helminth ES products can induce regulatory cells such as Tregs, Bregs and AAMs. Through the production of anti-inflammatory cytokines IL-10 and TGFβ and the increased expression of AAMs markers (i.e. Arg1), Th2 immune responses are suppressed thus promoting the parasite survival, in addition to reducing allergic effector mechanisms.

**(C) Allergic inflammation.** During airway inflammation, allergens evoke a type 2 immune response, similar to helminths, associated with high levels of IL-4, IL-5, IL-13 and IgE, in addition to infiltration of eosinophils. This cascade can lead to an increase in smooth muscle contractility, mucus production, eosinophilia and epithelial cell proliferation.

IL: interleukin; TSLP: thymic stromal lymphopoietin; Th2: T helper 2; ILC2: group 2 innate lymphoid cells; Ig: immunoglobulin; AAMs: alternatively activated macrophages; ES: excretory/secretory; Treg: regulatory T cell; Breg: regulatory B cell; TGF-β: transforming growth factor-β.

## 1.3 The *Heligmosomoides polygyrus* model

*Heligmosomoides polygyrus bakeri* (*H. polygyrus*), previously known as *Nematospiroides dubius*, is a natural intestinal parasite of mice sharing the same taxonomic subfamily as the human hookworms *Ancylostoma duodenale* and *Necator americanus. H. polygyrus* is widely used as a laboratory model to understand the pathology associated with human chronic helminth infections and most importantly to study immunomodulatory mechanisms in inflammatory diseases (Behnke, 1987; Robinson *et al.*, 1989; Behnke, Menge and Noyes, 2009; Donskow-Łysoniewska *et al.*, 2013; Filbey *et al.*, 2014).

Within 24 hours of *H. polygyrus* larvae ingestion, third-stage (L3) infective larvae migrate to the duodenum, where they invade the muscular layer and reside beneath the serosal membrane (Figure 1.2). The larvae develop into L4 stage worms in the muscularis externa by day 5, then emerge back into the intestinal lumen as adult worms approximately 8 days post-infection. Around the villi of the proximal intestinal epithelium, they coil and feed on the intestinal tissues, unlike their hookworm relatives which are blood feeders penetrating the epithelium to access blood (Bansemir and Sukhdeo, 1994). By day 10, adult worms mate and start shedding eggs with the faeces. The eggs hatch 36 hours later giving way to the first larval stages (L1 and L2). L3 infective larvae then develop in faeces within 7-8 days (Figure 1.2) (Reynolds, Filbey and Maizels, 2012; Johnston *et al.*, 2015). Similar to other nematode infections, *H. polygyrus* induce Th2 immune responses displaying an elevation of IL-4, IL-5, and IL-13 production as well as IgG1 and IgE antibodies (Reynolds, Filbey and Maizels, 2012).

During infection, the parasite releases products termed *H. polygyrus* excretory/secretory (HES), that have been found to protect against immunopathology in

experimental models of autoimmunity, allergy and colitis. Recent studies showed that secreted products from the parasitic nematode *H. polygyrus* can block IL-33 receptor expression (Buck *et al.*, 2014) and IL-33 production during *Alternaria alternata*-induced airway inflammation (McSorley *et al.*, 2014). It was then reported that following *alternaria* administration, *H. polygyrus* Alarmin Release Inhibitor (HpARI) secreted by the larvae and adult worm (Hewitson *et al.*, 2013) reproduce the IL-33 inhibitory effect suppressing type 2 responses and lung eosinophilia (Osbourn *et al.*, 2017). Furthermore, adult *H. polygyrus* persists in the host for several months and prolongs its survival through the activation and expansion of host regulatory cells, in particular Treg cells, mediating immunosuppressive mechanisms such as IL-10 production (Wilson *et al.*, 2005, 2010; Finney *et al.*, 2007; Rausch *et al.*, 2008; Grainger *et al.*, 2010; K. A. Smith *et al.*, 2016). More recently, TGFβ mimic (HP-TGM), a protein secreted by *H. polygyrus*, was shown to mimic TGFβ properties, inducing suppressive Treg cells (Johnston *et al.*, 2017).

Several other proteins with possible immunomodulatory function have been identified including venom-allergen-like (VAL) proteins, proteases and protease inhibitors, acetylcholinesterases, lysozymes and apyrases (Hewitson *et al.*, 2011). Secretion of these type of proteins has previously been found in other strongylids such as *H. contortus* and *Teladorsagia circumcincta* (Yatsuda *et al.*, 2003; Mulvenna *et al.*, 2009; Nisbet *et al.*, 2010; Hewitson *et al.*, 2011). Besides known immunomodulators, several other *H. polygyrus*-derived molecules have been identified. Among these are families which contain a large number of uncharacterized proteins termed novel secreted proteins (NSPs) whose function has yet to be outlined, and proteins of unknown function with homologues in other nematodes (Hewitson *et al.*, 2011).

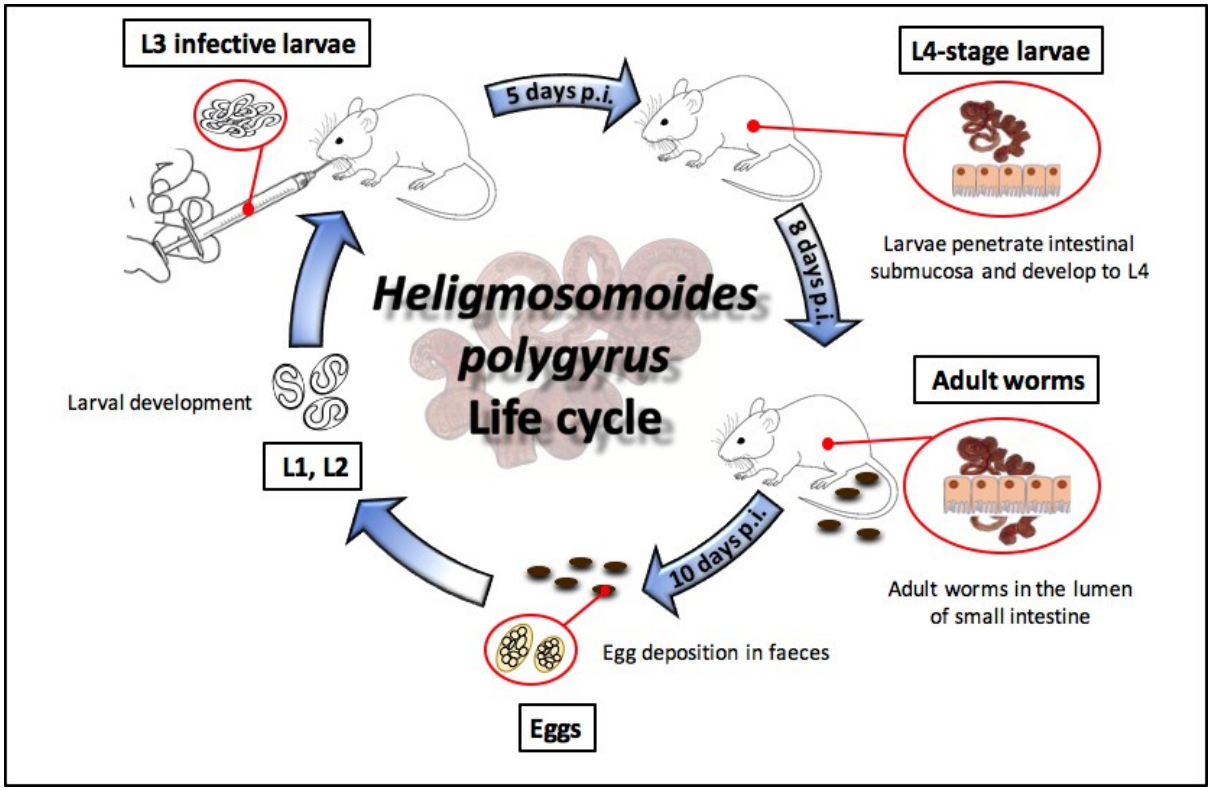


**Figure 1.2: Life cycle of *Heligmosomoides polygyrus***

Ingested third-stage (L3) infective larvae migrate to the intestine, develop into L4 stage worms then to adult worms. By day 10 p.i., adults start shedding eggs which hatch 36 hours later giving way to L1 and L2. L3 infective larvae develop in faeces within 7-8 days.
p.i.: post-infection.

## 1.4 Purinergic signalling

The "purinergic signalling pathway" was first discovered by Burnstock in the early 1970s, proposing a purinergic neuromuscular transmission mechanism in the central nervous system (CNS), which involved the synthesis, storage and release of the purine nucleotide ATP. In the late 1970s, Burnstock and Kennedy classified the purinergic receptors into types and subtypes (Burnstock *et al.*, 1970; Burnstock, 1972, 1978; Burnstock and Kennedy, 1985). Since then, purinergic signalling has been described in other organs and tissues, and more receptor subtypes have been identified. Many studies have demonstrated the importance

of extracellular ATP and targeted purinergic receptors as potential therapeutics to treat a wide range of diseases including cancer, inflammatory and immune disorders, autoimmune diseases, neurological and psychiatric illnesses, stroke, thrombosis, supraventricular tachycardia, visceral pain, chronic cough, hypertension, bladder disorders, neuropathic pain, and more (Burnstock, 2017).

**Extracellular ATP release**

The ATP molecule, besides being well known as an intracellular energy source for all living cells, serves also as a messenger between cells, making ATP essential to basic function and development of organs and tissues (Khakh and Burnstock, 2009). Under a normal healthy state, extracellular ATP concentration is undetectable. However, during pathological conditions, for example intracellular pathogen infection (Mempin *et al.*, 2013), tumours (Pellegatti *et al.*, 2008), and pro-inflammatory or allogeneic graft injury (Hart *et al.*, 2008; Zeiser *et al.*, 2016), extracellular ATP increases, reaching a concentration of a few hundred μM (Idzko, Ferrari and Eltzschig, 2014; Morciano *et al.*, 2017). Following tissue injury, stressed or apoptotic cells release ATP and other nucleotides from intracellular storage pools into the extracellular space (Surprenant and North, 2009; Cekic and Linden, 2016; Zimmermann, 2016) via connexin (Cx) or pannexin (Panx) cell-surface membrane hemichannels, such as Cx43 and Panx1 (Eltzschig *et al.*, 2006; Chekeni *et al.*, 2010; Esseltine and Laird, 2016; Dou *et al.*, 2018). The release of ATP then activate immune cells triggering pro-inflammatory immune responses (Ohta and Sitkovsky, 2001; Junger, 2011; Ayna *et al.*, 2012; Asgari *et al.*, 2013; Wang and Chen, 2018).

**Purinergic (P1 and P2) receptors**

Once in the extracellular compartment, nucleotides and nucleosides play a signal transduction role in different cellular responses through the activation of P1 and P2 purinergic receptors. These receptors are widely distributed in almost all cell types mediating physiological and pathophysiological responses (Khakh and Alan North, 2006; Surprenant and North, 2009). Studies have revealed the functions of each subtype using knockout mice, transgenic mice and selective receptor agonists and antagonists.

P2 receptors have been sub-classified into the ionotropic P2X receptors (P2XRs) (Brake, Wagenbach and Julius, 1994; Valera *et al.*, 1994) and metabotropic G protein-coupled P2Y receptors (P2YRs) (Burnstock and Kennedy, 1985). So far, seven P2XRs and eight P2YRs have been identified and characterized in mammals (Ralevic and Burnstock, 1998; Burnstock, 2007; Nishimura *et al.*, 2017). P2XRs ($P2X_{1-7}R$), activated by ATP, are plasma membrane ion channels selective for the monovalent cations $Na^+$ and $K^+$, and the divalent cation $Ca^{2+}$. Each P2XR subunit assembles in a trimeric homomer or heteromer complex to form seven different subtypes that share two transmembrane domains separated by a large glycosylated disulphide-rich extracellular loop and an intracellular N- and C- termini (Brake, Wagenbach and Julius, 1994; Valera *et al.*, 1994; Nicke *et al.*, 1998; North, 2002; Aschrafi *et al.*, 2004). The $P2X_1$ receptor has been shown to be expressed at sympathetically innervated smooth muscles, thus when activated, it can initiate contraction of smooth muscles such as in the vas deferens (Mulryan *et al.*, 2000). In addition, $P2X_1R$ is involved in platelet activation (Hechler *et al.*, 2003; Oury *et al.*, 2003), renal autoregulation (Inscho *et al.*, 2003) and neutrophil chemotaxis (Lecut *et al.*, 2009). $P2X_2$, $P2X_3$ and their co-

assembly heteromeric $P2X_{2/3}$ receptors were found to be commonly expressed on sensory neurons, where they play an important role in the initiation of sensory signalling pathways like gustatory signalling (Finger *et al.*, 2005; Eddy *et al.*, 2009; Hallock *et al.*, 2009), intestinal neurotransmission (Bian *et al.*, 2003; Ren *et al.*, 2003), and inflammatory and neuropathic pain (Cockayne *et al.*, 2000, 2005; Souslova *et al.*, 2000; Jarvis *et al.*, 2002). Moreover, the $P2X_4R$ is able to regulate synaptic plasticity as well as vascular endothelium tone and contractility of the cardiomyocytes. It is also implicated in neuropathic pain (Tsuda *et al.*, 2003, 2009; Sim, 2006; Ulmann *et al.*, 2008; Ulmann, Hirbec and Rassendren, 2010). Studies have shown that mice lacking the $P2X_4$ receptor gene have smaller-diameter arteries, no vascular re-modelling, and are hypertensive (Yang *et al.*, 2004; Shen, 2006; Yamamoto *et al.*, 2006; Shen *et al.*, 2009). As for the $P2X_5R$, it was shown to be essential for the activation of the ATP-mediated inflammasome and the production of IL-1β by osteoclasts (Kim et al., 2017). However, the activation of $P2X_7$ receptors results in the release of pro-inflammatory cytokines, and the activation of immune cells (Solle *et al.*, 2001; Labasi *et al.*, 2002; Ke *et al.*, 2003; Chessell *et al.*, 2005; Honore *et al.*, 2006).

P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11\text{-}14}$) belong to the family of G protein coupled receptors, and they contain seven hydrophobic transmembrane spanning domains joined by three extracellular and three intracellular loops (Erb *et al.*, 2006). Each receptor is activated by a variety of extracellular nucleotides such as ATP, ADP, UTP, UDP and UDP-glucose. P2YRs are demonstrated to regulate a range of physiological functions including proliferation, differentiation, secretion, cell adhesion, phagocytosis, nociception and cell migration. For instance, $P2Y_1R$ is activated by ADP and is expressed on the surface of

platelets and megakaryocytes (Soulet *et al.*, 2005). In contrast, the $P2Y_2$ receptor is activated by ATP and UTP, and is involved in the inhibition of bone formation (Orriss *et al.*, 2007), immune cell recruitment and phagocytosis, blood pressure regulation, and wound healing (Chen *et al.*, 2006; Rieg *et al.*, 2007; Boucher *et al.*, 2010; Müller *et al.*, 2010). The $P2Y_4R$ is highly selective for UTP. It can be activated by ATP but not nucleoside diphosphates, and is involved in intestinal $K^+/Cl^-$ secretion (Matos *et al.*, 2005). $P2Y_6R$ activated by UDP was shown to stimulate the release of cytokine/chemokine from macrophages (Bar *et al.*, 2008), whereas the $P2Y_{11}$ receptor activated only by ATP, can inhibit neutrophil apoptosis (Vaughan *et al.*, 2007) and induce the secretion of pancreatic $Cl^-$ (Nguyen *et al.*, 2001). Only ADP can activate $P2Y_2$ and $P2Y_{13}$ receptors, which are shown to be involved in platelet aggregation and in regulation of microglial activation, as well as dendritic cell activation (Haynes *et al.*, 2006), bone formation, liver uptake of HDL, and inhibition of ATP release from RBCs (Wang *et al.*, 2005). Finally, the $P2Y_{14}$ receptor, activated by UDP and UDP-glucose, can stimulate the release of IL-8 in epithelium gastric function, and is involved in stomach contractility (Müller *et al.*, 2005; Arase *et al.*, 2009).

P1 receptors, also called adenosine receptors (ARs), belong to the superfamily of G protein-coupled metabotropic receptors and contain seven hydrophobic transmembrane-spanning segments (Jespers *et al.*, 2018). Four distinct ARs have been identified: $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ (Urbina and Docampo, 2003) with $A_1$ and $A_{2A}$ receptors being the most sensitive as they can be activated in the nanomolar range (Jacobson and Gao, 2006). Adenosine, generated in the extracellular compartment, can stimulate or inhibit adenylyl cyclase activity via the activation of $A_{2A,B}$ or $A_{1,3}$ receptors respectively (Vallon, Mühlbauer and

Osswald, 2006; Fredholm *et al.*, 2011). ARs are widely distributed on most cells in the body and have been considered as a major target for drug development against pathological conditions of the cardiovascular, nervous, gastrointestinal and immune systems (Poulsen and Quinn, 1998; Silverman *et al.*, 2008; Massie *et al.*, 2010; Gessi *et al.*, 2011; Lopes, Sebastião and Ribeiro, 2011; Antonioli, Blandizzi, *et al.*, 2013; Chen, Eltzschig and Fredholm, 2013; Borea *et al.*, 2016; Haskó, Antonioli and Cronstein, 2018). $A_1R$ is highly expressed in CNS tissues (brain cortex, cerebellum and hippocampus), as well in the eye, adrenal gland and atria, where it can modulate several physiological mechanisms (Olah and Stiles, 1995; Poulsen and Quinn, 1998; Yuzlenko and Kieć-Kononowicz, 2006). Based on adenosine affinity, $A_2Rs$ were subdivided into the $A_{2A}$ (low affinity) and $A_{2B}$ (high affinity) receptors (Cieślak, Komoszyński and Wojtczak, 2008). $A_{2A}Rs$ are localised in the neurons, olfactory bulb, spleen, thymus and leukocytes (Schiffmann *et al.*, 1990; Svenningsson *et al.*, 1999; Fredholm, Cunha and Svenningsson, 2003), and are mainly involved in motor behaviour (Kuwana *et al.*, 1999; Matasi *et al.*, 2005). Their activation was also shown to have an anti-inflammatory effect in allergic lung inflammation (da Rocha Lapa *et al.*, 2013). However, $A_{2B}R$ was shown to be important in the initiating of type 2 immunity such as in allergic diseases and helminth infections (Karmouty-Quintana *et al.*, 2015; Philip *et al.*, 2017). These receptors were found to be expressed in airway smooth muscle cells, lung fibroblast cells, and mast cells. As for the $A_3$ receptor, it was shown to be expressed in the brain and endocrine tissues, as well as on eosinophils and neutrophils (Linden, 1994). In summary, P1 and P2 receptors have a crucial role in the immune response, heart and neurotransmission.

**Purinergic signalling in immune cells**

Several recent studies and reviews have described the widespread expression of purinergic receptors throughout different immune cells and highlighted the importance of these receptors in inflammation and immunomodulation (Di Virgilio and Vuerich, 2015; Beamer, Fischer and Engel, 2017; Burnstock, 2017; Di Virgilio, Sarti and Grassi, 2018).

In response to cell and tissue injury, extracellular ATP and other nucleotides are rapidly released through hemichannels, attracting and activating immune cells such as monocytes, DCs and neutrophils (Elliott *et al.*, 2009). ATP then binds to ionotropic P2XRs which become permeable to $Na^+$, $K^+$ and $Ca^{2+}$ (Figure 1.3). All P2XR subtypes have been identified to be expressed on immune cells, in particular $P2X_{1,4,7}Rs$ with $P2X_7$ receptor the most widely expressed and shown to be important in activating lymphocytes, granulocytes, macrophages and dendritic cells (Junger, 2011; Ayna *et al.*, 2012; Asgari *et al.*, 2013; Di Virgilio *et al.*, 2017).

The activation of metabotropic P2Y receptors, such as $P2Y_2$ (Chen *et al.*, 2006) and $P2Y_{14}$ (Barrett *et al.*, 2013), stimulates the chemotaxis and activation of phagocytes, and reduces anti-cAMP accumulation. Subsequently, in the presence of ecto-nucleotidases that hydrolyse ATP to adenosine, extracellular adenosine levels start to increase slowly, upregulating $A_{2A}R$ and $A_{2B}R$ on immune cells. These 2 adenosine receptors were shown to suppress inflammatory cytokine production (IL-12 and TNFα), enhance the production of IL-10 by monocytes and macrophages (Hasko & Cronstein, 2004), inhibit platelet aggregation,

inhibit phagocyte chemotaxis and activate protein Kinase A (PKA), thus limiting inflammation. It was also suggested that adenosine production activates Foxp3+ regulatory T cells (Tregs), suppressing the activation of DCs and effector T cells (Deaglio *et al.*, 2007). Therefore, targeting P2X, P2Y and P1 receptors is useful for reducing inflammation caused by different diseases. Clinically, P2 purinergic receptor (mainly $P2X_7R$) antagonists and $A_{2A}R$ agonists are being tested for the treatment of tissue inflammation in autoimmune diseases (Lang *et al.*, 2010) tissue transplantation (Vergani *et al.*, 2013) and in long-term inflammatory diseases (Cekic and Linden, 2016).

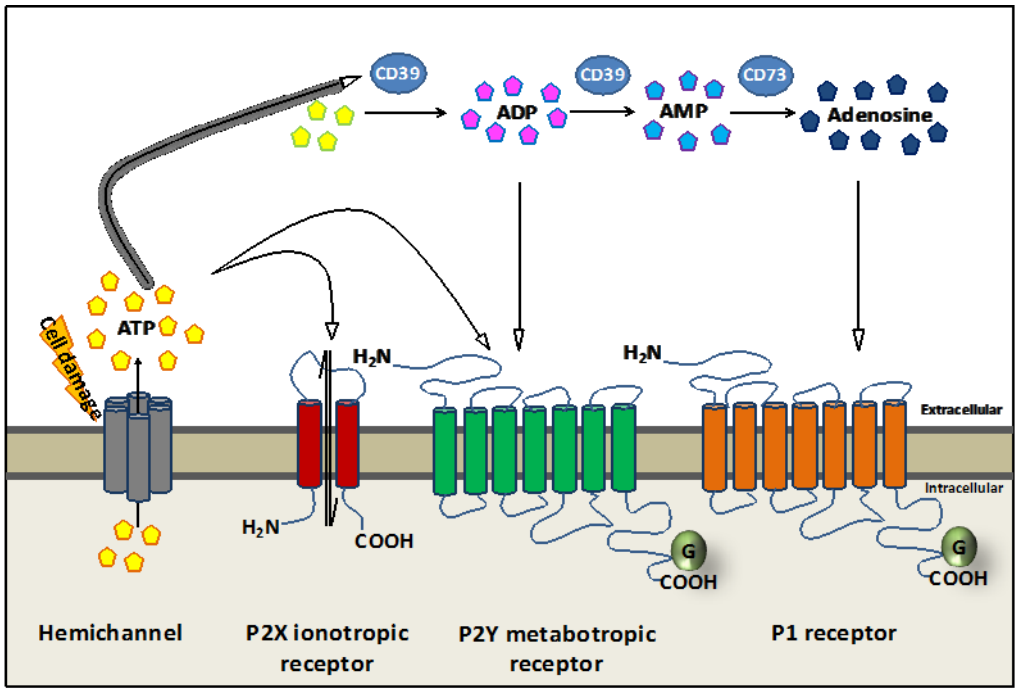


**Figure 1.3: Extracellular ATP signalling during inflammation**

## 1.5 Kinetoplastids and Trypanosomatids

Kinetoplastids belong to the class kinetoplastidae and are members of the flagellated protozoans. They are unicellular eukaryotic parasites, classified into two monophyletic groups, the uniflagellated trypanosomatids and the free-living biflagellated bodonids (Vickerman 1974; Hamilton *et al.*, 2004; Moreira, López-García and Vickerman, 2004).

The cellular features of kinetoplastids include: a nucleus, a golgi apparatus, an endoplasmic reticulum, a peroxisome-like organelle named a glycosome to perform glycolysis, a cytoskeleton made up of subpellicular microtubules, a paraxial rod which runs along the axoneme, one or two flagellae emerging from a flagellar pocket and supported by a paraflagellar rod and a basal body, in addition to a very distinguishing feature, the kinetoplast, which contains the mitochondrial DNA called kDNA (Hoare and Wallace, 1966; Hoare, 1967; McGhee and Cosgrove, 1980; Vickerman, 1994, 2008; Souza, 2002; Gadelha *et al.*, 2005; Field and Carrington, 2009). The kDNA is composed of minicircle and maxicircle DNA rings and is located within a single large mitochondrion. Kinetoplastid cells grow asexually within their host and divide by binary fission (Ray, 1989; Yurchenko *et al.*, 1999; Lukeš *et al.*, 2002; Hong and Simpson, 2003; Vargas-Parada, 2010).

The morphological form of these flagellated protozoans is defined by the position of the kinetoplast in relation to the nucleus (Figure 1.4). In the amastigote stage, the parasite is spherical with no free flagellum and the kinetoplast is near the nucleus, whereas in the promastigote stage, the kinetoplast is located in front of the nucleus to the anterior end,

and the flagellum, which is not attached to the cell body, is anterior to the nucleus. In the epimastigote stage, the kinetoplast is located centrally between the nucleus and the anterior end of the body and the flagellum is connected by an undulating membrane. However in the trypomastigote stage, the kinetoplast is located in a posterior position related to the nucleus, and the flagellum is attached to the entire length of the cell body by an undulating membrane which help in increasing the motility of the parasite (Hoare and Wallace, 1966).

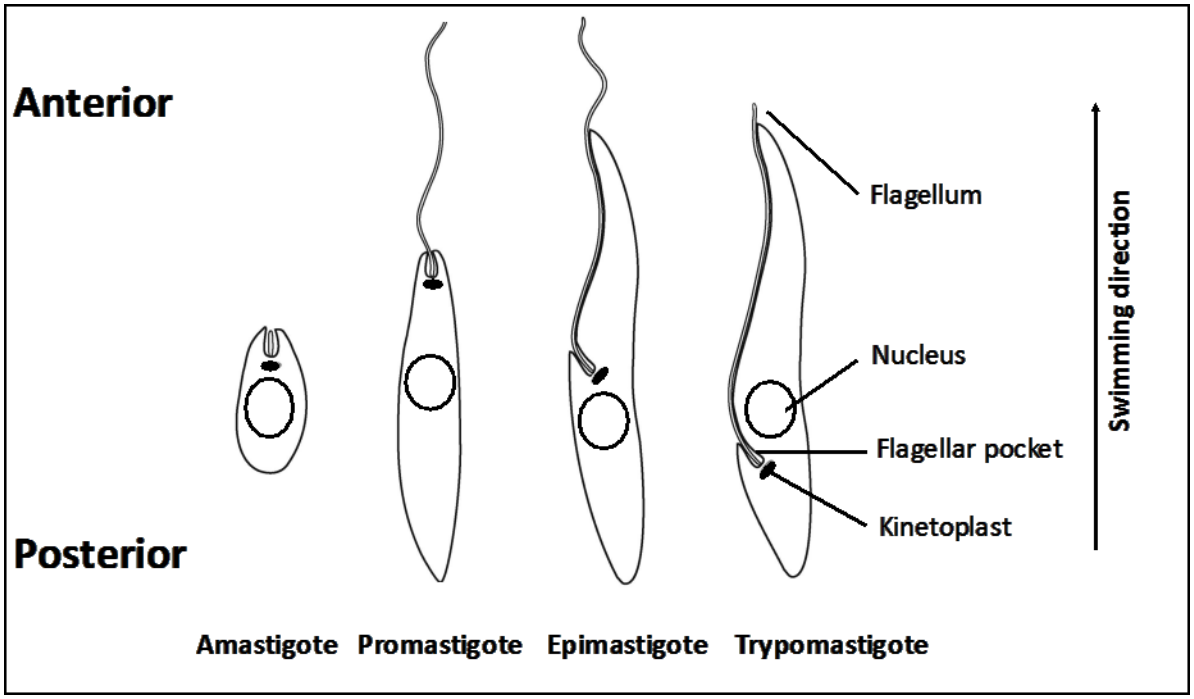


**Figure 1.4: Major Kinotoplastid morphological forms**

Trypanosomatids, the first organisms viewed in an electron microscope, belong to the genus *Trypanosoma* and the class Kinetoplastidae (Sleigh, 1989). These parasites include species and subspecies which cause various diseases in animals and humans (Stuart *et al.*,

2008). In humans, the most common serious diseases include Chagas disease caused by *Trypanosoma cruzi* (Urbina and Docampo, 2003; de Souza, 2007), human African trypanosomiasis or sleeping sickness caused by *Trypanosoma brucei* species (Fairlamb, 2003) and Leishmaniasis caused by *Leishmania* species (Croft and Coombs, 2003; Dujardin, 2006; Lukeš *et al.*, 2014). Two types of trypanosomes have been described: the stercorarian trypanosomes which are transmitted to their hosts via faeces of insect vectors, and the salivarian trypanosomes which are transmitted in the saliva of a biting insect (Tsetse fly) (Hoare, 1964, 1972).

Trypanosomatids have been of great interest in molecular biology, as they express genes in a unique way which differs from plants, fungi and animals (Figure 1.5). In the nucleus, multiple protein-coding genes are arranged in polycistronic transcription units, and bi-directional transcription is initiated between two divergent gene clusters by RNA polymerase II (Johnson, Kooter and Borst, 1987; Mottram, Murphy and Agabian, 1989; Campbell, Thomas and Sturm, 2003; Martínez-Calvillo *et al.*, 2003, 2010; Das, Banday and Bellofatto, 2008; De Gaudenzi *et al.*, 2011). Genes are co-transcribed then processed to individual mature mRNAs before translation via a process called trans-splicing. This process is performed by the addition of a capped 39-nucleotide long mini-exon from a spliced leader RNA (SL RNA) to generate a capped 5´ end, coupled with the cleavage and polyadenylation of the mRNA 3´ end (Kooter and Borst, 1984; Parsons *et al.*, 1984; LeBowitz *et al.*, 1993; Teixeira and daRocha, 2003). In the cytosol, mRNAs are degraded by the removal of the poly(A) tail then de-capped and digested in the 5´-3´ direction. Most of the gene expression

process in trypanosomes is regulated post-transcriptionally (Maslov and Simpson, 1994; Haile and Papadopoulou, 2007; Hajduk and Ochsenreiter, 2010).

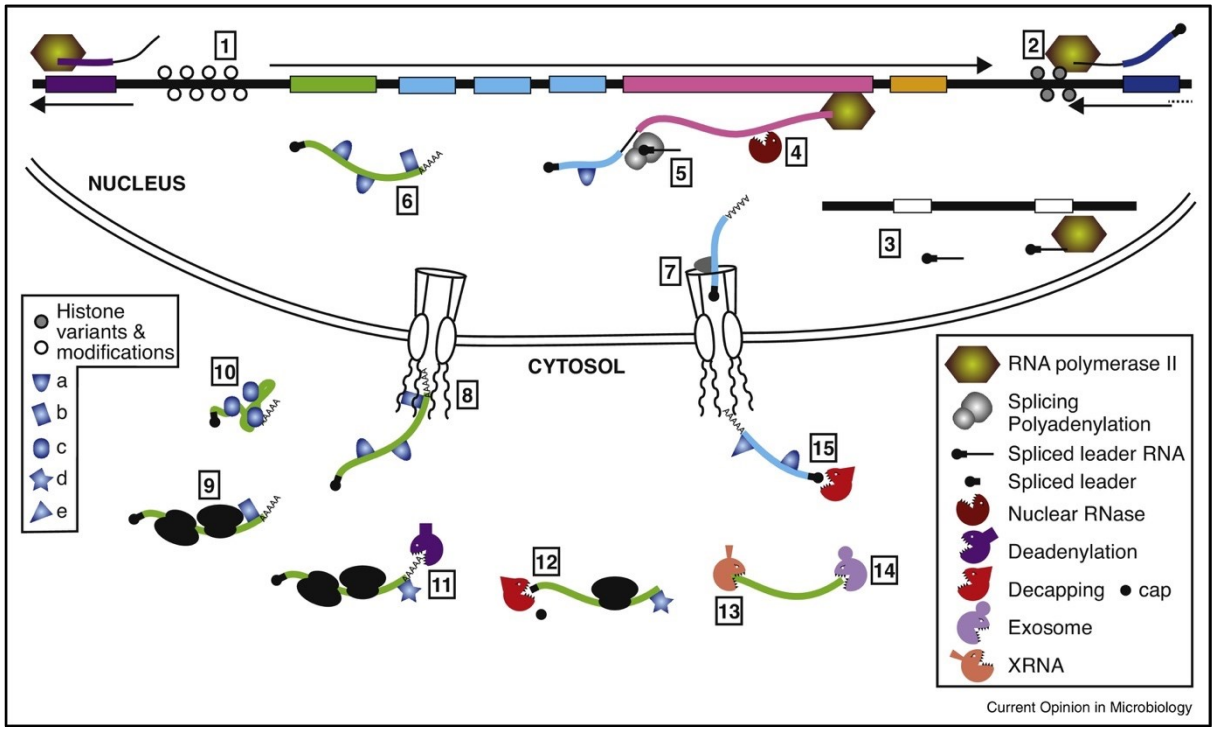


**Figure 1.5: Schematic representation of kinetoplastid gene expression**

(Figure from Clayton, 2016)

***Trypanosoma musculi***

*Trypanosoma musculi* (*T. musculi*), a stercorarian trypanosome, is a natural protozoan parasite of mice, closely related to *Trypanosoma lewisi*, which infects only rats (Taliaferro and Alesandro, 1971). Once introduced into the host, a single parasite can reproduce and initiate an infection. The two major proliferative stages of *T. musculi* are the epimastigote and the trypomastigote forms, with the latter being 28-32 µm in length and 2-3 µm in width when mature (Ashraf *et al.*, 2002; Hong *et al.*, 2017). It resides extracellularly

in the bloodstream and tissue fluids of the mouse, as well as in organs such as the liver, spleen, kidneys, lungs and in smaller amount in the intestine (Albright *et al.*, 1999). *T. musculi* causes a self-limiting infection that persists for a period of 3 weeks. The course of parasitaemia is characterized by a pre-patent phase (2-4 days), a logarithmic growth phase (5-7 days), and a parasitaemia plateau phase (5-7 days) which is followed by an immune parasite clearance from the vascular system (5-7 days) by an antibody-dependent cell-mediated process (Targett and Viens, 1975; Viens, Targett and Lumsden, 1975). After recovery from the infection, mice are resistant to reinfection and the parasites, though no longer detected in peripheral blood or other organs, have been shown to persist for the lifetime of mice in the vasa recta of the kidneys (KFs, Kidney forms) described as an immunologically privileged site (Viens *et al.*, 1972; Albright, Pierantoni and Albright, 1990; Monroy and Dusanic, 2000).

**Immune response to *T. musculi***

It is important to know how the parasite interacts with the host immune system and how it is controlled and eliminated. *T. musculi* infection causes an enlargement of the spleen and lymph nodes by 10 and 3 fold respectively, as well as the liver to a lesser degree when compared to naïve mice (Hirokawa *et al.*, 1981; Albright, Pierantoni and Albright, 1990). During the early course of *T. musculi* infection, acquired humoral immunity is suppressed, whereas the innate system controls but does not cure the infection. NK cells seem to control the infection through the secretion of cytokines, presumably TNFα and IFNγ, which activate

peritoneal space macrophages, but NK cells are unable to damage *T. musculi* directly (Albright, Jiang and Albright, 1997).

Reproduction of the parasites was inhibited during the plateau phase by a substance termed ablastin which was later shown to be an immunoglobin (Dusanic, 1975; Ormerod, 1975). Immunoglobulin, specifically IgG1 and IgG2a subtypes, binds first to trypanosomes, then to FcγR1/γ2b and FcγR2a receptors on macrophages (Vincendeau, Daeron and Daulouede, 1986; Wechsler and Kongshavn, 1986, 1988; Shaw *et al.*, 1992). Trypanosomes are killed either by phagocytosis or release of NO from macrophages. Previous studies have shown that *T. musculi* elimination is an antibody-facilitated, cell-dependent process requiring the assistance of trypanosome-specific antibodies, particularly IgG1, IgG2a and b, IgG3, Kupffer cells of the liver and spleen, and activated macrophages, leaving the host immune to reinfection (Vargas Del, Viens and Kongshavn, 1984; Wechsler and Kongshavn, 1986; Albright and Albright, 1991; Albright, J.W., Stewart, M.J., Latham, P.S., Albright, 1994; Albright, Jiang and Albright, 1997). Athymic or nude mice and T-cell deprived mice did not recover from *T. musculi* infection, showing that T lymphocytes play a vital role in the elimination of dividing parasites and the termination of the infection (Viens, Targett and Lumsden, 1975).

**Trypanosome purine salvage**

Purines are molecules of great importance in all living organisms (Figure 1.6). They play an essential role in nucleic acid synthesis and also act as signalling molecules, besides being

part of energy-requiring reactions and components of ATP, cyclic AMP, coenzyme A, GTP and NADH (El Kouni, 2003; Berg *et al.*, 2010). Purine nucleotides can be synthesized by *de novo* and/or salvage pathways.

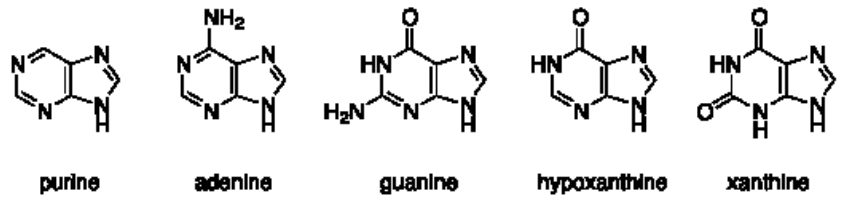


**Figure 1.6: The main purine molecules**

Nearly all parasitic protozoa, including *T. musculi* (Albright and Albright, 1988), are unable to synthesise purines *de novo,* instead they rely on purine salvage to supply their metabolic requirements (Marr, Berens and Nelson, 1978; Boonlayangoor, Albach and Booden, 1980; Berens *et al.*, 1981; Fish *et al.*, 1982; Schwartzman and Pfefferkorn, 1982; Hammond and Gutteridge, 1984). Therefore, they express nucleoside/nucleobase transporters and intracellular salvage enzymes to take up and synthesise purine bases from their hosts (Gutteridge and Davies, 1981; Allen and Ullman, 1994; De Koning and Jarvis, 1997; Van Rompay, Johansson and Karlsson, 2000; de Jersey *et al.*, 2011). This pathway attracted chemotherapeutic studies over the last twenty years as a potential drug target for different parasites, such as *Leishmania* and *Trypanosoma* (Boitz *et al.*, 2012; Li *et al.*, 2015; Doleželová *et al.*, 2018). Trypanosomes are shown to compete with their hosts for purines, transporting and concentrating adenosine via high affinity adenosine transporters (Carter and Fairlamb, 1993; Koning, Watson and Jarvis, 1998).

Adenine, adenosine, inosine, guanosine and sometimes hypoxanthine are imported via the P1 nucleoside transporter (Ortiz *et al.*, 2009), whereas P2 enables transport of adenine and adenosine (Mäser *et al.*, 1999) (Figure 1.7) and has been shown to be of great pharmacological importance as it can also transports trypanocidal drugs (Carter and Fairlamb, 1993; Carter, Berger and Fairlamb, 1995; Li *et al.*, 2015). All other nucleobases can be imported via the other 4 nucleobase transporters H1-H4, mainly H2 and H3 (De Koning, Bridges and Burchmore, 2005). Some trypanosomes such as *T. brucei* and *T. cruzi* have a preference for adenine and adenosine over other purines, as these have been shown to be taken up the fastest (Fish *et al.*, 1982; Vodnala *et al.*, 2008; Berg *et al.*, 2010).

Intracellularly, several enzymes are involved in the trypanosome purine salvage pathway including salvage enzyme transferases such as nucleoside hydrolases (Inosine-adenosine-guanosine NH and inosine-guanosine NH), phosphoribosyltransferases (6-oxopurine PRTase, adenine PRTase) methylthioadenine phosphorylase (MTAP) (Parkin, 1996; Versées *et al.*, 2001) and adenosine kinase (AK). In addition to interconversion enzymes like AMP deaminase, adenylosuccinate lyase (ADSL), adenylosuccinate synthetase (ADSS), guanine deaminase, GMP synthase (GMPS), GMP reductase and inosine-5´-monophosphate dehydrogenase (Figure 1.6). In the bloodstream form *T. brucei,* AK phosphorylates adenosine to AMP (Vodnala *et al.*, 2008) and some other enzymes packaged in glycosomes can be involved in purine salvage and pyrimidine biosynthesis (Opperdoes and Michels, 1993; Lüscher *et al.*, 2014; Doleželová *et al.*, 2018). In the purine salvage pathway of *P. falciparum*, adenosine and hypoxanthine are converted to AMP and GMP and

excess AMP is de-aminated back to IMP by AMP deaminase to constitute the purine nucleotide cycle (Albright and Albright, 1988).

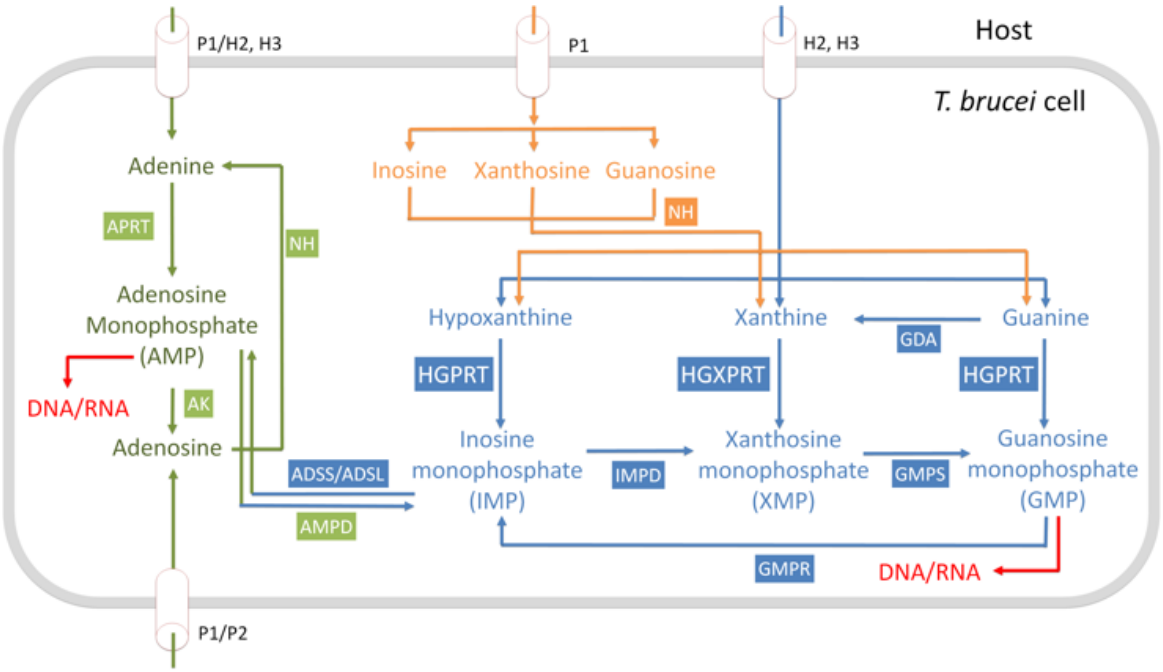


**Figure 1.7: Purine salvage pathway in trypanosomes**

(Figure from Doleželová *et al.,* 2018)

## 1.6 Ecto-nucleotidase enzymes

On the surface of most cells, including immune cells, there are enzymes belonging to a large family known as the ectonucleotidase family, responsible in controlling ATP and ADP levels in the extracellular milieu. These nucleotidase enzymes have been extensively characterized on the external surface of trypanosomatids, where they are expressed presumably to

guarantee the supply of purines which they cannot synthesis *de novo* (Fietto *et al.*, 2004). For instance, a recent study showed that purine starvation increased the activity of *Leishmania infantum* ecto-nucleotidase, suggesting that this enzyme play an important role in parasite nutrition and survival (Peres *et al.*, 2018). Various families of ecto-nucleotidase enzymes have been described in protozoan parasites.

E-type ATPases (extracellular ATPases) are membrane bound enzymes localized at the cell surface and hydrolysing mainly ATP besides other nucleoside tri- and diphosphates (Plesner, 1995; Kirley, 1997). An Ecto-ATPase, an E- type ATPase subclass, which can only hydrolyse ATP, but no other nucleoside di- or triphosphates and which is cation dependent ($Mg^{2+}$ or $Ca^{2+}$), has been shown to be present on the surface of protozoan parasites including *Leishmania tropica* (Meyer-Fernandes *et al.*, 1997), *Leishmania amazonensis* (Berrêdo-Pinho *et al.*, 2001), *Toxoplasma gondii* (Asai *et al.*, 1995; Nakaar *et al.*, 1998), *Entamoeba histolytica* (Barros *et al.*, 2000), *Trypanosoma cruzi* (Bernardes *et al.*, 2000; Bisaggio *et al.*, 2003; Meyer-Fernandes *et al.*, 2004), and *Tetrahymena thermophila* (Smith Jr., Kirley and Hennessey, 1997).

Ecto-nucleoside triphosphate diphosphohydrolases (ecto-NTPDases or ENTPDase), also known as CD39, are enzymes of the apyrase family characterized by the presence of five highly conserved domains known as “apyrase conserved regions” (ACR1 to ACR5). These ecto-enzymes can hydrolyse nucleoside tri- and diphosphates to nucleoside monophosphates, which are further catalysed to adenosine by an ecto-5´-nucleotidase (ecto-5´-NT) also known as CD73 (Plesner, 1995; Zimmermann, 1999, 2000, 2001). Ecto-5´-

NT and ecto-NTPDase hydrolysing a broad range of nucleoside tri- and diphosphates have been previously characterised in *T. cruzi.* The presence of an Mg ecto-NTPDase activity was also described in *T. brucei* and *L. amazonensis* (Fietto *et al.*, 2004; Gomes *et al.*, 2015). Adenosine signalling is terminated by adenosine deaminase (ADA) which converts adenosine to inosine (Langer *et al.,* 2008), or by the cellular uptake of adenosine by equilibrative or concentrative nucleoside transporters (ENTs or CNTs) to the intracellular compartment, where it is phosphorylated to AMP by adenosine kinase (ADK) (Baldwin *et al.*, 2004; Gray, Owen and Giacomini, 2004).

The shift from a pro-inflammatory to an anti-inflammatory environment, characterized by adenosine production via CD39/CD73, has been of great importance in the control of various pathologies of the immune system such as autoimmunity, infections and cancer (Takenaka, Robson and Quintana, 2016; Dou *et al.*, 2018).

# Aims of the study

The aims addressed by this study are as follows:

1. Expression of *Heligmosomoides polygyrus* secreted apyrases and characterisation of their enzymatic activity.
2. Expression of apyrases in *Trypanosoma musculi* in order to determine possible immunomodulatory effects.
3. In vivo administration of parasite apyrases in order to study their effect on type 2 immunity.

# CHAPTER 2

# Materials and Methods

## 2.1 Laboratory animals, husbandry and ethics statement

Six- to eight-week-old female BALB/c mice were purchased from Charles River UK or Harlan UK Laboratories and maintained at the Central Biomedical Services (CBS) facility at Imperial College London in accordance with Imperial guidelines. Mice were housed in groups of five in individually ventilated cages in controlled rooms (20-24°C, 55% relative humidity and 12:12 hours light to dark cycle) and were acclimated for 7 days upon delivery before any experimental manipulation. Animal procedures considered in this study were reviewed by the Imperial College Animal Welfare Ethical Review Body (AWERB) and performed under the UK Home Office Animals (Scientific Procedures) Act Personal Project Licence number 70/8193: 'Immunomodulation by helminth parasites'.

## 2.2 Parasites

**Propagation and maintenance of *Heligmosomoides polygyrus***

Female BALB/c mice were inoculated with 200 *H. polygyrus* infective third-stage larvae (L3) in 200 µl of distilled water by oral gavage using a stainless-steel feeding needle with a curved end. The suspension was mixed prior to infection as the larvae settle very quickly. Infections were verified by the presence of eggs in faecal samples 10 days post-infection.

### Collection of *H. polygyrus* eggs and larvae from infected mice

Infected mice were kept for 4 hours in a wire-bottomed cage with a tray beneath covered with a layer of moistened paper towels. Faecal pellets were collected in a 50 ml tube and soaked in a suitable volume of distilled water ($dH_2O$) (enough to cover the pellets) for 1 hour at room temperature before they were mashed into a smooth paste. The faecal slurry was then washed 3 times in 50 ml $dH_2O$ by centrifugation of the collection tube at 300 x *g*, 4$^{o}$C for 2 minutes, aspirating the supernatant down to the pellet and refilling the tube. After the last wash, the pellet was re-suspended with 50 ml $dH_2O$ and passed through a sieve into a new tube which was then centrifuged as before. The pellet was plated on the centre of 5-6 layers of wet Whatman 40 filter paper in a petri dish, leaving 2 cm at the edge of the filter paper clean. The plates were placed in a humid box in the dark at room temperature and kept moist throughout the incubation period by adding drops of water to the faecal culture and to the edge of the filter paper. From day 7 onwards, infective L3 were collected into a 15 ml tube by rinsing the petri dish and the edge of the filter paper with $dH_2O$ using a transfer pipette. The collected water was centrifuged at 250 x *g* for 2 minutes. The larvae were then washed three times in 15 ml $dH_2O$ and stored at 4°C in $dH_2O$ for up to six months.

### Recovery and culture of *H. polygyrus* adult worms

Adult *H. polygyrus* were recovered from the intestine 21 days post-infection using Baermann Apparatus as previously described (Johnston *et al.*, 2015). Adult worms were washed extensively in sterile Hanks' solution, then incubated for 3 weeks at 37°C/5% $CO_2$ in RPMI 1640 supplemented with 1% glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 100 µg/ml gentamicin (Johnston *et al.*, 2015).

### Collection of *H. polygyrus* secreted proteins

Culture supernatants from adult *H. polygyrus* were recovered twice per week, cleared through a 0.2 µm filter and replaced with an equal volume of medium. Secreted products were pooled (excluding the first collection after 24 hours) and concentrated through a 3,000 molecular weight cut-off (MWCO) Amicon membrane in an ultrafiltration device under nitrogen pressure. Secreted proteins were washed several times with 50 mM HEPES buffer (pH 7.5) using 3000 MWCO spin columns (vivaspin). Protein concentration was measured by Bradford assay.

## *Nippostrongylus brasiliensis*

### Infection of mice with N. brasiliensis

Mice were infected sub-cutaneously (s.c.) with 500 L3 *N. brasiliensis* in 200 µl sterile PBS using a 21-gauge needle.

### Faecal egg count

Each infected mouse was restrained for enough time to collect 3 to 4 faecal pellets. The faeces were weighted then soaked in 5 ml of $dH_2O$ for 1 hour at room temperature in a 15 ml tube. The mixture was then vortexed and 5 ml of water saturated sodium chloride (NaCl) solution was added. A McMaster Egg Slide (Hawksley) was filled with well shaken suspension and kept at room temperature for 3-5 minutes waiting for the eggs to float to

the top of the chamber before being counted. Faecal egg count was expressed as eggs per gram of faeces (EPG) using the following equation:

$$EPG = \frac{Cavrg./Vgrid * Vt}{M}$$

Where:

$\mathbf{C_{avrg.}}$ is the average count of eggs in the grid of chambers 1 and 2 in McMaster slide
$\mathbf{V_{grid}}$ is the volume under the grid of chambers 1 and 2 (0.30 ml)
$\mathbf{V_t}$ is the total volume of mixture (10 ml)
**M** is the mass of faeces (grams)

**Gut parasite burden**

Day 5 post-infection, mice were euthanized and the entire length of the small intestine of each mouse was removed and placed in a petri dish containing PBS. The intestine was teased open longitudinally using dissecting scissors with straight round blades, then wrapped in a double layer of cheesecloth and submerged in a 50 ml Falcon tube filled with 45 ml pre-warmed PBS. The excess of cheesecloth was trapped under the cap of the tubes, and tubes were incubated upright in a water bath at 37°C for 2-3 hours. After incubation, the intestines and the cheesecloth were examined under a dissecting microscope for any remaining worms, which were placed in the tube of the corresponding sample. The worms from each collection tube were transferred to a petri dish and counted.

## 2.3 Intranasal administration of substances to mice

Mice were lightly anaesthetized with isoflurane delivered by SurgiVet classic T3 Isoflurane funnel fill vaporizer (Smiths Medical) in an induction chamber with $O_2$ supplies (2 l/min)

entering the gas circuits downstream of the vaporizer. Animals were held in a supine position and 50 µl PBS containing substances (as indicated in individual experiments) were intranasally delivered dropwise to each nare of the mouse using a pipette (P100, Gilson). Mice were dosed with 10 µg of active or inactive apyrase purified from *Pichia pastoris* with or without 50 µg *Alternaria alternata* extract (Greer Laboratories, USA). Control mice received 50 µl PBS only or 10-20 µg BSA (bovine serum albumin) diluted in PBS.

## 2.4 RNA extraction and cDNA synthesis

Total RNA was isolated from *H. polygyrus* L4 stage larvae or adult worms by homogenising in 1 ml cold TRIzol reagent (Sigma), using a glass homogeniser. Samples were then centrifuged for 10 minutes to remove insoluble material. Following centrifugation, the clear supernatant was collected in a new tube and incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoproteins complex. Chloroform (0.2 ml per 1 ml of TRIzol) was added to the homogenised samples which were mixed by vortexing for 15 seconds and centrifuged for 15 minutes. The upper aqueous phase, containing RNA, was transferred to a fresh tube and incubated for 10 minutes at room temperature with 0.5 ml of isopropanol. Samples were spun for 10 minutes, and the RNA pellet was washed with 75% (v/v) ethanol, centrifuged for 5 minutes at 7,500 x *g*, then air-dried for 5-10 minutes at room temperature before being resuspended in 80 µl RNase-free water. RNA was treated with DNase I (Qiagen), followed by RNA clean-up using the QIAgen RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols. Total RNA concentration and purity were assessed by a NanoDrop spectrophotometer, and RNA quality was checked on a 1% (w/v) agarose gel by

electrophoresis. RNA extracted was stored at −80°C. All centrifugation steps were performed at 12, 000 x *g* at 4°C unless otherwise noted.

Following RNA extraction, cDNA was synthesised using Super Script III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Briefly, in a 20 µl final reaction volume, 1 µg of total RNA was incubated with 150 ng random hexamers and 5 mM dNTPs mix at 65°C for 5 minutes, then placed on ice for 1-2 minutes. Ribolock RNase Inhibitor (40 U), 0.1 mM dithiothreitol (DTT), first strand cDNA reaction buffer (5 x), and 200 U Reverse Transcriptase enzyme (RT) were then added and the mixture was placed in a thermocycler with the conditions as follow: 5 minutes at 25°C, one hour at 50°C and 15 minutes at 72°C. Reverse transcribed cDNA sample was stored at -20°C.

## 2.5 Polymerase Chain Reaction (PCR)

PCR was carried out in a 20 µl reaction volume using Q5 High-Fidelity DNA Polymerase (New England Biolabs) according to the manufacturer's instructions, in a thermocycler under the following conditions: initial denaturation at 98°C for 30 seconds, 35 Cycles of 98°C for 10 seconds, primers annealing temperature for 30 seconds, and 72°C for 30 seconds per kb; followed by a final elongation of 72°C for 2 minutes. All reactions were run with a no-template control to check for contamination of reagents, and a no-RT control to check for genomic DNA (gDNA) contamination. PCR products were resolved on a 1-2% (w/v) agarose gel stained with GelRed nucleic acid gel stain (Biotium) and visualised using a GelDOC-IT TS imaging system (UVP). Following PCR amplification, cDNA fragments were purified using the GenElute PCR clean-up kit (Sigma-Aldrich) according to the manufacturer's protocol.

## 2.6 Expression and purification of recombinant apyrase from *Pichia pastoris*

### Gene cloning and sequencing

Purified PCR products (*apy-1, 2, 3, 4* and *5*) and pPICZα-A plasmid (see appendix A.1 for plasmid map) were digested overnight with XbaI and KpnI restriction endonucleases, resolved on a 1% (w/v) agarose gel then recovered and purified using GenElute Gel Extraction Kit (Sigma-Aldrich). Before ligation, purified linearised plasmid was treated with FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) according to the manufacturer's instructions to prevent re-ligation. Digested PCR products were subcloned into pPICZα-A vector using T4 DNA ligase (New England Biolabs) at room temperature for one hour. The recombinant clones were transformed into *E. coli* DH5-α competent cells (Invitrogen), and the transformation mixture was spread onto Luria Broth (LB) agar plates supplemented with Zeocin (50 µg/ml). Plates were incubated overnight at 37°C. Individual colonies were picked and tested by colony PCR using Taq DNA polymerase in Thermopol buffer (New England Biolabs) in a thermocycler under the following conditions: initial denaturation at 95°C for 5 minutes; 30 cycles of: denaturation at 95°C for 30 seconds, primers annealing temperature for 30 seconds and elongation at 68°C for 1 minutes per kb; followed by a final elongation step for 5 minutes at 68°C. Selected positive transformants were cultured in 6 ml LB medium containing 50 µg/ml Zeocin, and incubated overnight at 37°C with shaking. Plasmid DNA was then isolated from these cultures following the GenElute Miniprep protocol (Sigma-Aldrich).

Diagnostic digests were carried out to identify which clones contained the insert of the correct size in the correct orientation, and these clones were sent to GATC Biotech for

sequencing. The sequence data was analysed using A Plasmid Editor (ApE) software. Once the insert was cloned and sequenced, 5–10 µg of plasmid DNA was generated for transformation following the GenElute plasmid Midiprep protocol (Sigma-Aldrich). Plasmid DNA was then linearized using SacI restriction enzyme, extracted with phenol/chloroform and recovered by ethanol precipitation.

**Apyrase expression in *Pichia pastoris***

The transformation of *Pichia* strain X-33 with the construct was performed following the EasySelect Pichia Expression protocol (Invitrogen). Yeast extract peptone dextrose (YPD) medium (500 ml) was inoculated with a single colony of *Pichia* and grown overnight at 30°C in a shaking incubator at 250 RPM. Cells were collected by centrifugation at 1,500 x *g* for 5 minutes at 4°C, washed with 250 ml sterile $dH_2O$ twice and with 20 ml sorbitol (1 M) once before being re-suspended in 1 ml sorbitol and stored on ice.

*P. pastoris* cells (80 µl) were transferred to an ice-cold 0.2 cm electroporation cuvette to which the concentrated linearized plasmid DNA (5-10 µg) was added. Samples were incubated on ice for 5 minutes, then pulsed at 1,500 V charging voltage, 25 µF capacitance and 200 Ω resistance. After electroporation, 1 ml of ice-cold 1 M sorbitol was added immediately to the cuvette. The mixture was transferred to a sterile 15 ml tube and incubated at 30°C without shaking for 2-3 hours before being spread on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) containing different concentrations of Zeocin (100, 250, 500 and 1000 µg/ml). Plates were incubated at 30°C until colonies formed.

**Isolation and purification of apyrase**

A single colony was selected to inoculate 25 ml BMGY medium (1% yeast extract, 2% peptone, 0.1 M potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, 400 ng/ml biotin, 1% glycerol) and grown overnight at 30°C with constant shaking at 200 RPM. Cells were then harvested by centrifugation at 3,000 x *g* for 5 minutes, re-suspended in 20 ml BMMY medium (same as BMGY, but with 1% methanol instead of glycerol) to induce expression and incubated at 30°C with constant shaking. To maintain induction of protein expression, methanol was added to a final concentration of 0.5% (v/v) every 24 hours for 2 days. Cells were then centrifuged as before, and the culture supernatant containing the recombinant protein was passed through 0.2 µm filter and concentrated using 30,000 MWCO spin columns (vivaspin). Recombinant protein was washed several times with 50 mM HEPES buffer (pH 7.5) and tested for enzyme activity. Once the expression was optimized, the expression protocol was scaled-up to produce more protein, increasing the culture volume and using larger baffled flasks. Following this, total yeast supernatant was concentrated through a 30,000 MWCO Amicon membrane in an ultrafiltration device, then dialysed overnight at 4°C against HEPES buffer (50 mM, pH 7.5) for activity assays, or buffer A (50 mM $NaH_2PO4$, 300 mM NaCl, 1 mM imidazole pH 8.0 in $dH_20$) for protein purification.

Recombinant 6 x His-tagged apyrase was purified using Superflow nickel nitrilotriacetic acid (Ni-NTA) resin affinity chromatography. Ni-NTA-agarose beads (GE Healthcare Lifesciences) were equilibrated with buffer A, then added to the dialysed concentrated yeast supernatant, and incubated for one hour with rotation at 4°C. The beads were loaded on a chromatography column (Bio-Rad) and allowed to sediment before

collecting the flow-through. The column was washed twice with 10 ml buffer A and twice with 10 ml buffer B (buffer A + 10 mM imidazole) to remove unbound proteins. Bound proteins were then eluted with 3-5 ml buffer C (buffer A + 200 mM imidazole) and dialysed against 2 changes with 50 mM HEPES buffer (pH 7.5) for 4 hours, then again with fresh buffer overnight to remove all imidazole. Purified proteins were then concentrated using 30 kDa vivaspin column to a volume of 200 µl and stored at -80°C until further use.

## 2.7 Endotoxin removal

Purified proteins used for in vivo purposes were cleared from pyrogens using Detoxi-Gel endotoxin removing columns (Thermo Fisher) then tested using Pierce™ LAL (Limulus Amoebocyte Lysate) Chromogenic Endotoxin Quantitation Kit, according to the manufacturer's protocols. Absorbance of samples and endotoxin standards was read at 450 nm on a FluoSTAR OPTIMA plate reader (BMG Labtech). A standard curve was generated using Fluostar OPTIMA software, and endotoxin levels were quantified in EU/ml.

## 2.8 Determination of protein concentration

Protein concentration was determined using the Coomassie (Bradford) Protein Assay Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, a standard curve was generated using serial dilutions of BSA at a concentration ranging from 0.025 mg/ml to 2 mg/ml. The assay was carried out in a 96 well flat bottom plate using 200 ul Coomassie assay reagent and 5 ul of standard, blank or protein samples added in triplicate.

Absorbance was measured at 600 nm using FLUOstar OPTIMA microplate reader (BMG Labtech) and protein concentration was determined.

## 2.9 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples (15 µl maximum volume) were mixed with 1x SDS-loading buffer (50 mM Tris-HCl pH 6.8, 0.5 M DTT (dithiothreitol), 2% v/v SDS, 10% v/v glycerol, 0.1% v/v bromophenol blue) and boiled at 105°C for 5 minutes. Samples and pre-stained ladder (ThermoFisher Scientific) were loaded onto 12% polyacrylamide gel (table 2.1) and electrophoresed in 1 x running buffer (2.5 mM Tris-base, 19.2 mM glycine, 0.01% SDS, pH 8.3) at 70 V until proteins run through the stacking gel, then voltage was increased to 100 V until the ladder reaches the bottom of the resolving gel. The gels were either stained with Coomassie blue stain or transferred to a polyvinylidene fluoride (PVDF) blotting membrane (Amersham Hybond, GE Healthcare).

| | **Resolving Gel** | | **Stacking Gel** | |
|---|---|---|---|---|
| | **Volume (10 ml)** | **Final Concentration** | **Volume (5 ml)** | **Final Concentration** |
| **30 % N,N'-Methylenebis (acrylamide)** | 4 ml | 12 % | 830 µl | 5 % |
| **Tris Buffer (1.5 M, pH 8.8)** | 2.5 ml | 375 mM | - | - |
| **Tris Buffer (1 M, pH 6.8)** | - | - | 625 µl | 125 mM |
| **10 % (w/v) SDS** | 100 µl | 0.1 % | 50 µl | 0.1 % |
| **10 % (w/v) ammonium persulphate (APS)** | 100 µl | 0.1 % | 50 µl | 0.1 % |

| | | | | |
|---|---|---|---|---|
| **N,N,N',N'-Tetramethylethylenediamine (TEMED)** | 10 µl | 0.1 % | 5 µl | 0.1 % |
| **$dH_2O$** | 3.3 ml | - | 3.4 ml | - |

**Table 2.1: Polyacrylamide gels recipe**

## 2.10 Coomassie blue staining of polyacrylamide gels

Following SDS-PAGE, gels were stained with Coomassie blue stain (50% v/v $dH_2O$, 10% v/v glacial acetic acid, 25% v/v methanol, 0.4% w/v Coomassie brilliant blue-R) overnight at room temperature with shaking, then washed in destain solution (65% v/v $dH_2O$, 10% v/v glacial acetic acid, 25% v/v methanol) the following day until blue protein bands were clearly seen.

## 2.11 Western blotting analysis

Proteins were transferred onto a PVDF membrane (pre-wet in 100% methanol for 30 seconds) in 1 x transfer buffer (2.5 mM Tris pH8.3, 19.2 mM glycine, 20% v/v methanol) for 90 minutes at 300 mA. Following transfer, the membrane was blocked for 1 hour at room temperature on a shaker using blocking buffer containing 5% (w/v) skimmed milk powder in 1 x Tris-Buffered Saline-Tween (TBS-T: 50 mM Tris-base, 150 mM NaCl, 0.1% Tween 20, pH 7.6). The membrane was washed for 5 minutes in TBS-T, repeated 3 times, then probed overnight at 4 °C with murine anti-c-myc primary antibody diluted 1:1000 in blocking buffer. The membrane was washed again as before, then incubated with goat anti-mouse Ig-

horseradish peroxidase (HRP) secondary antibody (1:1000) for one hour on a shaker at room temperature. The membrane was then washed, and protein bands were visualized using enhanced chemiluminescence (ECL) Western Blotting Detection Reagents (Amersham Bioscience). Chemiluminescence was detected using a LAS-3000 Fuji Imager.

## 2.12 Measurement of enzymatic activity

Enzymatic activity was determined using a phosphate colorimetric assay kit (Abcam) assaying inorganic phosphate ($P_i$) released from nucleotides with reference to a standard curve generated using a range of inorganic phosphate standards. All reactions were carried out in triplicate in a 96 well flat bottom plate (Sterilin) in a final volume of 40 µl HEPES buffer (25 mM HEPES, 150 mM NaCl, pH 7.5) containing 2 mM nucleotide substrates (unless otherwise stated) and the enzyme (apyrase or 5-nucleotidase). Plates were incubated for 10 minutes at room temperature, and the reaction was stopped by the addition of 160 µl $dH_2O$ and 30 µl phosphate reagent to each well. Samples were incubated in the dark for 30 minutes before measuring the absorbance at 600 nm using FLUOstar OPTIMA microplate reader (BMG Labtech). Enzyme activity was expressed as nmol of $P_i$ generated per µg of protein per hour.

To determine divalent cation dependence, between 0 mM and 10 mM $CaCl_2$, $MgCl_2$ or $ZnCl_2$ were added to the HEPES buffer. To evaluate optimum pH, reactions were carried out in a buffer containing 25 mM Bis-Tris propane and 150 mM NaCl with a pH ranging from 5.0 to 10 in increments of 0.5 pH units. To determine kinetic constants (Km and Vmax), enzymatic activity was carried out in HEPES buffer containing different ATP concentrations

(0-10 mM), and values were calculated by Graphpad Prism 7.0 software using non-linear regression analysis fitted to the Michaelis–Menten equation.

## 2.13 Gene expression in *Trypanosoma musculi*

### Gene cloning and sequencing

PCR product, digested with NheI and BamHI restriction endonucleases, was subcloned into the linearized pSSUsp plasmid (see appendix A.3 for plasmid map), and transformed into *E. coli* DH5-α competent cells (Invitrogen) as described previously using 100 µg/ml Ampicillin instead of Zeocin. Once the insert was cloned and the correct insertion confirmed by sequencing, 10 µg of plasmid DNA was generated and linearised with ScaI restriction enzyme. DNA was then extracted with phenol/chloroform, recovered by ethanol precipitation and re-suspended in 10 µl Tris-HCl (10 mM pH 8.0).

### Preparation of macrophage-conditioned medium

Murine macrophages (RAW 264.7) were maintained at 37°C/ 5% $CO_2$ in complete Dulbecco's Modified Eagle's Medium (cDMEM) supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin). Conditioned media were collected from cultured macrophages prior to confluency and centrifuged at 300 x *g* for 6 minutes. Supernatants were passed through a 0.2 µm filter and stored at -20°C.

**In vitro culture of *T. musculi***

*T. musculi* were cultured at 37°C/ 5% $CO_2$ in medium containing 50% macrophage-conditioned medium and 50% McCoy's 5A medium supplemented with L-glutamine, 25 mM HEPES, 10% heat-inactivated FCS, 2 mM sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.2 mM L-cysteine and 0.1 mM 2-mercaptoethanol.

**Generation of transgenic cell lines**

Approximately 5 x $10^7$ wild type (WT) trypanosomes were collected and washed with sterile PBS containing 1% glucose (PBS-G), centrifuged at 1,000 x *g* for 10 minutes, and re-suspended carefully in 100 µl room temperature transfection buffer (90 mM sodium phosphate, 5 mM potassium chloride, 50 mM HEPES, 0.15 mM calcium chloride, pH 7.3). Trypanosomes were transferred to a 0.2 cm electroporation cuvette to which the concentrated linearized plasmid DNA was added, and immediately transfected by electroporation using Nucleofector II from Amaxa Biosystem (Lonza). The cell suspension was gently transferred into 30 ml of pre-warmed medium with no selection drugs and cultured at 37°C/5% $CO_2$. After 24 hours recovery, transfectants were selected by the addition of 25 µg/ml blasticidin, and fresh selection medium was added every 2-3 days. After 7-10 days, blasticidin-resistant clonal cell lines were isolated by serial dilution and maintained in 20 µg/ml blasticidin.

**Isolation of *T. musculi* gDNA and total RNA**

Genomic DNA (gDNA) was isolated from wild type and transgenic *T. musculi* using QIAgen DNeasy Blood and Tissue Kit (QIAgen), and total RNA was extracted using Mammalian Total RNA Miniprep Kit (GenElute) following the manufacturer's instructions. RNA was reverse transcribed as described previously.

***T. musculi* lysate and secreted proteins**

WT and transgenic *T. musculi* were collected, washed twice with PBS and grown in a medium containing 50% McCoy's 5A complete medium (described previously but without heat-inactivated FCS) and 50% DMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. After 24-48 hours, trypanosomes were centrifuged at 1,000 x *g* for 10 minutes. Culture supernatants containing total secreted proteins were filtered, concentrated and washed with 50 mM HEPES buffer, and trypanosomes (pellet) were lysed in ice-cold lysis buffer (50 mM HEPES pH 7.5, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA and protease inhibitor cocktail).

**Enzymatic activity of *T. musculi* secreted proteins**

Protein concentration was determined by Bradford assay, and nucleotidase activity of total secreted proteins was assessed by colorimetric method (phosphate assay kit, Abcam) described previously. Results were expressed in nmol of $P_i$ generated per µg of total secreted proteins per hour.

**Ecto-NTPDase activity in *T. musculi***

Live parasites were washed twice with 0.9% NaCl and incubated for 1 hour at 37°C/ 5% $CO_2$ in reaction buffer (50 mM HEPES, 116 mM NaCl, 5.4 mM KCl, 5 mM $MgCl_2$, 5.6 mM D-glucose, pH 7.4) containing 5 mM nucleotide substrate, in a final volume of 250 µl (1 x $10^8$ parasites/ml). The reaction was initiated with the addition of specific substrate and terminated by addition of 250 µl ice-cold HCl (0.2 M). The cell suspensions were centrifuged, and $P_i$ was measured in aliquots of the supernatant using a phosphate assay colorimetric kit (Abcam). Parasites were counted before and after the assay to check if any cell lysis occurred. Enzymatic activity, expressed as nmol of $P_i$ released by 1 x $10^8$ parasites in one hour, was calculated by subtracting non-specific hydrolysis that was detected in the absence of cells, in the absence of cells and substrate, or after adding the parasites once the reaction was stopped.

Ecto-nucleotidase activity was further analysed in the presence of various inhibitory agents. Activity was measured in reaction buffer containing 5 mM ATP in the presence of 1 mM sodium fluoride or 3 µM ammonium molybdate (acid phosphatase inhibitors), 1 mM levamisole (alkaline phosphatase inhibitor), 100 µM DIDS (4,40-diisothiocyanatostilbene 2,20-disulfonic acid) or Suramin (ecto-ATPase inhibitors), or 1 mM vanadate (ecto-NTPDase inhibitor).

**Parasite infection**

In order to adapt in vitro *T. musculi* to growth in vivo, trypanosomes were passaged through irradiated mice until parasitaemias reach $10^6$ parasites/ml of blood. Irradiated (1 Gy) female

BALB/c mice were infected intraperitoneally with *T. musculi,* and their blood was sub-passaged every 7-10 days in new irradiated mice. After the third passage, trypanosomes were isolated from the blood by cardiac puncture, diluted in sodium citrate buffer (PBS, 3.2% w/v sodium citrate, 1% w/v glucose), counted, and 2 x $10^5$ were injected intraperitoneally into experimental mice. Parasitaemias were monitored by microscopy analysis of tail vein blood samples diluted in ACK lysis buffer (155 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) and expressed as number of parasites per ml of blood. In vivo growth curves were performed by counting the parasites and converting the average to log equivalent values.

## 2.14 Generation of inactive apyrase

The expression of inactive apyrase by *P. pastoris* or *T. musculi* was carried out in the same way as for the active form. Inactivation of the apyrase catalytic activity was achieved by altering glutamate 147 to glutamine (E147Q) (Jiayin Dai *et al*., 2004), using the Q5 site-directed mutagenesis kit (New England Biolabs) according to the manufacturer's instructions. Specific primers with the desired mutation were designed and used in a PCR reaction that amplifies the entire vector. A methylation-dependent endonuclease (DpnI) was used to remove the original un-mutated plasmid without affecting the mutated one. Colonies were sequenced to confirm the desired modification, and phosphate assays were performed to assess the loss of enzymatic activity.

## 2.15 Tissue processing

### Bronchoalveolar lavage (BAL)

Mice were euthanized, and the trachea was exposed and cannulated with an 18-gauge needle catheter. Lavage was performed twice by slowly injecting 1 ml cold PBS containing 0.2% BSA and 2 mM EDTA. BAL fluid was then aspirated, collected in a 15 ml Falcon tube, and centrifuged at 300 x *g* for 5 minutes. The supernatant was recovered and stored at -20°C until assayed. Cytokines levels were measured in the supernatants of BAL fluid by ELISA according to the manufacturer's instructions.

### Preparation of lung single-cell suspensions

Mice were euthanized, and the trachea was exposed and cannulated with an 18-gauge needle catheter connected to a 3 ml syringe and injecting 1.5 ml dispase II (Sigma) digest solution (5 mg/ml) until lungs were inflated. The trachea was tied off with a suture and lungs were gently extracted from the thorax and placed into a 5 ml polystyrene bijou tube containing another 1.5 ml of digest solution. Lungs were incubated for 25 minutes at room temperature then for a further 30 minutes at 37°C. Subsequently, the heart and mediastinal tissues were snipped away and discarded, and lungs were mechanically dissociated in 7 ml serum-free DMEM supplemented with 100 units/ml DNAse I and 25 mM HEPES. Samples were then incubated for 10 minutes at room temperature on a rocking shaker. After this time, DNAse-treated samples were pushed through a 100 µm Falcon cell strainer (BD Biosciences) into a sterile tube using the plunger end of a 2 ml syringe, then washed with

cDMEM and centrifuged at 450 x *g* for 6 minutes at 4°C. The cell suspension was treated with ACK lysis buffer for 5 minutes at room temperature to lyse red blood cells, then neutralised with cDMEM and centrifuged again as before. Cells were re-suspended in a known volume of cDMEM, passed through 70 µm cell strainer and total viable cells were counted.

**Preparation of spleen single-cell suspensions**

Spleens were isolated and placed on ice into a 5 ml polystyrene bijou tube containing 1 ml cDMEM. Tissues were processed into a single-cell suspension by pushing the spleen through a 100 µm cell strainer. The cell suspension was centrifuged at 450 x *g* for 5 minutes, treated with ACK lysis buffer for 5 minutes at room temperature and neutralised with cDMEM medium. After being centrifuged, cells were re-suspended in a known volume of cDMEM, passed through 70 µm cell strainer and counted.

**Cell counting**

Samples were diluted in 0.2% trypan blue (in PBS) and total live cells were counted by microscopy using a haemocytomer. The total number of cells in each sample was determined as follow:

$$Total\ Cell\ Number = C_{avrg} * 10^4 * DF * V_t$$

Where:
$C_{avrg}$ is the average count of cells in the 4x4 grids of the haemocytomer
DF is the dilution factor in trypan blue
$V_t$ is the total volume of sample.

**Serum collection**

Blood was collected by cardiac puncture in blood collection tubes containing serum separating gel. Tubes were centrifuged at 4000 x *g* for 10 minutes, and serum was stored at -80°C until analysed.

## 2.16 Fluorescence Activated Cell Sorting (FACS) analysis

Negative controls (Unstained or live/dead) received FACS buffer (1% FCS, 25 mM HEPES, 1 mM EDTA diluted in PBS) only, and suitable fluorescence minus one (FMO) samples were considered where needed. All incubations were made at 4°C in the dark, unless otherwise stated.

**Extracellular cell staining**

After single-cell suspensions were made and cells were counted, 2 x $10^6$ cells/well were plated in a 96 well V bottomed plate, centrifuged at 900 x *g* for 2 minutes at 4°C, and washed with 200 µl cold PBS. Cells were centrifuged as before, then stained for 20 minutes with Live/Dead Zombie Aqua (Biolegends, diluted in PBS) to exclude dead cells from subsequent analysis. Cells were then washed with FACS buffer, centrifuged, and incubated for 20 minutes in Fcγ receptor block (BD Biosciences, diluted in FACS buffer) to prevent non-specific binding to Fc receptors. Cells were washed and pelleted as before prior to being stained for 30 minutes with the appropriate fluophore conjugated antibody cocktails (listed

in table 2.2). Following staining, cells were washed then re-suspended in 150 µl of FACS buffer and stored at 4°C in the dark until analysis.

**Intracellular cell staining**

For intracellular staining, cells stained with the appropriate cell surface markers were fixed with 200 µl Cytoperm/Cytofix (BD Biosciences) for 20 minutes at room temperature in the dark. Cells were centrifuged again as before, re-suspended in 200 µl permeabilisation buffer (BD Biosciences), then centrifuged and incubated for 30 minutes with Fc block. After this time, cells were washed by adding 100 µl permeabilisation buffer, centrifuged as previously and stained with 50 µl fluorophore antibody cocktails diluted in permeabilisation buffer. After 30 minutes incubation, cells were centrifuged then re-suspended in 150 µl FACS buffer and stored in the dark at 4°C until analysed by flow cytometry.

For Intracellular cell staining of cytokines (such as IL-5 and IL-13), single-cell suspensions were plated in a 96 well V bottomed plates and incubated at 37°C/5% $CO_2$. After 2 hours, cells were centrifuged as before, and re-suspended in cDMEM containing phorbol myristate acetate (PMA, 100 ng/ml) and ionomycin (1 µg/ml) and incubated at 37°C/ 5% $CO_2$ for one hour. Samples were then incubated for a further 3 hours in the presence of Golgi-Plug (Brefeldin-A, BD Biosciences) diluted in cDMEM and added to each well. After this time, cells were centrifuged as before, washed once with PBS and stained with live/dead and extracellular markers as described previously, before being fixed for intracellular cell staining.

| Antigen recognised | Fluophore/conjugate | Clone number | Dilution | Manufacturer |
|---|---|---|---|---|
| **CD3** | APC-Cy7 | 145-2C11 | 1:150 | Biolegend |
| **CD4** | AF700 | RM4-5 | 1:100 | eBioscience |
| **CD8** | PB | 53-6.7 | 1:200 | eBioscience |
| **CD11b** | PE | M1170 | 1:600 | BD Bioscience |
| **CD11c** | PE-Cy7 | N418 | 1:1600 | Biolegend |
| **CD19** | PE | 6D5 | 1:300 | Biolegend |
| **CD39** | PE-Cy7 | Duha59 | 1:200 | Biolegend |
| **CD44** | PerCP-Cy5.5 | IM7 | 1:200 | Biolegend |
| **CD45** | AF700 | 30-F11 | 1:400 | Biolegend |
| **CD62L** | PE | MEL14 | 1:200 | eBioscience |
| **CD73** | PE | TY/11.8 | 1:200 | Biolegend |
| **CD127** | APC | A7R34 | 1:100 | Biolegend |
| **CD278 (ICOS)** | Pe-Cy7 | C398.4A | 1:100 | Biolegend |
| **F4/80** | APC-Cy7 | BM8 | 1:200 | Biolegend |
| **Foxp3** | PerCp-Cy5.5 | FJK-16S | 1:200 | eBioscience |
| **Gr1** | PB | RB6-*C5 | 1:400 | Biolegend |
| **IL-5** | APC | TRFK5 | 1:100 | BD Pharmingen |
| **IL-13** | PE-Cy7 | EBIO13A | 1:100 | eBioscience |
| **Siglec F** | APC | E50-2440 | 1:1600 | BD Pharmingen |
| **ST2** | PerCP-CY5.5 | RMST2 | 1:50 | eBioscience |
| **CD16/CD32** | Unconjugated | 2.4g2 | 1:500 | TONBO Bioscience |

**Table 2.2: Flow cytometry antibodies**

### Data analysis and cell gating

Data were acquired using an LSR-Fortessa flow cytometer (BD Biosciences) and analysed with FlowJo software (TreeStar). Compensations and voltage setup were performed prior to each experiment using fluochrome-labelled beads (eBioscience) and unstained cells, and a

total of 100,000 to 1,000,000 events were collected. Forward scatter (FSC) and sideway scatter (SSC) parameters were used to exclude doublets, debris and cell fragments and AQUA Live/Dead stain was used to define live cells. Gating strategies for different cell populations are shown below (figures 2.1-2.3).

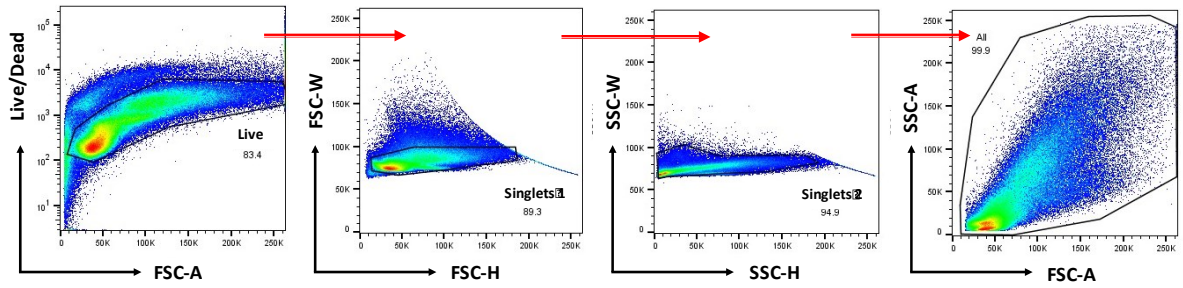


**Figure 2.1: Staining and gating strategy for live, single-celled lymphocytes**
FSC-H, FSC-W and FSC-A: Forward scatter (FSC), height (H), width (W) and aperture (A).
SSC-H, SSC-W and SSC-A: Sideways scatter (SSC), height (H), width (W) and aperture (A).

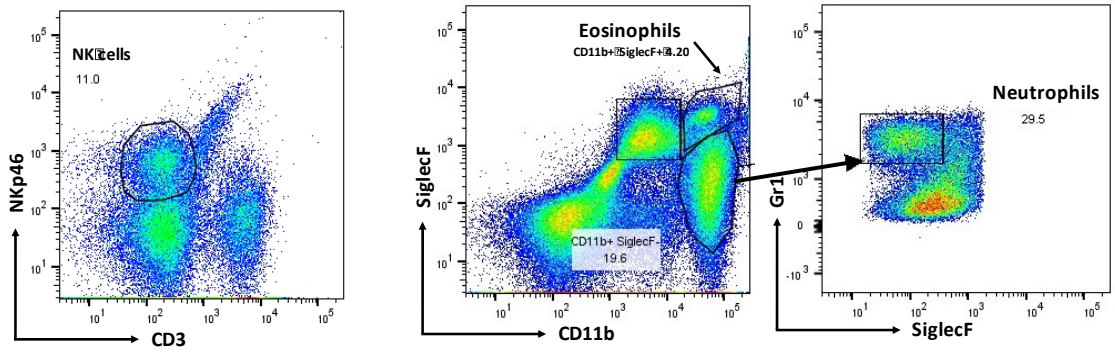


**Figure 2.2: Staining and gating strategy for NK cells (NKp46+), eosinophils (CD11b+SiglecF+) and neutrophils (CD11b+SiglecF-Gr1+)**
After gating for live and single cells, different cell populations were selected.

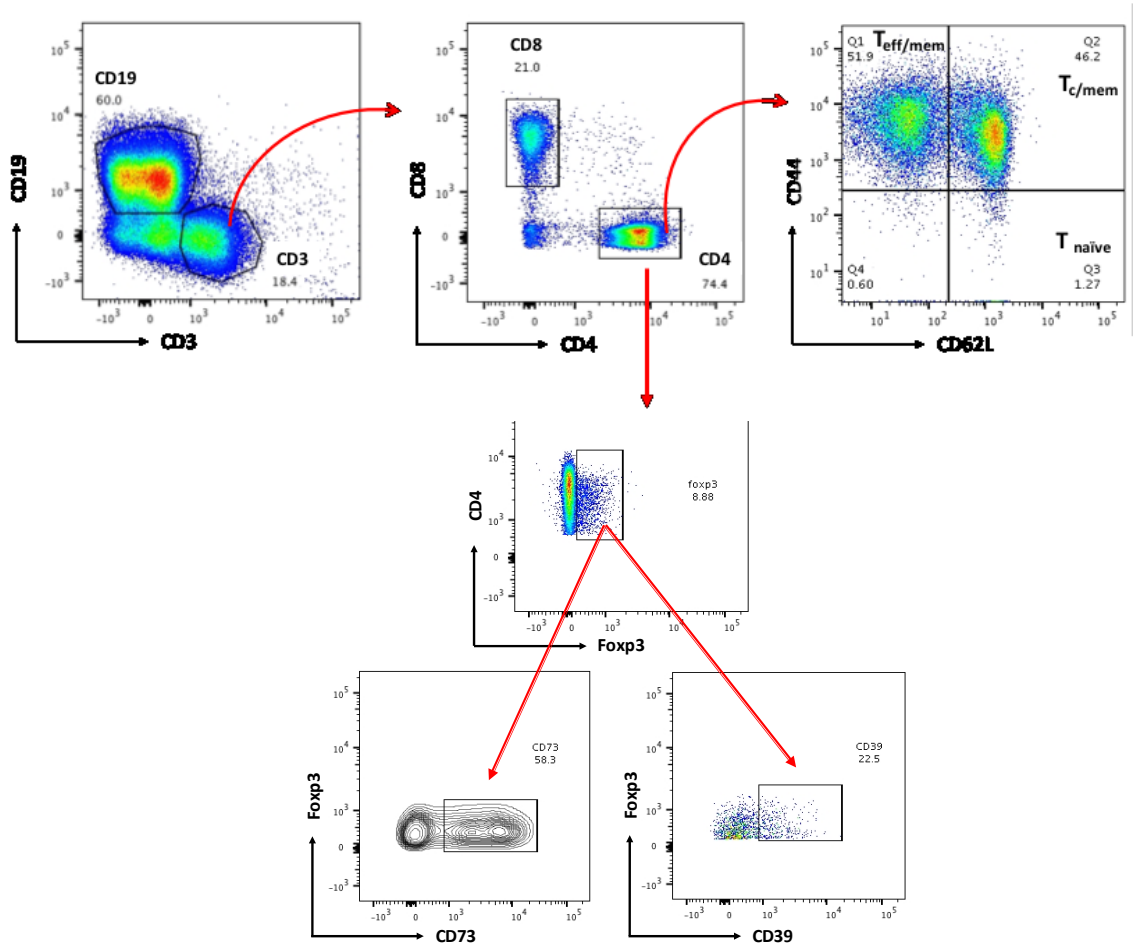


**Figure 2.3: Staining and gating strategy for B (CD19+) and T (CD3+) lymphocytes, T cell subsets (CD4+ and CD8+), CD4+ T cell activation markers, and CD4+Foxp3+ T cell subsets (Foxp3+CD39+ and Foxp3+CD73+)**

After gating for live and single cells, different cell populations were selected.

## 2.17 Enzyme Linked Immunosorbent Assay (ELISA)

**Cytokine ELISA**

Spleen cells were seeded into a 96 well flat-bottom plate with 2 x $10^6$ cells/ well and stimulated with 10 µg anti-CD3/anti-CD28 for 24 hours. Cell culture supernatants were recovered by centrifugation (500 x *g*, 5 minutes), carefully pipetted into a fresh plate and stored at –20°C until assayed.

Cytokine ELISA was carried out using BD BioLegend kits or R&D systems according to the manufacturer's recommendations. Nunc 96 maxisorp plates (Thermoscientific) were coated with capture antibody in 0.5 M carbonate-bicarbonate buffer and incubated overnight at 4°C. The capture antibody was aspirated, and the plates were washed 3 times with washing buffer (PBS, 0.05% v/v Tween 20). Plates were then incubated with blocking buffer (PBS, 1% w/v BSA) for one hour at room temperature. Plates were washed as before and 50 µl of samples and standards at the appropriate dilutions were loaded in triplicates and incubated for 2 hours at room temperature. Plates were further washed then incubated with biotinylated detection antibody for 2 hours. Streptavidin-coupled horseradish peroxidase (HRP, 50 µl) was added after washing the plates which were then incubated in the dark for 30 minutes. The plates were further washed and TMB (3,3',5,5'-Tetramethylbenzidine) substrate was added before stopping the reaction with 1 M $H_2SO_4$. Absorbance was measured at 450 nm and 540 nm in a FLUOstar OPTIMA microplate reader (BMG Labtech), and levels of cytokines were determined after subtracting the absorbance at 540 nm from the absorbance at 450 nm.

**Antibody subtype ELISA**

Parasite-specific antibody were measured by ELISA coating Nunc plates with 5 µg/ml of wild type *T. musculi* protein extract in 0.1 M carbonate buffer overnight. To determine end-point titres, sera from infected mice were used in doubling dilutions, and plates were incubated with HRP-conjugated goat anti-mouse antibodies (Invitrogen) diluted in blocking buffer: IgM (1:10000), IgG1 (1:12000), IgG2a (1:8000), IgG2b (1:2000) and IgG3 (1:8000).

## 2.18 Quantitative real-time PCR (qPCR)

Total RNA was extracted from spleenocytes using GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich) and converted to cDNA using Superscript III (Invitrogen) according to the manufacturer's instructions. The qPCR reactions were carried out using QuantiTect SYBR Green PCR Master Mix (Qiagen) in a 7500 Fast Real-time PCR thermocycler (Applied Biosystems) under the following conditions: 30 seconds denaturation at 95°C, 30 seconds annealing at 60°C and 30 seconds elongation at 72°C for 40 cycles. All reactions were run in duplicates including a no-template and no-RT controls to check for contamination.

Relative gene expression of NOS2, Chi3l3, SOCS1, MRC1, ARG1 and RELMα was calculated by the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) using HPRT, B2m and HMBS as housekeeping genes. Ct values were calibrated to the median of control samples infected with *T. musculi* mutant Apy-3.

| Gene symbol | Gene Name | Primer Sequence (5'-3') | Annealing Temperature (°C) |
|---|---|---|---|
| **B2m** | Beta-2 microglobulin | **Forward**: CTCACACTGAATTCACCCCCA<br>**Reverse:**CATGTCTCGATCCCAGTAGACG | 60 |
| **HMBS** | Hydroxy-methylbilane synthase | **Forward**: AGGTCCCTGTTCAGCAAGAA<br>**Reverse:** CATTAAGCTGCCGTGCAACA | 60 |
| **HPRT** | Hypoxanthine guanine phosphoribosyl transferase | **Forward:** ACAGGCCAGACTTTGTTGGA<br>**Reverse:** ACTTGCGCTCATCTTAGGCT | 60 |
| **ARG1** | arginase-1 | **Forward:** AAAGGCCGATTCACCTGAGC<br>**Reverse:**CTGAAAGGAGCCCTGTCTTGTA | 60 |
| **Chi3l3 (YM1)** | chitinase-like 3/YM1 | **Forward:** AAGTTGAAGGCTCAGTGGCT<br>**Reverse:**GTAGATGTCAGAGGGAAATGTCT | 60 |
| **MRC1** | mannose receptor C-type-1 | **Forward:** GGAGGGTGCGGTACACTAAC<br>**Reverse:** TCAGTAGCAGGGATTTCGTCTG | 60 |
| **NOS2** | nitric oxide synthase-2 | **Forward:** CCGGCAAACCCAAGGTCTAC<br>**Reverse:** CTGCTCCTCGCTCAAGTTCA | 60 |
| **RELMα** | resistin-like molecule-alpha | **Forward:**TCTTGCCAATTCCAGCTAACTATC<br>**Reverse:** GCCACAAGCACACCCAGTAG | 60 |
| **SOCS1** | suppressor of cytokine signaling-1 | **Forward:** CAACGGAACTGCTTCTTCGC<br>**Reverse:** AGCTCGAAAAGGCAGTCGAA | 60 |

**Table 2.3: List of primers used for qPCR**

## 2.19 Nitric Oxide (NO) and arginase activity assays

Spleen single-cell suspensions were incubated for 48 hours in DMEM after being stimulated with 10 µg/ml *T. musculi* extract. Supernatants were recovered, and nitrite level was assayed by Griess assay (Promega) according to the manufacturer's instructions. Briefly, a standard curve was generated by serial dilutions of the stock nitrite solution (0.1 M) at concentrations ranging from 100 µM to 1.56 µM. The assay was carried out in a 96 well flat-bottom plate adding 50 µl sulphanilamide reagent to 50 µl sample or standard. The plate was incubated for 10 minutes at room temperature in the dark, then 50 µl of N-1-

naphthylethylenediamine dihydrochloride (NED) solution was added to each well, and the plate was incubated again as before. Absorbance was measured at 540 nm using FLUOstar OPTIMA microplate reader (BMG Labtech), and nitrite concentration was calculated from the mean of triplicate values.

Cells were lysed with 0.1% (v/v) Triton X-100 containing protease inhibitors. Urea standards, at a concentration ranging from 160 µg/ml to 1.25 µg/ml, were prepared in $MnCl_2$ buffer (10 mM $MnCl_2$, 25 mM Tris-HCl, 0.1% v/v Triton X-100) using serial dilutions of 10 mg/ml urea stock solution. Cell lysates were incubated for 10 minutes at 55°C in 10 mM $MnCl_2$, 50 mM Tris-HCl (pH 7.5), then further incubated for one hour at 37°C after the addition of L-Arginine (500 mM, pH 9.7). To stop the reaction, 800 µl acid mix ($H_2SO_4$: $H_3PO_4$: $H_2O$ at a ratio of 1:3:7) was added, followed by 40 µl of 9% ISPF (α-isonitrosopropiophenone). Samples and standards were incubated for 30 minutes at 95°C, then for 10 minutes at room temperature in the dark. After this time, samples were plated in triplicate in a 96 well flat-bottom plate, and absorbance was measured at 450 nm. Urea concentration was calculated from the mean of triplicate values.

## 2.20 Statistical analysis

Data were expressed as the mean ± SEM and analysed using GraphPad Prism 7.0 (GraphPad Software). Significance differences were calculated using non-parametric Mann-Whitney test, one-way ANOVA Kruskal-Wallis test, or parametric two tailed non-paired student's t-test (unless otherwise stated). Results were considered to be statistically significant when p value was lower than 0.05: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

# CHAPTER 3

# Characterisation of the recombinant and native secreted *Heligmosomoides polygyrus* apyrases

## 3.1 Introduction

Apyrases (EC 3.6.1.5), also known as nucleoside triphosphate-diphosphohydrolases (NTPDases), are nucleotide-metabolising enzymes that can hydrolyse a broad range of nucleoside triphosphates and diphosphates to monophosphates. The latter can then be hydrolysed by 5´-nucleotidase (5´-NT) to nucleosides (Figure 3.1) (Zimmermann *et al.*, 1992; Champagne *et al.*, 1995; Zimmermann, 1996). Their enzymatic activities are usually dependent on divalent cations such as $Ca^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Mg^{2+}$, or $Zn^{2+}$ (Meyerhof, 1945; Plesner, 1995; Komoszyński and Wojtczak, 1996).

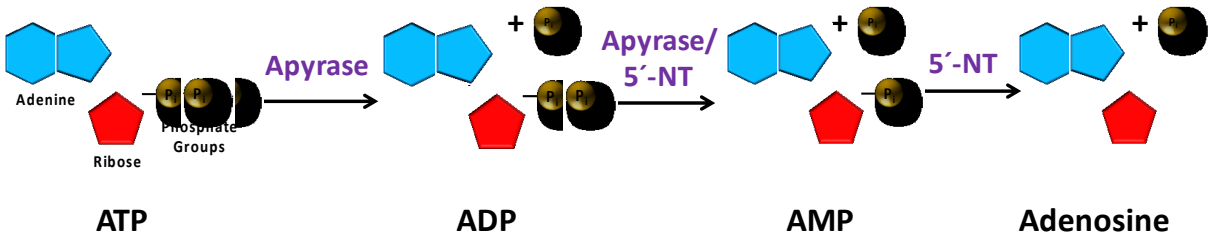


**Figure 3.1: ATP hydrolysis pathway**
A structural illustration of the hydrolysis of ATP, ADP and AMP by apyrase and 5´-NT to produce adenosine.
ATP: Adenosine triphosphate; ADP: Adenosine diphosphate; AMP: adenosine monophosphate; $P_i$: inorganic phosphate.

Apyrases can be classified into five distinct families. The ecto-apyrases are membrane-bound, with their catalytic domain exposed on the cell surface. These include the human apyrase referred to as CD39, NTPDase 1, 2, 3 and 8 (Plesner, 1995; Kaczmarek *et al.*, 1996; Komoszyński and Wojtczak, 1996). They are characterised by the presence of five

conserved domains of amino acids called “apyrase conserved regions” (ACRs) essential for enzyme function (Handa and Guidotti, 1996; Smith and Kirley, 1999b; Joan H. F. Drosopoulos *et al.*, 2000; Smith *et al.*, 2002). The endo-apyrases, with their catalytic domain localized intracellularly, are intracellular enzymes but can be secreted as well (Zimmermann, 1999). There is also a family referred to as organelle-located (for example Golgi apparatus) apyrases which are not known to be secreted (Biederbick *et al.,* 2000; Komoszynski and Wojtczak, 1996; Wang and Guidotti, 1998, Zimmermann *et al.*, 2000). The secreted apyrases are another family which is also called Cimex-type apyrases, as they were first discovered in a blood-feeding insect, the bed bug *Cimex lectularius* (Valenzuela *et al.*, 1998; Smith *et al.*, 2002; Hughes, 2013). The 5´-nucleotidase (5´-NT) enzymes (Champagne *et al.*, 1995) are another family of apyrases which include CD73.

Functionally, secreted and membrane-bound apyrases have been previously documented to play important roles in many biological processes in a vast range of organisms including animals, plants and parasitic helminths (Komoszynski and Wojtczak, 1996). They have been shown to be involved in blood platelet aggregation (Colman, 1990; Côté *et al.*, 1992; Marcus and Safier, 1993), blood pressure regulation (Zimmermann, 1996), blood fluidity maintenance (Smith *et al.*, 2002), membrane permeability (Komoszynski and Wojtczak, 1996) and homeostasis (Bernardes *et al.*, 2000). Furthermore, apyrases have been shown to play a role in several other mechanisms such as pathogen-host interaction (Bisaggio *et al.*, 2003; Matin and Khan, 2008), parasite virulence (Berrêdo-Pinho *et al.*, 2001), purine salvage (de Souza Leite *et al.*, 2007), cell lipid and protein glycosylation, plant growth, eye development and oncogenesis (Knowles, Isler and Reece, 1983; Smith and Kirley, 1999a; Murphy and Kirley, 2003; Knowles and Li, 2006; Massé *et al.*, 2007; Knowles, 2011). In

addition to regulating many physiological and metabolic processes, apyrases have also been proposed as key regulators of neurotransmission (Battastini *et al.*, 1991; Sarkis and Salto, 1991; Wang, Rosenberg and Guidotti, 1997; Zimmermann, Zebisch and Sträter, 2012).

Moreover, apyrases are thought to play key roles in the immune system. Extracellular nucleotides, mainly ATP, are released in the extracellular space from damaged, stressed or apoptotic cells. Extracellular ATP binds to purinergic P2 receptors expressed on the cell surface of immune cells and triggers pro-inflammatory immune responses. Apyrases can be immunomodulatory, by converting the potent pro-inflammatory ATP to ADP then to AMP which could be converted to the anti-inflammatory molecule adenosine. The latter was shown to suppress inflammatory cytokine production (IL-12 and TNFα) and to enhance the release of IL-10 by macrophages (Hasko & Cronstein, 2004). It has also been shown that adenosine can induce regulatory T cells (Tregs) thus suppressing the activation of Dendritic cells (DCs) and effector T cells (Deaglio *et al.*, 2007). Tregs express the membrane-bound enzymes CD39 and CD73, which show apyrase and 5´-NT activity respectively, and they act in concert to produce adenosine which can suppress T cell activation (Antonioli, Pacher, *et al.*, 2013). In the intestine, the release of ATP by intestinal bacteria activates DCs to secrete the pro-inflammatory cytokines IL-6 and IL-23 resulting in the induction of inflammatory Th17 cells, and promotes the conversion of Treg cells into effector Th17 cells. These two processes seem likely to be limited by apyrase enzymes as the ATP would be broken down (Atarashi *et al.,* 2008; Schenk *et al.,* 2011). Similarly, ATP-dependent dendritic cell activation is essential for Th2-dependent lung pathology in asthma models, and this can be inhibited through the administration of an apyrase (Idzko *et al.,* 2007).

In a proteomic analysis, four apyrases (Apy-1 – Apy-4) have been identified in the excretory/secretory products of adult *Heligmosomoides polygyrus,* with Apy-1 consisting of three minor isoforms (Hewitson *et al.*, 2011). Subsequently, L4 stage *H. polygyrus* have been shown to secrete Apy-3 and low levels of a fifth L4-specific apyrase, termed Apy-5 (Hewitson *et al.*, 2013). Biochemical characterisation of *H. polygyrus* apyrases was not yet undertaken. Thus, in order to investigate the function of these apyrases and study their immunomodulatory effects, they need to be either purified in native form or cloned and expressed. This study aimed to clone, express and characterise the apyrases secreted by the L4 and adult worms via heterologous expression.

The methylotrophic yeast *Pichia pastoris* is a single-celled eukaryote used successfully as an expression system which can generate high amounts of recombinant proteins (Cereghino *et al.*, 2002; Krainer *et al.*, 2012; Ahmad *et al.*, 2014). One of the vector series used for *P. pastoris* expression is pPICZα (Appendix A1) which has the very strong, methanol inducible alcohol oxidase (AOX1) promoter (Weidner, Taupp and Hallam, 2010; Bawa *et al.*, 2014), and where methanol serves as the main carbon source while inducing expression (Ellis *et al.*, 1985). This vector also contains an α-factor secretion signal for secretion of the recombinant protein, an antibiotic (Zeocin) resistance gene and a C-terminal peptide containing the c-myc epitope and the polyhistidine (6xHis) tag for detection and purification of the recombinant protein (Weidner, Taupp and Hallam, 2010; Yang *et al.*, 2013).

## 3.2 Results

### *H. polygyrus* apyrase genes were successfully cloned into pPICZαA and expressed in *Pichia pastoris*

In order to biochemically characterise the apyrases secreted by L4 stage (Apy-5) and adult (Apy-1-4) *H. polygyrus*, the mature protein-coding sequences were amplified by RT-PCR, cloned into a yeast expression vector, then expressed as secreted proteins in the methylotrophic yeast *P. pastoris.* Apy-1.3, one of the three minor sequence variants of Apy-1, was expressed in *Pichia,* but for convenience it will be referred as Apy-1 in this thesis.

Total RNA was first isolated from L4 and adult *H. polygyrus*, reverse transcribed to cDNA which was then amplified by PCR excluding the signal peptide of the corresponding gene, using forward and reverse primers specific for each apyrase and including suitable restriction sites (XbaI and KpnI). Amplification yielded fragments with the expected size of ~1000 bp (Figure 3.2). All five apyrase genes were then cloned into the pPICZαA plasmid downstream of the *Saccharomyces cerevisiae* α-mating secretion factor, and transformant cells were selected by Zeocin resistance. Positive clones were identified by colony PCR, then sequenced to confirm that the genes were successfully cloned in frame with the C-terminal peptide tag into the expression vector and to ensure that no mutations were present in the gene sequence or key features of the plasmid. Having confirmed the correct sequence of the constructs with no mutations present, the plasmid DNAs were linearized by digestion with SacI to promote integration into the yeast genome, then purified by phenol/chloroform extraction and transformed into *P. pastoris* strain X-33. *Pichia* transformants (5-10 for each

apyrase gene) were selected using Zeocin in a small-scale expression study, and the total culture supernatants were analysed by SDS-PAGE for expression of apyrases.

Results showed a protein of approximately 50-55 kDa for Apy-1 and Apy-4, and a more diffuse band of 55-60 kDa for Apy-2, Apy-3 and Apy-5 as visualized by Coomassie brilliant blue staining (Figure 3.3A). A western blot was performed using a primary anti-c-myc mouse monoclonal antibody and a secondary goat anti-mouse horseradish peroxidase (HRP) antibody, which confirmed that the bands correspond to recombinant proteins with the c-myc tag (Figure 3.3B). For all five apyrases, bands ranging in apparent molecular weight between 50 and 60 kDa indicated the expression of the recombinant His-tagged proteins. In addition, no bands were observed in culture supernatants of WT *P. pastoris*.

The molecular weight of the recombinant proteins appeared to be larger than the expected size from the cDNA sequence (Table 3.1). This suggests that the proteins are likely to be glycosylated, causing the shift on SDS-PAGE. Therefore, in order to investigate the possible presence of glycan residues, an enzymatic deglycosylation reaction was performed using PNGase F enzyme and analysed by SDS-PAGE and Coomassie Brilliant Blue staining. Post-PNGase F treatment, protein bands shifted from 50-60 kDa to about 40 kDa, the expected molecular mass of the native apyrases (Figure 3.4). This confirms that the recombinant enzymes expressed in *P. pastoris* were glycosylated with N-linked oligosaccharides.

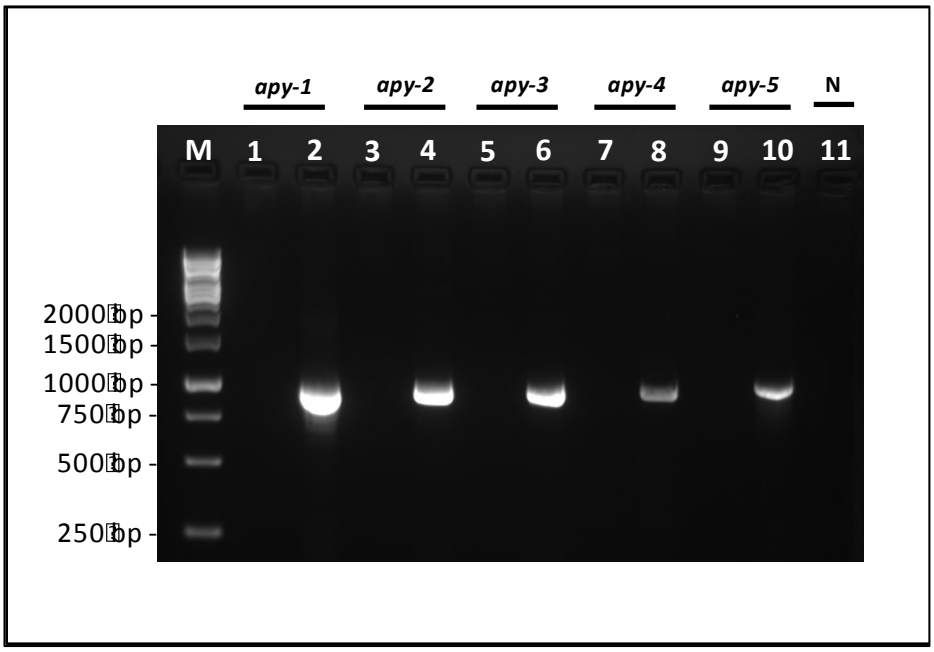


**Figure 3.2: Amplification of *H. polygyrus* apyrase cDNAs by RT-PCR**
Apyrase cDNAs were amplified by RT-PCR using RNA from *H. polygyrus* L4 and adult worms. Specific primers were used to amplify the full-length coding sequence (minus signal peptide) for each gene (*apy-1, apy-2, apy-3, apy-4,* and *apy-5*) and introduce 5´ KpnI and 3´ XbaI restriction sites. Amplification products were separated on 1.5% (w/v) agarose gels, stained with GelRed stain and visualized on a UV trans-illuminator.
Lanes 2, 4, 6, 8, 10: RT-PCR products from *apy-1, apy-2, apy-3, apy-4* and *apy-5.*
Lanes 1, 3, 5, 7, 9: No-RT (no reverse transcriptase) controls for each gene.
Lane 11: Negative control sample (N) in which template cDNA was not included in PCR reaction.
Lane M: Molecular weight markers (1 kb DNA ladder).

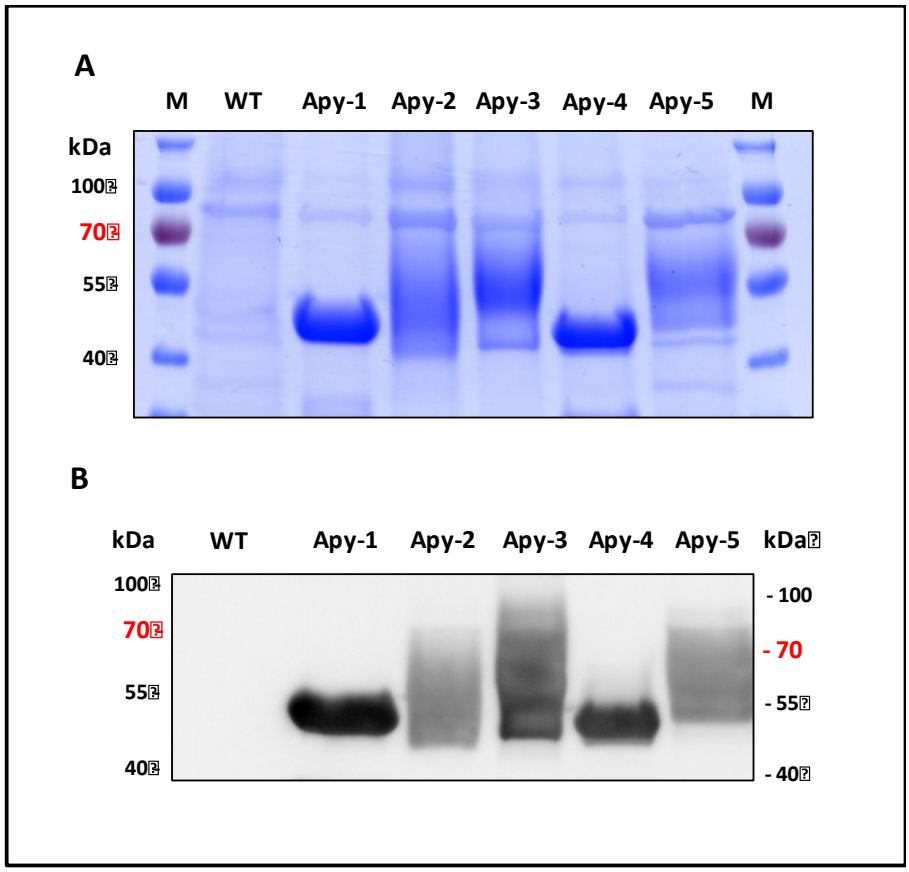


**Figure 3.3: Expression of apyrases in *Pichia pastoris***
**Panel A:** Culture supernatants from *P. pastoris* clones expressing Apy-1, 2, 3, 4, and 5. Samples were resolved on a 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue.

**Panel B:** Western blot of the same samples probed with mouse anti-c-myc epitope antibody.
WT: Culture supernatant from *P. pastoris* wild-type strain *X-33.*
M: Molecular mass marker shown in kilodaltons.

| Apyrase | Molecular Weight (kDa) |
|---|---|
| Apy-1 | 39.8 |
| Apy-2 | 41.2 |
| Apy-3 | 40.1 |
| Apy-4 | 40.0 |
| Apy-5 | 40.2 |

**Table 3.1: Molecular weight in kDa**
Expected molecular weight of expressed apyrases from the nucleotide plasmid sequence.

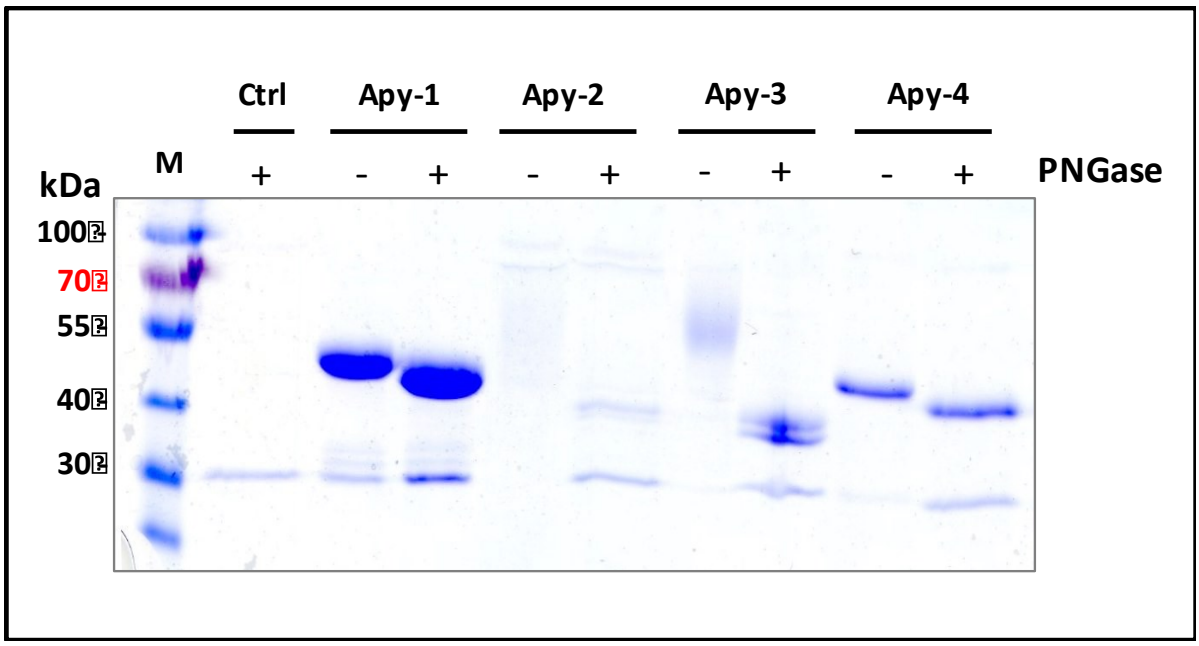


**Figure 3.4: Coomassie-stained SDS-PAGE gel demonstrating apyrases pre- and post-treatment with PNGase F**

Yeast culture supernatants were treated with PNGase-F for one hour, subjected to SDS-PAGE and analysed by Coomassie Blue staining.

M: Molecular mass marker shown in kilodaltons.

**Recombinant secreted apyrases require $Ca^{2+}$ and hydrolyse NTPs and NDPs**

Following confirmation of apyrase expression by *P. pastoris,* the biochemical characterisation of the yeast total supernatants was performed using a phosphate assay kit which measures the inorganic phosphate ($P_i$) released as described previously (see Materials and Methods). The enzymatic activity of the recombinant apyrases was analysed, testing the dependence on divalent cations, the optimum pH and the substrate specificity of the recombinant apyrases. No nucleotidase activity was detected with WT yeast culture supernatant.

The effect of divalent cations on the catalytic activity of the recombinant apyrases was tested using ATP as a substrate in the presence of calcium, magnesium or zinc as co-factors. Results showed that the addition of 5 mM $Ca^{2+}$ dramatically enhanced the hydrolysis of ATP. However, equivalent $Mg^{2+}$ and $Zn^{2+}$ concentrations caused an inhibition of ATP hydrolysis with all apyrases (Figure 3.5). Furthermore, the effect of calcium concentrations in the range of 1-10 mM was tested, and maximal activity was observed at the concentration of 10 mM (Figure 3.6). No activity was detected in the absence of $Ca^{2+}$. The data suggest that the apyrase activities are completely dependent on the presence of calcium.

The influence of pH on ATP hydrolysis was then studied between pH 5.0 to 10.5 in the presence of 5 mM $Ca^{2+}$. The catalytic activity was low at pH 5-6, then started to rise and plateaued at pH values greater than 7.0. The data suggest that all enzymes are active in alkaline conditions with optimal activities over a broad pH range between the values of 7.5

and 10.0 (Figure 3.7). All subsequent experiments were performed at pH 7.5 and in the presence of 5 mM $CaCl_2$.

The enzyme activity was further characterized examining the substrate specificity of recombinant proteins. At a concentration of 2 mM, nucleoside tri-, di- or monophosphates were used as substrates in the presence of 5 mM $CaCl_2$. Inorganic phosphate, indicative of nucleotide hydrolysis, was detected in all samples when triphosphates and diphosphates were used as substrates. However, no activity was detected in the presence of AMP or other monophosphates (results not shown). In addition, apyrases showed no distinct preference for any nucleotide. Among the nucleoside triphosphates, the highest relative activities were observed with ATP and UTP, with less than 20% difference between substrates. Lower activities were observed with CTP and GTP. When nucleoside diphosphates were used as substrates, apyrases showed a preference for ADP and UDP. CDP and GDP were hydrolysed at lower rates of that shown for the preferred diphosphate substrates (Figure 3.8).

Overall, the recombinant enzymes were shown to exhibit true apyrase activity, hydrolysing only di- and triphosphates. They thus belong to the Ca-dependent apyrase family and are active over a broad pH range.

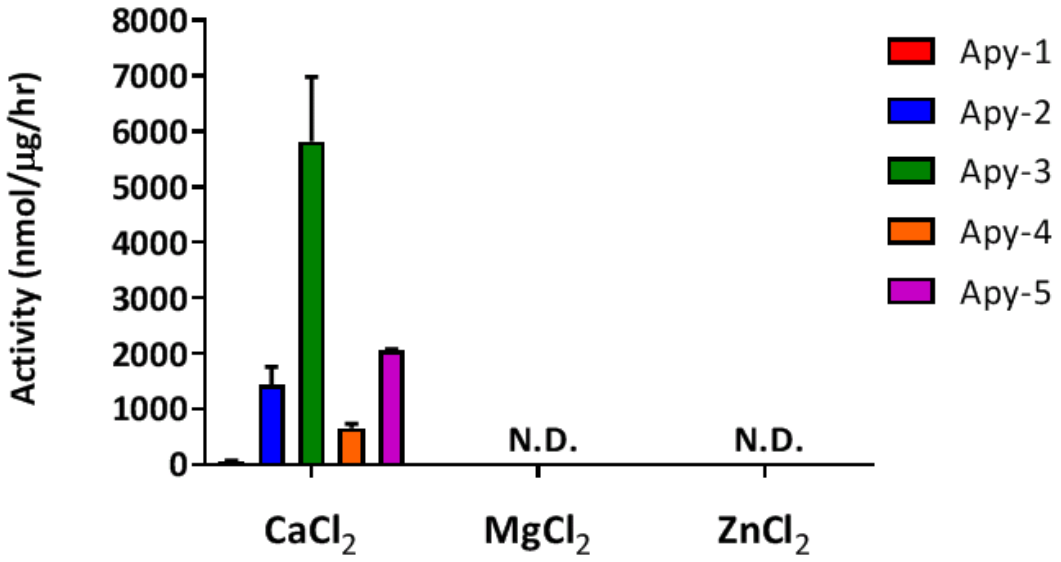


**Figure 3.5: Cation dependence of secreted apyrases**

The effect of divalent cations on ATP hydrolysis (2mM) of apyrases in *P. pastoris* culture supernatants was evaluated in the presence of 5 mM $Ca^{2+}$, $Mg^{2+}$ or $Zn^{2+}$. Bars represent the mean + S.D. of two independent experiments carried out in triplicate. N.D., Not detected.

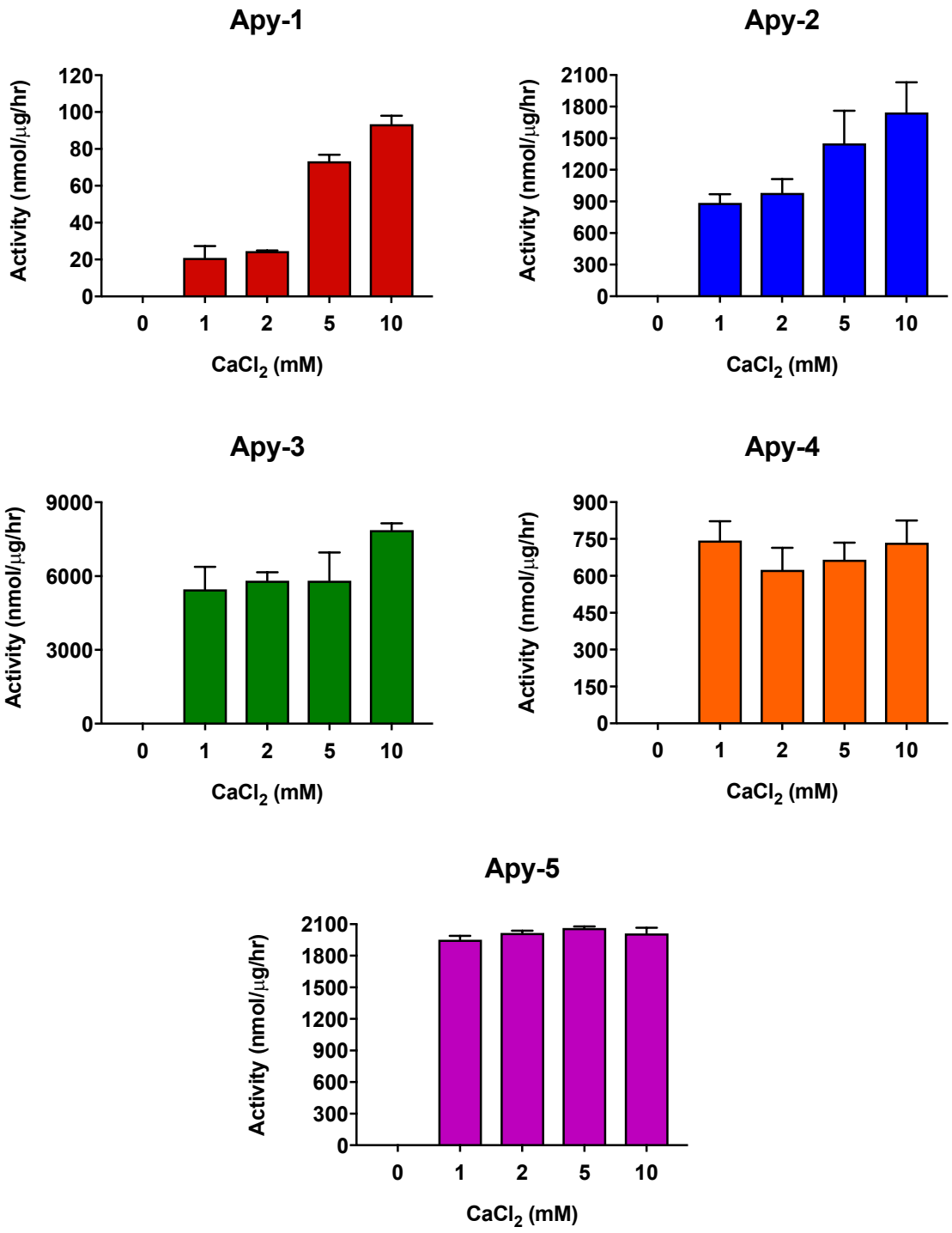


**Figure 3.6: Calcium dependence of secreted apyrases**

The enzymatic activity of apyrases in *P. pastoris* culture supernatants was assessed with 2 mM ATP substrate at different concentrations of $CaCl_2$ as indicated. Bars represent the mean + S.D. of two independent experiments carried out in triplicate.

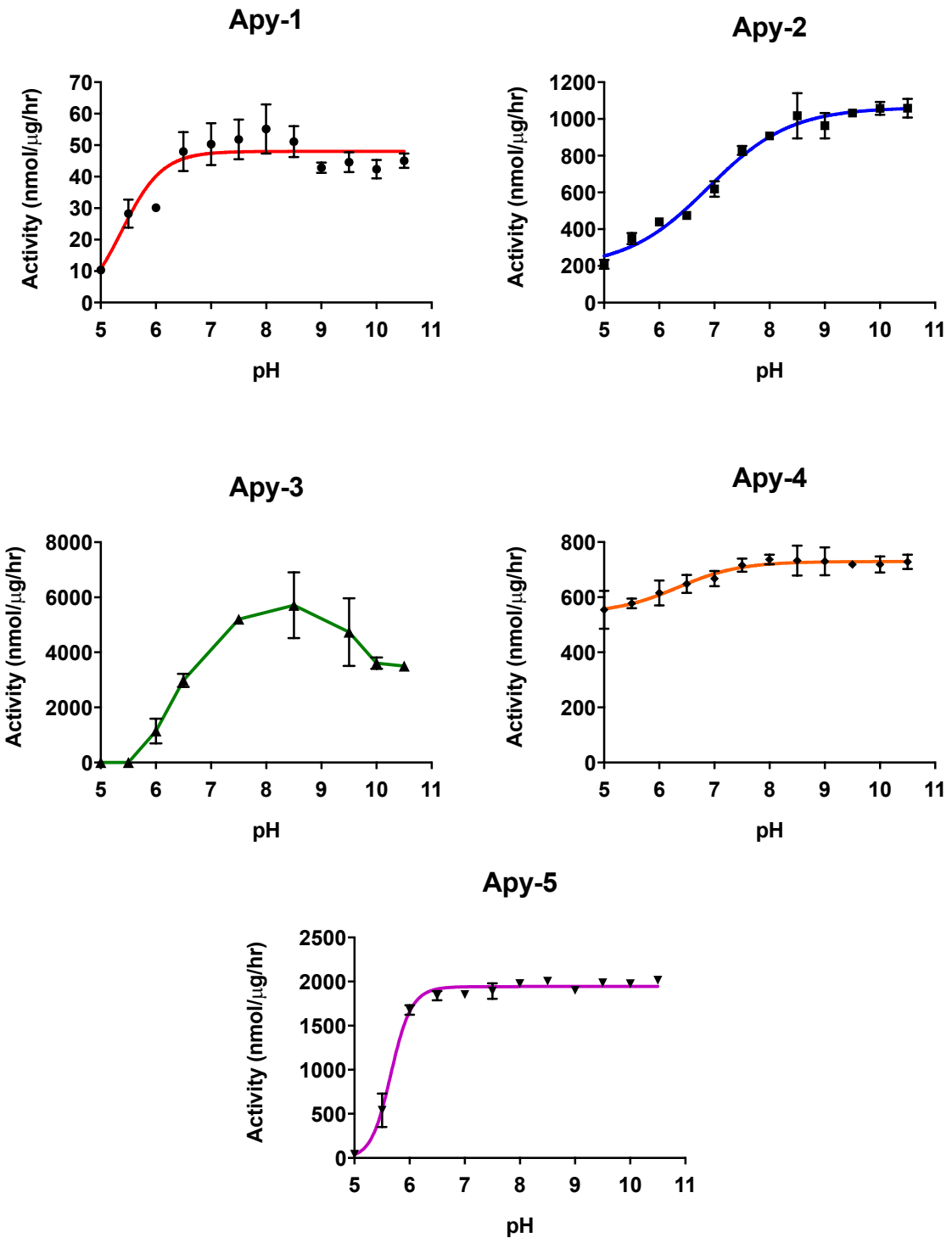


**Figure 3.7: pH dependence of secreted apyrases**

The enzymatic activity of apyrases in *P. pastoris* culture supernatants was assessed with 2 mM ATP substrate as a function of pH in the presence of 5 mM $Ca^{2+}$. Data are expressed as the mean ± S.D. for two independent experiments carried out in triplicate.

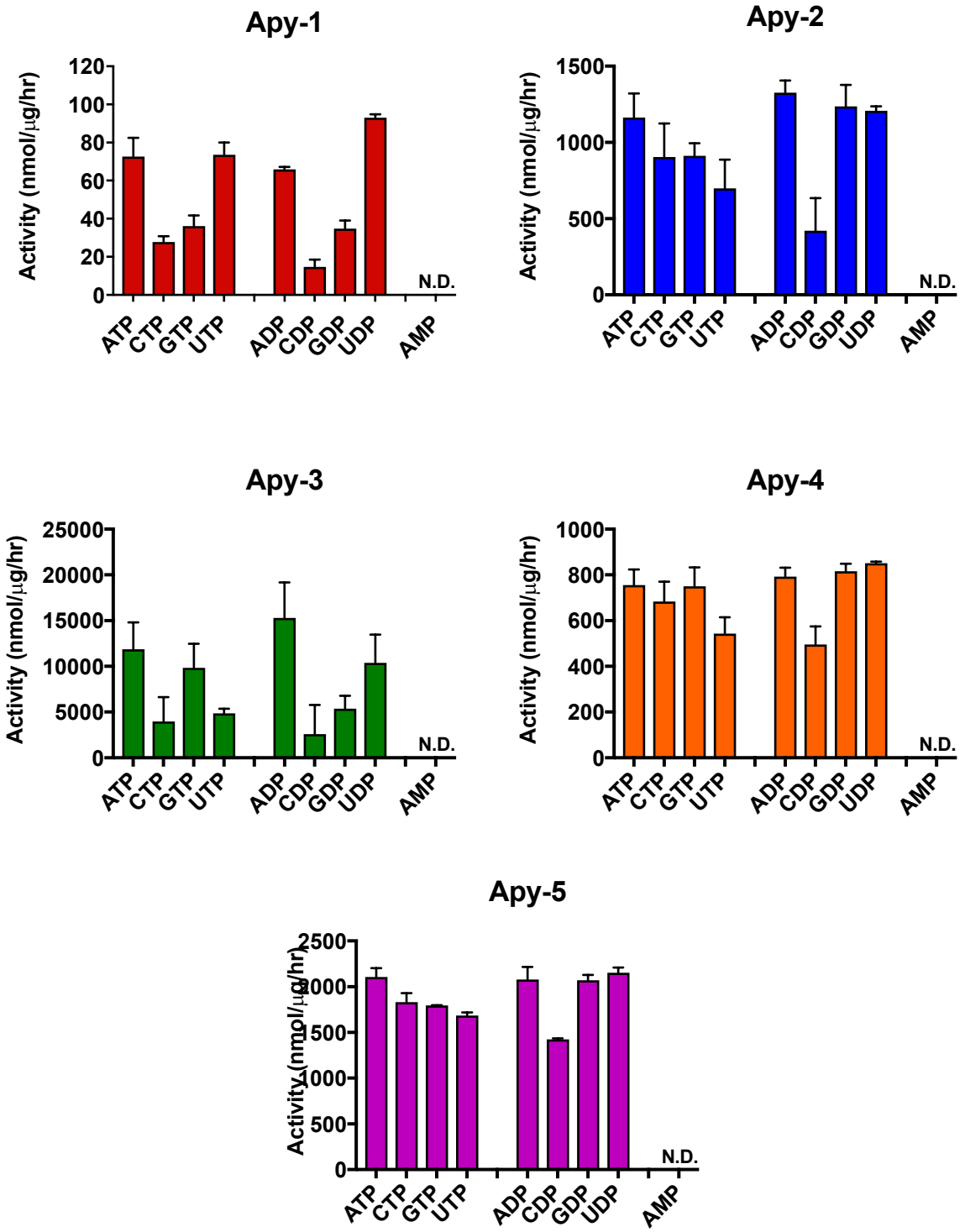


**Figure 3.8: Substrate specificity of secreted apyrases**

The enzymatic activity of apyrases in *P. pastoris* culture supernatants was measured using nucleoside triphosphates, diphosphates or monophosphates, at a concentration of 2 mM in the presence of 5 mM $Ca^{2+}$. The data shown represent the mean + S.D. of two independent experiments performed in triplicate. N.D., Not detected.

**Biochemical characterisation of purified apyrases**

Since the expression of active apyrases was optimised, the *Pichia* expression protocol was scaled up in order to produce more proteins for purification. The yeast culture supernatants expressing the five 6xHis-tagged proteins were filtered, concentrated and purified by His-tag affinity chromatography. Enzymatic properties of the purified apyrases were studied using a colorimetric phosphate release assay. The enzymes had a molecular mass between 50 and 60 kDa based on migration in SDS polyacrylamide gels (Figure 3.9A) and reacted to the anti-c-myc antibody in an immunoblot analysis (Figure 3.9B).

The apyrase catalytic activities saturated at a calcium concentration of about 0.05 mM $CaCl_2$ (Figure 3.10). No higher activity was observed as the concentration of calcium increased, and no activity was detected in the absence of calcium. This indicates that the purified proteins were strictly dependent on $Ca^{2+}$. The activity of increasing amount of the purified enzymes determined the enzymatic saturation of each apyrase as shown in Figure 3.11. Additionally, the activity with ATP was tested using pH values in the range 5.0-10.0 (Figure 3.12) and showed a broad pH spectrum similar to the yeast culture supernatants expressing apyrases. Furthermore, increasing ATP concentrations (0-4 mM) determined the substrate saturation (Figure 3.13). The activities increased with increasing concentrations up to 200 µM, which was shown to be sufficient to saturate the enzymes. The kinetic parameters for ATP hydrolysis using substrate concentrations were also calculated. Lineweaver–Burk plot (inset Figure 3.14) was used to determine the $K_m$ (Michaelis constant) and $V_{max}$ (maximum velocity) values summarized in table 3.2. The turnover number ($K_m/v_{max}$) of apyrases against ATP was also calculated. The nucleotidase activity of recombinant

saturated apyrases was then evaluated using a variety of substrates at a concentration of 1 mM, in a buffer containing 0.5 mM $Ca^{2+}$, at a pH of 7.5. As shown in figure 3.15, the $Ca^{2+}$-dependent apyrases exhibited a broad substrate specificity.

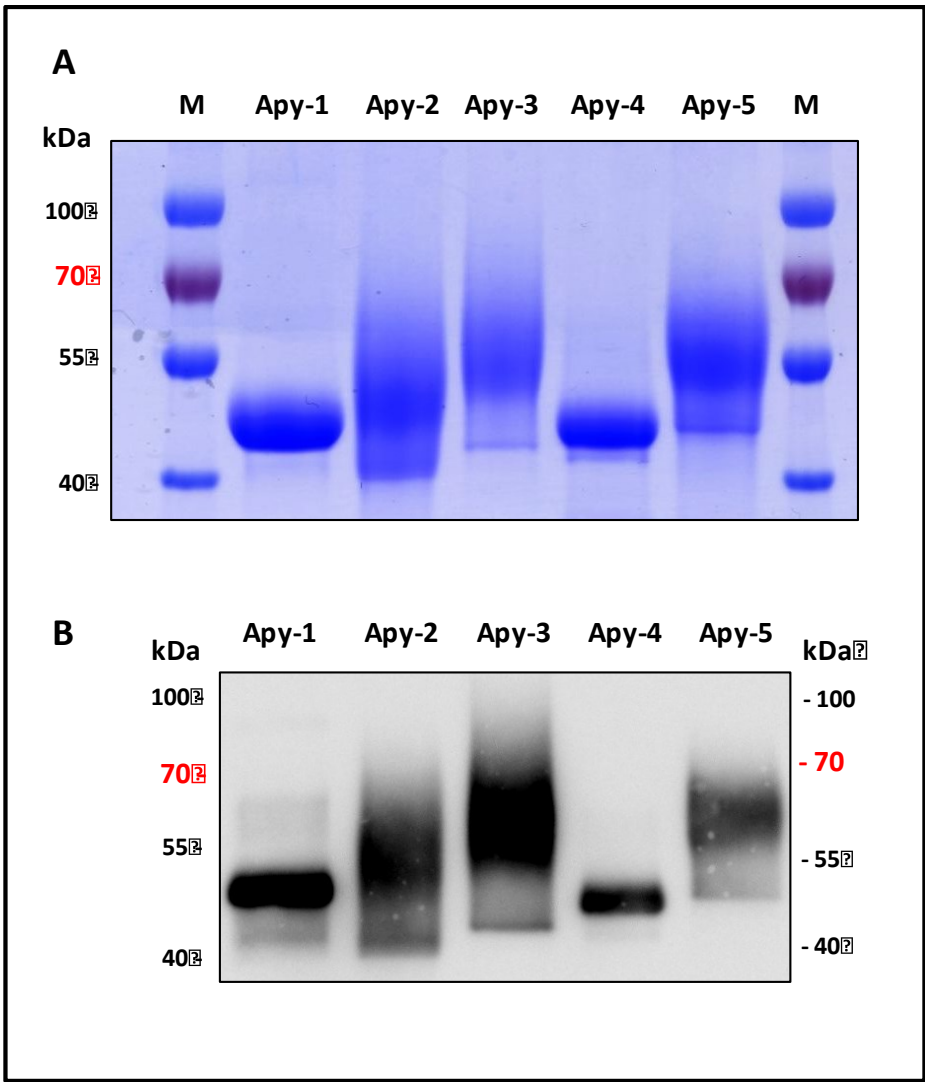


**Figure 3.9: Purification of apyrases from *P. pastoris***

**Panel A:** Coomassie-stained SDS-PAGE gel demonstrating apyrases following purification from *Pichia* supernatant.

**Panel B:** Western blot of the same samples probed with mouse anti-c-myc epitope antibody.
M: Molecular mass marker shown in kilodaltons.

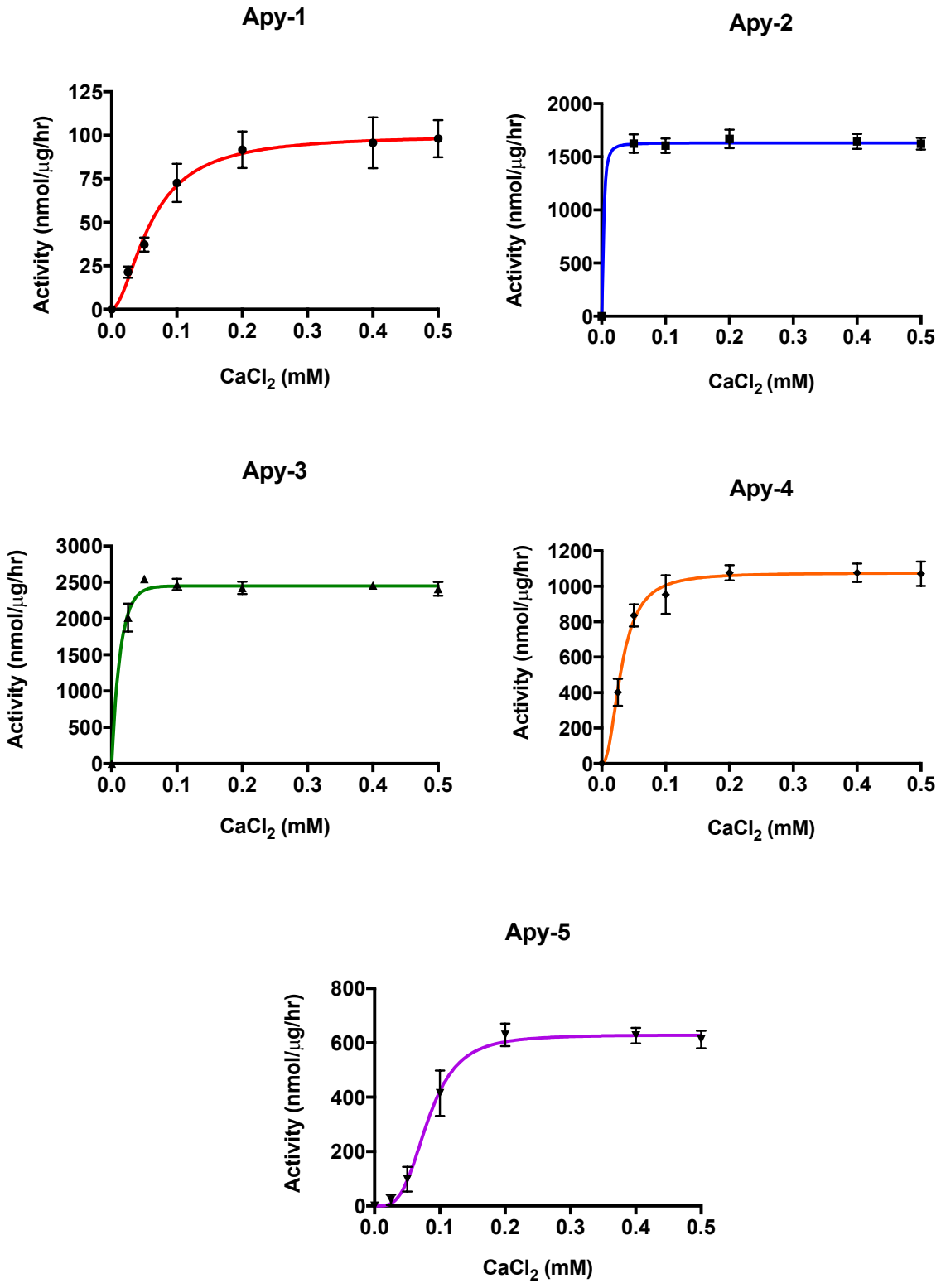


**Figure 3.10: Calcium dependence of recombinant apyrases**
Calcium dependence was determined using ATP (2 mM) as substrate with different concentrations of $CaCl_2$ (0-0.5 mM). Data represent the mean ± S.D. of three independent experiments carried out in triplicate.

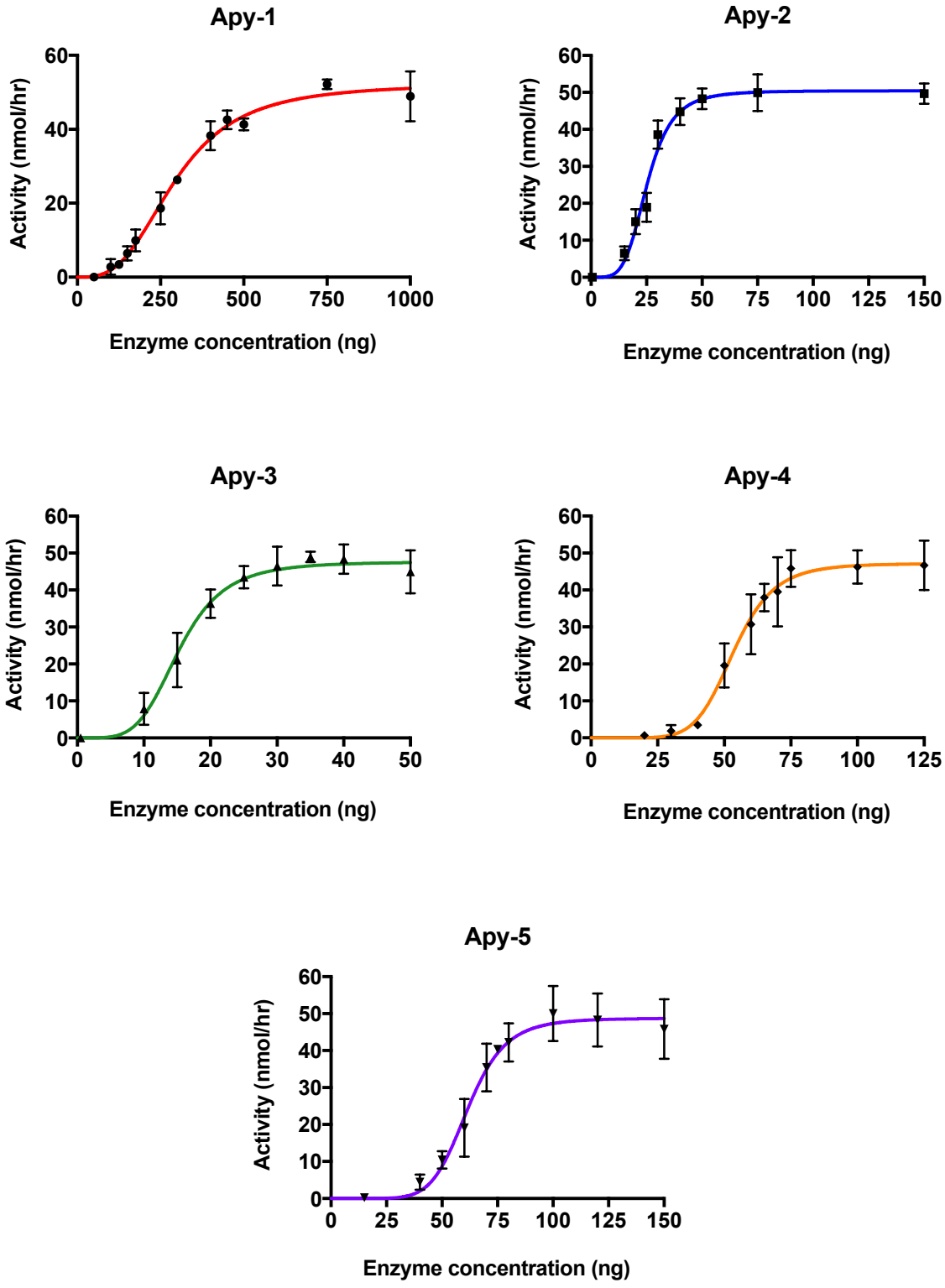


**Figure 3.11: Effect of enzyme concentration on catalytic activity**
Enzyme activity was measured using 2 mM ATP as substrate in the presence of 0.5 mM $Ca^{2+}$. Data represent the mean ± S.D. of three independent experiments carried out in triplicate.

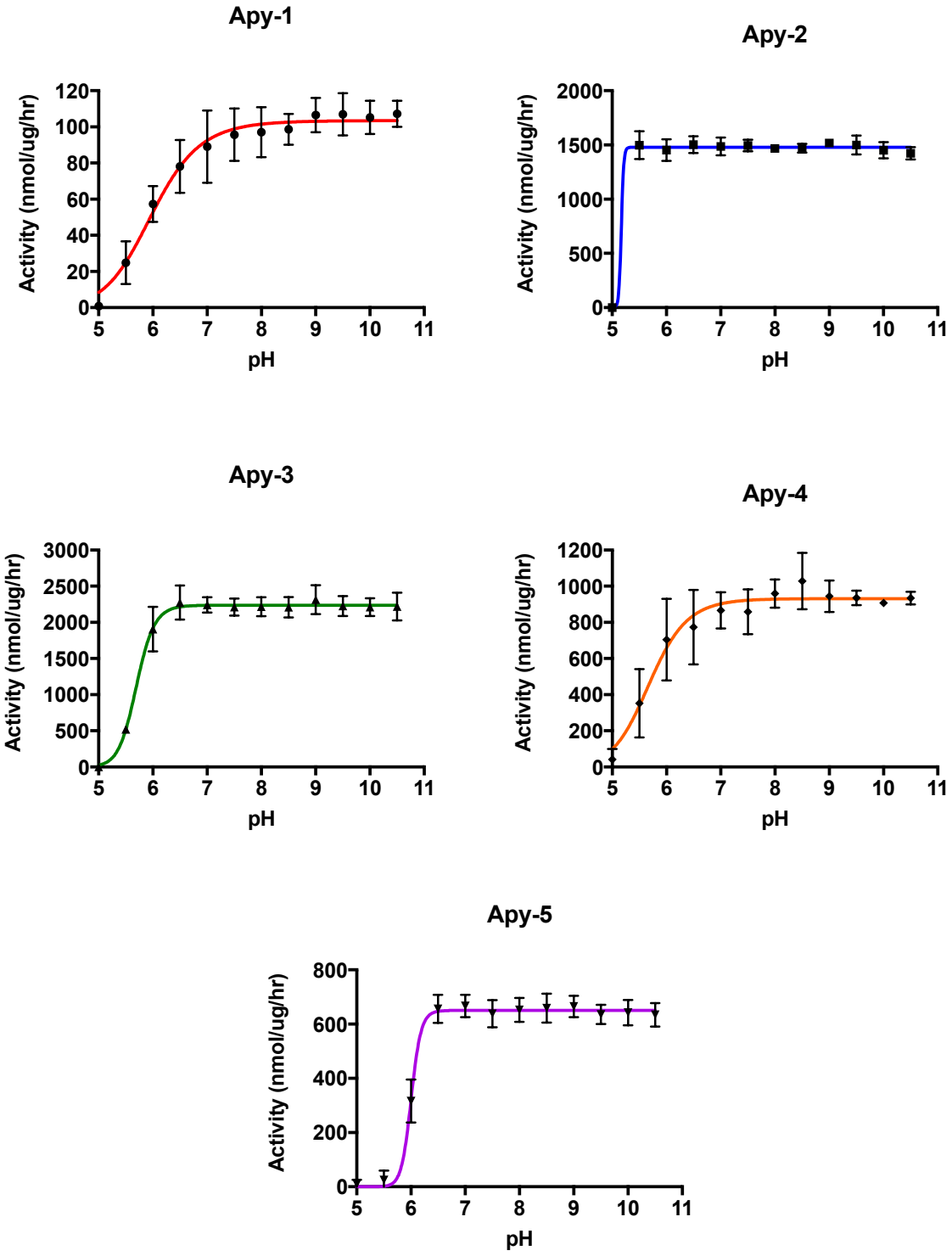


**Figure 3.12: Optimum pH of recombinant apyrases**
The pH-dependence of purified enzymes was assessed using ATP (2 mM) as substrate in the presence of 0.5 mM $Ca^{2+}$. Data represent the mean ± S.D. of three independent experiments performed in triplicate.

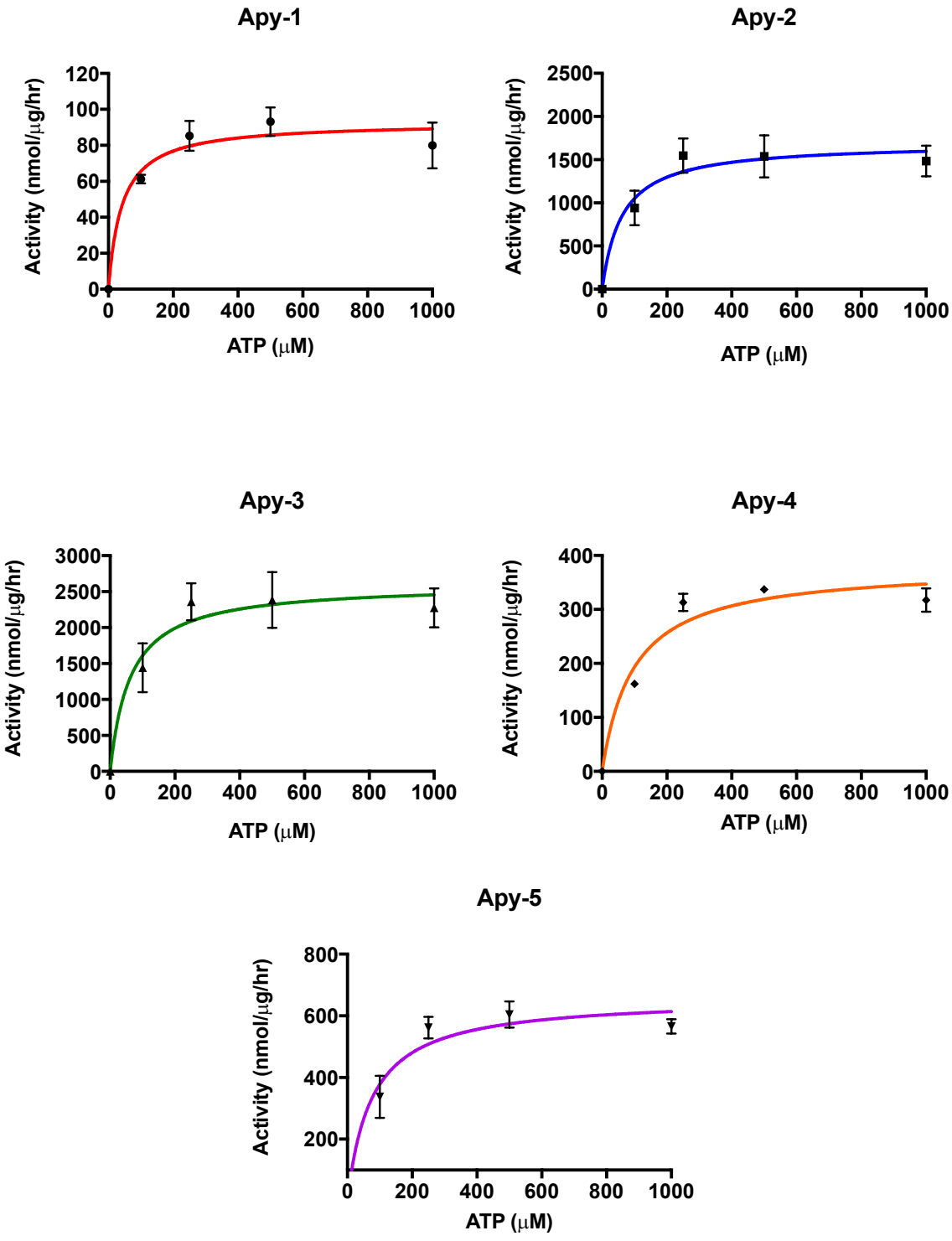


**Figure 3.13: Michaelis-Menten plots for recombinant apyrases**
Substrate concentration dependence of the rate of ATP hydrolysis by recombinant apyrases are illustrated at pH 7.5 and in the presence of 5 mM $Ca^{2+}$. Experimental values shown represent the mean ± S.D. of three independent experiments performed in triplicate.

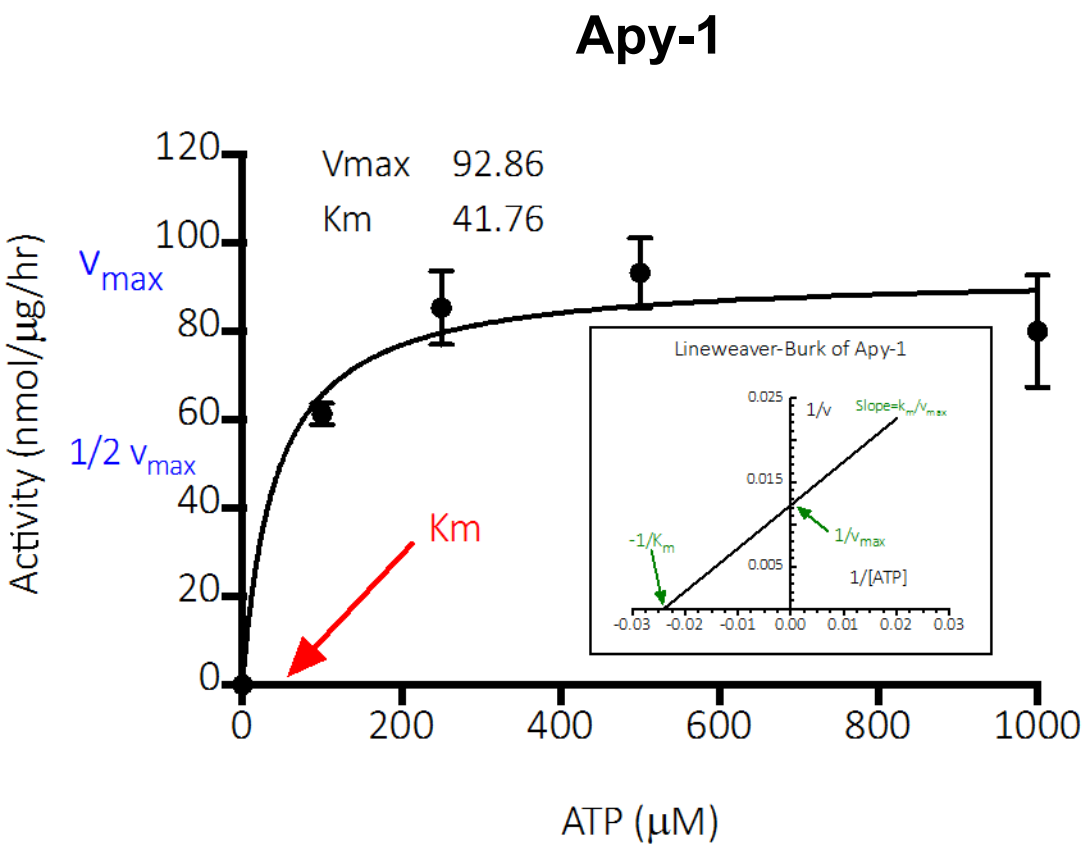


**Figure 3.14: Representative Michaelis–Menten and Lineweaver–Burk plot in inset for Apy-1**

| Apyrase | Substrate | Km (µM) | $V_{max}$ (nmol $hr^{-1}$) | $V_{max}$ /$K_m$ |
|---|---|---|---|---|
| **Apy-1** | ATP | 42 ± 2.3 | 92.9 ± 7.6 | 2.21 x $10^3$ |
| **Apy-2** | ATP | 61 ± 2.5 | 1694 ± 127.7 | 2.78 x $10^4$ |
| **Apy-3** | ATP | 62 ± 2.4 | 2607 ± 188.4 | 4.20 x $10^4$ |
| **Apy-4** | ATP | 97 ± 3 | 381 ± 27.8 | 3.93 x $10^3$ |
| **Apy-5** | ATP | 75 ± 1.9 | 659.8 ± 36 | 8.80 x $10^3$ |

**Table 3.2: Kinetic parameters of the recombinant apyrases**
Kinetic constants (Km and vmax) of all apyrases were calculated for ATP as substrate, using non-linear regression analysis.

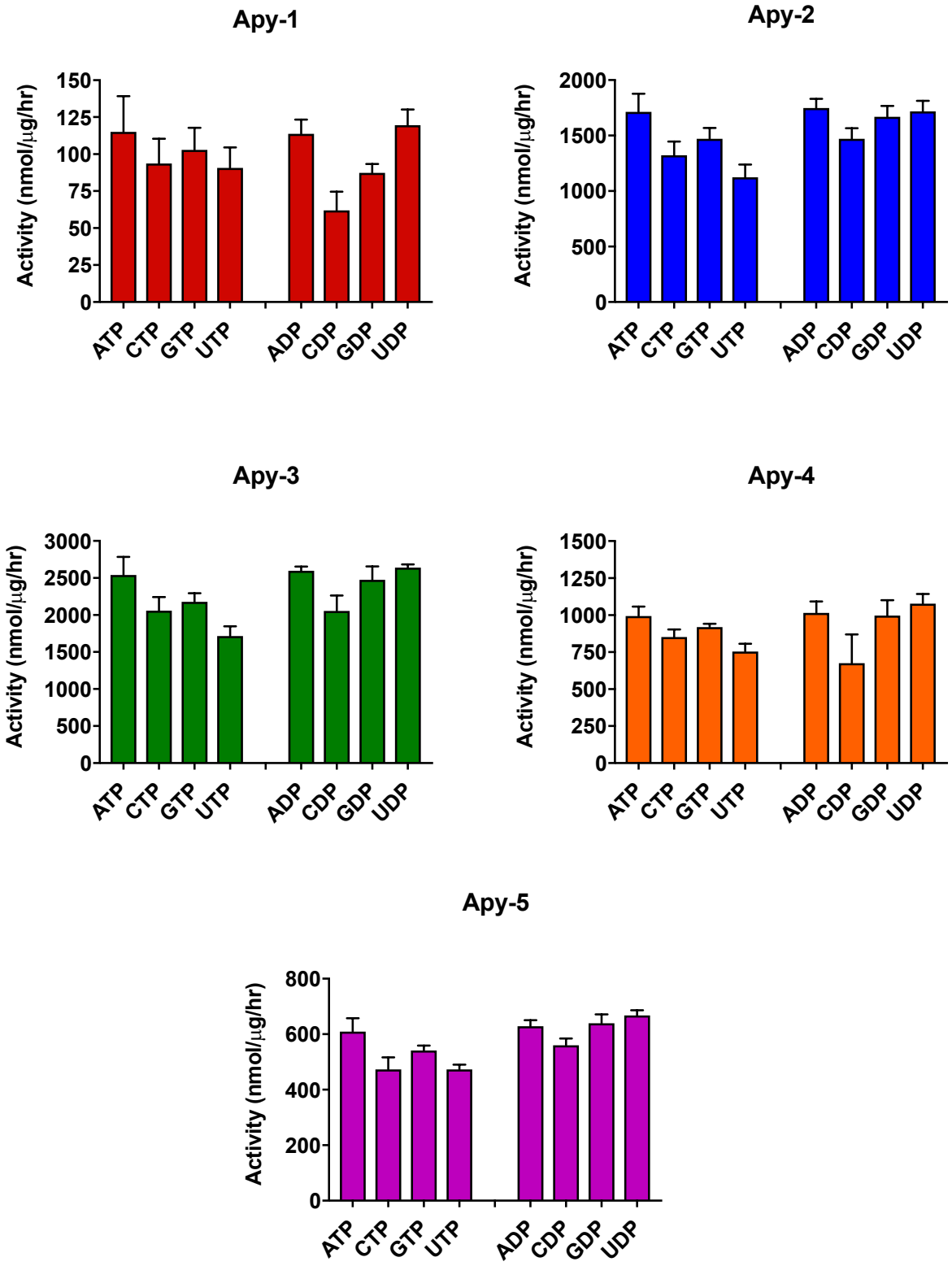


**Figure 3.15: Substrate specificity of recombinant apyrases**
The substrate specificity was determined using nucleoside triphosphates or diphosphates at a concentration of 2 mM in the presence of 0.5 mM $Ca^{2+}$. The data represent the mean + S.D. of three independent experiments carried out in triplicate.

**Characterisation of *H. polygyrus* secreted products**

The activity of adult *H. polygyrus* total secreted proteins was also examined. Enzymatic activity levels were comparable to those found in recombinant proteins. *H. polygyrus* products contain enzymes capable of catalysing the hydrolysis of nucleoside tri- and diphosphates, with no activity against monophosphates, exhibiting true apyrase activity. The activity, optimal over a broad pH range between 7.5 and 10.5, was dependent on the presence of calcium (Figure 3.16 A and B). As for substrate specificity, HES products showed no distinct preference for any nucleotide, similar to that observed with recombinant purified apyrases (Figure 3.16C).

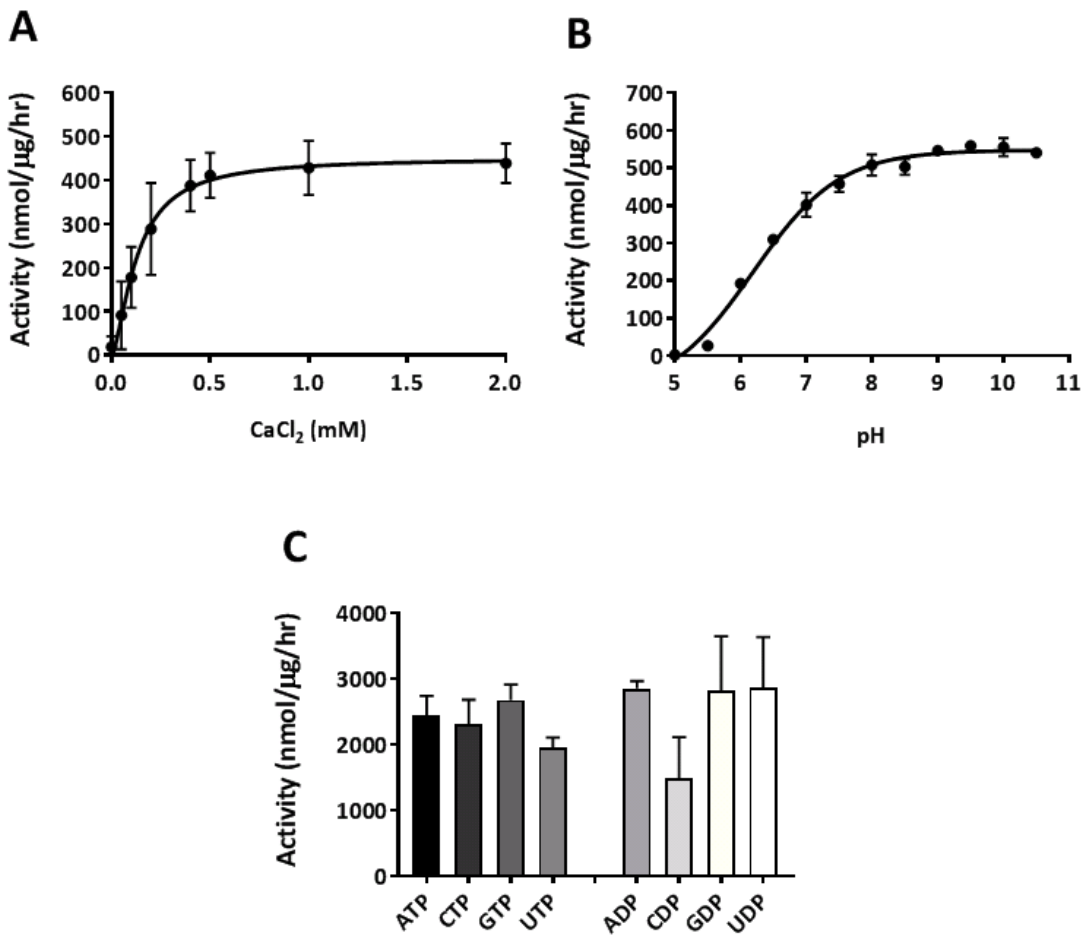


**Figure 3.16: Characteristics of apyrase activity in adult *H. polygyrus* secreted products**

**Panel A:** Assays for calcium dependence were performed in a buffer containing incremental concentrations of $CaCl_2$ (0-2 mM), where ATP (2 mM) was used as a substrate.

**Panel B:** pH dependence was determined using a pH range from 5.0-10.5, in the presence of 0.5 mM $Ca^{2+}$ and 2 mM ATP.

**Panel C:** The preference for different substrates at a concentration of 2 mM was assessed in the presence of the cofactor calcium (0.5 mM).

Data shown represent the mean ± S.D. of three independent experiments performed in triplicate.

## 3.3 Discussion

Helminth parasites have been documented to secrete molecules that can modulate the host immune system in order to prevent elimination of the parasite, but also reducing symptoms of immunopathological diseases in the host. An effective approach to gain insight into the potential functions of these molecules is their heterologous expression in an appropriate vehicle. The biochemical properties of *H. polygyrus* secreted apyrases haven't been examined yet, thus studying these enzymes is essential in order to further understand their role *in vivo*. In the current study, secreted apyrases from the parasitic nematode *H. polygyrus* were successfully expressed in a heterologous system and biochemically characterised in terms of cation dependence, optimal pH and substrate specificity. The native secreted products from the adult worms were also characterised in the same manner.

The methylotrophic yeast *P. pastoris* was used as an expression host, as it is suitable for the production of large amounts of secreted proteins (Tschopp *et al.*, 1987; Heimo, Palmu and Suominen, 1997; Macauley-Patrick *et al.*, 2005; Guo and Ma, 2008; Ahmad *et al.*, 2014). *Pichia* is known to secrete a very low level of native proteins; therefore, the heterologous protein will constitute the majority of the total proteins in the growth medium (Barr *et al.*, 1992). In addition, secreted products from the wild type *P. pastoris* used in this study did not contain any nucleotidase activities, as was previously shown (Gounaris, Selkirk and Sadeghi, 2004), thus any nucleotide hydrolysis will be due to the recombinant enzymes. A slight difference was shown among the apyrases in terms of substrate preference, ion dependence and optimal pH.

It is highly likely that there is a binding site for calcium within the apyrases, as the activity of the recombinant proteins was greatly enhanced by the addition of calcium; however, no activity was detected in the absence of $Ca^{2+}$, or in the presence of increasing concentrations of $Mg^{2+}$ and $Zn^{2+}$. *H. polygyrus* secreted apyrases can be therefore classified as Ca-dependent apyrases. NTPDases characterised so far are usually activated by either $Mg^{2+}$ or $Ca^{2+}$ divalent cations (Handa and Guidotti, 1996; Robson, Sévigny and Zimmermann, 2006; Zimmermann, Zebisch and Sträter, 2012). For instance, apyrases secreted by other parasitic nematodes such as *Ostertagia ostertagi* (Zarlenga *et al.*, 2011) and *Teladorsagia circumcincta* (Nisbet *et al.*, 2011) have also been shown to be dependent on calcium. Furthermore, the activity of apyrases identified in the saliva of blood-feeding arthropods and belonging to the *Cimex* family was reported to be strictly $Ca^{2+}$-dependent (Valenzuela *et al.*, 1998, 2001), whereas the activity of CD39 apyrases can be active with $Ca^{2+}$ or $Mg^{2+}$. Other cation-dependent apyrases secreted by helminth parasites such as *T. spiralis* (Gounaris, 2002), and arthropods including ecto-ATP diphosphohydrolase of the trypanosomatid parasite *Herpetomonas muscarum muscarum* (Alves-Ferreira *et al.*, 2003) showed a $Mg^{2+}$-dependence.

Biochemical characterisation of the recombinant apyrases showed a broad pH optimum ranging from approximately 6.5 to 10.0. This suggests that these enzymes could be active in quite different environments that occur during the *H. polygyrus* life cycle and cannot be inactivated by changes in pH of the host intestinal lumen. On the other hand, the release of inflammatory mediators as a result of the immune response during a *H. polygyrus* infection may affect the local pH, another reason why apyrases might operate at different pH conditions. The apyrases of most arthropods including *Ochlerotatus*

*triseriatus* (Say), *Ochlerotatus hendersoni* (Cockerell), and *Aedes aegypti*, have an optimal pH between 7.0 and 10.0 (Ribeiro *et al.*, 1984; Ribeiro, Vaughan and Azad, 1990; Marinotti, De Brito and Moreira, 1996; Mans *et al.*, 1998; Reno and Novak, 2005).

Interestingly, purified recombinant apyrases seem to possess a broad substrate specificity. These enzymes catalysed the hydrolysis of all tested nucleoside tri- and diphosphates, but not monophosphates, thus displaying true apyrase activity (Champagne *et al.*, 1995; Zimmermann, 1999; Valenzuela *et al.*, 2001). This activity against distinct substrates, in particular adenine and uridine substrates, is possibly impacting various signalling pathways. They are likely to modulate the host immune system through interfering with the purinergic signalling by breaking down the inflammatory ATP/ADP to anti-inflammatory AMP. The latter being a precursor for the production of adenosine via CD73 expressed on the surface of immune cells, will result in suppression of immune responses (Atarashi *et al.*, 2008; Fletcher *et al.*, 2009). A recent study showed that during a *H. polygyrus* infection, extracellular ATP activates purinergic P2 receptors on the surface of mast cells which mediate the production of IL-33 (Shimokawa *et al.*, 2017). Thus, apyrases might suppress the release of this cytokine by reducing levels of extracellular ATP (McSorley *et al.*, 2014; Osbourn *et al.*, 2017).

Extracellular nucleotides, are often released during inflammation by damaged cells and function as a signalling molecule through the activation of purinergic P2 receptors, causing the release of pro-inflammatory cytokines. For example, ADP binds to P2 purine receptors on platelets, causing blood platelet aggregation (Marcus *et al.*, 2005; Knowles, 2011; Antonioli, Blandizzi, *et al.*, 2013). Other nucleotides including ATP, ADP, UTP and UDP

have been shown to activate purinergic P2Y receptors (Von Kugelgen and Wetter, 2000). UTP can activate P2Y2 and P2Y4 receptors present on macrophages and dendritic cells (Matos *et al.*, 2005; Müller *et al.*, 2010), whereas UDP can activate the P2Y6 receptor expressed on monocytes leading to the production of the pro-inflammatory cytokine IL-8 (Von Kugelgen and Wetter, 2000; Warny *et al.*, 2001; Wang *et al.*, 2004; Xu *et al.*, 2018). In addition, the activation of P2Y6 on intestinal epithelial cells was shown to increase the intracellular $Ca^{2+}$ and cAMP, thus stimulating the production of $Cl^-$ ions causing fluid secretion and eradicating parasites from the intestine (Köttgen *et al.*, 2003). However, adenosine activates P1 purinergic receptors, exhibiting anti-inflammatory effects through inhibiting the production of TNF-α, IL-6 and IL-8 (Antonioli *et al.,* 2013; Failer *et al.,* 2002).

Apyrases from bed bugs, i.e. *Cimex* species (Valenzuela *et al.*, 1998, 2001) and several parasitic nematodes (Andersen *et al.*, 2007; Nisbet *et al.*, 2011; Zarlenga *et al.*, 2011) were reported to hydrolyse ATP and ADP most efficiently. For instance, in the salivary glands of blood-feeding arthropods, apyrases were reported to hydrolyse both ATP and ADP but not AMP, restraining inflammation and thrombosis and thus facilitating the blood-feeding process (Strobel *et al.*, 1996; Valenzuela *et al.*, 1998, 2001; Charlab *et al.*, 1999; Berrêdo-Pinho *et al.*, 2001; De Jesus *et al.*, 2002; Andersen *et al.*, 2007; Uccelletti *et al.*, 2008). Apyrases from *O. triseriatus, O. hendersoni*, *A. aegypti* (Ribeiro *et al.*, 1984; Champagne *et al.*, 1995) and *Aedes albopictus* (Marinotti, De Brito and Moreira, 1996) have been shown to dephosphorylate ATP and ADP, with a preference for ATP. However, apyrases of the tick *Ornithodoros moubata* and both anthropophilic species *Simulium metallicum* and *Simulium ochraceum* were shown to have a preference for ADP over ATP in contrast to other *Simulium* species tested (Ribeiro, Endris and Endris, 1991). On the other hand, the activity of

nucleotide-metabolising enzymes from *Trichinella spiralis* was found to be highest with UDP (Gounaris, 2002). *T. spiralis* 5´-nucleotidase was shown to catalyse the hydrolysis of nucleoside di- and monophosphates, with no significant hydrolysis of any nucleoside triphosphate, showing both an apyrase and a 5´-nucleotidase activity (Gounaris, Selkirk and Sadeghi, 2004). In addition, there is little to no ATP hydrolytic activity in total secreted products of *T. spiralis*, suggesting that they do not express or secrete conventional apyrase enzymes (Gounaris, 2002). This probably reflects different requirement in term of nucleotide hydrolysis linked to the intracellular niche and the life cycle of the parasite.

Additionally, nucleotide-metabolising enzymes have been found on the cell surface of some protozoan parasites such as *Toxoplasma gondii* (Silverman *et al.*, 1998), *Leishmania amazonensis* (LeBel *et al.*, 1980; Berrêdo-Pinho *et al.*, 2001), *Leishmania tropica* (Peres-Sampaio, Palumbo and Meyer-Fernandes, 2001), *Trichomonas vaginalis* (Cross *et al.*, 1993; De Jesus *et al.*, 2002), *Entamoeba histolytica* (Barros *et al.*, 2000), *Trypanosoma cruzi* (Bisaggio *et al.*, 2003; Fietto *et al.*, 2004), *Trypanosoma brucei* (de Souza Leite *et al.*, 2007), *Tritrichomonas foetus* (De Jesus *et al.*, 2002) and *Cryptosporidium parvum* (Manque *et al.*, 2012). *Schistosoma mansoni* possesses ecto-apyrases on their surfaces as well, capable of hydrolysing exogenous ATP and ADP (Levano-Garcia *et al.*, 2007; Bhardwaj and Skelly, 2009; A. A. Da'dara *et al.*, 2014). A common feature of parasitic nematodes, blood-feeding insects and protozoans could thus be the ability to control levels of local pro-inflammatory ATP and pro-thrombotic ADP via secreted and ecto apyrases, thereby inhibiting inflammation and platelet aggregation, and protecting the parasites from the host immune system (Manque *et al.,* 2012; Da'dara *et al.*, 2014).

During *H. polygyrus* infection, L4 are embedded in the muscularis externa of the intestinal submucosa, and the adult worms reside in the lumen, coiling around the villi of the proximal intestinal epithelium (Bansemir and Sukhdeo, 1994; Telford *et al.*, 1998; Maizels *et al.*, 2012). Although the L4-specific apyrase, Apy-5, is from a different life cycle stage, the enzyme showed a similar activity profile to the apyrases secreted by adult worms. In addition, this study showed that native apyrases secreted by adult *H. polygyrus* shared several common features with the recombinant enzymes, in terms of optimum pH, nucleotide specificity and cation dependence, confirming the secretion of active apyrases by *H. polygyrus,* in addition to the fact that they do not secrete a 5´-nucleotidase.

In conclusion, previous proteomic analysis indicated the secretion of five apyrases by the nematode *H. polygyrus.* This study elucidated biochemical properties of these secreted apyrases. The enzymes described here are a group of calcium-dependent apyrases with a broad optimum pH ranging from 6.5 to 10, and a broad substrate specificity, catalysing the hydrolysis of both nucleoside tri- and diphosphates to the final product monophosphate. *H. polygyrus* apyrases most likely act as immunomodulators and could potentially be therapeutically useful for immune disorders, but this has yet to be demonstrated experimentally. Additionally, around 374 proteins have been identified in *H. polygyrus* secreted products, many with unknown function; hence the importance to compile a comprehensive list of the immunomodulatory secreted proteins.

# CHAPTER 4

# Expression of Apy-1, Apy-3 and 5´-NT in *Trypanosoma musculi* and their effect on infection

## 4.1 Introduction

Methods in functional genomics such as knockout strategies by RNA interference (RNAi) or transgenesis, have been difficult to achieve in many parasitic helminths (Kalinna and Brindley, 2007; Dalzell *et al.*, 2011; Selkirk *et al.*, 2012). An alternative and possible technique to gain insights into cellular functions of specific parasitic helminth genes is heterologous expression. Bacteria, yeast, fungi, insect cells, mammalian cells and transgenic animals have been used as protein expression models. Heterologous host organisms, such as the soil nematode *Caenorhabditis elegans*, have been used previously to investigate the function of genes from *Haemonchus contortus*, *Onchocerca volvulus* and *Strongyloides stercoralis* parasitic nematodes (Grant, 1992; Britton and Murray, 2002; Massey *et al.*, 2006), however it is not a suitable model for studying immunomodulation as it has a free-living lifestyle.

Kinetoplastid parasites have been successfully used for analysis of gene function in transfected organisms or for protein expression *in vitro*. They are characterised by a genome with overlapping polycistronic transcriptional units and express genes by read-through transcription. Kinetoplastids are easily genetically manipulated and can produce high levels of exogenous proteins (Cruz and Beverley, 1990; Coburn *et al.*, 1991; Clayton, 1999; Beverley, 2003). The intracellular kinetoplastid parasite *Leishmania mexicana* was described as an applicable expression system to assay immunomodulatory effects of helminth molecules (Gomez-Escobar *et al.*, 2005; Maizels *et al.*, 2008), but as the parasite is intracellular, it may not be suitable for all proteins secreted by extracellular helminths. The

systemic, non-pathogenic *Trypanosoma theileri*, a member of the kinetoplastid family, was developed as a vehicle to deliver vaccine antigens (Mott *et al.*, 2011).

*T. musculi* has been genetically manipulated in our lab to express and secrete *N. brasiliensis* AChE *in vivo*, and was shown to be a suitable vehicle to study immunomodulatory proteins (Vaux *et al.*, 2016). There are many reasons for *T. musculi* being an excellent in vivo expression model to investigate immunomodulatory effects by helminth genes. *T. musculi* is a natural parasite very specific to mice, that can't even infect rats (Albright and Albright, 1991) and is not a zoonotic pathogen (Zhang *et al.*, 2018), which makes this organism safe to use. Being an extracellular parasite makes it suitable to study the effect of various parasitic helminth secreted proteins *in vivo*, as these proteins are normally delivered extracellularly to interact with the host immune system. *T. musculi* possess a simple, reproducible pattern of infection that lasts 3 weeks and is cleared by an antibody-dependent immune response (Albright and Albright, 1991); so any immunomodulatory effect that may alter the parasitaemia can be easily seen. Finally, *T. musculi* can be easily genetically manipulated by transfecting DNA using Amaxa Nucleofector technology (MacGregor *et al.*, 2013) and selecting transfectants with blasiticidin antibiotic (Vaux *et al.*, 2016).

## 4.2 Results

**Expression and secretion of Apy-1 by transgenic *T. musculi* results in higher parasitaemia and delay in clearance of infection**

In order to generate transgenic *T. musculi* secreting an exogenous gene, the coding sequence of the gene of interest was amplified by RT-PCR and expressed as a secreted protein using a previously designed expression cassette. The vector contains a blasticidin-selection gene, regions of the 5´- and the 3´-end of the SSU rRNA gene, paraflagellar rod (PFR) and tubulin intergenic (IG) regions, in addition to the coding sequence for the N-terminal signal peptide of *T. musculi* Binding Protein (BiP/GRP78) found in the endoplasmic reticulum (ER) to direct secretion (see appendix A.4 for vector map) (Vaux *et al.*, 2016).

Following isolation of total RNA from adult *H. polygyrus*, cDNA was generated by reverse transcription and used as a template to amplify *apy-1* yielding to an amplification fragment with the expected size of 950 bp (Figure 4.1A). *Apy-1* was then inserted into the expression cassette by ligation, and transformant cells were selected by ampicillin resistance. After confirming that the gene was successfully cloned in frame into the expression vector via colony PCR and diagnostic digest, and that there were no mutations in the gene sequence or key features of the plasmid via sequencing, the construct was linearized by digestion with ScaI restriction enzyme and used to transfect *T. musculi* by electroporation. Transfectants were selected by blasticidin, and the incorporation of *apy-1* into the *T. musculi* genome was confirmed by PCR using specific primers (Figure 4.1B).

At the protein level, the expression of Apy-1 by the transgenic line was revealed by western blot analysis, showing a protein band at 50 kDa in the cell lysate and a slightly higher mass in the supernatant, suggesting possible glycosylation of the secreted protein. No bands were shown in WT trypanosomes (figures 4.1C). Following confirmation of Apy-1 expression by *T. musculi,* the apyrase activity of wild type and transgenic trypanosome secreted products was assayed to test whether the recombinant protein secreted by transgenic line was enzymatically active, and whether the wild type trypanosomes secrete an apyrase or any other nucleotide-metabolising enzymes. Is it noteworthy that Tm Apy-1 (grey bars) secreted products can hydrolyse nucleoside tri- and diphosphates at substantially greater levels than Tm WT (black bars) (Figure 4.2), indicating that transgenic trypanosomes are secreting an active Apy-1 with the same substrate specificity as the native enzyme (see chapter 3).

After the expression and secretion of an active apyrase by transgenic *T. musculi,* the immunomodulatory property of the transgenic line was studied *in vivo*. But first, the growth *in vitro* of Tm Apy-1 was compared to Tm Luc (trypanosomes engineered to express cytosolic luciferase) for 4 days. Tm Apy-1 showed a trend for growing faster than controls, although the difference was only statistically significant at day 2 (Figure 4.3A). Female BALB/c mice were then infected intraperitoneally with $2 \times 10^5$ trypanosomes, and parasitaemia was monitored every 1-2 days until parasites disappeared from the blood. The pattern of infection was similar in Tm Luc and Tm Apy-1 infected mice, reaching a peak around day 8, plateauing for 5-6 days and clearing few days later. However, the numbers of Tm Apy-1 parasites were approximately double that of Tm Luc throughout much of infection, with a significant difference from day 4 to day 10, and a clearance delayed from the circulation by 1 day (Figure 4.3B).

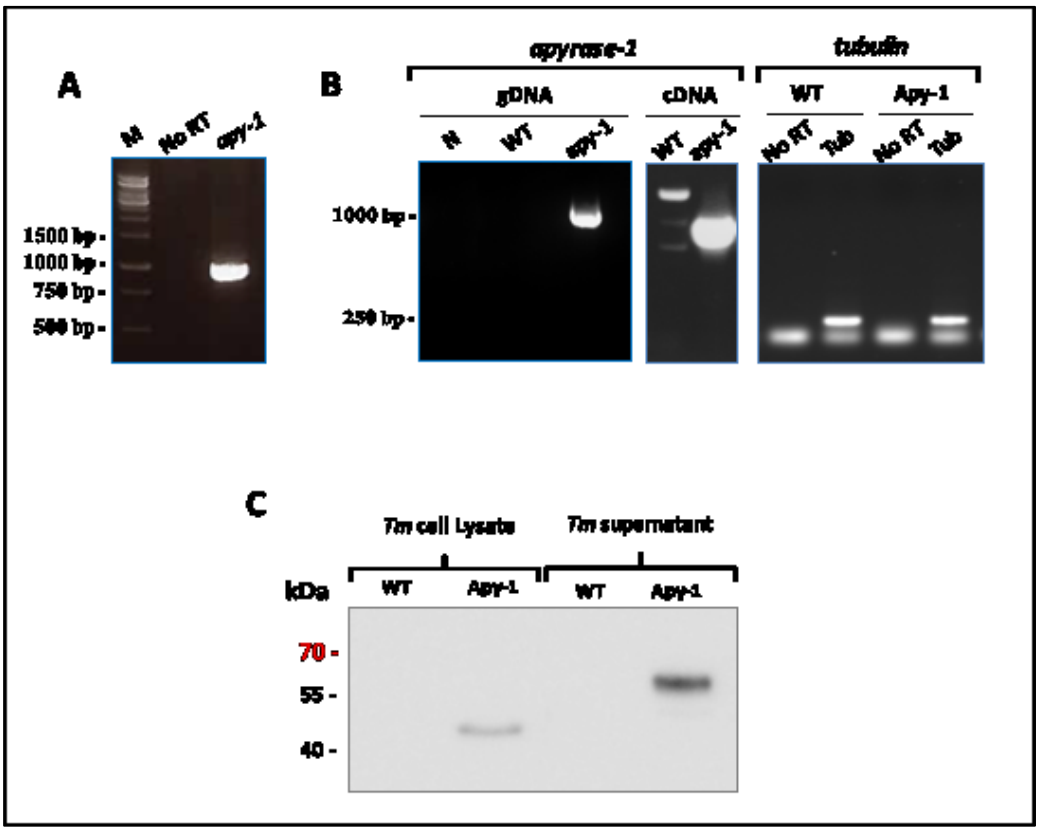


**Figure 4.1: Apy-1 expression by transgenic *T. musculi***
**(A) Amplification of *H. polygyrus* Apy-1 by RT-PCR.** *Apy-1* was amplified from adult *H. polygyrus* cDNA by RT-PCR using specific primers (expected size = 1,000 bp). PCR products were separated on 1 % (w/v) agarose gel, stained with GelRed stain and visualised on a UV trans-illuminator.

**(B) Incorporation of Apy-1 into the *T. musculi* genome.** Genomic DNA (gDNA) and total RNA converted to cDNA, were isolated from wild type (WT) *T. musculi* and transgenic *T. musculi* expressing Apy-1, and used for PCR with Apy-1 forward and reverse primers (expected size = 1,000 bp). The α tubulin gene (tub) was amplified as a positive control for gDNA quality (expected size = 251 bp). Fragments were resolved on a 1 % (w/v) agarose gel.

**(C) Western Blot against *H. polygyrus* Apy-1.** Proteins from cell lysate (5 x $10^5$ trypanosomes) and concentrated culture supernatants from WT and Apy-1 *T. musculi* were resolved on a 12 % (v/v) SDS polyacrylamide gel, blotted onto a PVDF membrane and probed with anti-c-myc epitope antibody followed by a goat anti-mouse horseradish peroxidase (HRP) secondary antibody. Chemiluminescence was detected using an LAS-300 Fuji Imager.
Lane M: Molecular weight markers (1 kb DNA ladder).
Lane No RT: no reverse transcriptase control sample.
Lane N: Negative control in which gDNA template was not included in PCR reaction.

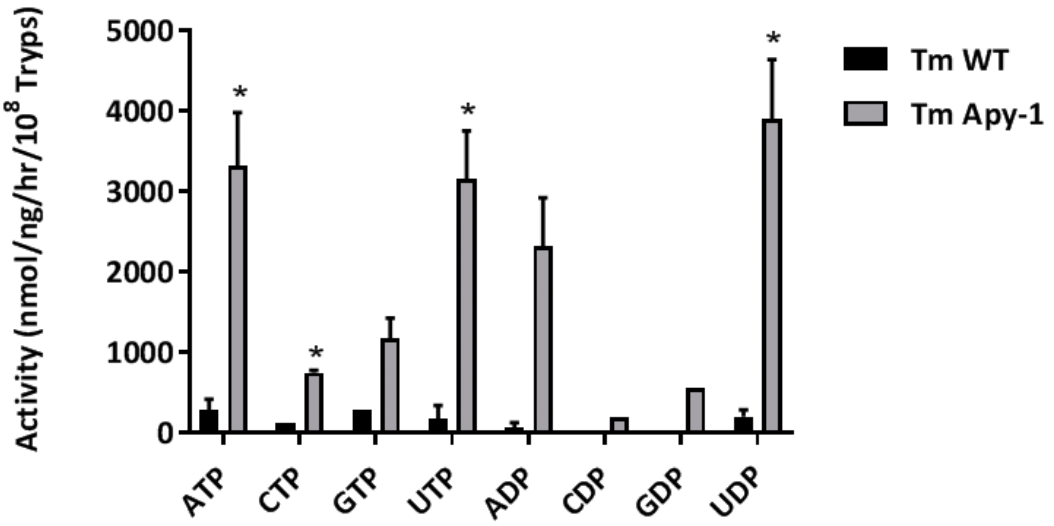


**Figure 4.2: Substrate specificity of transgenic *T. musculi* secreted products**
Apyrase activity of secreted products from *T. musculi* expressing Luciferase (Luc; black bars) or Apy-1 (grey bars) was assessed with 2 mM substrate in the presence of 5 mM $Ca^{2+}$. Data shown represent the mean + S.D. of three independent experiments carried out in triplicate. *p<0.05, **p<0.01, ***p<0.001.
Activity is expressed in nmol/ng of total secreted proteins/hr/$10^8$ trypanosomes.

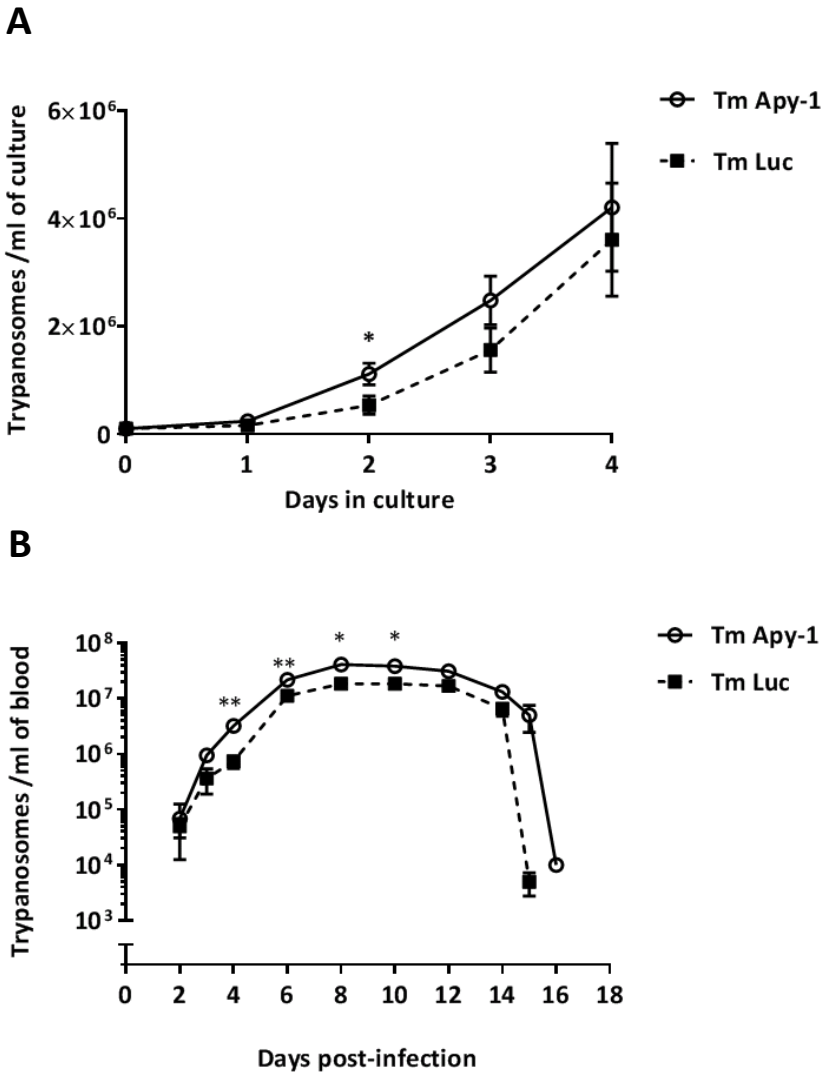


**Figure 4.3: Growth of transgenic *T. musculi* in vivo and in vitro**

**(A) Comparative *in vitro* growth curve**.

Growth of Luc and Apy-1 *T. musculi* in culture starting from 1 x $10^5$ parasites $ml^{-1}$. Data points represent mean values ± S.D. obtained from four independent experiments performed in duplicate.

**(B) Parasitaemia during *T. musculi* infection.**

BALB/c female mice were infected with 2 x $10^5$ *T. musculi* expressing Luciferase (Luc) or Apy-1 at day 0. Parasitaemias were followed by microscopy analysis of tail vein blood samples diluted in blood lysis buffer and expressed as number of parasites (in thousands) per ml of blood (limit of detection = 5 x $10^4$ trypanosomes/ml). Each point represents the mean ± S.D. of two independent experiments (n=5 in each experiment). The error bars for some data points are smaller than the symbol size.

*p<0.05, **p<0.01, ***p<0.001.

### *T. musculi* ecto-nucleotidase activity

Enzymes that catabolise exogenous nucleotides were not previously described in *T. musculi,* therefore examining the presence of these enzymes on the surface of trypanosomes is required, since most protozoan parasites possess ecto-nucleotidases. To do so, live trypanosomes were cultured *in vitro* in a medium containing ATP, and nucleotidase activity was analysed, showing that *T. musculi* were able to hydrolyse exogenous ATP (Figure 4.4A). The number of live trypanosomes before and after the assay was the same, indicating that ATP hydrolysis did not occur as a result of cell lysis. To eliminate the possibility that ATP hydrolysis was due to secreted enzymes, or to phosphate background from substrates, trypanosomes were incubated in the absence of any substrate, and the latter was tested in the absence of parasites. Supernatants from these control samples failed to show non-specific hydrolysis, confirming the presence of an ecto-nucleotidase.

The substrate specificity was also characterised by evaluating the ability of trypanosomes to hydrolyse other nucleotides. Figure 4.4A shows an evident substrate preference for ATP followed by GTP and CTP. A very low hydrolysis rate was generated with UTP and all diphosphates, along with negligible to no activity when monophosphate nucleosides were used as substrates. These results indicate that there are no 5´-nucleotidase enzymes on the surface of *T. musculi* and suggest the presence of an ecto-ATPase that can hydrolyse nucleoside tri- and/or diphosphates, but not monophosphates. To confirm this hypothesis, the effect of known extracellular impermeant inhibitors on ATP hydrolytic activity was evaluated in live trypanosomes. Sodium fluoride (NaF) and ammonium ($NH_4$) molybdate, two potent inhibitors of acid phosphatases (Fernandes *et al.*,

1997; Dutra *et al.*, 1998), and levamisole, an inhibitor of alkaline phosphatases (Van Belle, 1976), had no effect on the ATP hydrolysis (Figure 4.4B). Since these results rule out the possibility that the hydrolysis is due to an acid or alkaline phosphatase, it is likely that this ecto-enzyme is an ecto-ATPase. The presence of vanadate, an inhibitor of p-type ATPase (Sodré *et al.*, 2000; Miranda *et al.*, 2005), resulted in a 42% reduction in ATP hydrolysis compared to control. Subsequently, the addition of suramin, an ecto-ATPase inhibitor (Hourani and Chown, 1989; Ziganshin *et al.*, 1995; Meyer-Fernandes *et al.*, 1997), and DIDS (4,40-diisothiocyanatostilbene 2,20-disulfonic acid) which is a selective inhibitor of ecto-ATPase as well (Knowles, 1988; Barbacci *et al.*, 1996; Meyer-Fernandes *et al.*, 1997) resulted in a 50 and 44% inhibition of ATP hydrolysis rate, respectively. These data confirm that ATP hydrolysis is catalysed by an ecto-ATPase present on the surface of *T. musculi.*

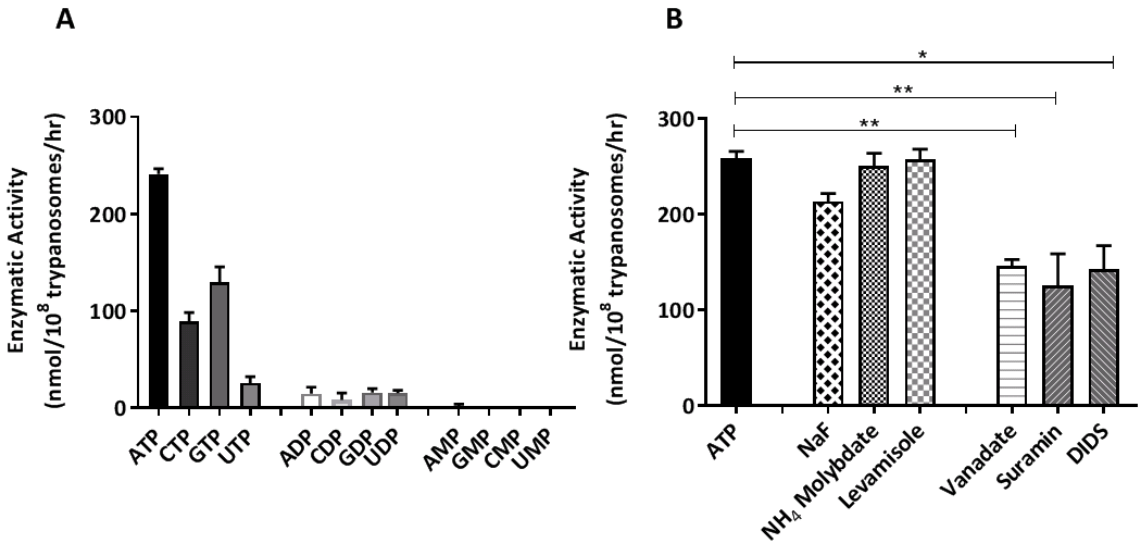


**Figure 4.4: *T. musculi* ecto-nucleotidase activity**

**(A) Substrate specificity**. Ecto-nucleotidase activity of wild type *T. musculi* was assessed in the presence of 5 mM triphosphates, diphosphates or monophosphates.

**(B) The effect of inhibitory agents on *T. musculi* ecto-nucleotidase activity.** Enzymatic activity was measured in reaction buffer (described under Materials and Methods) containing 5 mM ATP in the presence of various inhibitory agents.

The data shown represent the mean of enzyme activity + S.D. of three independent experiments performed in triplicate. Statistical significance was calculated using Mann-Whitney test (*$p < 0.05$, **$p < 0.01$).

**Expression and secretion of Apy-3 and 5´-NT by transgenic *T. musculi***

In light of the results described in chapter 3, Apy-3 was expressed in *T. musculi,* as it was shown that Apy-1 was the least active apyrase and Apy-3 the most active one. Adult *H. polygyrus* and *T. spiralis* cDNA were used as a template to amplify *apy-3* and *5´-NT*, respectively. PCR products were the expected size of 1000 bp for *apy-3* and 1500 bp for *5´-NT* (Figure 4.5A). Genes were then inserted into the expression cassette, and transformant cells were selected by ampicillin resistance. Colony PCR, diagnostic digest and sequencing were performed to confirm that the genes were successfully cloned in frame into the expression vector and that there were no mutations in the gene sequence.

As the ideal control for an active protein would be its inactive form, inactivation of Apy-3 catalytic activity was generated using specific primers with the desired mutation in a PCR reaction that amplifies the vector containing *apy-3*, altering glutamate 147 to glutamine (E147Q) (Dai *et al.*, 2004). After confirming that the desired mutation was introduced via sequencing, all three constructs containing *apy-3*, mutant *apy-3* or *5´-NT* were linearized by digestion with ScaI restriction enzyme and used to transfect *T. musculi* by electroporation, selecting transfectants with blasticidin. The expression of the three proteins by transgenic lines was revealed by western blot analysis showing a protein band in the supernatant at 60 kDa for active and mutant Apy-3, and at 85 kDa for 5´-NT. No bands were shown in cell lysates (Figure 4.5B).

In order to test if Apy-3 and 5´-NT were enzymatically active, and mutant Apy-3 was inactive, trypanosome secreted products were assayed. Mutated Apy-3 transgenic line

showed the same apyrase activity as wild type trypanosomes, confirming that mutant Apy-3 was enzymatically inactive (Figure 4.6A). It is clear that an active apyrase was highly detected in secreted products of Apy-3 transgenic trypanosomes compared to those expressing mutated Apy-3, and that this active apyrase had almost the same substrate specificity as the native enzyme (see chapter 3). On the other hand, Tm 5´-NT hydrolysed diphosphates and monophosphates at substantially greater levels than Tm WT (Figure 4.6B), indicating that transgenic trypanosomes secreted an active form of the enzyme.

The growth *in vitro* of *T. musculi* expressing active Apy-3 was compared to those expressing 5´-NT, mApy-3 (mutant Apy-3) and a mix of the two transgenic cells (50:50 active Apy-3 and 5´-NT), and observed to exhibit a significant difference only at day 2 (Figure 4.7A). Female BALB/c mice were then infected intraperitoneally with 2 x $10^5$ trypanosomes, and parasitaemia was monitored every 1-2 days until parasites disappeared from the blood. Tm 5´-NT, Tm mix and Tm mApy-3 exhibited almost the same course of parasitaemia, except that Tm 5´-NT infection was cleared from the circulation one day earlier. However, the course of parasitaemia of Tm active Apy-3 was substantially higher at day 4, and from day 10 to day 15, compared to mApy-3. Moreover, clearance was delayed from the circulation by 2 days (Figure 4.7B).

Given that the secretion of apyrase by transgenic trypanosomes would be predicted to increase extracellular AMP, and assuming that the host 5´-NT will hydrolyse AMP to adenosine to supply the purine salvage pathway, the effect of adenosine and AMP on the growth of wild type trypanosomes was tested *in vitro*. The addition of 100 µM adenosine or AMP had no effect on the *T. musculi* growth when compared to controls (Figure 4.8).

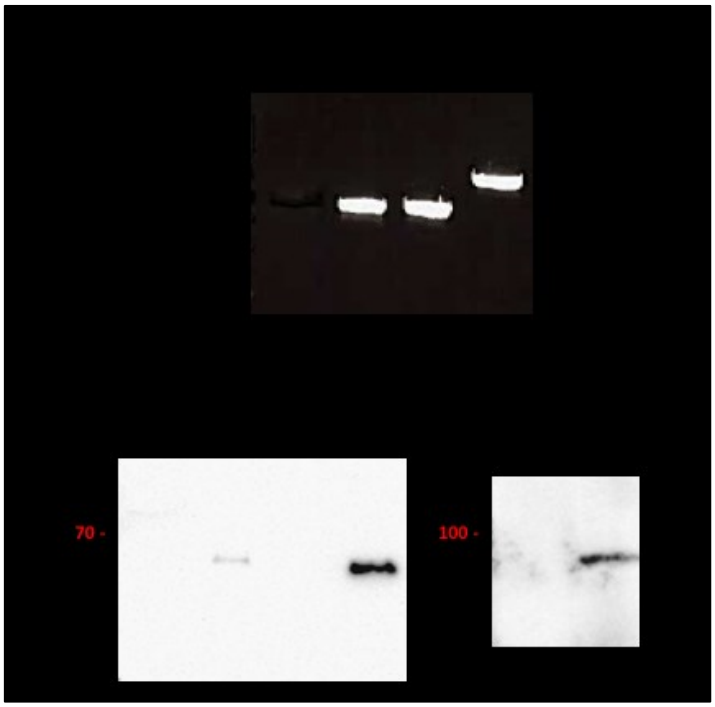


**Figure 4.5: Mutant Apy-3, Apy-3 and 5´-NT expression by transgenic *T. musculi***
**(A) Agarose gel electrophoresis of RT-PCR amplified products of *H. polygyrus apy-3, mutant apy-3* and *T. spiralis 5´-NT***. *Apy-3, mutant apy-3* and *5´-NT* were amplified by RT-PCR using specific primers. PCR products were separated on 1.5 % (w/v) agarose gel, stained with GelRed stain and visualised on a UV trans-illuminator.

**(B) Western Blot against Apy-3, mutant Apy-3 and 5´-NT.** Cell lysate (5 x $10^5$) and culture supernatants (sup.) from transgenic *T. musculi* were resolved on a 12 % (v/v) SDS polyacrylamide gel, blotted onto a PVDF membrane and probed with anti-c-myc epitope antibody followed by a goat anti-mouse horseradish peroxidase (HRP) secondary antibody. Chemiluminescence was detected using an LAS-300 Fuji Imager.
Lane No-RT: no reverse transcriptase control sample.
mApy-3: mutant Apy-3

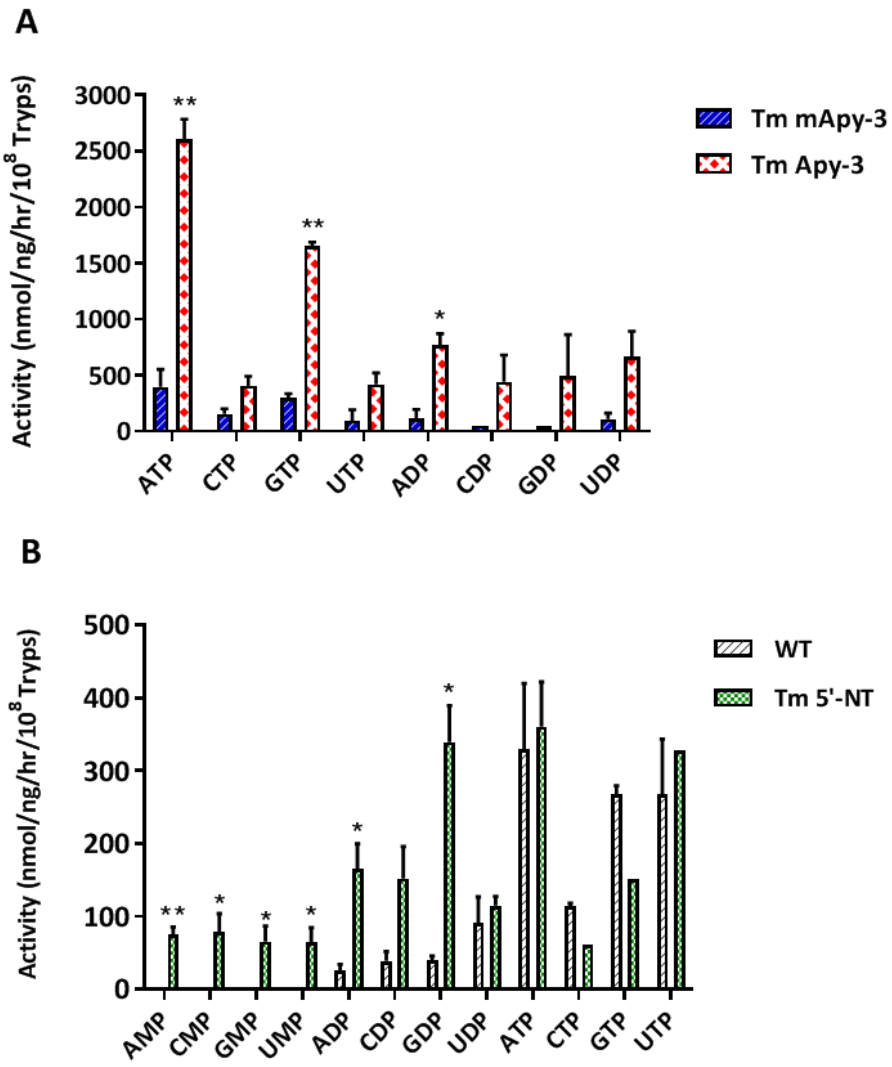


**Figure 4.6: Substrate specificity of WT and transgenic *T. musculi* secreted products**

Apyrase (A) and 5´-Nucleotidase (B) activities of secreted products from mutant Apy-3 (blue bars), Apy-3 (red bars), WT (black bars) and 5´-NT (green bars) were assessed with 2 mM substrates in the presence of 5 mM $Ca^{2+}$. Data shown represent the mean + S.D. of two independent experiments carried out in triplicate.

Activity is expressed in nmol/ng of total secreted proteins/hr/$10^8$ trypanosomes.

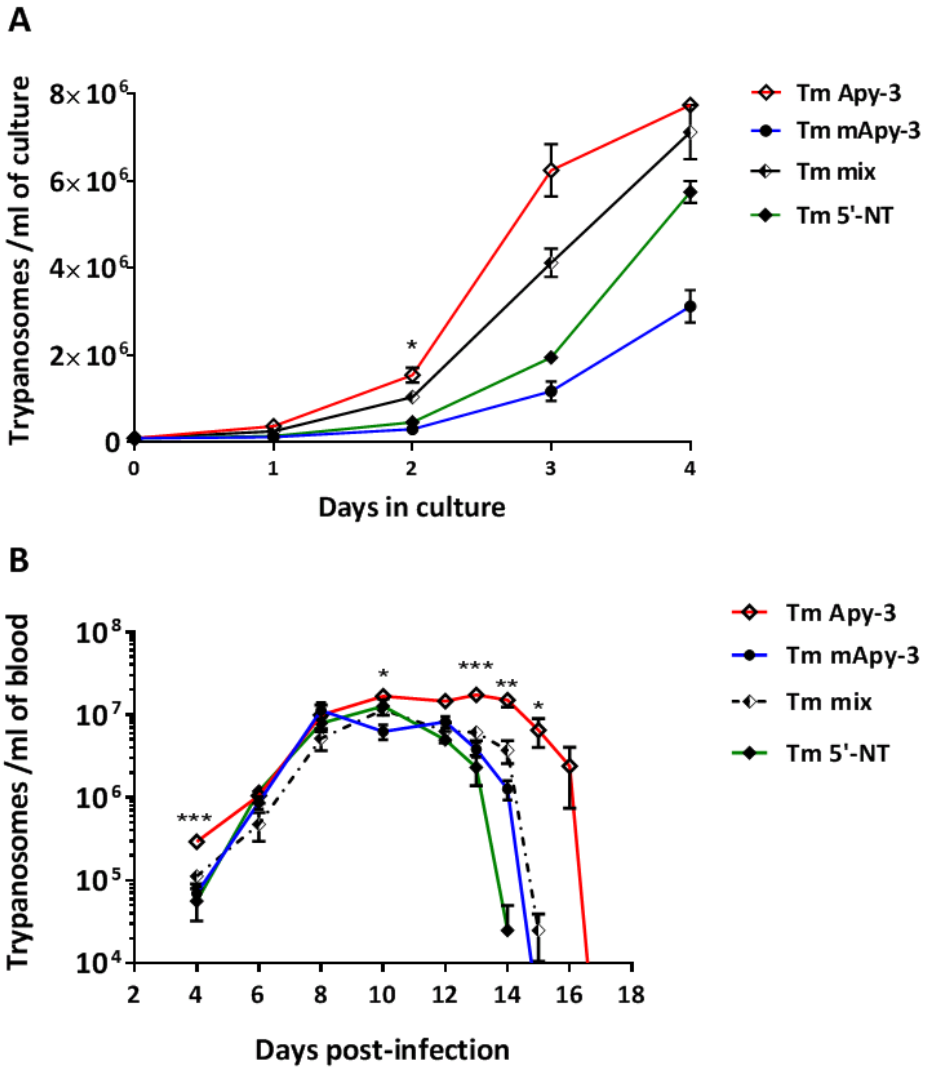


**Figure 4.7: Growth of transgenic *T. musculi* in vitro and in vivo**
**(A) Comparative *in vitro* growth curve of transgenic *T. musculi.***
Growth of *T. musculi* expressing mutant Apy-3 (mApy-3; blue), 5´-NT (green), Apy-3 (red) and a 50:50 mix of Apy-3 and 5´-NT (black dotted line) in culture starting from 1 x $10^5$ parasites $ml^{-1}$. Values expressed in number of parasites per ml of culture represent the mean ± S.D. of three independent experiments performed in triplicate. The error bars for some data points are smaller than the symbols.

**(B) Parasitaemia during transgenic *T. musculi* infection.**
Female BALB/c mice were infected on day 0 with 2 x $10^5$ *T. musculi* expressing Apy-3 (red), mutant Apy-3 (blue), 5´-NT (green) or a 50:50 mix of Apy-3:5'-NT (black dotted line). Parasitaemias were followed by microscopy analysis of tail vein blood samples diluted in blood lysis buffer and expressed as number of parasites (in thousands) per ml of blood (limit of detection = 5 x $10^4$ trypanosomes/ml). Each point represents the mean ± S.D. of 5 individual mice per group (*$p < 0.05$, **$p < 0.01$).

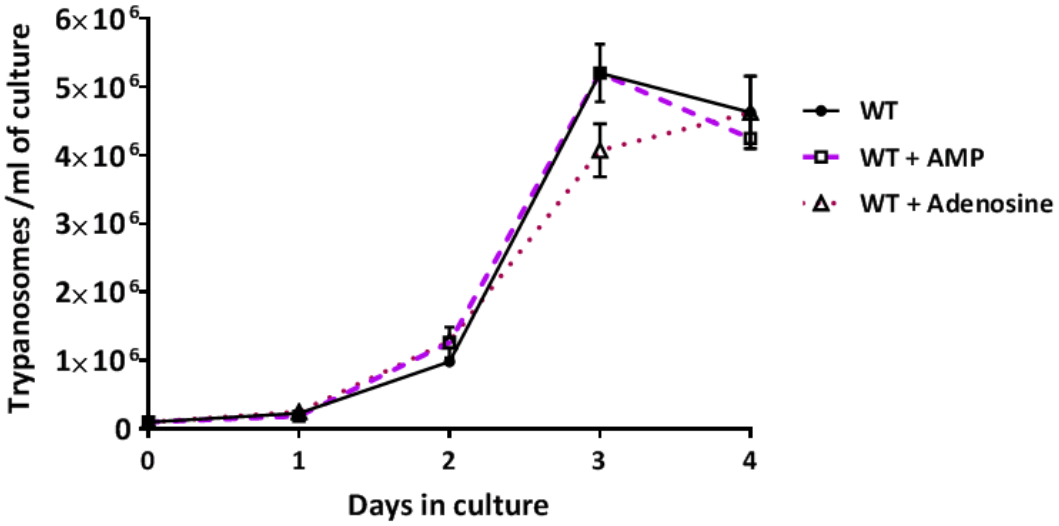


**Figure 4.8: Growth of *T. musculi* in the presence of AMP or adenosine**
Wild type (WT) *T. musculi* were grown in culture starting from 1 x $10^5$ parasites $ml^{-1}$ in the presence of 100 µM AMP or adenosine. Parasites were counted and fresh AMP or adenosine were added daily. Values expressed in number of parasites per ml of culture represent the mean ± S.D. of two independent experiments performed in triplicate. The error bars for some data points are smaller than the symbols.

**Spleen cellular composition, cytokine levels, antibody responses and macrophage activation markers during transgenic *T. musculi* infection**

In order to address the possible immunomodulatory properties of the recombinant proteins secreted by the transgenic trypanosomes, spleen cellular composition was tested at day 13 post-infection. A decrease in leukocyte populations was observed in the spleens of mice infected with Tm Apy-3, 5´-NT or mix when compared to controls, although this decrease was only significant with Tm 5´-NT and Tm mix infections (Figure 4.9).

There was a significantly lower number of B cells, T cells and CD8+ T cells in the spleens during Tm 5´-NT, Apy-3 and mix infections, in addition to fewer CD4+ T cells which were only significant in the spleen of mice infected with Tm 5´-NT (figure 4.10). A further analysis of the CD4+ T cell population was performed to differentiate between cells expressing regulatory T cells (CD4+Foxp3+), naïve CD4+ T cells (CD44-CD62L+), effector/memory CD4+ T cells (CD44+CD62Llow) and central memory CD4+ T cells (CD44+CD62L+). No difference was observed in central memory CD4+ T cells, whereas significantly lower numbers of effector memory phenotype were observed in spleens during Tm 5´-NT, Apy-3 and mix infections when compared to controls (Figure 4.11). There was a significantly lower level of regulatory T cells (Tregs) in Tm 5´-NT infection when compared to controls, and less Tregs were expressing CD73 and CD39 markers (Figure 4.12). However, the numbers were the same in Tm mApy-3 and Tm Apy-3 infections.

Furthermore, T cell cytokine production was measured during transgenic *T. musculi* infections. There was no significant difference in the amount of TNFα, IFNγ, and IL-4

produced by splenocytes from mice infected with Tm Apy-3, 5´-NT, and mix when compared to Tm mApy-3. However, enhanced IL-5 and IL-13 production, although not significant, was observed during Tm Apy-3 infection, accompanied by lower levels during Tm 5´-NT infections (figure 4.13). On the other hand, antibody end-point titres for IgG subclasses and IgM were performed at day 13 post-infection and no significant differences were observed (Figure 4.14).

Macrophage activation and polarisation markers (NOS2, Arg1, Ym1, Socs1, Mrc1 and Relmα) expressed by spleen cells were investigated by Griess assay and qPCR. No significant difference was observed in the expression of NOS2, however Arg1 expression was significantly upregulated in Tm Apy-3 and Tm mix infections. The expression of the alternative activation marker Ym1 was significantly downregulated in Tm 5´-NT and Tm mix infected mice, whereas Mrc1 expression was significantly upregulated in these same infections. However, Socs1 and Relmα level of expressions were intact in all infections (Figure 4.15). In addition, spleen cells were stimulated with *T. musculi* extract, but the levels of nitric oxide and arginase measured were below the limits of detection.

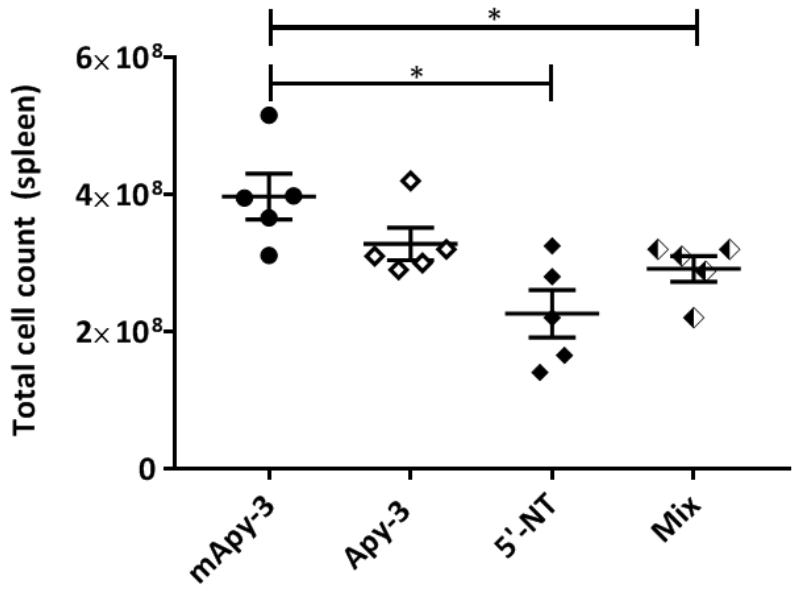


**Figure 4.9: Total spleen cell numbers from mice infected with transgenic *T. musculi***
Female BALB/c mice infected with 2 x $10^5$ *T. musculi* were harvested at day 13 post-infection and single cell suspensions made. Cells treated with RBC lysis buffer (see Materials and Methods) were stained with trypan blue and live cells were counted. Data are represented as mean +/- S.E.M (n= 5 mice/group). Statistical significance was calculated using Mann-Whitney test (*$p < 0.05$, **$p < 0.01$).

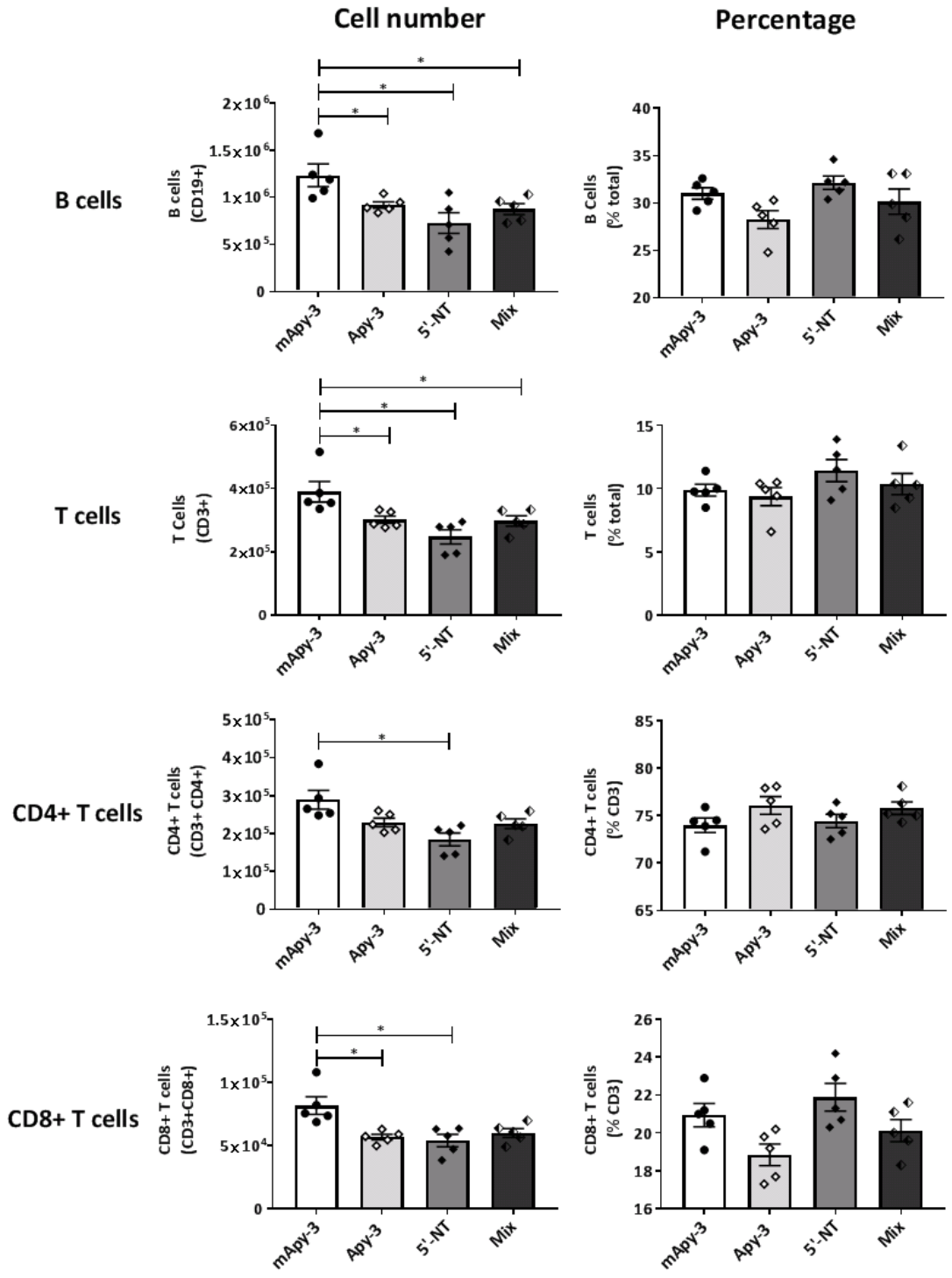


**Figure 4.10: Total number and proportion of B cells, T cells, CD4+ T cells, and CD8+ T cells in the spleen of mice infected with transgenic *T. musculi***

Female BALB/c mice infected intraperitoneally with 2 x $10^5$ *T. musculi* expressing Apy-3, mutant Apy-3, 5´-NT, or a 50:50 mix of Apy-3 and 5´-NT, were harvested at day 13 post-infection. Spleen single-cell suspensions were stained and analysed by flow cytometry for the number (left panels) and percentage (right panels) of B cells (CD19+), T cells (CD3+), CD4+ T cells (CD3+CD4+), and CD8+ T cells (CD3+CD8+). Data are shown as mean +/- SEM (n=5 mice/ group) (*$p < 0.05$, **$p < 0.01$).

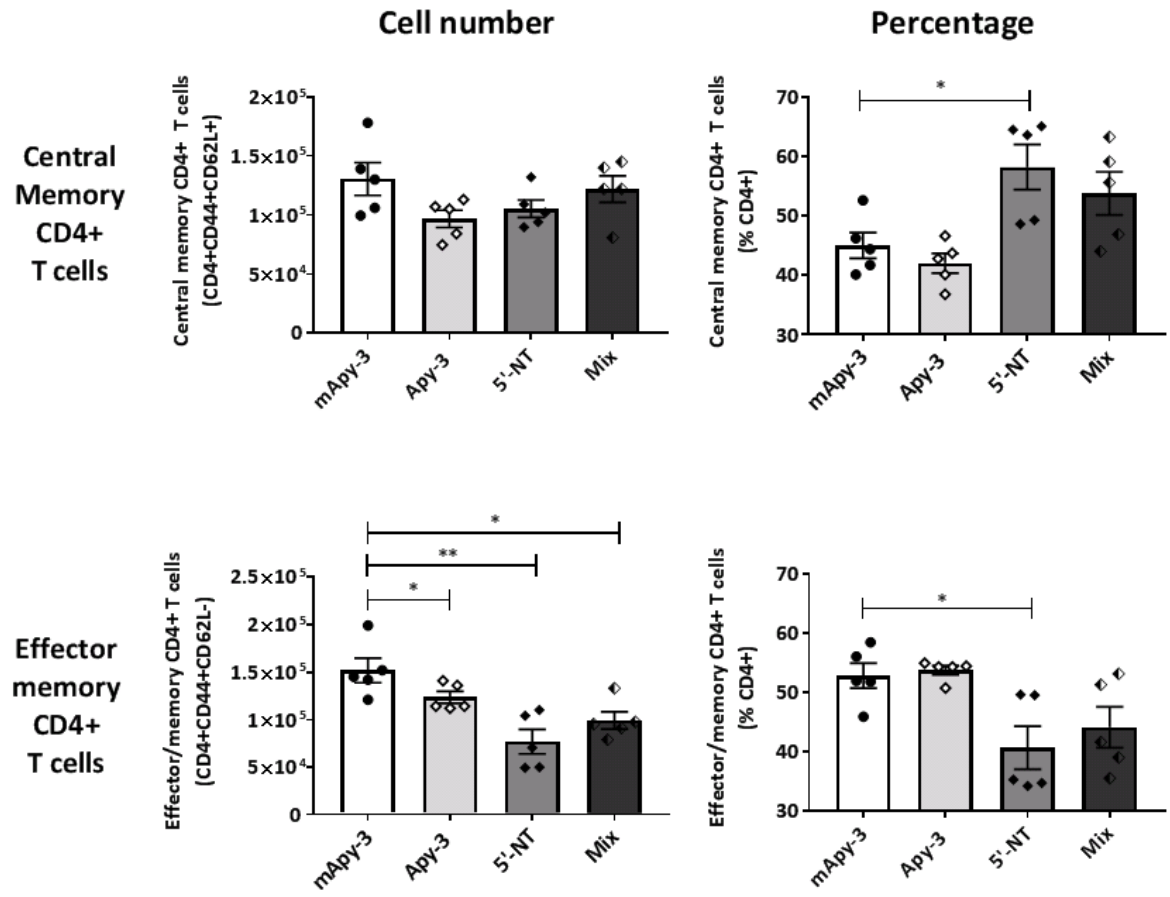


**Figure 4.11: Total number and proportion of CD4+ T cells expressing central memory or effector/memory phenotype in the spleen of mice infected with transgenic *T. musculi***
Female BALB/c mice infected intraperioneally with 2 x $10^5$ *T. musculi* expressing Apy-3, mutant Apy-3, 5´-NT, or a 50:50 mix of Apy-3 and 5´-NT, were harvested at day 13 post-infection. Single-cell spleen suspensions were stained and analysed by flow cytometry for the number (left panels) and percentage (right panels) of CD4+ T cells expressing a central memory (CD44+CD62L+) or effector/memory (CD44+CD62Llo) phenotype. Data are shown as mean +/- SEM (n=5 mice/ group). (*$p < 0.05$, **$p < 0.01$).

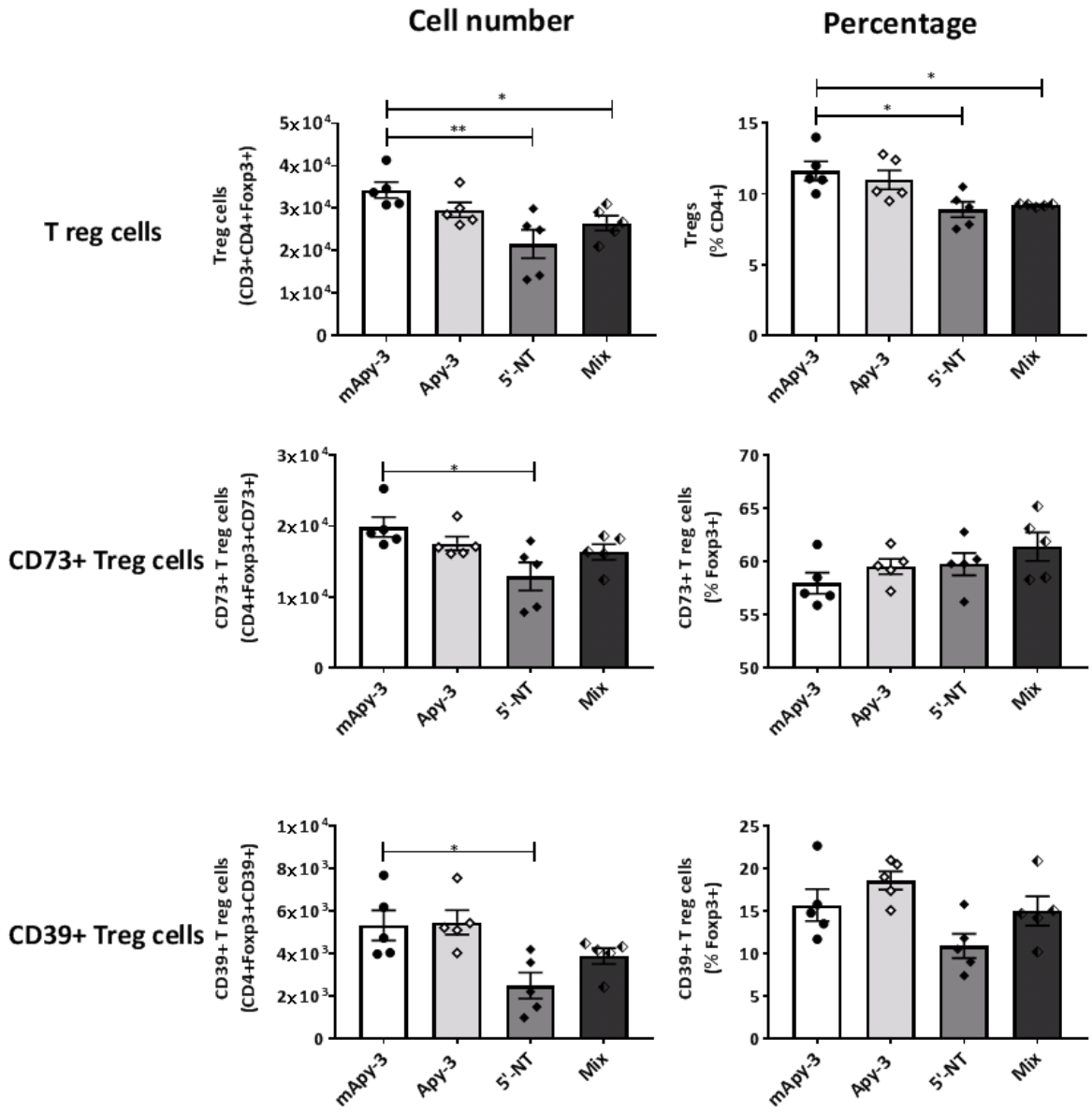


**Figure 4.12: Total number and proportion of Foxp3+, CD73+ and CD39+ cells in spleen CD4+ populations of mice infected with transgenic *T. musculi***

Female BALB/c mice infected intraperioneally with 2 x $10^5$ *T. musculi* expressing Apy-3, mutant Apy-3, 5´-NT, or a 50:50 mix of Apy-3 and 5´-NT, were harvested at day 13 post-infection. Spleen single-cell suspensions were stained and analysed by flow cytometry for the number (left panels) and percentage (right panels) of Foxp3+ within CD4+ cells. Foxp3+ populations were further analysed for the expression of intracellular CD39 and CD73. Data are shown as mean +/- SEM (n=5 mice/ group). (*$p < 0.05$, **$p < 0.01$).

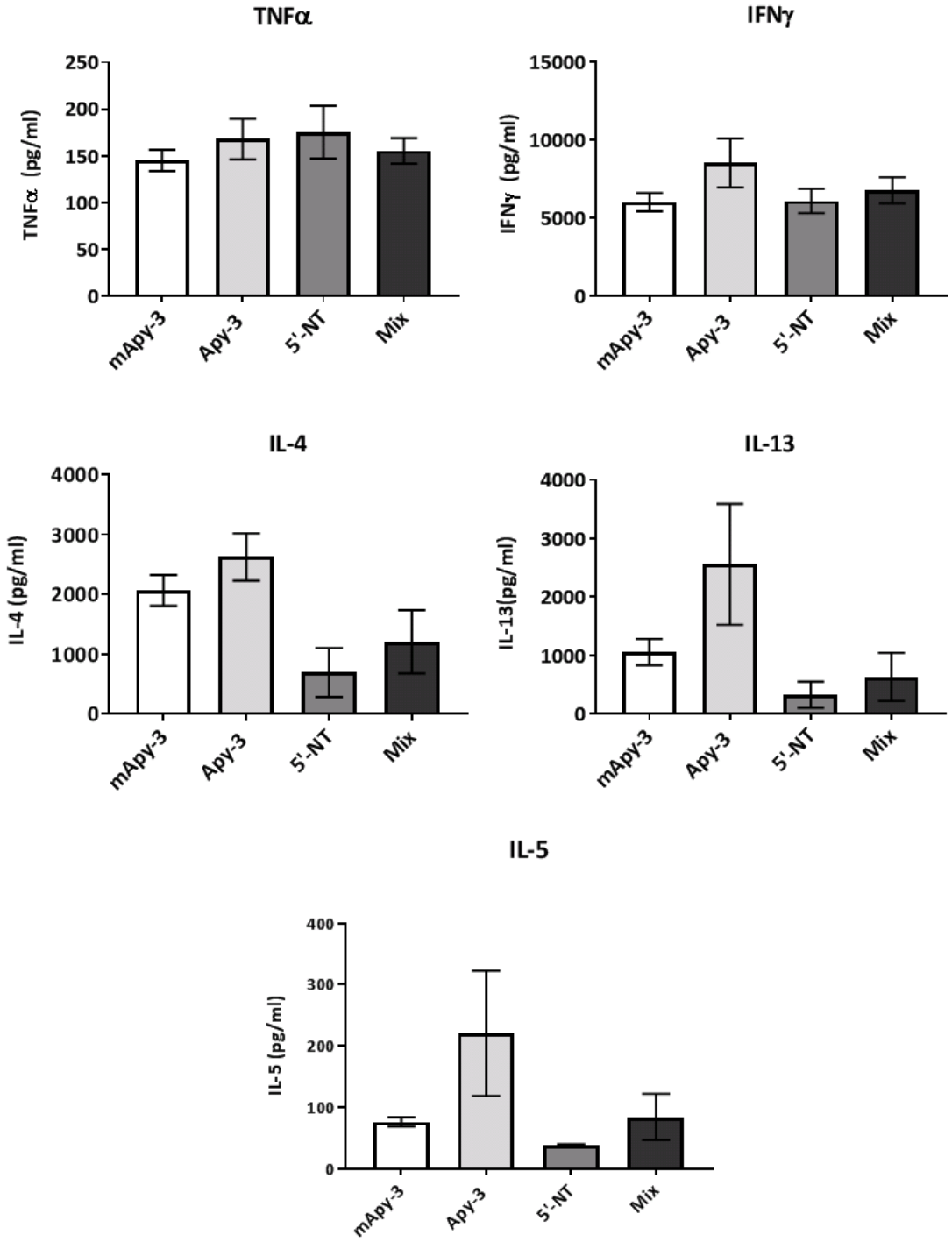


**Figure 4.13: Cytokine production by splenocytes at day 13 post-infection with transgenic *T. musculi***

Spleen cells from mice infected intraperioneally with 2 x $10^5$ *T. musculi* expressing Apy-3, mutant Apy-3, 5´-NT, or a 50:50 mix of Apy-3 and 5´-NT, were cultured at a density of 1 x $10^6$ $ml^{-1}$ and stimulated with anti-CD3/CD28 for 48 hours. The levels of interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin-4 (IL-4), IL-13 and IL-5 produced in the culture supernatant were determined by ELISA. Data are shown as mean +/- SEM (n=5 mice/ group). (*$p < 0.05$, **$p < 0.01$).

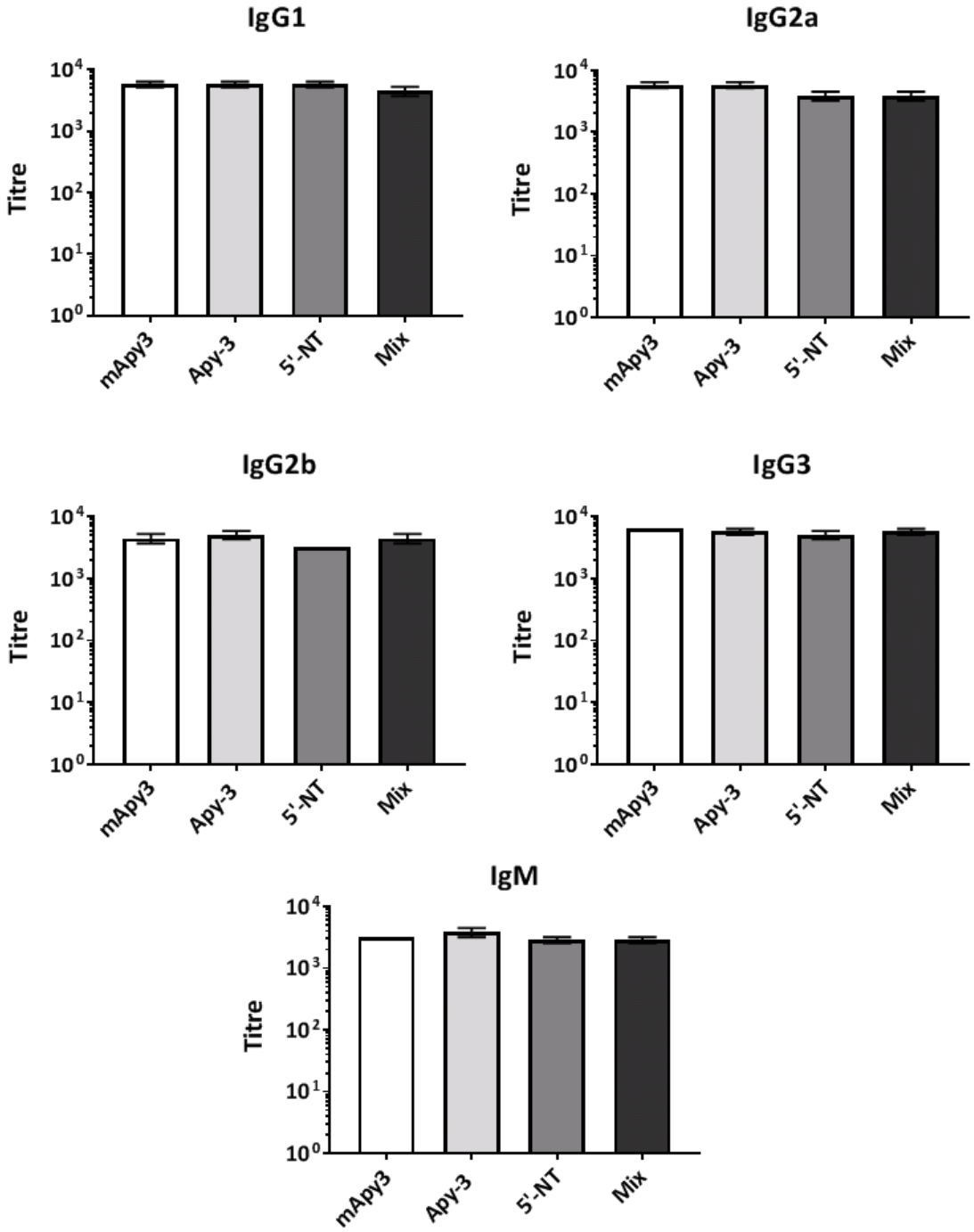


**Figure 4.14: Specific anti-trypanosome antibody responses (end-point titres) at day 13 post-infection with transgenic *T. musculi***

Female BALB/c mice infected intraperioneally with 2 x $10^5$ *T. musculi* expressing Apy-3, mutant Apy-3, 5´-NT, or a 50:50 mix of Apy-3 and 5´-NT, were harvested at day 13 post-infection, and serum was isolated. IgG1, IgG2a, IgG2b, IgG3 and IgM antibody titres were measured by ELISA against wild type *T. musculi* extracts. Results are expressed as the mean of antibody endpoint titres +/- SEM (n=5 mice/ group). (*$p < 0.05$, **$p < 0.01$).

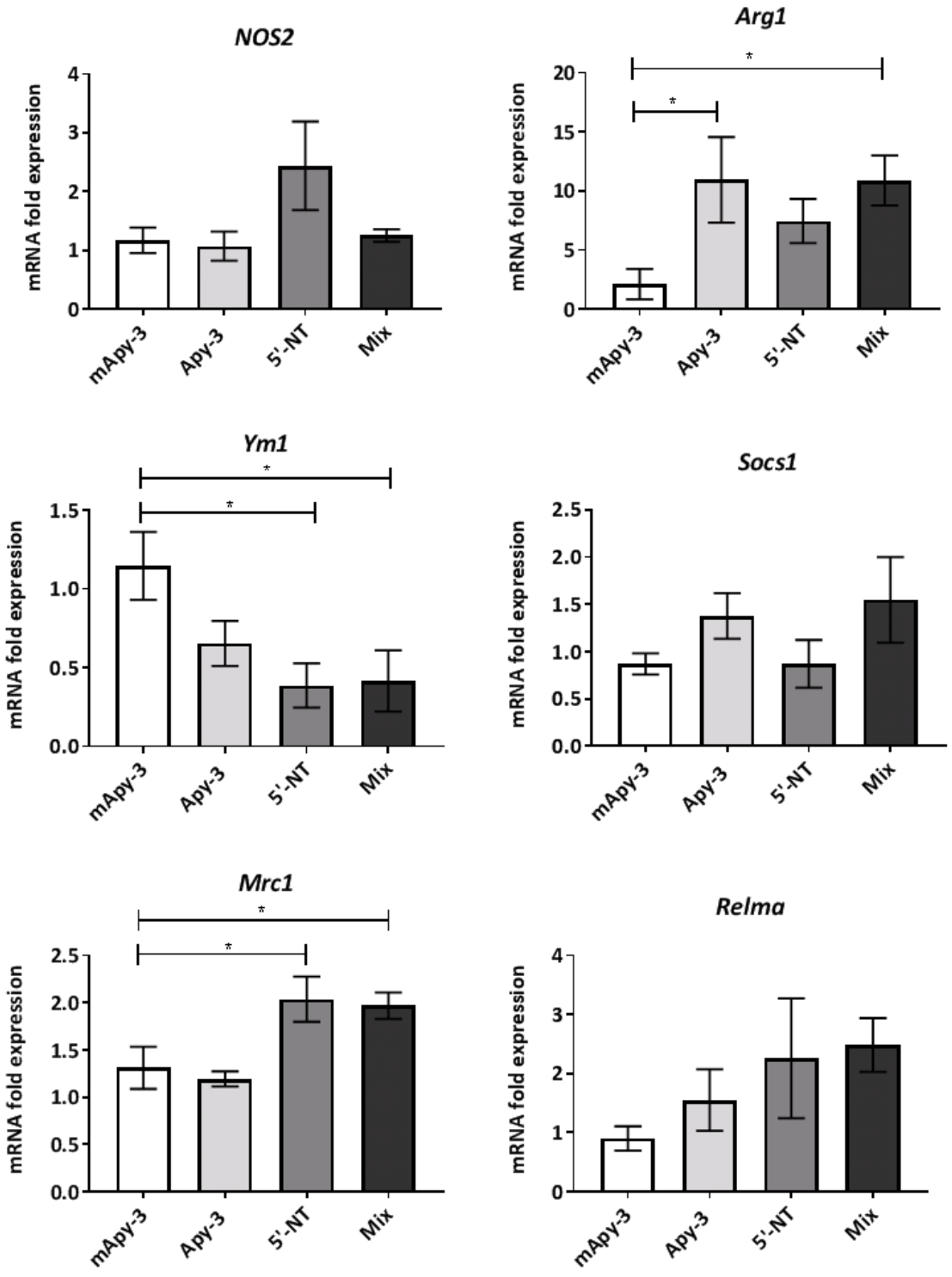


**Figure 4.15: Expression of macrophage polarisation markers in splenic macrophage population of mice infected with transgenic *T. musculi* at day 13 post-infection**

Expression of NOS2 (iNOS), Ym1, Arginase, Socs1, Mrc1 and Relmα was measured by quantitative real-time PCR and shown as fold-change relative to the median of mice infected with *T. musculi* mutant Apy-3. Data are represented as mean +/- SEM (n=5 mice/ group). (*$p < 0.05$, **$p < 0.01$).

## 4.3 Discussion

Helminth parasites secrete a wide range of immunomodulatory molecules which can influence the host immune response in a way to prevent the clearance of the parasite, but also suppress symptoms of immune dysregulatory diseases of the infected host. These molecules, contained in excretory/secretory (ES) products, often exert their effects by suppressing type 2 immune responses, thus constraining the symptoms associated with Th2-related diseases. They target host cells in many distinct ways, inducing IgG4 along with a decrease in IgE, IL-4 and IL-5, and an increase in IL-10 levels (Hewitson, Grainger and Maizels, 2009; Allen and Maizels, 2011; McSorley *et al.*, 2014). A large number of studies have examined the effects of helminth infections, e.g. *Nippostrongylus brasiliensis*, *Heligmosomoides polygyrus* and *Trichinella spiralis* on a range of mouse models of immunopathological diseases. These studies indicate that infection with helminth parasites downregulates the immune system and can protect against development of immune-mediated diseases such as colitis, allergy and type-1 diabetes. From here comes the interest in these molecules as a target for future human therapies (Erb, 2009; Hewitson *et al.*, 2011; Donskow-Łysoniewska *et al.*, 2013).

However, genetic manipulation of helminth parasites is limited. For instance, inducing gene knockdown via RNA interference (RNAi) in *H. polygyrus* has been unsuccessful (Lendner *et al.*, 2008). An alternative approach to study parasite gene function within a host is heterologous expression into an appropriate vehicle. Heterologous expression systems have been previously used to analyse gene function in transfected organisms or *in vitro*. The intracellular kinetoplastid parasite *Leishmania mexicana* was described as an applicable

expression system to assay immunomodulatory effects of helminth molecules, such as *Brugia malayi* ALT proteins (Gomez-Escobar *et al.*, 2005; Maizels *et al.*, 2008), but as the parasite is intracellular, it may not be suitable for all proteins secreted by extracellular helminths. The systemic, non-pathogenic extracellular *Trypanosoma theileri,* a member of the kinetoplastid family, was developed as a vehicle to deliver vaccines against pathogens to cattle targeting multiple infections, including major cattle-borne zoonoses (Mott *et al.*, 2011). *T. theileri* persists at very low levels for the lifetime of the host, and thus may not be a suitable vehicle to study the effect of an exogenous protein on the host immune response over a short period of time. Other studies have suggested the use of the non-colonising bacterium *Lactococcus lactis* as a vaccine delivery system to treat helminth infections (Medina *et al.*, 2010; Berlec *et al.*, 2015; Durmaz *et al.*, 2016), and immunopathologies such as colitis via the secretion of IL-10 (Steidler *et al.*, 2000, 2011; Braat *et al.*, 2006; Foligne *et al.*, 2007).

Although these previously described heterologous systems have been proven to be useful for the expression of exogenous genes, the aim here is to study the effect of the gene of interest and genes of unknown functions on the immune responses when the expressed protein is delivered extracellularly, as in a worm infection. *T. musculi* has been shown to be an excellent in vivo expression model to investigate immunomodulatory effects by helminth genes, as it is an extracellular natural mouse parasite which can deliver the exogenous proteins to various host tissues. It is easy to genetically manipulate, with a simple, reproducible pattern of infection that lasts for three weeks, allowing study of the host-pathogen interactions through monitoring changes in parasitaemia. It has been successfully

used as a vehicle for expression and secretion of *N. brasiliensis* acetylcholinesterase (AChE) in mice by Vaux *et al.* (2016).

In this study, the immunomodulatory functions of *H. polygyrus* apyrases (Apy-1 and Apy-3) and *T. spiralis* 5´-NT have been examined via heterologous expression in *T. musculi.* Apyrases secreted by *H. polygyrus* are hypothesised to inhibit ATP-mediated inflammation and ADP-mediated thrombosis by catabolising ATP and ADP to AMP, thus helping the parasite to neutralise the host's immune system and promote persistence within the host. *T. spiralis* 5´-NT was shown to catalyse the hydrolysis of nucleoside di- and monophosphates to the anti-inflammatory molecule adenosine which activates P1 receptors on immune cells and are therefore predicted to suppress immune responses (Gounaris, Selkirk and Sadeghi, 2004). Thus, one major approach to consider is that apyrases and 5´-NT might selectively target P2 and P1 receptors respectively, reducing inflammation and suppressing the immune system; hence the importance of further studying the effects of these parasitic molecules on the host immune system. The *H. polygyrus apy-1* gene was first inserted into the expression vector, and when the construct was transfected into trypanosomes via electroporation, RT-PCR confirmed that the gene was found to be expressed and integrated into the *T. musculi* genome. Western blot results then confirmed the presence of the protein in the cell lysate and the supernatant of the transgenic lines and not of the WT trypanosomes, confirming the secretion of the recombinant protein. The latter might have been glycosylated while being translocated into the ER, which could explain the presence of a slightly higher mass in the supernatant.

No previous studies have examined the presence of ecto-nucleotidase enzymes on the external surface of *T. musculi,* neither the secretion of an apyrase. Thus, apyrase activity assays were performed on both the WT and transgenic trypanosomes. Very little nucleoside tri- and diphosphate hydrolysis was detected in WT *T. musculi* secreted products. This could be due to an enzyme belonging to one of the four classes: phosphatase, phosphodiesterase, ATP diphosphohydrolyse, or apyrase (A. A. Da'dara *et al.*, 2014). In contrast, the transgenic trypanosomes showed the secretion of an active apyrase, with a substrate specificity reflecting that of Apy-1 expressed in *P. pastoris* (see chapter 3).

Moreover, the data here confirm the presence of an ecto-ATPase sensitive to the impermeant inhibitor DIDS and suramin, which are inhibitors of ecto-ATPases (Hourani and Chown, 1989; Ziganshin *et al.*, 1995). The activity could not be attributed to the presence of an acid- or alkaline phosphatase, since the ATP hydrolysis was insensitive to sodium fluoride, ammonium molybdate and levamisole, potent inhibitors of phosphatase activities. Since the ecto-ATPase described here did not hydrolyse AMP and monophosphates, in addition to being insensitive to ammonium molybdate, the 5´-NT inhibitor (Gottlieb and Dwyer, 1983), the possibility of the presence of an ecto-5´-nucleotidase was discounted. The presence of ecto-nucleotidases have been described in several parasites including protozoans, and was shown to exert important roles including cellular adhesion and termination of purinergic signalling (Plesner, 1995). Studies have suggested that the Mg-dependent ecto-ATPase expressed on the surface of *Entamoeba histolytica* (Barros *et al.*, 2000)*, Leishmania amazonensis* (Berrêdo-Pinho *et al.*, 2001) and *Trypanosoma cruzi* (Silber *et al.*, 2002) is considered a marker of pathogenesis for these parasites as the virulent promastigotes and trypomastigotes have a much higher ATPase activity than the avirulent ones. In addition, it is

known that trypanosomatids such as *T. musculi, T. cruzi, L. amazonensis* and other protozoans are unable to synthesise purines de novo, and thus they rely on purine salvage pathways from the external environment as source of these nutrients. The presence of ecto-nucleotidase enables these parasites to hydrolyse nucleotides to the respective nucleosides to support purine salvage (Albright, Pierantoni and Albright, 1990; Berrêdo-Pinho *et al.*, 2001; Meyer-Fernandes *et al.*, 2004; De Koning, Bridges and Burchmore, 2005).

The effect of Apy-1 was then studied in vivo in order to gain some understanding of its potential role in *H. polygyrus.* When the parasitaemia of mice infected with *T. musculi* secreting active Apy-1 was compared to Tm-Luc infection, Tm Apy-1 was found to grow faster, with a prolonged infection and delayed clearance. These results suggest that the changes to survival rates could results from either a physiological and/or an immunological advantage. Secretion of an active apyrase by the transgenic trypanosomes, along with the presence of the ecto-ATPase on the surface of *T. musculi*, will likely increase the capacity of hydrolysing the extracellular potent pro-inflammatory ATP. This is released upon tissue damage, which would clearly be caused by migrating helminth parasites. This has been considered for *Schistosoma mansoni* (Smith and Von Lichtenberg, 1974; Bloch, 1980; Bhardwaj and Skelly, 2009), and would also be relevant for *H. polygyrus*, although it is unclear whether *T. musculi* causes significant tissue damage. Hydrolysis of ATP could result in the accumulation of extracellular nucleosides available for the trypanosomes to take up if working in concert with host 5´-NT, thus supplying the *T. musculi* purine salvage pathways (Albright, Pierantoni and Albright, 1990). On the other hand, apyrase might be interfering with early control of the infection, impeding host immunity which would favour the multiplication of the parasite, hence the difference in parasitaemia.

Prior to studying the reason behind the faster growth, a more active apyrase (Apy-3) secreted by *H. polygyrus* was expressed in *T. musculi* together with 5´-NT secreted by *T. spiralis*, as Apy-1 was shown to be the least active apyrase (see chapter 3), and 5´-NT will result in the accumulation of the anti-inflammatory molecule adenosine. In addition, the catalytic activity of Apy-3 was inactivated, and a mutant form of Apy-3 was expressed in *T. musculi* to be used as a control for the active form of the enzyme. Results confirmed the secretion of enzymatically active Apy-3 and 5´-NT and inactive mutant Apy-3 (mApy-3).

The effect of these enzymes was first studied *in vitro*, and it was found that Tm Apy-3 grow slightly faster than the control Tm mApy-3. All strains eventually reached the same level of growth after few days due to nutrient depletion, however the difference observed is in rate of growth. This might be due to the presence of nucleoside tri- or diphosphates such as ATP and ADP, in the medium or serum (Creek *et al.*, 2013; Doleželová *et al.*, 2018), which were hydrolysed to nucleosides and supported the faster growth of the transgenic trypanosomes secreting 5´-NT and Apy-3 in culture. The transgenic trypanosomes were therefore grown in the absence of 10% FCS and conditioned medium (data not shown), and all transgenic lines replicated at the same rate, although depletion of FCS is growth limiting since it is essential in supplying nutrients to support trypanosome growth to higher densities (Creek *et al.*, 2013; Doleželová *et al.*, 2018). Different FCS and conditioned medium batches were tested for the presence of ATP, and results confirmed that some batches contain very small amounts of ATP. This might be the reason behind the slight faster growth of transgenic trypanosomes, as apyrase and 5´-NT will hydrolyse the ATP to adenosine, supporting purine salvage and growth.

Given that the secretion of apyrase or 5´-NT by the transgenic lines should increase extracellular AMP and adenosine *in vivo*, the addition of an appropriate low concentration of adenosine and AMP in the culture medium would favour the growth of *T. musculi in vitro*. High (mM) concentrations are toxic to trypanosomes (Taliaferro and Alesandro, 1971; Geiser *et al.*, 2005; Lüscher *et al.*, 2014). Data here showed that supplementing the medium with AMP or adenosine had no effect on the growth of trypanosomes. However, on the basis of these results, we cannot conclude that Apy-3 and 5´-NT do not play a beneficial physiological role for trypanosomes in *vivo*.

When the effect of these proteins was monitored *in vivo*, the course of parasitaemia of Tm Apy-3 was found to be substantially higher at day 4, again with a prolonged infection and a delayed clearance when compared to the control. However, Tm 5´-NT and Tm mix parasites exhibited a similar course of parasitaemia as the control until late in infection when Tm expressing 5´-NT were cleared from the bloodstream a day earlier than the control. These differences might suggest that the faster growth of Tm apyrase could be due to modulation of innate immune responses, whereas the early clearance of Tm 5´-NT and the prolonged infections of Tm Apy-3 could be due to modulation of the adaptive immune responses (Figure 4.7B). Albright and Albright (1999) suggest that early control of trypanosome infection is induced by NK cells which might activate macrophages via the secretion of IFNγ, thus numbers and phenotype of macrophages and NK cells would be more informative.

Following the generation of *T. musculi* transgenic lines secreting inactive Apy-3, active Apy-3 and 5´-NT, and monitoring the course of parasitaemia *in vivo* for three weeks, it

was essential to study the possible immunomodulatory functions of apyrase and 5´-NT, screening for potential effects these proteins may have on the host immune response by determining antibodies, cytokine levels and leukocyte populations at day 13 post-infection.

Purinergic signalling plays an essential role in regulating the immune system. Extracellular ATP (eATP), released during inflammation, functions as a signalling molecule through the activation of purinergic P2 receptors, inducing an inflammatory response by recruiting innate immune cells and causing the release of pro-inflammatory cytokines and inflammatory mediators such as nitric oxide (NO) (Junger, 2011).

During Tm Apy-3 infection, extracellular ATP would be predicted to be degraded to AMP via secreted Apy-3 and *T. musculi* ecto-ATPase. AMP could then serve as a substrate to CD73 for the extracellular generation of adenosine. With Tm 5´-NT infected animals, extracellular adenosine would be predicted to be generated via the secreted 5´-NT. Once adenosine is produced in the extracellular compartment, it will activate P1 purinergic receptors expressed on the surface of a wide range of immune cells including T and B lymphocytes, macrophages, dendritic cells and neutrophils. Depending on the concentration of extracellular adenosine, different adenosine receptors might be activated, due to the difference in receptor affinity (Bours *et al.*, 2006). *In vivo* studies using adenosine receptor knockout mice suggested that A2A receptors are the most dominant P1 receptors in inducing lymphocyte responses. It was previously shown that the activation of these receptors inhibited the production of IL-2 (Naganuma *et al.*, 2006), IFNγ and IL-4 by naïve CD4+ T cells (Lappas, Rieger and Linden, 2005). In addition, IL-2 production by CD8+ T cells

was inhibited (Erdmann *et al.*, 2005), however the production of IL-4 and IL-5 cytokines produced by cytotoxic CD8+ T cells was not influenced.

B and T cell numbers were lower in mice infected with Tm Apy-3, 5´-NT and mix. A lower number of CD4+, CD8+ and effector CD4+ T cells was also observed with Tm Apy-3 and Tm 5´-NT infections, in addition to a lower number of Tregs, CD73+ and CD39+ Tregs with Tm 5´-NT infection. These data suggest that hydrolysis of ATP by apyrase and 5´-NT introduced by transgenic *T. musculi* might have resulted in accumulation of extracellular adenosine which then activated adenosine receptors expressed on B cells (Mainly $A_1$, $A_{2A}$ and $A_3$), resulting in downregulation of activation and proliferation of B cells. This suppression might be mediated via $A_3$ adenosine receptors, which upon activation with adenosine, can prevent functional activation of B cells (Saze *et al.*, 2013). B cells are necessary for proliferation and expansion of CD4+ T cells and Tregs. Other studies suggest that B cells can mediate a dual regulatory activity effect on T cells, either upregulating CD4+ and CD8+ T cells or downregulating CD73 expression, thus inhibiting T cell proliferation and cytokine production. This was shown to be directed by 5´-AMP and adenosine, products of ATP hydrolysis in the extracellular compartment. However, activated B cells were shown to upregulate CD39 expression thus increasing the production of 5´-AMP, which is an $A_1$ adenosine receptor agonist (Rittiner *et al.*, 2012), and suppressing activated T cells (Tu *et al.*, 2008; Chen *et al.*, 2009; Sakowicz-Burkiewicz *et al.*, 2012; Saze *et al.*, 2013). On the other hand, adenosine deaminase (ADA) present on activated T effector cells can terminate adenosine signalling by hydrolysing adenosine to the purine nucleoside inosine, that has a longer half-life than adenosine (Welihinda *et al.*, 2018). However, activated B cells can oppose this effect via

adenosine kinase by phosphorylating adenosine to 5´-AMP, which can bind to $A_1$ receptors on activated T effector cells and inhibit their function (Saze *et al.*, 2013).

Foxp3+ Tregs are known to co-express the ecto-nucleotidase enzymes CD39 and CD73, which are responsible for increasing pericellular adenosine (Kobie *et al.*, 2006; Deaglio *et al.*, 2007; Dwyer *et al.*, 2007; Mandapathil *et al.*, 2009; Schuler *et al.*, 2011). During inflammation, extracellular ATP activates $P2X_7$ receptors on Treg cells, inhibiting their differentiation and suppressive function while stimulating T cell differentiation towards pro-inflammatory Th17 cells (Piconese *et al.*, 2009; Schenk *et al.*, 2011; Takenaka, Robson and Quintana, 2016). However, adenosine activates $A_{2A}$ receptors on Tregs in an autocrine signalling loop, upregulating their expression and increasing their immunoregulatory activity (Borsellino *et al.*, 2007; Zarek *et al.*, 2008; Ohta *et al.*, 2012; Antonioli, Pacher, *et al.*, 2013; Ohta and Sitkovsky, 2014; Faas, Sáez and de Vos, 2017). CD39 activity is induced on activation of T cell receptors (TCRs) expressed on Treg cells. It was also shown that Tregs secrete CD39 and CD73 in exosomes that can suppress effector T cell proliferation and IL-2 secretion through the activation of $A_{2A}$ receptors expressed on T effector cells (Deaglio *et al.*, 2007; Smyth *et al.*, 2013).

Cytokine responses were intact in all infections, except for IL-5 and IL-13 which were higher, although not significantly so, in Tm Apy-3 infections. Studies have shown that activation of the A2B receptor by adenosine downregulates IL-5 and IL-13 production by group 2 innate lymphoid cells (ILC2) (Csóka *et al.*, 2017). This was not evident in the current study, in which slightly higher levels of these cytokines were detected. No significant changes were seen in TNFα and IFNγ levels, nor in iNOS expression or NO production, so it is

less likely that TNF is contributing to the premature clearance seen in Tm 5´-NT. However, injection of TNF induces rapid clearance of *T. musculi,* and TNF is known to induce iNOS expression in macrophages, thus controlling the parasite *in vivo* (Kongshavn and Ghadirian, 1988; Liew, Li and Millott, 1990; Silva *et al.*, 1995; Fonseca *et al.*, 2003). In addition, IFNγ stimulates macrophages to produce NO which was shown to kill *T. musculi in vitro* (Vincendeau and Daulouède, 1991; Albright *et al.*, 1994). No changes in titres of IgG subclasses and IgM were observed here, although IgG1, IgG2b and predominantly IgG2a were shown to be the antibodies associated with *T. musculi* clearance (Olivier, Tijssen and Viens, 1986; Vincendeau, Daeron and Daulouede, 1986; Wechsler and Kongshavn, 1986).

Monocytes are polarised into classically activated macrophages (M1) or alternatively activated macrophages (M2 or AAMs), depending on the cytokine environment. Studies have shown that M1 macrophages are pro-inflammatory, whereas M2 macrophages suppress inflammation and promote tissue remodelling (Csoka *et al.*, 2012; Zanin *et al.*, 2012). During parasitic helminth infections, such as filarial and gastrointestinal nematode infections, the expression of AAM markers (Arg1 and Ym1) are upregulated (Nair et al., 2005; Taylor *et al.*, 2006).

In this study, the expression of macrophage polarisation markers (NOS2, Arg1, Ym1, Socs1, Mrc1 and Relmα) by splenocytes from *T. musculi*-Apy-3 and/or *T. musculi*-5´-NT infected mice were compared to *T. musculi*-mutant Apy-3 infected mice. The accumulation of adenosine due to ATP hydrolysis by *T. musculi* expressing active Apy-3 and/or 5´-NT led to a higher expression of Arg1 and a lower expression of Ym1 when compared to *T. musculi* expressing mutant Apy-3. Adenosine is known to induce polarisation to an M2 phenotype

characterized by high expression of arginase-1, TIMP-1, and mgl-1, but not Ym1 and Fizz1 (Csoka *et al.*, 2012), in addition to low expression of pro-inflammatory cytokines such as TNFα, IL-6, IL-8 and IL-12. The regulatory effects of adenosine are mediated by the activation of $A_{2A}$ and $A_{2B}$ receptors on macrophages (HASKO, 2000; Majumdar and Aggarwal, 2003; Fortin *et al.*, 2005; Garcia *et al.*, 2007; Gessi *et al.*, 2007; Addi *et al.*, 2008; Haskó and Pacher, 2012; Koscso *et al.*, 2012; Antonioli, Pacher, *et al.*, 2013; Ferrante *et al.*, 2013). For instance, activation of the $A_{2A}$ receptor mediates inhibition of TNFα production (HASKO, 2000; Kreckler *et al.*, 2006; Ryzhov *et al.*, 2007), and activation of the $A_{2B}$ receptor inhibits production of NO and IL-12 by macrophages infected with *Leishmania*, thus enhancing parasite persistence in the host (Figueiredo, Souza-Testasicca and Afonso, 2016). In addition, $A_{2B}$ receptors have been shown to be involved in promoting macrophage differentiation into an M2 phenotype which are important for defence against extracellular parasites (Noël *et al.*, 2004), as well as increasing IL-4 or IL-13 induced macrophage activation (Patel *et al.*, 2014).

Several recent studies suggested that adenosine activates $A_{2B}$ receptors which upregulate IL-33 release and drive type 2 immunity during helminth infection including *H. polygyrus* (Chen *et al.*, 2012; Csoka *et al.*, 2012; Gause, Wynn and Allen, 2013; Hung *et al.*, 2013). In this study, there was a non-significant trend for less IL-4 being produced by splenocytes from mice infected with Tm 5´-NT. This decrease might contribute to the lower expression of Ym1, although macrophages from Tm Apy-3 and Tm 5´-NT infections showed higher expression of arginase, another marker of M2 macrophages.

In summary, Tm Apy3 grew faster than the control Tm Luc or Tm mApy3. There were no changes in titres of IgG subclasses, and no lower production of pro-inflammatory cytokines and mediators such as TNFα, IFNγ, and NO. Lower levels of IL-4 were observed with Tm 5´-NT, coupled with lower Ym1 expression, however significant higher expression of Arg1 was seen with Tm Apy-3 infections, along with higher levels of IL-5 and IL-13. The changes might be due to a combination of factors both immunological and physiological. These data suggest that the secretion of an apyrase or 5´-NT by *T. musculi* might be contributing in modulating the host immune response in addition to supplying the purine salvage pathway.

# CHAPTER 5

# Effect of apyrase on *Nippostrongylus brasiliensis* infection and allergic inflammation

## 5.1 Introduction

ATP is well known for its pivotal intracellular roles in many biological processes such as chemical energy, motility, cell division and biosynthesis. Its presence extracellularly was initially believed to be due only to cell damage (Burnstock, 1999; Khakh and Burnstock, 2009). However, it was reported that under physiological conditions and at low micromolar concentrations, extracellular ATP acts as a signalling molecule for nonadrenergic, noncholinergic neurotransmission, in addition to other responses such as cardiac function, muscle contraction and relaxation, vascular tone, endothelium-dependent vasodilation, calcium-dependent histamine secretion from mast cells, prostacyclin production and cell growth (Wolf and Berne, 1956; Keller, 1966; Burnstock, 1972; Dahlquist, Diamant and Krüger, 1974; Needleman, Minkes and Douglas, 1974; Burnstock and Meghji, 1981; Boeynaems and Galand, 1983; Gordon, 1986; Schwiebert and Zsembery, 2003).

In the respiratory system, extracellular ATP contributes to airway homeostasis in normal conditions. It is released via connexin or pannexin hemichannels, ion channels or vesicular exocytosis (Bodin and Burnstock, 2001; Lazarowski, 2012) from airway and alveolar epithelial cells, lung fibroblasts, pulmonary artery endothelial cells and airway smooth muscle (ASM) cells in response to mechanical stretch and compression (Takahara *et al.*, 2014). ATP activates P2X and P2Y purinergic receptors in both autocrine and paracrine epithelial cell signalling (Lazarowski and Boucher, 2009), regulating airway surface hydration and epithelial mucociliary clearance for lung defence against inhaled pathogens (Tarran *et al.*, 2005; Button, Picher and Boucher, 2007; Button *et al.*, 2013). ATP can also up-regulate transepithelial salt and fluid transport (Mason, Paradiso and Boucher, 1991; Benali *et al.*,

1994), mucin secretion (Kim and Lee, 1991; Canwen *et al.*, 1993; Lethem *et al.*, 1993; Benali *et al.*, 1994; Tarran *et al.*, 2005; Lazarowski and Boucher, 2009), surfactant secretion (Patel *et al.*, 2005), intracellular $Ca^{2+}$ mobilization (Mason, Paradiso and Boucher, 1991), hydration of mucus and respiratory mucosa (Benali *et al.*, 1994), and airway epithelial cells ciliary beat frequency (Brown *et al.*, 1991; Saano *et al.*, 1992). The concentration of ATP released extracellularly is balanced and kept at low micromolar level via ecto-nucleotidase enzymes (CD39 and CD73).

In response to tissue damage and inflammation, the extracellular ATP concentration can dramatically increase, alerting the immune system. Thus, besides its physiological roles, ATP can act as a danger-associate molecular pattern (DAMP) contributing to the pathophysiology of various lung and airway diseases including asthma, cystic fibrosis, pulmonary fibrosis, chronic obstructive pulmonary disease (COPD), lung cancer and lung injury (Mohsenin and Blackburn, 2006; Idzko *et al.*, 2007; Esther *et al.*, 2008; Mortaz *et al.*, 2009, 2010; Polosa and Blackburn, 2009; Willart and Lambrecht, 2009; Lommatzsch *et al.*, 2010; Riteau *et al.*, 2010). The release of ATP activates purinergic receptors expressed on a wide range of immune cells such as macrophages, dendritic cells, lymphocytes, eosinophils and neutrophils, triggering a pro-inflammatory immune response and tissue degradation (Junger, 2011; Ayna *et al.*, 2012; Asgari *et al.*, 2013; Gallo and Gallucci, 2013; Pittman and Kubes, 2013; Wang and Chen, 2018).

Asthma is an inflammatory disease of the airways characterized by bronchial hyper-responsiveness (AHR), airflow obstruction, increased mucus secretion and

bronchoconstriction. Environmental and genetic factors contribute to this disease, which is associated with many symptoms in both adults and children (Robinson *et al.*, 1992; Howarth, 1995; Kim, Dekruyff and Umetsu, 2010; Zambalde *et al.*, 2016; Thiriou *et al.*, 2017; Li *et al.*, 2018). During allergic asthma, extracellular ATP initiates a T helper 2 (Th2) immune response via the activation of P2 purinergic receptors, mainly $P2Y_2R$ expressed on dendritic cells and eosinophils, and triggers the production of reactive oxygen species (ROS), eosinophil infiltration, and increased serum level of IgE (Dichmann *et al.*, 2000; Ferrari *et al.*, 2000; Idzko *et al.*, 2001, 2003, 2007; Mohanty *et al.*, 2001; Song, Vijayaraghavan and Sladek, 2006; Müller *et al.*, 2010; Kobayashi *et al.*, 2015; Zech *et al.*, 2015). Activated Th2 cells drive chronic inflammation via the secretion of cytokines such as IL-4 which initiates IgE synthesis, IL-5 which induces the recruitment and activation of eosinophils, and IL-13 which promotes overproduction of mucus and airway smooth muscle contraction (Wills-Karp *et al.*, 1998; Khurana Hershey, 2003; Kuperman and Schleimer, 2008; Akdis *et al.*, 2011; Romeo *et al.*, 2014; Chung, 2015; Kobayashi *et al.*, 2017; Dickinson *et al.*, 2018).

However, type 2 innate lymphoid cells (ILC2s) and airway epithelium have recently been shown to be of great importance, as these cells are associated with the initiation of asthma. The increased level of the “danger signal” ATP activates purinergic receptors and sustains increased intracellular $Ca^{2+}$ concentration (Burnstock, 2006; Lüthi *et al.*, 2009; Kouzaki *et al.*, 2011; Barlow *et al.*, 2012; Bartemes *et al.*, 2012; Halim *et al.*, 2012; Kim *et al.*, 2012; Lambrecht and Hammad, 2012; Wolterink *et al.*, 2012). Studies suggested that this P2 purinergic pathway induces the secretion of IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) cytokines from airway epithelial cells, promoting ILC2 proliferation and activity, and leading to a Th2-type immune response independent of adaptive immunity (Guo *et al.*,

2009; Paul and Zhu, 2010; Kouzaki *et al.*, 2011; Ober and Yao, 2011; Ohno *et al.*, 2012; Kobayashi *et al.*, 2013; Makrinioti *et al.*, 2014; McSorley *et al.*, 2014; S. G. Smith *et al.*, 2016; Yanagibashi *et al.*, 2017). Thus, both innate and adaptive immune responses are associated with the development of asthma.

*Alternaria alternata* (*A. alternata* or ALT) is a fungal allergen clinically associated with asthma in humans (O'Hollaren *et al.*, 1991; Downs *et al.*, 2001; Saglani, 2017), and used as a murine model to study the immune response during Th2-mediated inflammation (Denis *et al.*, 2007; Kobayashi, Iijima and Kita, 2008; Kobayashi *et al.*, 2009; Moosavi *et al.*, 2009; Kouzaki *et al.*, 2011; Gil *et al.*, 2014; Iijima *et al.*, 2014). The administration of ALT extract induces rapid release of IL-33 from epithelial cells, followed by the activation of ILC2s and the production of IL-5 and IL-13 (Kouzaki *et al.*, 2011; Bartemes *et al.*, 2012; Hardman, Panova and Mckenzie, 2013; Gold *et al.*, 2014; Halim *et al.*, 2014; Snelgrove *et al.*, 2014; Kabata *et al.*, 2015; Stier *et al.*, 2017).

A Th2 immune response is also induced in infection with helminth parasites such as the intestinal nematode *Nippostrongylus brasiliensis.* The alarmin cytokine IL-33 has been shown to promote both airway inflammation and immunity to nematode infection (Humphreys *et al.*, 2008; Liew, Pitman and McInnes, 2010; Neill *et al.*, 2010a; Kouzaki *et al.*, 2011; Bartemes *et al.*, 2012; Yasuda *et al.*, 2012). Studies have suggested that helminth parasite secreted products possess anti-allergic properties, partly through inhibiting the release of IL-33 in order to promote parasite survival in the host (McSorley *et al.*, 2014). Thus it is important to study immune regulation pathways during helminth infection for developing allergic disease therapies (Postigo, Guisantes and Martínez, 2016).

## 5.2 Results

### Lung cellular composition and cytokine production following administration of *H. polygyrus* recombinant Apy-1 during *N. brasiliensis* infection

#### Experimental design, worm burden and faecal egg count

*H. polygyrus* enzymatically active and inactive recombinant Apy-1 expressed in *Pichia pastoris* (see Chapter 3) were purified, then subjected to endotoxin removal and tested by an LAL-assay. Inactive recombinant Apy-1 was produced via heat-inactivation, and nucleotidase assays confirmed the loss of activity. Mice were infected subcutaneously with *N. brasiliensis* in PBS (naïve groups received PBS only), then intranasally dosed for 3 consecutive days with recombinant enzymes, PBS or BSA (bovine serum albumin) as controls (Figure 5.1). After the mice were euthanised at day 5 post-infection, the intestinal worms and the faecal eggs were enumerated (Figure 5.2). The worm burden showed a possible trend of higher number of parasites in animals dosed with active Apy-1 when compared to the other groups, but this was not statistically significant. Furthermore, the pattern for faecal egg counts was the same in mice treated with Apy-1 when compared to PBS control group only.

#### Eosinophils and neutrophils

At the same time, the lung cellular composition was tested and cytometrically analysed. An increase in leukocyte populations was observed in the lungs of mice treated with active Apy-1 in both naïve and infected groups, although this increase was only significant with non-

infected animals (Figure 5.3). Intranasal dosing with active recombinant Apy-1 significantly enhanced eosinophilia (CD11b+SiglecF+) in the lungs of naïve mice compared to BSA and inactive enzyme controls (Figure 5.4). A similar increase was observed in neutrophils (CD11b+SiglecF-CD11c-Gr1+), macrophages (CD11b+F4/80+) and pDendritic cells (CD11b+CD11c+) in the lungs of mice treated with active Apy-1 when compared to PBS and BSA control animals. As for the infected groups, an increase in these cells, although not statistically significant, was shown following Apy-1a treatment (Figure 5.4, 5.5).

**B and T cells**

There was a significantly higher number of B cells, T cells and NK cells in the lungs during Apy-1a treatment in both naïve and infected groups (Figure 5.6), in addition to greater CD4+ T cells, which were only significantly higher in the lungs of infected animals (Figure 5.7). A further analysis of the CD4+ T cell population was performed to differentiate between cells expressing central memory CD4+ T cells (CD44+CD62L+), effector/memory CD4+ T cells (CD44+CD62Llow), naïve CD4+ T cells (CD44-CD62L+) and regulatory T cells (Tregs) (CD4+Foxp3+).

There was no difference in the numbers of Tregs in the lungs of naïve groups treated with inactive and active apyrases when compared to PBS and BSA controls (Figure 5.8). No difference was also observed in naïve CD4+ T cells during *N. brasiliensis* infection, but significantly higher numbers of effector/memory and central memory phenotype were observed in lungs of Apy-1a treated animals (Figure 5.9). T cell cytokine production was measured in both naïve and infected groups. There was no significant difference in the

amount of IL-5 and IL-13 produced by lungs from the naïve groups, but significantly lower IL-13 and slightly lower IL-5 levels were observed during *N. brasiliensis* infections of mice treated with Apy-1a (Figure 5.10).

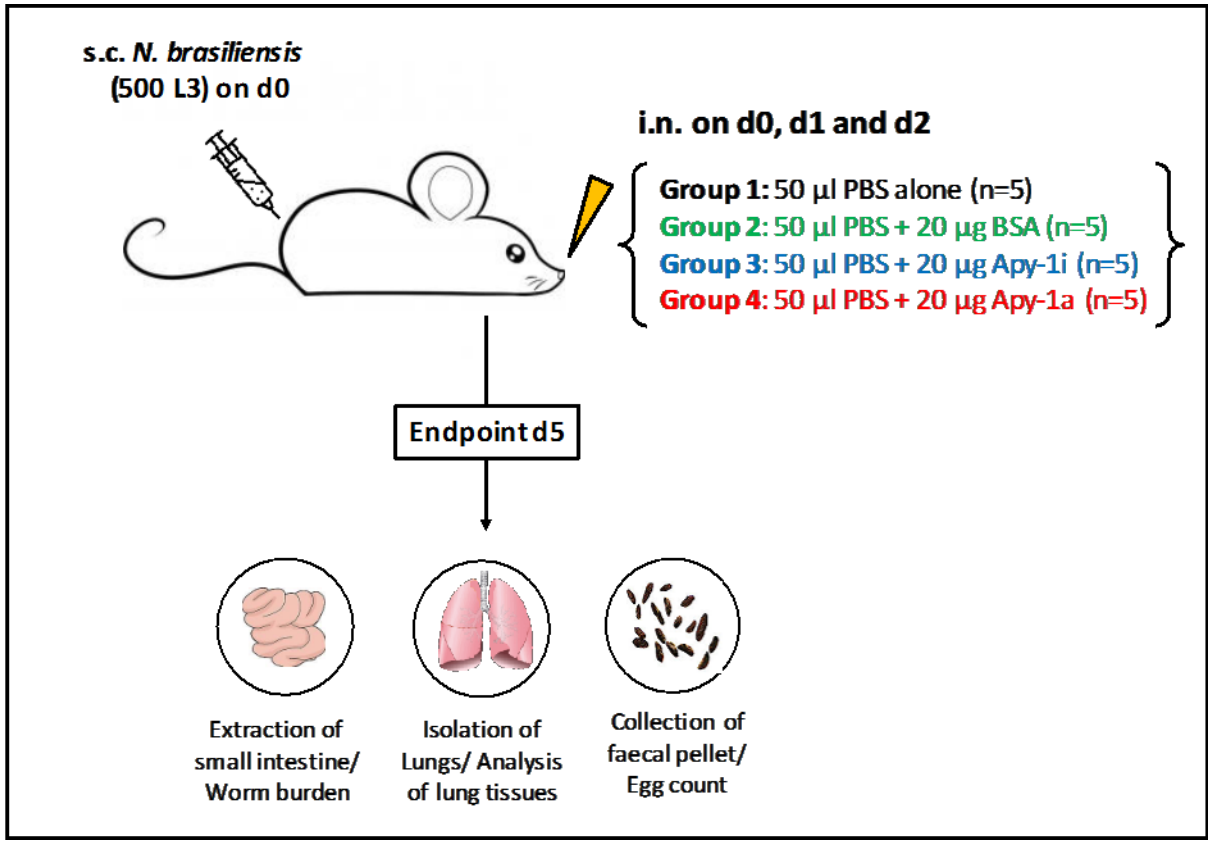


**Figure 5.1: Schematic illustration of the experimental design**
Female BALB/c mice were infected by sub-cutaneous injection (s.c.) with 500 L3 *N. brasiliensis* in PBS at day 0. Naïve mice received the same volume of PBS. Naïve and infected mice were dosed intranasally (i.n.) for 3 consecutive days (days 0, 1 and 2 post-infection) with recombinant enzymatically active Apy-1 (Apy-1a), enzymatically inactive Apy-1 (Apy-1i), BSA (bovine serum albumin) or vehicle control (PBS). Mice were euthanised at day 5 post-infection.

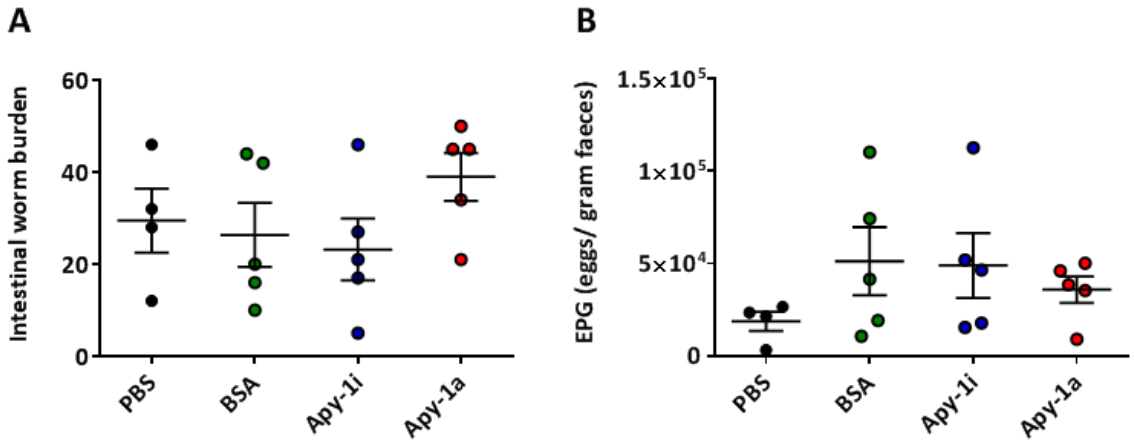


**Figure 5.2: Worm and egg burden of *N. brasiliensis* infected mice following intranasal administration of recombinant Apy-1**

(A) Adult worm recovery from the small intestine of mice at day 5 post-infection.
(B) Faecal egg count expressed as eggs per gram of faeces at day 5 post-infection (EPG).
Data are represented as mean +/- S.E.M (n=5 mice/group).

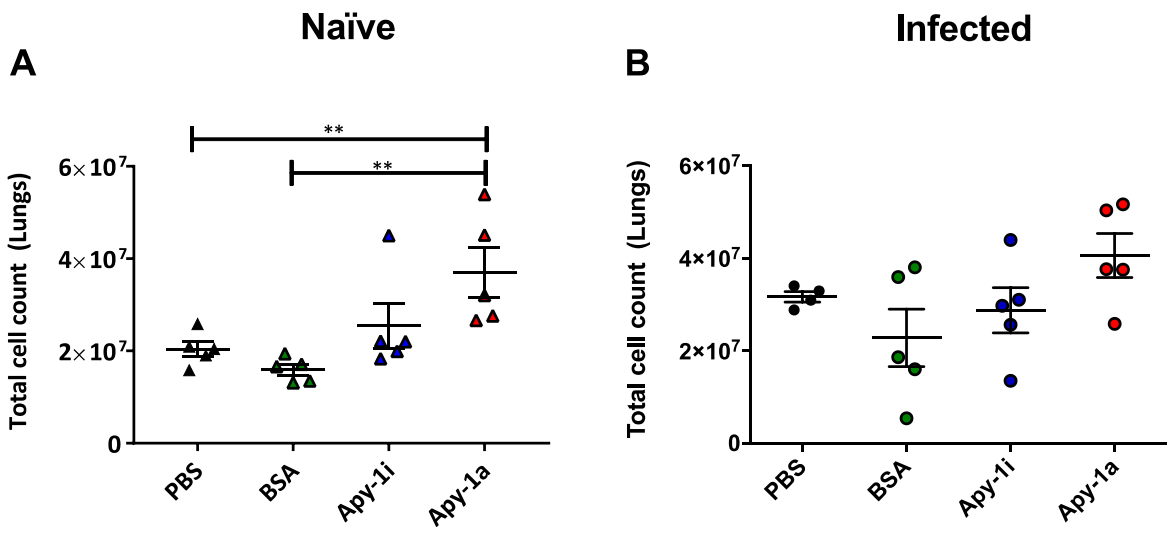


**Figure 5.3: Total lung cell count**

Naïve (A) and *N. brasiliensis*-infected (B) mice were harvested at day 5 post-infection. Single cell suspension of lungs were made and viable cells counted. Data are represented as mean +/- S.E.M (n=5 mice/group). Statistical significance was calculated using Mann-Whitney test (*$p < 0.05$, **$p < 0.01$). PBS: vehicle control; BSA: bovine serum albumin; Apy-1i: recombinant enzymatically inactive Apy-1; Apy-1a: recombinant enzymatically active Apy-1.

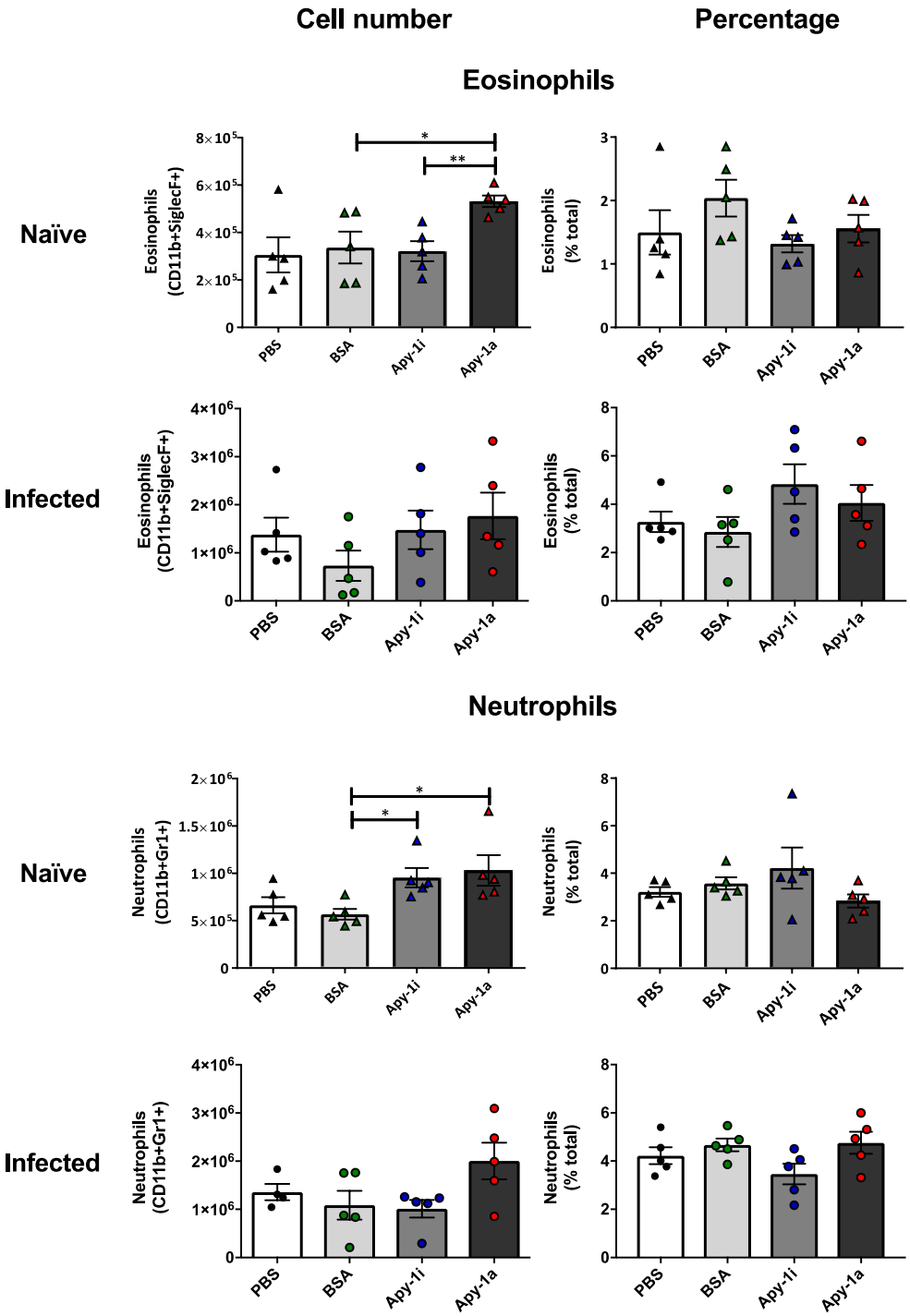


**Figure 5.4: Total number and proportion of eosinophils and neutrophils in the lungs of mice following intranasal administration of recombinant Apy-1**

Naïve and *N. brasiliensis*-infected mice were harvested at day 5 post-infection. Lung single-cell suspensions from each treated group were stained and analysed by flow cytometry for the number (left panels) and percentage (right panels) of eosinophils (CD11b+SiglecF+) and neutrophils (CD11b+Gr1+). Data are shown as mean +/- SEM (n=5 mice/ treated group) (*$p < 0.05$, **$p < 0.01$). PBS: vehicle control; BSA: bovine serum albumin; Apy-1i: recombinant enzymatically inactive Apy-1; Apy-1a: recombinant enzymatically active Apy-1.

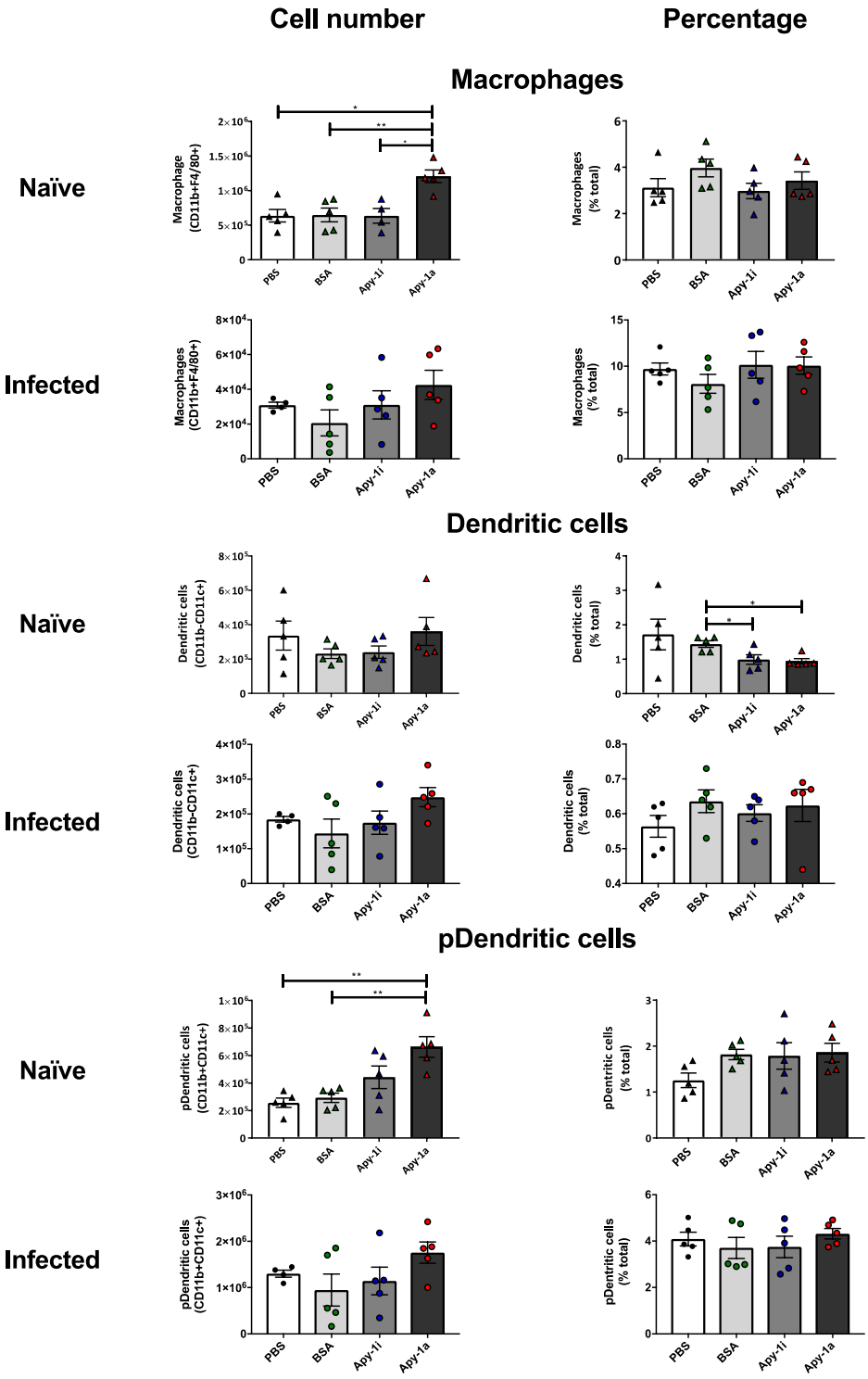


**Figure 5.5: Total number and proportion of macrophages and dendritic cells in the lungs of mice following intranasal administration of recombinant Apy-1**

Naïve and *N. brasiliensis*-infected mice were harvested at day 5 post-infection. Lung single-cell suspensions from each treated group were stained and analysed by flow cytometry for the number (left panels) and percentage (right panels) of macrophages (CD11b+F4/80+), dendritic cells (CD11b-CD11c+) and plasmacytoid dendritic cells (CD11b+CD11c+). Data are shown as mean +/- SEM (n=5 mice/treated group) (*$p < 0.05$, **$p < 0.01$). PBS: vehicle control; BSA: bovine serum albumin; Apy-1i: recombinant enzymatically inactive Apy-1; Apy-1a: recombinant enzymatically active Apy-1.

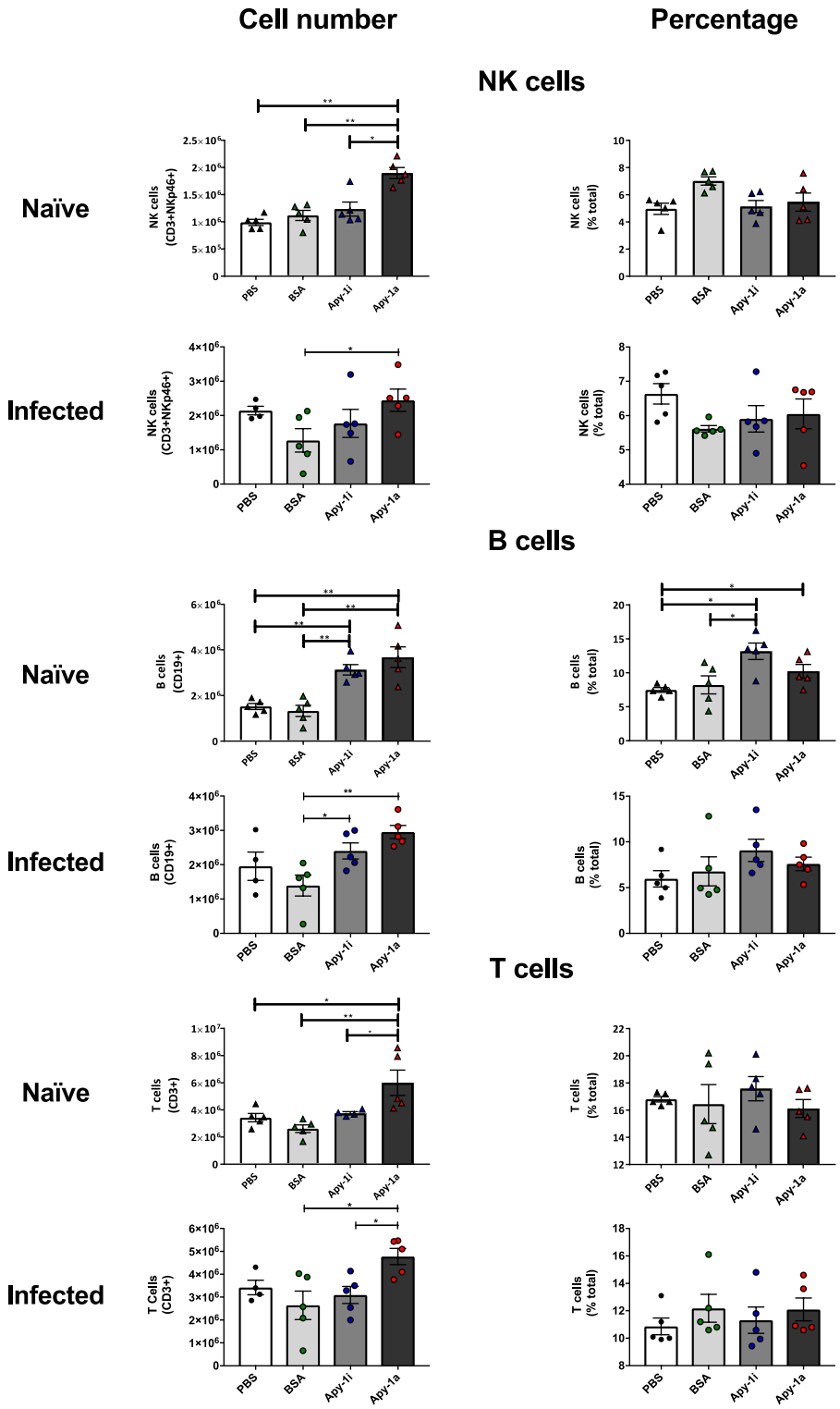


**Figure 5.6: Total number and proportion of NK cells, B cells, and T cells in the lungs of mice following intranasal administration of recombinant Apy-1**

Naïve and *N. brasiliensis*-infected mice were harvested at day 5 post-infection. Lung single-cell suspensions from each treated group were stained and analysed by flow cytometry for the number (left panels) and percentage (right panels) of B cells (CD19+), T cells (CD3+) and NK cells (Nkp46+). Data are shown as mean +/- SEM (n=5 mice/ treated group). (*$p < 0.05$, **$p < 0.01$). PBS: vehicle control; BSA: bovine serum albumin; Apy-1i: recombinant enzymatically inactive Apy-1; Apy-1a: recombinant enzymatically active Apy-1.

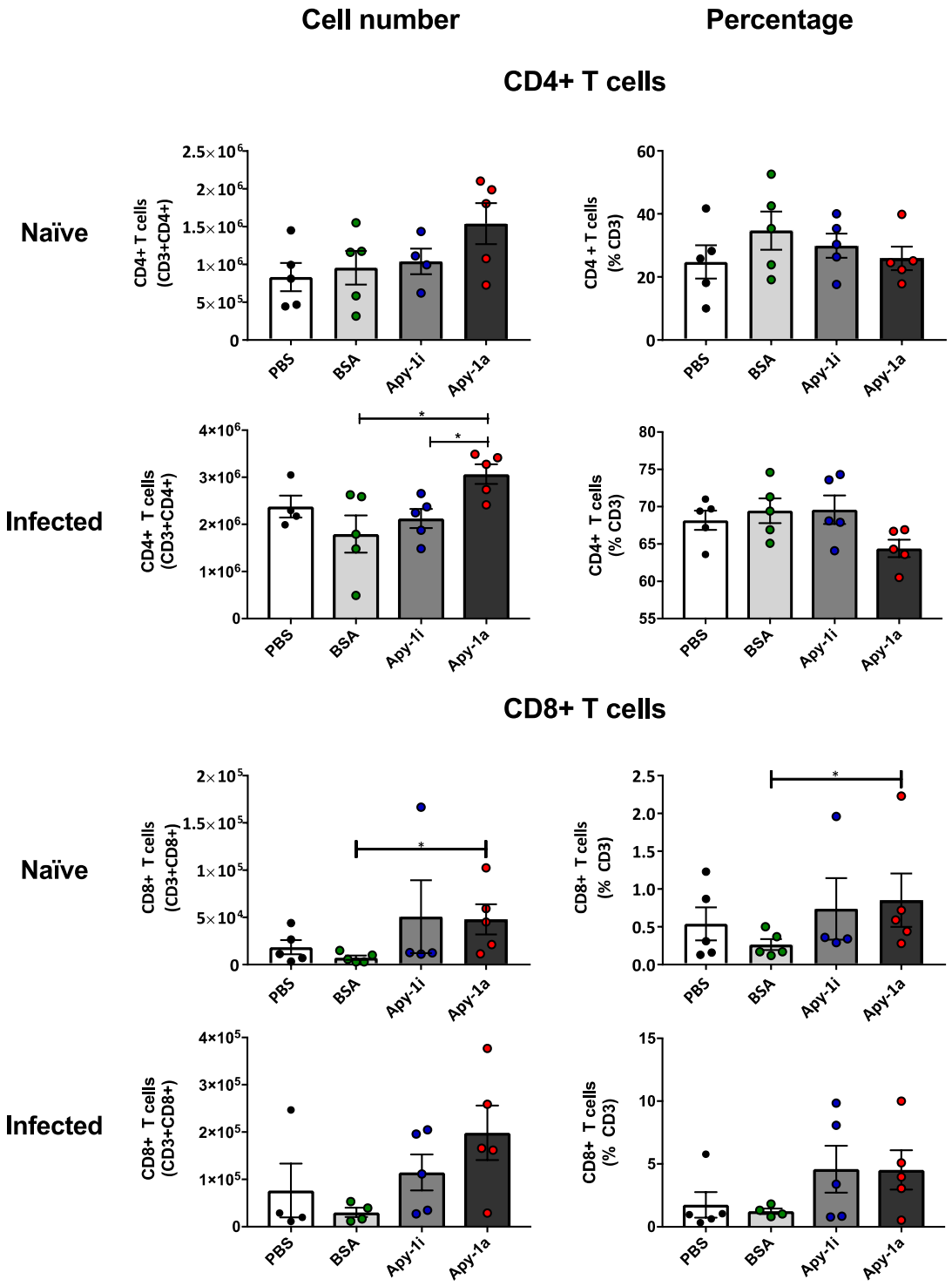


**Figure 5.7: Total number and proportion of CD4+ and CD8+ T cells in the lungs of mice following intranasal administration of recombinant Apy-1**

Naïve and *N. brasiliensis*-infected mice were harvested at day 5 post-infection. Lung single-cell suspensions from each treated group were stained and analysed by flow cytometry for the number (left panels) and percentage (right panels) of CD4+ T cells (CD3+CD4+), and CD8+ T cells (CD3+CD8+). Data are shown as mean +/- SEM (n=5 mice/ treated group). (*$p < 0.05$, **$p < 0.01$). PBS: vehicle control; BSA: bovine serum albumin; Apy-1i: recombinant enzymatically inactive Apy-1; Apy-1a: recombinant enzymatically active Apy-1.

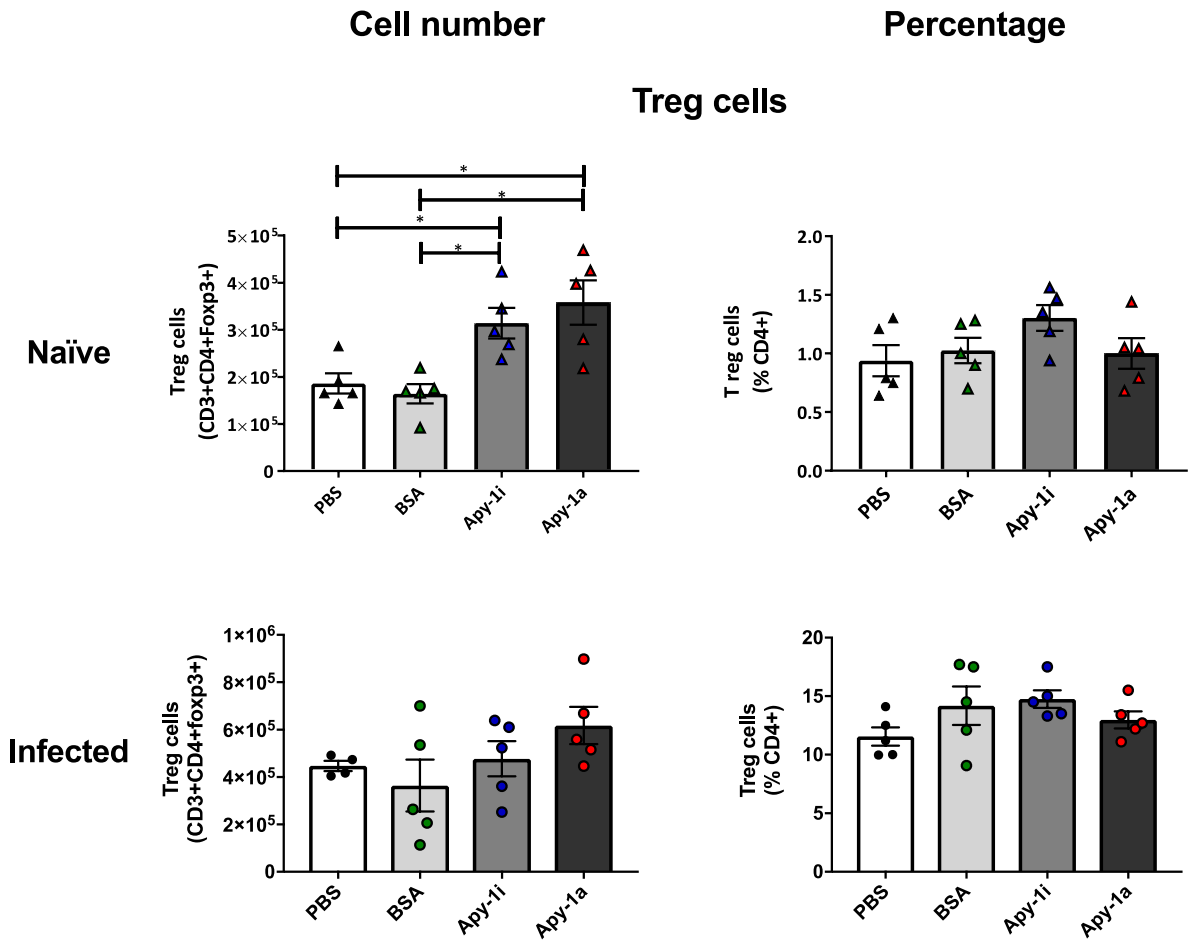


**Figure 5.8: Total number and proportion of Foxp3+ in lung CD4+ populations of mice following intranasal administration of recombinant Apy-1**

Naïve and *N. brasiliensis*-infected mice were harvested at day 5 post-infection. Lung single-cell suspensions from each treated group were stained and analysed by flow cytometry for the number (left panels) and percentage (right panels) of Foxp3+ within CD4+ cells. Data are shown as mean +/- SEM (n=5 mice/ treated group). (*$p < 0.05$, **$p < 0.01$).

PBS: vehicle control; BSA: bovine serum albumin; Apy-1i: recombinant enzymatically inactive Apy-1; Apy-1a: recombinant enzymatically active Apy-1.

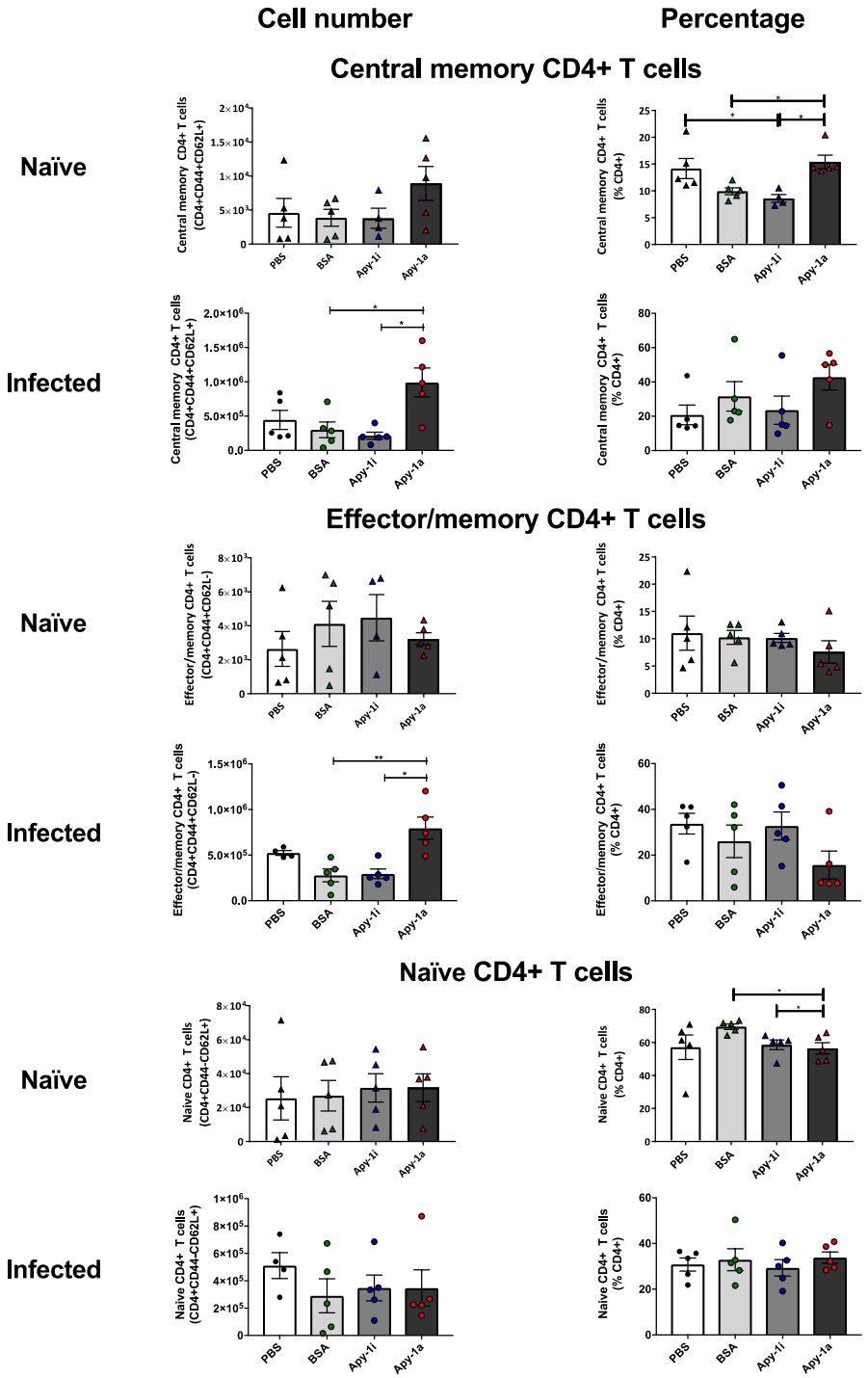


**Figure 5.9: Total number and proportion of CD4+ T cells expressing naïve, central memory or effector/memory phenotype in the lungs of mice following intranasal administration of recombinant Apy-1**

Naïve and *N. brasiliensis*-infected mice were harvested at day 5 post-infection. Lung single-cell suspensions from each treated group were stained and analysed by flow cytometry for the number (left panels) and percentage (right panels) of CD4+ T cells expressing a naïve (CD44loCD62L+), central memory (CD44+CD62L+) or effector/memory (CD44+CD62Llo) phenotype. Data are shown as mean +/- SEM (n=5 mice/ treated group). (*$p < 0.05$, **$p < 0.01$). PBS: vehicle control; BSA: bovine serum albumin; Apy-1i: recombinant enzymatically inactive Apy-1; Apy-1a: recombinant enzymatically active Apy-1.

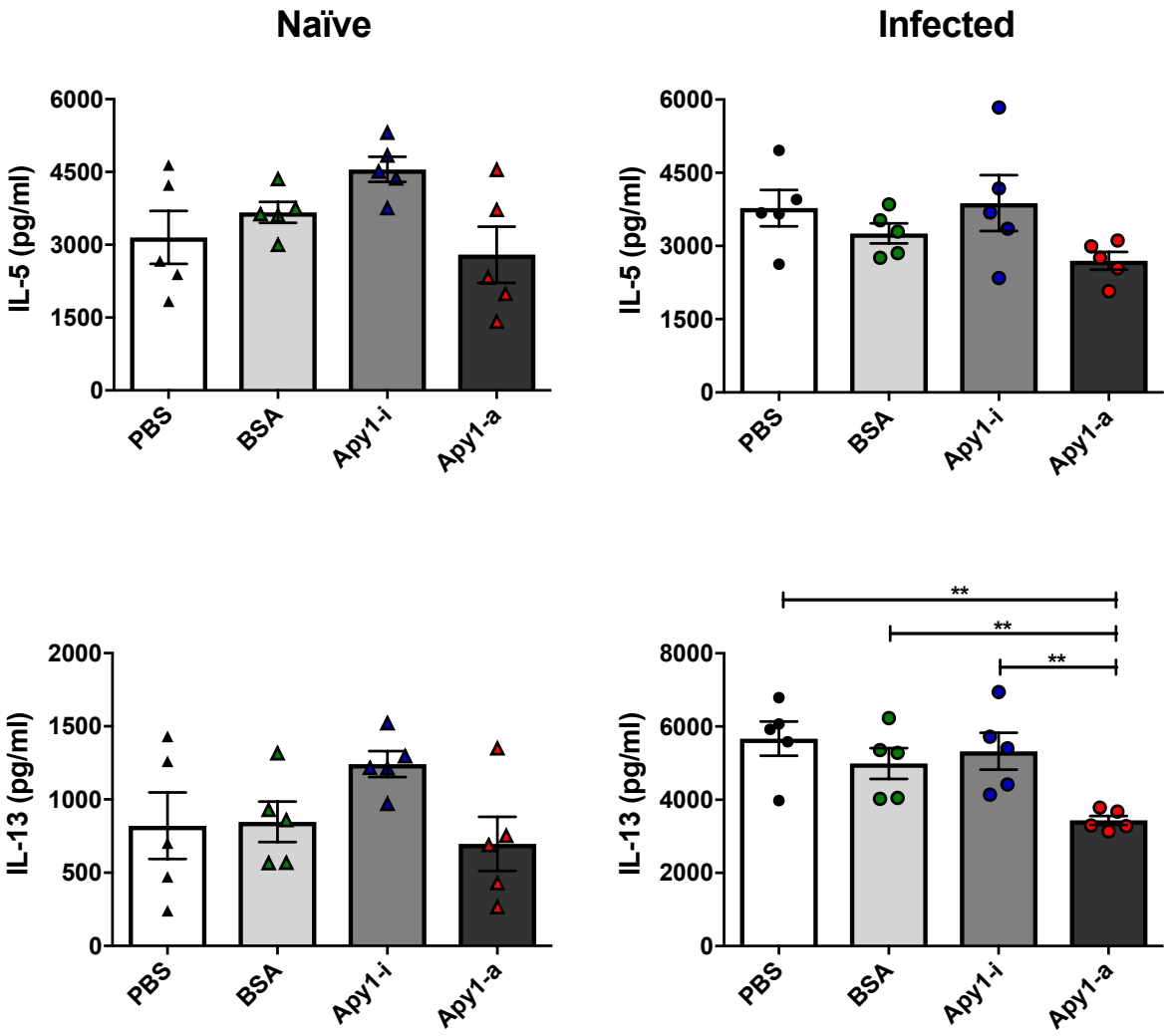


**Figure 5.10: Cytokine production by lung cells of mice following intranasal administration of recombinant Apy-1**

Lung cells from naïve and *N. brasiliensis*-infected mice were stimulated with PMA/ionomycin for 24 hours. The levels of IL-5 and IL-13 produced in the culture supernatants were tested by ELISA. Data are shown as mean +/- SEM (n=5 mice/ group). (*$p < 0.05$, **$p < 0.01$).

PBS: vehicle control; BSA: bovine serum albumin; Apy-1i: recombinant enzymatically inactive Apy-1; Apy-1a: recombinant enzymatically active Apy-1.

## Cellular composition and cytokine production in the lung and airways after administration of *H. polygyrus* recombinant Apy-3 during *Alternaria*-induced allergic airway inflammation

### Experimental design and total cell count

Samples of *H. polygyrus* enzymatically active and inactive recombinant Apy-3 expressed in *Pichia pastoris* (see Chapter 3) were purified then subjected to endotoxin removal and tested by an LAL-assay. Inactive recombinant Apy-3 was produced using Q5 site-directed mutagenesis (see Materials and Methods) and nucleotidase assays confirmed the loss of activity. Mice were intranasally dosed with *Alternaria alternata* (ALT) alongside a single dose of recombinant enzyme or PBS. Inflammation was induced over 24 hours, then lung tissues and BAL fluid cells were tested and cytometrically analysed (Figure 5.11). An increase in leukocyte populations was observed in the lungs and BAL samples of mice exposed to *Alternaria* extract compared to the PBS only control group. There were no significant difference between the ALT:PBS and ALT:Apy-3i control groups in the lung or BAL total cell count, however ALT:Apy-3a treated animals displayed a significant increased cell count in the lungs compared to PBS control group (Figure 5.12).

### Eosinophils and neutrophils

Animals treated with ALT had a significant increase in eosinophils and neutrophils in the lungs and BAL compared to those dosed with PBS, indicating that the allergic airway model was successful. The numbers of both cells in the lungs were enhanced by addition of active, but not inactive apyrase (Figure 5.13).

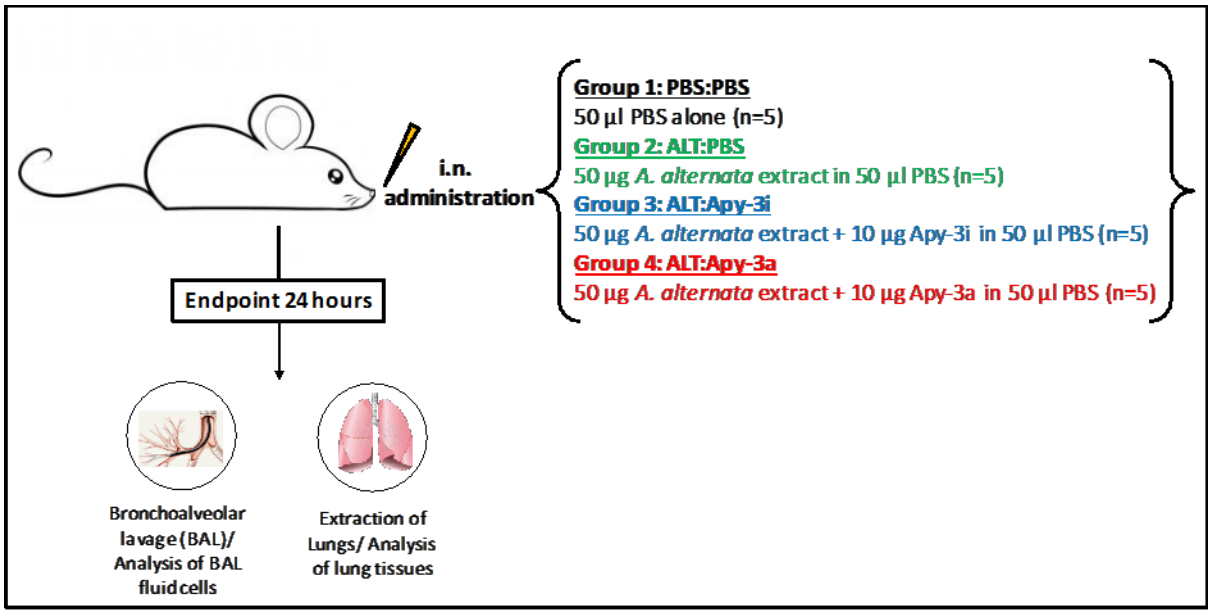


**Figure 5.11: Schematic illustration of the *Alternaria* experimental design**

Female BALB/c mice were dosed intranasally (i.n.) with *Alternaria alternata* (ALT) and recombinant enzymatically active Apy-3 (Apy-3a) or enzymatically inactive Apy-3 (Apy-3i), vehicle control (PBS only) or ALT only. Mice were euthanised after 24 hours, and lung tissues and BAL fluid cells were analysed.

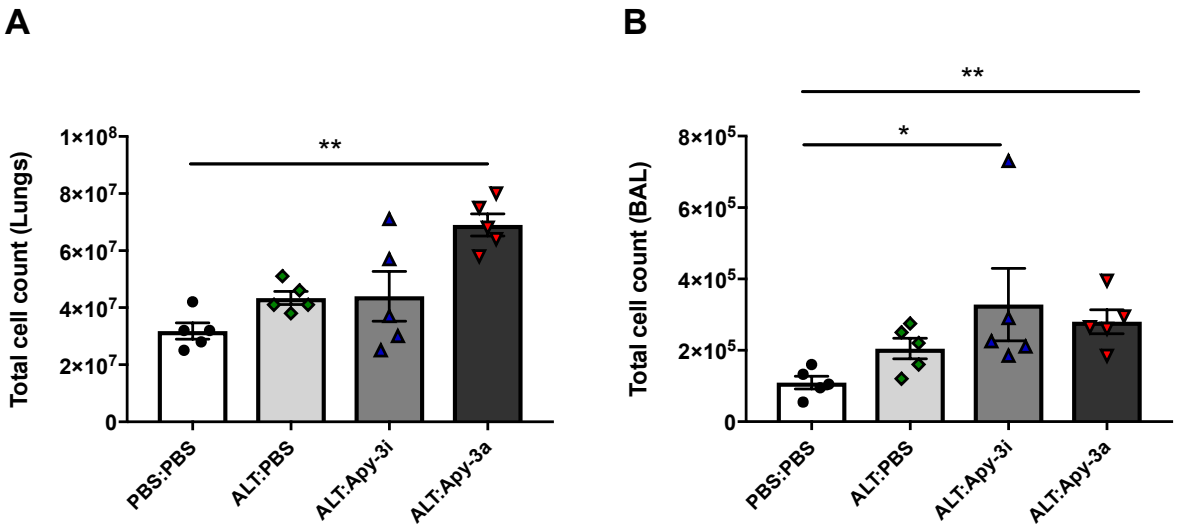


**Figure 5.12: Total lung (A) and BAL (B) cell count**

Mice were harvested 24 hours post-intranasal administration. A single cell suspension of lungs and BAL fluid was made and viable cells counted. Data are represented as mean +/- S.E.M (n=5 mice/group). Statistical significance was calculated using Mann-Whitney test (*$p < 0.05$).

Apy-3i: recombinant enzymatically inactive Apy-3; Apy-3a: recombinant enzymatically active Apy-3.

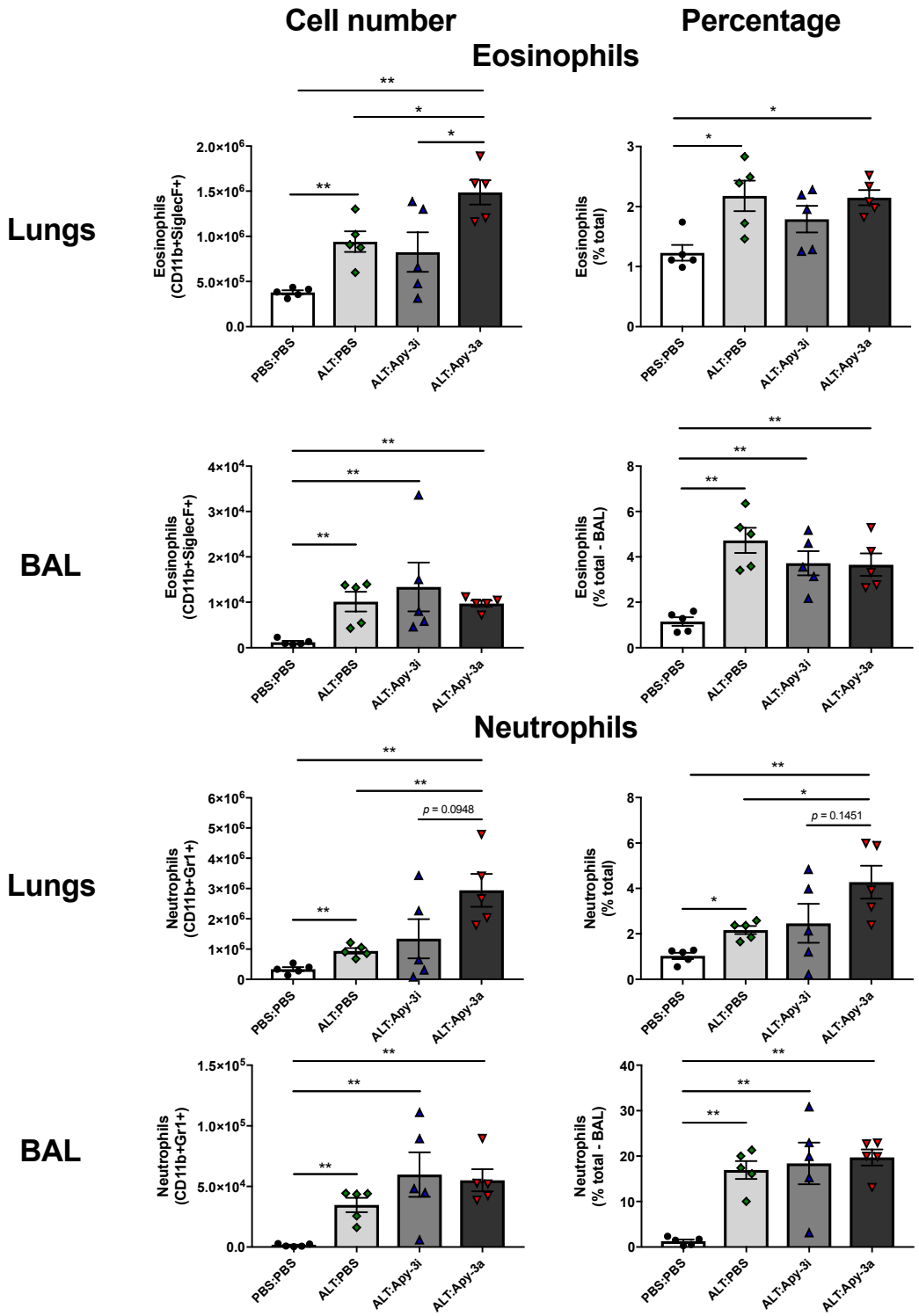


**Figure 5.13: Total number and proportion of eosinophils and neutrophils in the lungs and BAL of mice following intranasal administration of ALT and recombinant Apy-3**

Mice were harvested 24 hours post-intranasal administration. Lung and BAL single-cell suspensions from each treated group were stained and analysed by flow cytometry for the number (left panels) and percentage (right panels) of eosinophils (CD11b+SiglecF+) and neutrophils (CD11b+Gr1+). Data are shown as mean +/- SEM (n=5 mice/ treated group) (*$p < 0.05$, **$p < 0.01$). Apy-3i: recombinant enzymatically inactive Apy-3; Apy-3a: recombinant enzymatically active Apy-3.

**Type 2 innate lymphoid cells (ILC2s)**

Group 2 innate lymphoid cells (ILC2s), which lack the lineage markers (surface markers for T, B, NK cells, and monocyte/macrophage lineage) (Moro *et al.*, 2010; Neill *et al.*, 2010b; Price *et al.*, 2010), have been shown to play a key role in the early immune responses to helminth parasites, as well as in the pathophysiology of allergic diseases such as asthma (Kabata *et al.*, 2015). Recently, ILC2 population responsive to IL-25 and expressing KLRG1 (Killer cell lectin-like receptor G1) have been termed as inflammatory ILC2 (iILC2), distinct from IL-33 responsive ILC2s which express ST2 (a component of the IL-33 receptor) and reside naturally in the lung (nILC2) (Huang *et al.*, 2015; Huang and Paul, 2016).

Following ALT treatment, the expression of ICOS, ST2 and KLRG1 on ILC2s was increased, but this was unaffected by addition of either active or inactive apyrase (Figure 5.14). The number of total ILC2 (CD45+Lin-CD127-ICOS+), nILC2 (ST2+KLRG1-/lo) and iILC2 (ST2-/loKLRG1+) in the lungs of active Apy-3 treated animals was significantly higher compared to inactive control group (Figure 5.15). Additionally, active Apy-3 dosing led to a trend of increased intracellular IL-5 production by ILC2s. The same pattern was observed with IL-13 production in all ALT treated mice, and was only significant in the lungs of ALT:PBS group (Figure 5.16-17).

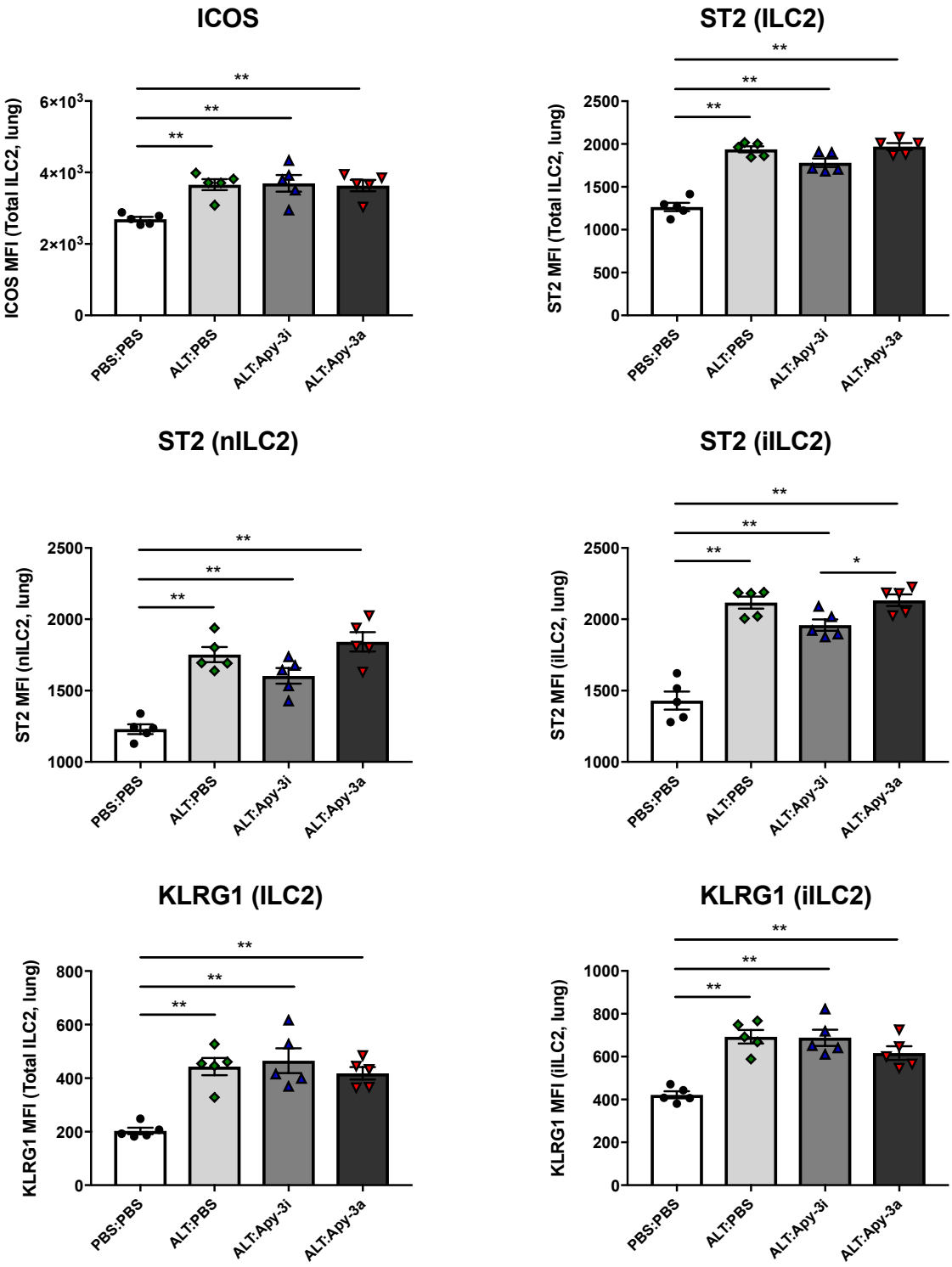


**Figure 5.14: ILC2 phenotype in the lungs of mice following intranasal administration of ALT and recombinant Apy-3**

Mice were harvested 24 hours post-intranasal administration. Lung single-cell suspensions from each treated group were stained and analysed by flow cytometry for ILC2 extracellular staining for ICOS, ST2 and KLRG1. Data are shown as mean +/- SEM (n=5 mice/ treated group) (*$p < 0.05$, **$p < 0.01$). Apy-3i: recombinant enzymatically inactive Apy-3; Apy-3a: recombinant enzymatically active Apy-3.

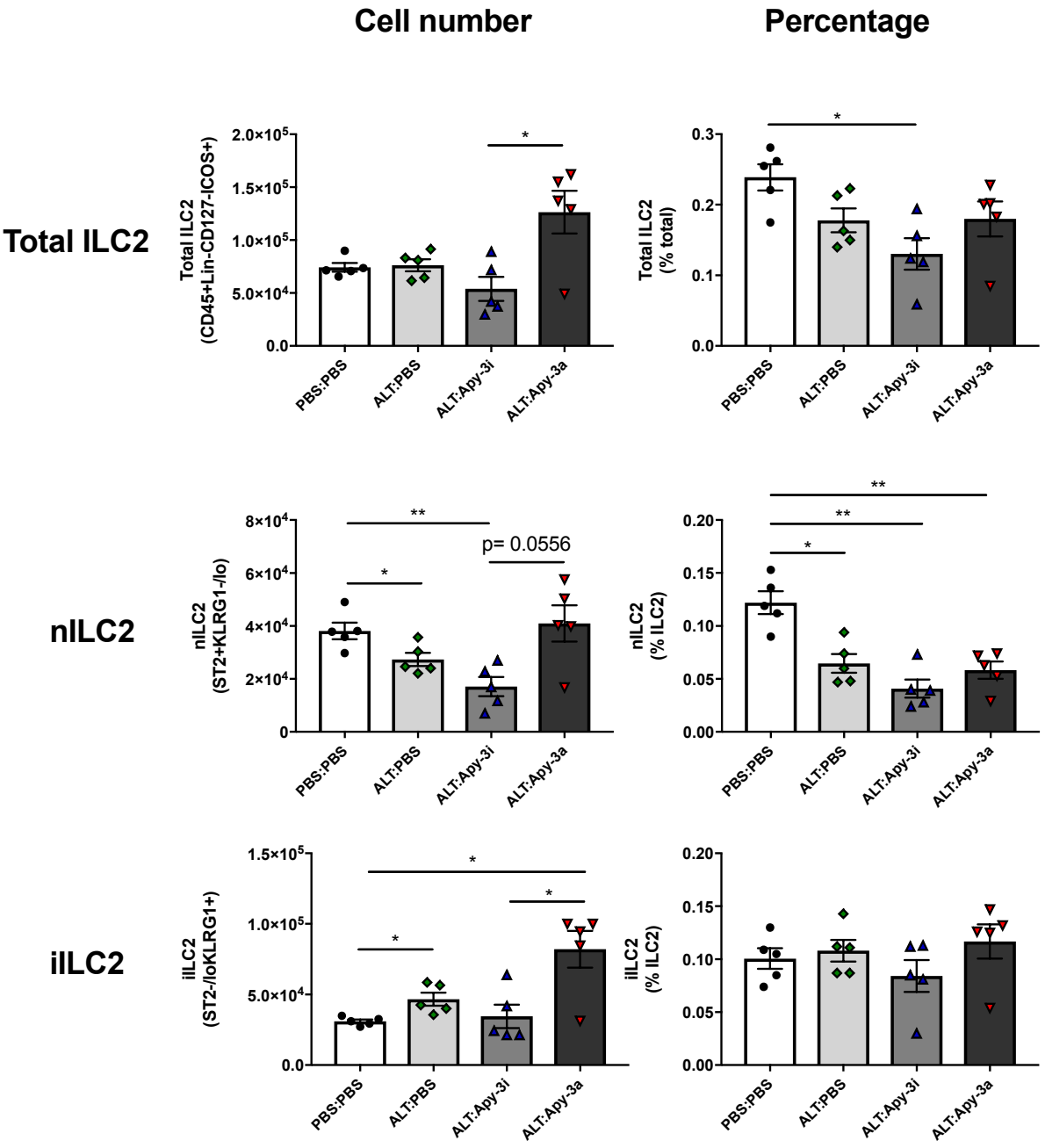


**Figure 5.15: Total number and proportion of ILC2 in the lungs of mice following intranasal administration of ALT and recombinant Apy-3**

Mice were harvested 24 hours post-intranasal administration. Lung single-cell suspensions from each treated group were stained and analysed by flow cytometry for the number (left panels) and percentage (right panels) of total ILC2 (CD45+Lineage-CD127-ICOS+), nILC2 (ICOS+ST2+KLRG1-/lo), and iILC2 (ICOS+ST2-/loKLRG1+). Data are shown as mean +/- SEM (n=5 mice/ treated group) (*$p < 0.05$, **$p < 0.01$). Apy-3i: recombinant enzymatically inactive Apy-3; Apy-3a: recombinant enzymatically active Apy-3.

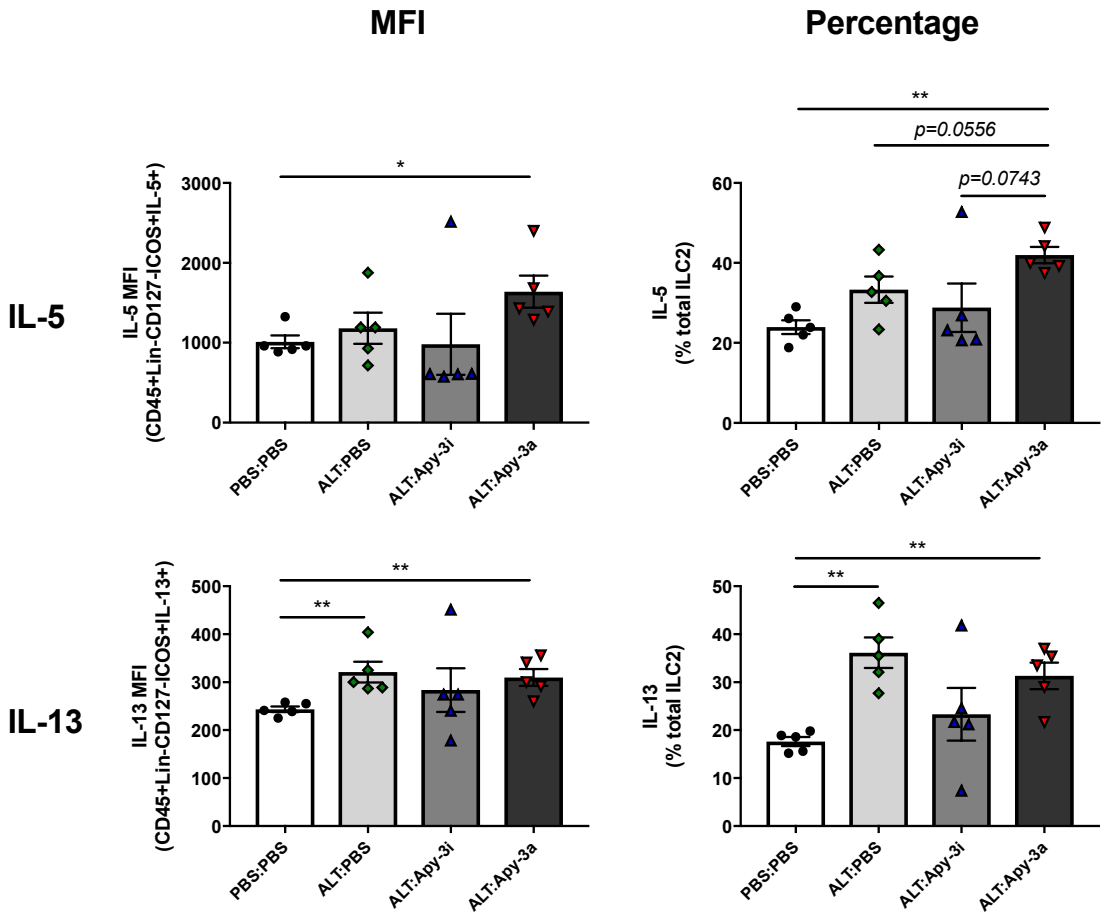


**Figure 5.16: Total number and proportion of total ILC2 expressing IL-5 and IL-13 in the lungs of mice following intranasal administration of ALT and recombinant Apy-3**

Mice were harvested 24 hours post-intranasal administration. Lung single-cell suspensions from each treated group were stained and analysed by flow cytometry for the geometric mean fluorescence intensity (MFI) (left panels) and percentage (right panels) of IL-5 and IL-13 within total ILC2 cells. Data are shown as mean +/- SEM (n=5 mice/ treated group) (*$p < 0.05$, **$p < 0.01$, ***p=<0.001). Apy-3i: recombinant enzymatically inactive Apy-3; Apy-3a: recombinant enzymatically active Apy-3.

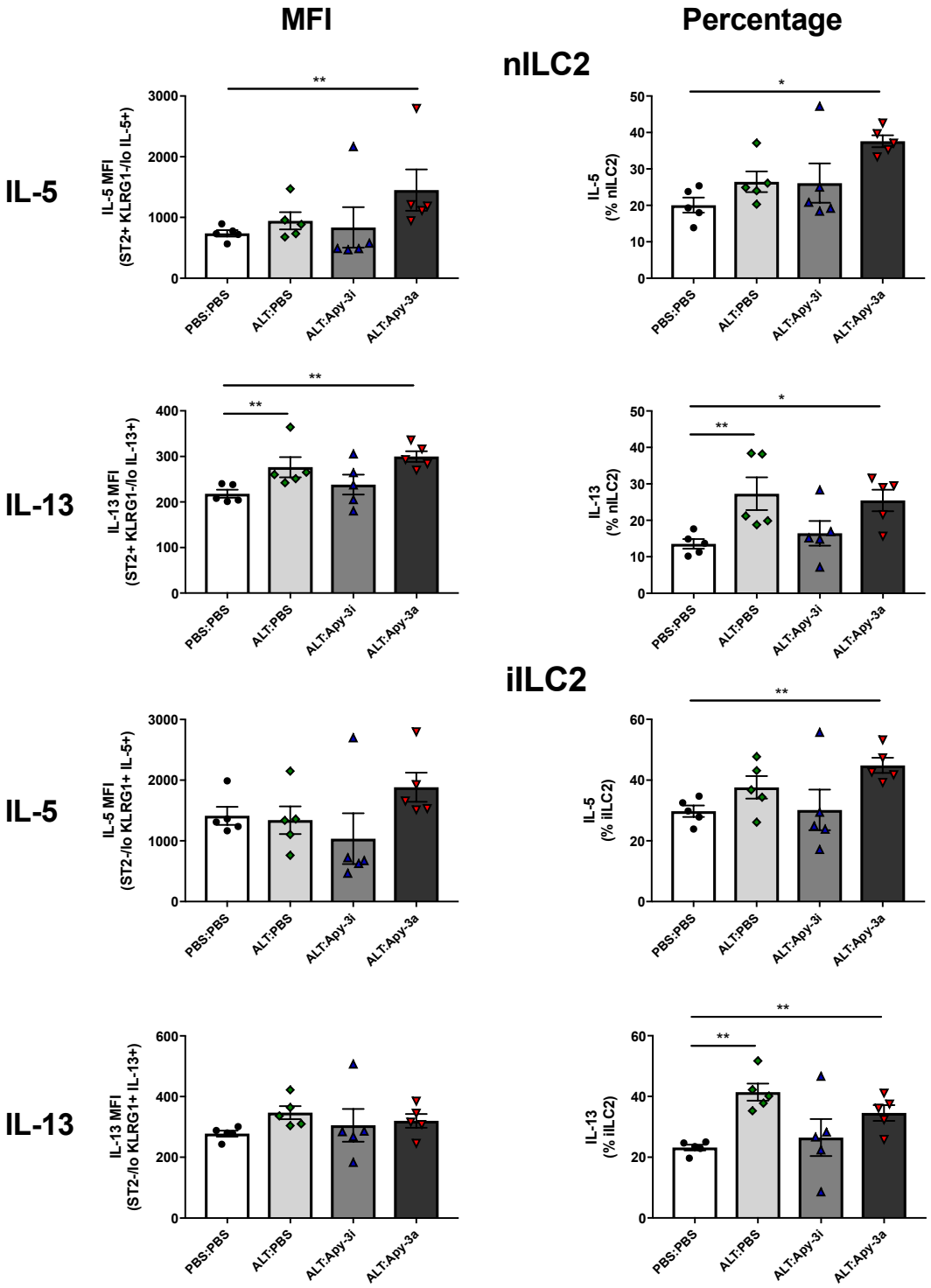


**Figure 5.17: Total number and proportion of nILC2 and iILC2 expressing IL-5 and IL-13 in the lungs of mice following intranasal administration of ALT and recombinant Apy-3**

Mice were harvested 24 hours post-intranasal administration. Lung single-cell suspensions from each treated group were stained and analysed by flow cytometry for the geometric mean fluorescence intensity (MFI) (left panels) and percentage (right panels) of IL-5 and IL-13 within nILC2 and iILC2 cells. Data are shown as mean +/- SEM (n=5 mice/ treated group) (*$p < 0.05$, ***p=<0.001). Apy-3i: recombinant enzymatically inactive Apy-3; Apy-3a: recombinant enzymatically active Apy-3.

## 5.3 Discussion

*H. polygyrus* secreted products were shown to block production of the alarmin cytokine IL-33 during allergic *Alternaria*-induced inflammation, suppressing eosinophilia and ILC2 pathways (Kouzaki *et al.*, 2011; McSorley *et al.*, 2014). Thus, this parasitic nematode possesses molecules that can act against the development of type 2 immune response to infection and allergy (McSorley *et al.*, 2014). As demonstrated in this thesis, apyrase exerts an important role in the termination of purinergic signalling via the hydrolysis of the pro-inflammatory ATP to AMP. The aim of this chapter was to study the effects of active apyrases secreted by *H. polygyrus* during type 2 immune scenarios.

Administration of active Apy-1 showed a trend for higher numbers of parasites in the gut of mice infected with *N. brasiliensis*, although the difference did not reach statistical significance. Apyrases are most likely to hydrolyse extracellular ATP produced in response to tissue damage after the larvae have migrated through the lung and airways. However, this possible mechanism was not translated in terms of eosinophil numbers, as any expected reduction due to the eosinophil killing ability was not observed. In contrast, an increase in eosinophils, neutrophils, dendritic cells, macrophages and T cells was shown following apyrase treatment in both naïve and infected groups. These data indicate that there might be some impurity in the enzyme preparations, although they went through endotoxin-removing columns. Interestingly, a trend for decreased IL-5 with reduced IL-13 production was observed during *N. brasiliensis* infections of mice treated with Apy-1a, suggesting a possible role for apyrase in the regulation of IL-5 and IL-13 cytokine responses in this type 2 immunity model.

In order to understand if apyrase activity had any effect on ILC2s during a non-parasite related experimental model of type 2 immunity, BAL and lung cells were analysed after 24 hours of *Alternaria*-induced airway inflammation. Enhanced eosinophilia and neutrophilia were observed in all ALT treated groups when compared to the PBS only control group, in addition to an increased ILC2 expression of ICOS and ST2. This confirms that the intranasal administration of substances and ALT extract were successful and in line with expectations for the *Alternaria* model (Bartemes *et al.*, 2012).

Several studies have reported that during asthma pathogenesis, ATP plays a role in the recruitment and activation of immune cells including eosinophils, lymphocytes and DCs, in addition to the release of alarmins such as IL-33 which in turn activates the production of ILC2-mediated Th2 cytokines (Ferrari *et al.*, 1997, 2006, Idzko *et al.*, 2002, 2003; Pelleg and Schulman, 2002; Bours *et al.*, 2006; Bartemes *et al.*, 2012, 2014; Idzko, Ferrari and Eltzschig, 2014). When extracellular ATP levels increase in the airways, the cardinal features of asthma such as eosinophilic airway inflammation and bronchial hyper-responsiveness increase, however in the presence of P2 purinergic receptor antagonists, Th2 immune responses are inhibited (Idzko *et al.*, 2007). In this study, locally derived apyrase was anticipated to reduce the levels of extracellular ATP, preventing its binding to purinergic receptors on immune cells and thus suppressing IL-33 release. Among the results observed, the apyrase-treated group showed a higher expression of ST2 (Figure 5.14-15), suggesting that a similar level of IL-33 was released regardless of the apyrase activity. This goes against the hypothesized type 2 suppressive effects of the apyrase.

IL-33 release mechanisms are not well understood, but it is known to be released in response to cell death, mechanical and oxidative stress, or through ATP purinergic signalling. It was recently shown that *Alternaria* allergen proteases and endogenous calpains released from damaged airway epithelial cells proteolytically increase the IL-33 alarmin functional activity, independently from serine proteases released from immune cells such as mast cells, neutrophils and cytotoxic lymphocytes (Scott *et al.*, 2018).

In conclusion, the present study demonstrates that *H. polygyrus* apyrase has no effect on the suppression of type 2 immune responses under the experimental conditions employed. This was unexpected, as apyrases hydrolyse pro-inflammatory ATP, which is an essential factor for generation of IL-33 and subsequent initiation of type 2 responses. It is quite possible that intranasal administration of the enzyme is not suitable or sufficient to hydrolyse ATP in localised microenvironments in the airways to the extent that an effect can be measured. Suppression of apyrase expression by parasites, if possible, would provide an alternative means to address this question.

# CHAPTER 6

# Conclusions and Future Work

Several studies have highlighted the importance of helminth secreted products in preventing and treating immune dysregulatory diseases such as allergy and autoimmunity. These secreted molecules have been shown to affect a variety of pathways, ultimately suppressing immune responses. Thus, in order to develop new therapeutics, it is necessary to understand the interaction between the parasite and the host immune system and to gain insights into the cellular and molecular mechanism of action of helminth secreted effector molecules. Given the numerous immunoregulatory effects of helminth infection, it is highly likely that many proteins secreted by the intestinal parasitic nematode *H. polygyrus* target the immune system; hence the importance to compile a comprehensive list of the immunomodulatory proteins through assessing the function of isolated molecules. Of these products, apyrases, previously identified by proteomic analysis, most likely act as immunomodulators, but this remains to be demonstrated experimentally. These nucleotide-metabolising enzymes can hydrolyse inflammatory ATP, changing nucleotide availability, and thus potentially disturbing purinergic signalling pathways of immune cells. The characterisation and the study of the immunological effects of these apyrases is therefore essential.

During this study, the five apyrases secreted by the nematode *H. polygyrus* were biochemically characterised. Apy1-5 were successfully cloned and expressed in the heterologous expression system *Pichia pastoris*, and enzymatic activities were performed using a colorimetric assay which detect inorganic phosphate released. The enzymes belonged to a group of calcium-dependent apyrases with a broad optimum pH ranging from 6.5 to 10 and a broad substrate specificity, catalysing the hydrolysis of both nucleoside tri- and diphosphates to the final product monophosphate. The activity of *H. polygyrus* total

secreted products was also tested, and enzymatic activities were comparable to those shown by recombinant proteins. After biochemical characterisation, *in vivo* studies were performed in order to reveal if any immune modulation was displayed by apyrases, and if they could show potential for future therapy of immune disorders.

The murine unicellular organism *Trypanosoma musculi* has been previously genetically modified by transfection and used as a heterologous system for the expression of parasitic helminth secreted proteins to study gain of function (Vaux *et al.*, 2016). In this thesis, the extracellular trypanosome was used as an *in vivo* vehicle for the expression of *H. polygyrus* apyrases and *T. spiralis* 5´-NT, and the immunological function of the transgenes was studied in order to investigate any possible immunomodulatory properties. The expression of active apyrase and 5´-NT in trypanosomes was successful, and both exhibited a substrate specificity similar to that of recombinant enzymes expressed in *Pichia.* When mice were infected with *T. musculi* expressing apyrase (apy-1 or apy-3), the trypanosomes grew faster compared to a control line. The immune cell numbers and cytokine profile in the spleen were tested and no changes were detected in pro-inflammatory cytokines and mediators such as TNF-α, IFN-γ, and NO; however significantly higher expression of Arg1 was seen, along with higher levels of IL-5 and IL-13. Both immunological and physiological factors appear to be responsible for these changes, suggesting that the secretion of an apyrase or 5´-NT by *T. musculi* might be modulating the host immune response in addition to affecting the purine salvage pathway in trypanosomes.

The effect of apyrase on type 2 immunity was also examined in this thesis. It was hypothesised that by hydrolysing ATP, apyrases might inhibit release of IL-33 with

subsequent reduction in IL-5 and IL-13 cytokine production. However, of the cells studied in the lung and airways during an acute model of allergic inflammation, intranasal administration of apyrase did not seem to have an effect in regulating immunological responses. Pro-inflammatory ATP triggers the release of the alarmin cytokine IL-33, which in turn regulates the activation of ILC2s. In this study, the activation and function of pulmonary ILC2s were not affected by the presence of an active apyrase. It is quite possible that the apyrase administered in these experiments was not able to access localised microenvironments in order to hydrolyse ATP and cause a measurable effect.

Drawing these conclusions together, the remaining question to be answered is why various parasitic nematodes including *H. polygyrus,* evolved to secrete a family of apyrases, and what effect these nucleotide-metabolising enzymes have on the host immune response.

The work presented in this thesis suggests that apyrase may not regulate type 2 immune responses, at least in the models that I have tested, however this does not negate other possible immunomodulatory functions of *H. polygyrus* secreted apyrases. Further work to probe the precise function of these apyrases is required. This could involve developing the *T. musculi* heterologous expression system. For instance, the fusion of a ligand to the protein which can bind a fluorescent molecule injected during transgene infection or tagging apyrase with a GFP reporter could be used in order to detect any protein interaction with immune cells by flow cytometry. Alternatively, the use of a luciferase-based *in vivo* imaging technique with a tetracycline inducible system would allow tracking the parasite location and targeting transgene expression during infection.

Suppression of apyrase expression by parasites, if possible, would also provide an alternative means to elucidate the true function and potential benefits of secreted apyrases.

Another important step is the investigation of parasitic helminth secreted proteins which belong to families of unknown function, as the discovery of anti-inflammatory or immunomodulatory molecules holds potential for future therapeutic drugs. Measuring parasitaemia of a *T. musculi* transgenic line expressing a gene of unknown function and looking at any perturbations in parasitaemia can be indicative of immunomodulation. Thus, screening novel proteins using a *T. musculi*-based system could be useful in order to know which gene to study further via measuring immune cell number, cytokine and antibody subtype production. The recombinant protein can also be isolated and intranasally administered into models of allergy or autoimmune diseases to assess whether this protein is an immunomodulator.

# CHAPTER 7

# References

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# CHAPTER 8

# Appendix

## A.1 *Heligmosomoides polygyrus* apyrases sequence

**Apy-1:**

ATGAGCCCTTTGCCAGTGGGAGAATGCACTGTTGACGCATCGGACAGAACGTTAAACATCATTGCCA
TCACTGACATGGATAAGGAATCCAGGACATCACCTGAGGGTTACACGTGGAGAGCGTTGACAAGAA
AGGGACAGCTGTTTGTGAAGGACGAACTAGCAAAGCTTGAAGTGACATGGGATCTCTCGTCTGACC
AAAATTTAACTACGCACCTAAACATGAAAGGACGAGCCATGGAGATGTCGGACTTGGTGAAATTTA
ACGGTCACTTGCTTTCGCCTGACGACAAAACCGGAATGATCTTTGAAACTGAAGACAAGAAGGCAAT
TCCATGGCTGTTCCTCAATTCTGGGCCAGGGAACACGGAAAGCGGAATGAAGGCGGAGTGGATGA
CGTTGAAAGACGACCAGCTGTATGTTGGCGGTCACGGAATGGCGTATAGGAACAAAAAGGGAGAG
ATTTTCAGCACGGATGCAATGTGGATAAAAATCATATCTCATGACGGAGTTGTCAAAAGCGTAGACT
GGACTGATGTCTATGACGAAATTGCACAAGCAGCTGGTGCAACATCGCCAGGATACCTGACCCACG
AAGCTGTGCAATGGTCGGAGATACACAAGAGATGGTTCTTCCTTCCAAGAAAATACTCAACTGAAAT
CTATAACGACGAATTGGACGAGATCAGGGGAACTAACCTACTAATTACCGCGGATGAATCTATGGA
AGACATCCAAGTTGTGAAAATTGGAGAACTAACACATCCAGATCGTGGATACTCGGCTTTCGATTTT
GTACCAGGAACCTGCGATAGCGTGATTTTAGCCCTTAAATCAATGGAGCATGATGGATCGACAGAG
AGTTATATCACTGCCCTTGATACTAATGGCAATGTACTTCTGGAAGACCAACCCCTCGATGGCGACCT
CAAATTCGAAGGATTGTACTTTTTGTGA

**Apy-2:**

ATGGCAGTTATCAAGCCCAGAAAGATCGACCTCAGTGCAAACAAACTTGGCCAGAAATCTACCAGAT
TCACTACACTACCTGATGGCTCAACTGAATACGAAATAATGGCCATCACGGACAACGACAAGTCTTC
GGTGGTCACGGCAGGCAGCACGTGGCAAGCTGTGACAAGAAAGGGAAAGCTGACTTTGAACAAGG
ATAAGACCCAGGCTAATGTAGTCTGGGATGCCAACGCCGACCAAACGGTTCAGTCAGGACTGAACT
ATAAAGGAAGAGCAATGGAGCTGTCGGATTTGGTGAATTTCAACGGGCATGTCATTTCACCTGACG
ATAAAACCGGACTGGTTTACGAGATCAAGGGCAACCAGGCAATTCCATGGATATTTCTAAATGCTGG
ACCTGGAAATACTACAGATGCCATGAAAGCCGAGTGGATGACTTTGAAAGACGGAGAGGTTTACAT
TGGAGGACATGGAACGGAATACATCGATCAAAATCAGCAAGTGGTCAACAGGTATGCAATGTGGAT
CAAGGTTGTATCGCCCGATGGACTTATTAAGCACGTCGACTGGAGGCGCAACTTCAACAGAATTCGA
AACGCTGCAGGGTTCCCCTTCCCCGGCTACCTCACCCATGAGGCAGCTCAATGGTCGGACATTCACA
AACGCTGGTTCTTCCTTCCGCGAAAACAGTCCAAGGAAATTTACGATGAAGCCAAAGACGAGACAA
GAGGTAGCAATCTTCTCATCTCTACAAACAGCTACATGATGGATCTGAAAAAGGTGGAAGTCGGACC
ACTGACTGATCCGTCCAAAGGCCACTCCGCGTTCCAGTTCGTTCCAGGCACTAACGACGAAATCATT
GTAGCTCTGAAAACGAGAGAAAGCGGAGGAACCACGAGCAGCTTCATCACGGTGTTCGATATTACG
GGAAAAATCATTCTTAGTGACCAGCAGCTCGCTGGCAATCACAAATTCGAAGGGTTATACTTTATTT
AA

**Apy-3:**

ATGGCTGCCCCTATGCCTCAGGAGCTAATCTGCGTTCCCGATGTAGTAGACAGAACGTATGATCTGA
TTGCTATCACTGACATGGACAAAGACGCTGGCGGAAGCCCATCAGACTGGACATGGCGGGCAGTCA
AAAGGAAGGGGCAGCTGACGATAAGCGGAGACGGTGAGAAGATCGCCGTGAATTGGGATCCTTCT
GCTGATCAAAATGTCACCACACACCTGAACGTGAAAGGACGAGCGATGGAGCTGTCAGACCTGGCA

CTATTCAATGGCCATCTGATTTCACCTGACGACAAAACCGGAATGATCTACCAGATAGAAGGCCAGA
AGGCTATTCCGTGGGTGTTTTTGAACTCGGGGCCTGGAAATACTACAAACGGCATGAAAGCGGAGT
GGATGGCTTTGAAGGACGGCCAGCTCTACGTCGGTGGACATGGGACGGAGTACAGAGCGAAGGAT
GGATCAATTGTGAGTACGGATTCTATGTGGATAAAAATCGTCTCGGCTGATGGTGCTGTTCAACATA
AGGACTGGACCAAAAATTACGAGAAGATTCGAGACGCAGCAGGATTTCCAGCACCTGGCTACCTCA
CACATGAGGCAGTGCAGTGGTCGAAAGTACACCACAGGTGGTTTTTCATCCCCAGAAGGCACTCCA
AGAAGATCTATGATGAAGAAGCCGACGAGCGCAGAGGCAGCAATAAAATAATTAGTGCGGACGAA
AATTTCGACGACTTCTACACGATAGACATTGGGGCGCTGAAGGATCGCAAGCGAGGATTCTCGGCG
TTCGCCTTTATCCCAGGCACCTGTGATGAGCTGGCTCTCGGCATTAAATCGGTGGAGTTTAAGGGCA
CAACGGAGAGCTACATCACCGCATTCGATCTTATGGGCAACATCTATCTTGAGGATCAGCGACTGCA
GGGTAACCTTAAATTCGAGGGACTGTACATTGTCAAAAAGTGA

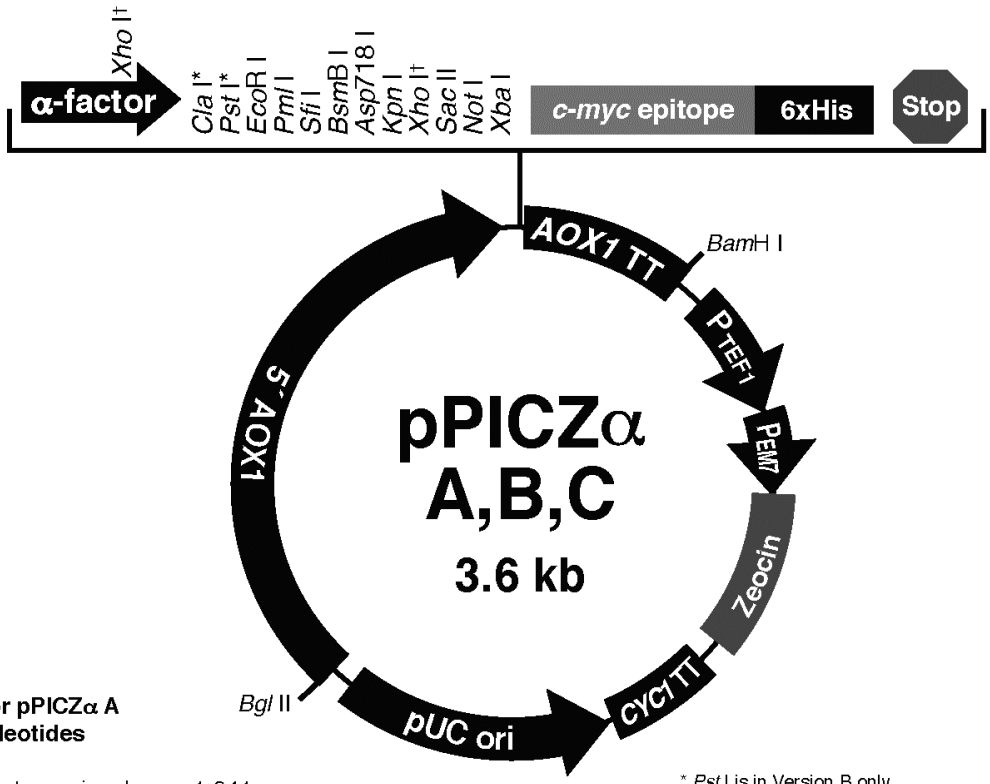
**Apy-4:**

ATGACATCATCGTCGTCACTGACATGGACAAGGACTCCAGAACCTCGGAATGGACATGGCGAGCAC
TGACAAGGAAGGGAATGGCATTTATGAAAAATGAACGATCGAAAGTTGAAGTAACATGGGATCCTT
CGTCTGATCAGAATGTAACCACGCATTTGAACATGAAAGGCCGAGCAATGGAGTTATCGGACTTGG
TGAAGTTTAACGGTCACTTGCTATCACCTGACGACAAGACTGGAATGATCTATGAGATAGAAGGAA
ACAAGGCGATTCCATGGATATTCCTGAACTCTGGACGAGGGGATACCACAAGTGGAATGAAGGCTG
AATGGATGACATTGAAGGATGGCCAGCTTTACGTTGGCGGTCATGGAATGGAGTACAGAGACAAAA
CCGGAAAAGTTTATAGCAGAGATGCTATGTGGATAAAAATCGTGTCGCCTGAAGGAGTTGTTAAGC
ATGTGGACTGGACAAGTAATTATAACGACATTGCACGCGCGGCTGATGTAACATCACCAGGTTACCT
CACACATGAAGCAGTACAGTGGTCTGAGATACACAAGCGATGGTTCTTCCTTCCAAGAAAATACTCC
ATAGAGGCGTACGACGAAAAAGCGGATGAGCGAAAGGGCACTAATCTACTCATCACCGCCGACGA
GACTTTCTCCGAAATCAGTGTGCTTGAAATAGGAGATCGAATTCATACAGAACGCGGATACTCAGCT
TTCGACTTTATGCCAGGAACTTGCGATACAGTGATTCTAGCGCTTAAGTCGATGGAACTTGAAGACA
ATACAGCAAGTTACATTACAGCCTTCAATACTGAAGGCACCATCCTCCTGGAGGACCAGCCCCTTGA
CGGTGACCTCAAGTTTGAAGGACTGTACTTTTTGTGA

**Apy-5:**

ATGTGGTCGCTGTCACTCTACATGGTCCTATTATTCACCATTGAACTTCTTCAAACCACCCTAGCTGCC
CCTTTGACTCCGCCTGAGGAGTGCATTCCTGAAGGAGGAGAGCGAACGCTCAAACTGATAGCGATC
ACTGACCTAGACAAAAAGGCTAGAACATCCGTTACGGATTGGACATGGAGAGCAGTGAAAAGGAG
CGGAGAGCTGCATCTGAACACAGAAAAGGGGGAGATTGGTGTGACTTGGGACCCCTCTTCCGATCA
GAATGTTACCACACAACTGAATGTGAAAGGACGAGGAATGGAGCTGTCCGACCTGGCCAATTTCAA
TGGTCACCTTCTCTCACCTGATGACAAAACCGGAATGATCTACCAAATAGAAGGCAAAAAGGCTATT
CCATGGGTCTTCCTCAACTCTGGACCTGGTAACACCACGAGCGGTATGAAGGCGGAGTGGATGACT
TTGAAGGACGGTCGGCTCTACGTCGGAGGACATGGAACGGAGTACAGAGGCAAAAACGGAGAAGT
CTTAAGCACAGACCCTATGTGGATAAAAATCGTCTCGCCAAGCGGAGCTGTCGAACACAAAGACTG
GACCGATGTCTACAAAAAGATTCGTCAGGCGGCTGGTTTTCCAGCACCGGGTTACCTCACTCATGAG
GCGGTGCAGTGGTCAGACATTCACCAGAAATGGTTCTTCCTTCCGAGAAAGCATTCCAAGCACGTTT
ATGACGAGGCTAAGGACGAGCGCAGAGGCAGCAATCTGTTAATTAGCGCGGACGATAACATTGAA
AATATCCAAGTGGTAAAGGTCGGCGAACTGGACAATCGTAAGCGTGGATACGCTGCCTTTGAATTT
GTACCAGGCACCTGTGACTACATGATCGTAGCAATCAAATCGAAGGAAATTGAAGACTCTACGGAA
AGCTATATCACAGTATTCGATATAAATGGCAACGTTCTTCTTGATGACCAAAAACTGGAGGGAAGCC
TCAAGTTTGAGGGTCTGTACTTTGTATGA

## A.2 Vector diagram of pPICZα




**Comments for pPICZα A**
**3593 nucleotides**

5´ *AOX1* promoter region: bases 1-941
5´ *AOX1* priming site: bases 855-875
α-factor signal sequence: bases 941-1207
Multiple cloning site: bases 1208-1276
*c-myc* epitope: bases 1275-1304
Polyhistidine (6xHis) tag: bases 1320-1337
3´ *AOX1* priming site: bases 1423-1443
*AOX1* transcription termination region: bases 1341-1682
*TEF1* promoter: bases 1683-2093
EM7 promoter: bases 2095-2162
*Sh ble* ORF: bases 2163-2537
*CYC1* transcription termination region: bases 2538-2855
pUC origin: bases 2866-3539 (complementary strand)

* *Pst* I is in Version B only
*Cla* I is in Version C only

†The two *Xho* I sites in the vector allow the user to clone their gene in frame with the Kex2 cleavage site, resulting in expression of their native gene without additional amino acids at the N-terminus.

**Figure A.1: pPICZα vector map**
Figure from EasySelect™ Pichia Expression Kit manual 2010 (Invitrogen, USA).

## A.3 Apy-1 cloned into pPICZαA

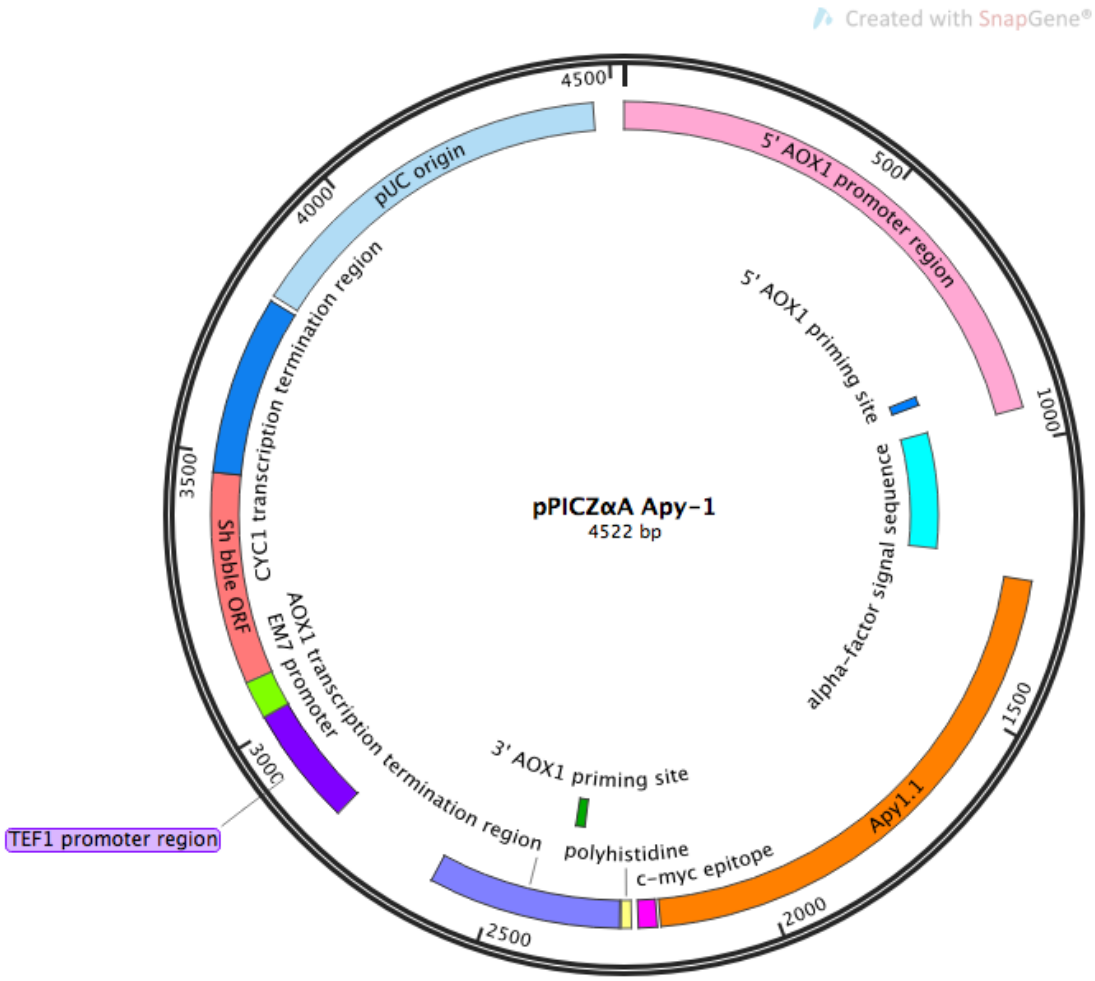



**Figure A.2: Plasmid map of *apy-1* cloned into the *Pichia pastoris* vector pPICZαA**
Figure created with SnapGene viewer.

## A.4 Apy-3 cloned into pSSU

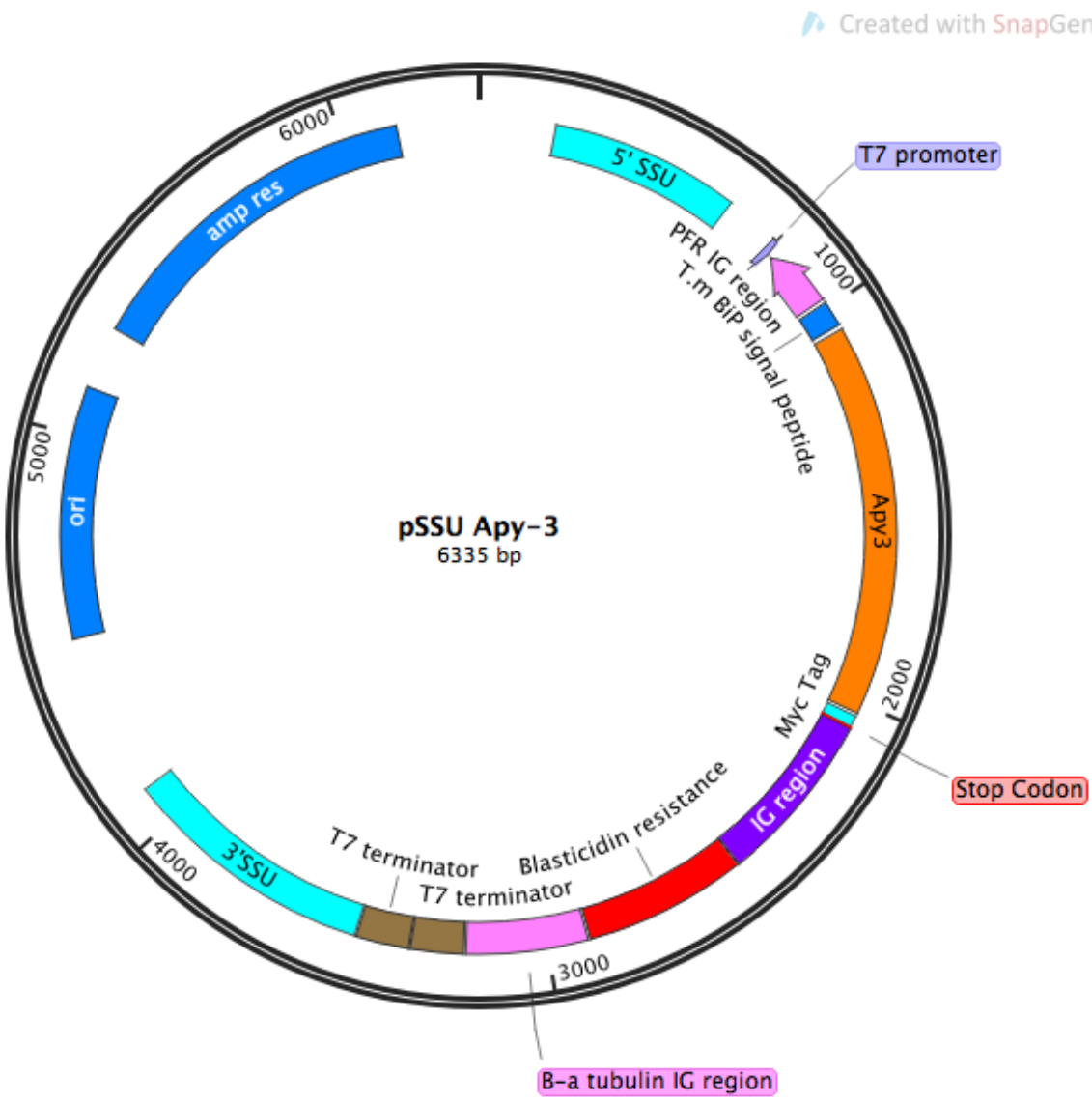



**Figure A.3: Plasmid map of *apy-3* cloned into the *Trypanosoma musculi* vector pSSU**
Figure created with SnapGene viewer.