

The Role of Hedgehog Signalling in the Biology of Eosinophils

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The Role of Hedgehog Signalling in the Biology of Eosinophils

A thesis submitted to the University of Bedfordshire, in fulfilment of the requirements for the degree of Master of Science by Research

by

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Institute of Biomedical and Environmental Science and Technology (iBEST)

October 2021

Word Count: 17599

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Abstract

Eosinophils are central to T-helper 2 (Th2) immune responses and allergy and asthma pathogenesis. Their degranulation in response to allergen is a cause of airway hypersensitivity and remodelling in allergic airway disease. Previous work showed active Hedgehog/Gli Signalling via Sonic Hedgehog (SHH) in the lungs of asthmatic murine models. Murine eosinophils can transduce SHH signals, however functional effects of SHH signalling on eosinophils remain unclear. The aim of this project is to elucidate these effects. Therefore, the HL60 myeloid cell line was differentiated into a human eosinophilic population (HL60-eos) via Sodium Butyrate treatment. HL60-eos cells were cultured in the presence or absence of (1) recombinant SHH ligand or (2) GANT61, a Gli antagonist and therefore Hh signalling inhibitor. Cellular phenotype and genotype were studied via qPCR, ELISA, Flow Cytometry, and cytochemical staining. We found that culture with SHH upregulates EPX and TGF-β expression in HL60-eos cells. EPX encodes Eosinophil Peroxidase, a constituent of eosinophilic granules and responsible for cell damage during degranulation. TGF-β1 encodes the cytokine TGF-β, important for lymphocyte regulation, eosinophil chemotaxis, fibrosis, and wound healing. Further investigation is needed to characterise the eosinophilic response to SHH/GANT61 when immunostimulated, as they would be in vivo during an immune response.

Acknowledgements

A huge thank you to Dr Anna Furmanski and Dr Eleftheria Diakogiannaki for their invaluable support throughout this project. A special thanks to Dr Furmanski for her endless encouragement, as well as unwavering patience at my myriad of questions and the endless bombardment that was my emails! Thank you also for introducing me to the complex and wonderful world of Hedgehog signalling, I have truly enjoyed every minute!

To my family, I thank you for your support and apologise for the endless ramblings of various theories and plans I made, I surely would have gone mad by this point without you. Lastly, a massive thank you to my partner, Samantha, of whom has been a great support throughout this journey. You have supported me far more than you'll realise, and I would have had a considerably harder time completing this without you by my side!

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1.0 Introduction

1.1 What is Asthma?

As one of the most prevalent diseases worldwide, asthma affects millions of people on a global scale (Braman, 2006). It reduces the quality of life in most cases, and for some asthma proves to be fatal, causing three deaths daily in the UK alone (Asthma UK, 2019). Mild to moderate asthma is typically well controlled with inhaled bronchodilators and/or corticosteroids (Cazzola et al, 2012), however for some these treatments are ineffective (Chapman and McIvor, 2009). This currently costs the NHS around £1 Billion per year in treatments, and so new drug targets are required to lower the effect on patient's lives and reduce the cost on the NHS (Mukherjee et al., 2016).

Asthma is defined as a reversible airway obstruction due to chronic inflammation. This causes symptoms of wheezing and coughing, with an increase in airway mucus production. Asthma itself is a heterogenous disease, which complicates our understanding of the pathophysiological mechanisms that trigger or sustain the condition, as every patient will present symptoms to a varying degree. It can be broadly divided into two categories: allergic and non-allergic asthma.

Non-allergic asthma is triggered by environmental factors, such as extreme temperature, extreme humidity, and rapid changes in humidity and/or temperature. It can also be triggered by respiratory infections, causing irritation and inflammation in the airways (Busse, Lemanske and Gern, 2010). This causes a response to the otherwise harmless trigger event, resulting in the inflammation of airways and presentation of further symptoms based on severity of response. This form of asthma is also called intrinsic asthma or non-eosinophilic asthma.

Allergic asthma is triggered in the presence of an allergen, a non-pathogenic antigen that stimulates an immune response and is an otherwise harmless molecule. Examples of such allergens include house dust mites and pollen (Miller, 2018). Allergic, eosinophilic, or Th2-type asthma is driven by the T-helper cell immune responses, central to which are eosinophils.

1.2 T-helper 2 response in asthma

During an asthmatic attack, an allergen, usually inhaled, is detected by an antigen presenting cell (APC) which engulfs the allergen and presents it on surface MHC molecules.

This is then detected by naïve T-helper 0 (Th0) cells, which in response to allergen, differentiate into T-helper 2 (Th2) cells. Th2 cells are thought to be responsible for the anti-helminth/anti-parasite responses (Allen and Sutherland, 2014) but are also involved in allergy. It is this Th2 response that causes the inflammation and related symptoms in asthma (Komai et al., 2003). When activated, Th2 cells produce and secrete Interleukin 4 (IL4), Interleukin 5 (IL5), and Interleukin 13 (IL13). IL4 and IL13 are responsible for the stimulation of mast cells, which leads to histamine release and local inflammation; increased production and secretion of mucin (causing increased mucus production); increased IgE synthesis by B cells; and further amplification of the Th2 response (Komai et al., 2003). IL5 is primarily responsible for the recruitment of eosinophils and eosinophil activation (Sur et al., 1996). Once recruited to the site of inflammation, eosinophils potentiate many of the responses that lead to asthmatic pathology.

1.3 Eosinophils

Eosinophils are acidophilic granulocytes found predominantly in blood. A part of the myeloid lineage, they differentiate from myeloblast cells into Eosinophil lineage-committed precursor cells (EoPs), which are known to mobilise to allergen-exposed sites during allergic responses. However, these EoPs lack granules, so their usefulness at the sites of exposure is as-of-yet unknown (Tang et al., 2018). It has been hypothesised that as well as providing local new sources of 'fresh' mature eosinophils, undergoing a final differentiation in the presence of IL5, EoPs are involved in eosinophilopoetic processes at these sites by secreting IL5 themselves. (Sehmi et al., 2016).

Eosinophils are primarily responsible for the destruction of multicellular pathogens, such as helminths, by degranulating onto the surface of the pathogen, and it is their large eosinophil-specific granules that distinguish eosinophils from other granulocytes such as neutrophils. These granules consist of a membrane-bound matrix, in which is a dense crystal structure (Shamri, Xenakis and Spencer, 2010). The granule's crystal structure and matrix themselves contain numerous different toxic mediators, such as Major Basic Protein (MBP), Eosinophil cationic protein (ECP), and Eosinophil Peroxidase (EPX). These all disrupt the cell membrane of the pathogen, causing structural failure and eventual death of the multicellular pathogen (Bass and Szejda, 1979). These degranulation events are regulated by a complex combination of cytokines but is triggered predominantly by the binding of

substrate to the pattern recognition receptors TLR-2 and TLR-9 (Wong, Cheung, Ip and Lam, 2007). This causes a fusing of the eosinophil's membrane to the pathogen cell surface, releasing the granules non-specifically onto the pathogen.

During an infection by a pathogen, or at sites of allergic events, eosinophils will be recruited through the binding of a ligand or signalling molecule (e.g., Eotaxin, monocyte-chemotactic protein 3 and 5, RANTES) to the eosinophil-membrane bound C-C chemokine receptor 3 (CCR3) (Resnick and Weller, 1993). It has also been shown that IL5 upregulates the chemotactic functions of primary human eosinophils (Warringa et al., 1992).

Eosinophils are an often-overlooked cell type in comparison with leukocytes, for example lymphocytes, despite their direct involvement in immune defence, homeostasis, and their involvement in asthma and other common diseases.

1.4 Eosinophils in Asthma

During an asthmatic response in the lung (Fig. 1.1), in the absence of a pathogen, eosinophils are recruited to the site of allergen-activated Th2 cells, are activated by IL-5 and degranulate in the airways, releasing their toxic mediators. This damages lung epithelial cells, causing the classical symptoms of airway hyperresponsiveness and increased mucus production. The eosinophils also release cytokines, causing the recruitment of more eosinophils to the area (IL-5), further Th2 activation (IL-13) and airway remodelling/fibrosis

(TGF-β) (Possa et al., 2013, Figure 1.1).

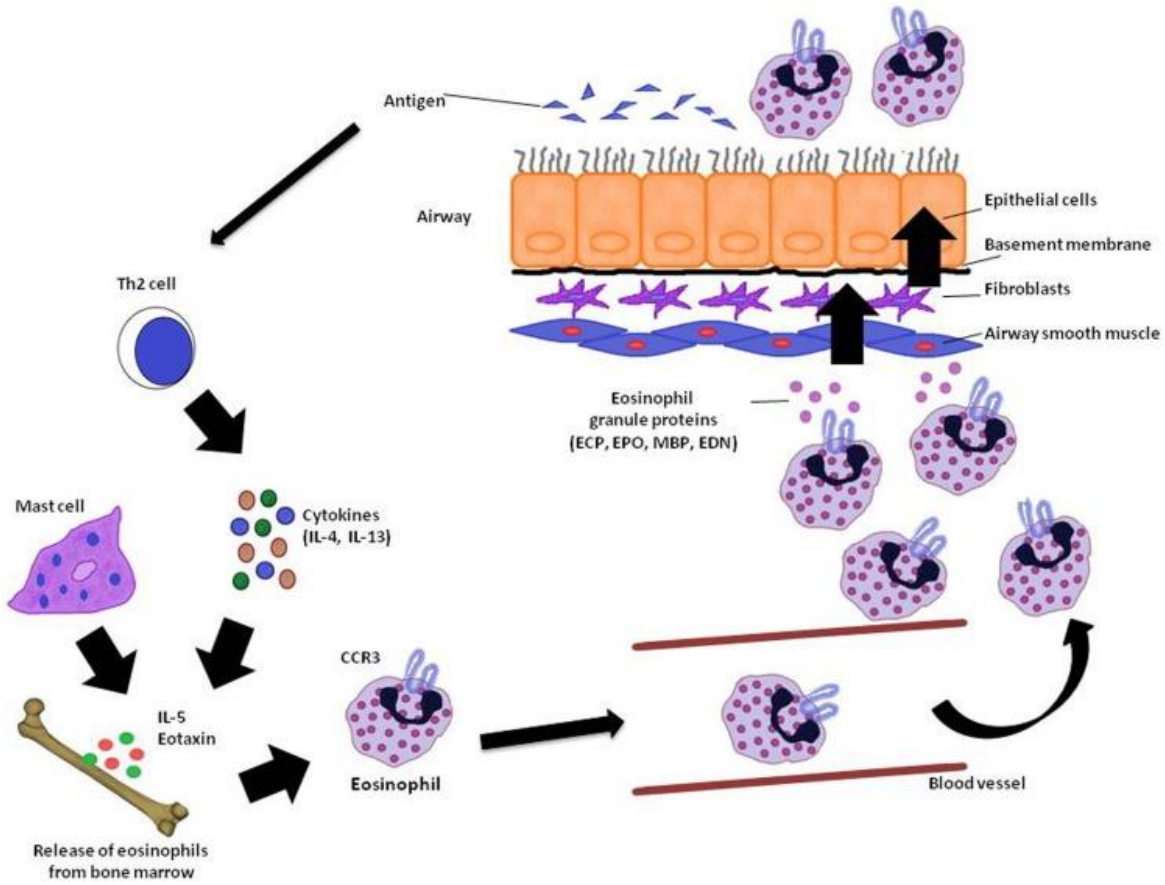

Figure 1.1: Eosinophil mechanism of recruitment in allergic asthma (Adapted from Possa et al., 2013). Release of eosinophils from the bone marrow is stimulated by Th2 cytokines, eotaxin, and IL5. The eosinophils release cytokines and granule proteins: Eosinophil cationic protein (ECP), Eosinophil Peroxidase (EPO), Major Basic Protein (MBP), Eosinophil-derived neurotoxin (EDN).

1.5 Current Treatment and approaches to allergic asthma

The current treatments for asthma are more reactive in nature than proactive. The most common treatment is to administer bronchodilators such as the short-term bronchodilator Albuterol, and the long-term Formoterol. The short-term bronchodilators are used during an asthma attack to alleviate symptoms. These are effective for low severity attacks but may not provide enough symptom alleviation during a chronic attack. The long-term bronchodilators are given as a prophylactic, but their efficacy is not absolute. Also, most chronic life-threatening cases of asthma occur with the elderly, and as the treatment

must be applied twice daily, this can be an issue in patients with multiple disorders and treatments (Cazzola and Matera, 2008).

More recently, new asthma treatments, including monoclonal antibody (mAb) therapies have been investigated, including the efficacy of a novel treatment to reduce the eosinophil response in asthma (Kelly et al., 2017). Here, Mepolizumab, an anti-IL5 antibody, was given intravenously to participants with mild allergic asthma 1 month before and immediately before eliciting airway inflammation via segmental allergen challenge by depositing either saline, or saline with allergen into separate bronchi of the lungs. Blood, bronchoalveolar lavage (BAL), and biopsies were taken 48hr after the allergen challenge. While the control groups showed a typical allergic response of increased numbers of circulating eosinophils, BAL eosinophilia, and eosinophil peroxidase release, the IL5 neutralisation elicited by Mepolizumab before allergen challenge removed the rise in circulating eosinophils and IL3 receptor expression and reduced the BAL eosinophilia and eosinophil peroxidase deposition (Kelly et al., 2017). Whilst this shows promise, the allergic response was still present, albeit in a suppressed form. This means that IL5 is not the only important molecule in the allergic response. This means that the asthmatic cascade can still occur and thus could still progress to life-threatening degrees without intervention, further proving the need for a treatment that could target Th2 allergic responses only, so as not to render the response useless in case of genuine immune threats, e.g parasitic infection.

Other novel treatments include the inhibition of: IL4 signalling - a key Th2 cytokine (Kau and Korenblat, 2014), IgE binding (Samitas, Delimpoura, Zervas and Gaga, 2015) responsible for priming mast cells, and anti-thymic stromal lymphopoietin (Matera, Rogliani, Calzetta and Cazzola, 2020). Whilst these have shown some promise in their respective trials in many patients with asthma who have eosinophilic airway inflammation, as well as having an effect in patients with Chronic Obstructive Pulmonary Disorder (COPD), not all patients respond equally, despite similarities in their clinical, inflammatory, and functional characteristics. It is this heterogeneity that results in the need for reliable and reproducible treatments as well as biomarkers for mapping and monitoring the pathways during an inflammatory response (Bel and ten Brinke, 2017).

1.6 Hedgehog Signalling

Hedgehog (Hh) proteins are intercellular molecules responsible for controlled embryonic differentiation and maintenance of stem cell niches (Ingham, 2001). Hedgehog proteins share a common signalling pathway within mammals (Figure 1.2), which have three Hh protein ligands: Sonic Hedgehog (SHH), Desert Hedgehog (DHH), and Indian Hedgehog (IHH). In the absence of extracellular Hh protein, there is no Hh ligand bound to the cell surface G-protein coupled receptor (GPCR) known as Patched (PTCH). This causes the inhibition of another membrane bound GPCR known as Smoothened (SMO). When SMO is inhibited, Intracellular molecules known as Gli transcription factors are converted into their repressor forms (GliR). An accumulation of GliR in the cell nucleus inhibits the expression of Hh pathway target genes (Carballo, Honorato, de Lopes and Spohr, 2018). In the presence of Hh ligand, the Hh protein binds to PTCH, allowing SMO to be activated. This converts Gli transcription factors into their activated form (GliA), causing Hh target genes to be expressed (Tamaoki, 2004), including members of the pathway itself such as Gli1 (Yang, Xie, Fan and Xie, 2009). Hh signalling has been shown to have multiple roles in the development of leukocytes, (Rowbotham, Hager-Theodorides, Furmanski and Crompton, 2007; Crompton, Outram and Hager-Theodorides, 2007; Rowbotham et al., 2008; Hager-Theodorides et al., 2009; Outram et al., 2009; Rowbotham et al., 2009; Drakopoulou et al., 2010; Furmanski et al., 2011; Furmanski et al., 2013; Solanki et al., 2017; Mengrelis et al., 2019). In mature naïve T-cells, these target genes are responsible for assisting the differentiation and activation of Th2 cells (Furmanski et al., 2013; Yánez et al., 2019). Little is known to what extent, if any, Hh signals affects gene expression or function in eosinophils, however.

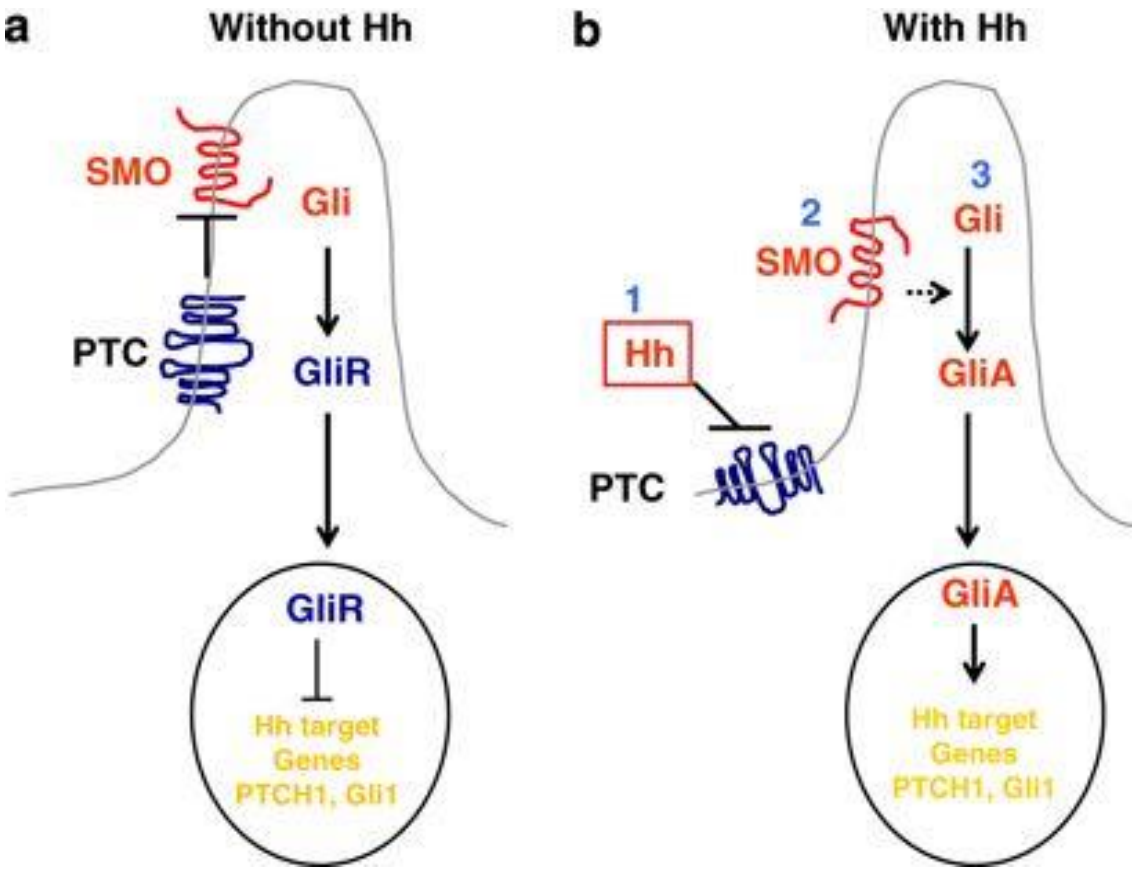

Figure 1.2: Hh signalling pathway in the absence and presence of Hh ligand. Abbreviations: Patched (PTC), Smoothened (SMO), Gli transcription factors (Gli), Gli transcription activation factors (GliA), Gli transcription repression factors (GliR) (Yang, Xie, Fan and Xie, 2009).

1.6.1 Hh signalling in embryogenesis

Of all fields it has been studied in, Hh signalling is most well characterised in its role in embryogenesis. This pathway function was first identified in the 1980's in drosophila through the discovery of lethal knock out genes, and the loss of ventral and bilateral patterning in drosophila embryos (Nüsslein-Volhard and Wieschaus, 1980). It has also been shown that embryos with mutations in the Hh genes (SHH, DHH, IHH) show non-viable phenotypes, ranging from incorrect limb development (Jiang and Struhl, 1995), to holoprosencephaly (Roessler et al., 1996). It has also been shown that SHH-knockout murine embryos exhibit reduced thymocyte development. This prevents the full committal of the thymocytes to the T-cell lineage, resulting in a lower number of T cells (Shah et al., 2004).

1.6.2 Hh signalling in postnatal tissue homeostasis and repair

As well as having such an important impact on embryogenesis, the Hh pathway remains active in some tissues throughout all stages of life, through its maintenance of stem cell niches as well as performing a regulatory role in tissue repair. For example, the Hh pathway is involved during the anagen phase of hair growth, SHH expression is readily seen in the lower epithelial cells of the hair follicle, with *GLI1* and *PTCH1* being upregulated also (Brownell et al., 2011). Of more immediate clinical significance is the involvement of Hh signalling in the maintenance of hematopoietic stem cells. These cells refresh the myeloid and lymphoid cell lines. However, in the presence of upregulated concentrations of Hh ligand, any carcinogenetic stem cells are also potentiated, with the ligand acting as a 'survival signal' (Dierks et al., 2008). Inhibition of the pathway has been shown to slow tumorigenesis and general disease progression in some leukaemias (Zhao et al., 2009).

Hh ligands are also expressed in mature tissues such as lung epithelial cells, with an upregulation of Hh ligand expression seen as a response to local injury/damage (Liu et al., 2013) and allergen challenge in mice (Standing et al., 2017; Wang et al., 2019). This upregulation has also been shown to increase cell proliferation and modifications to lung tissue at the site of injury, intrinsically linking the Hh pathway to lung airway remodelling (Krause et al., 2010).

1.7 Hedgehog Signalling in Th2 responses and asthma

Through the use of Genome-wide association studies (GWAS), several Hh related genes have been found to be asthma-associated such as *HHIP*, a regulatory component of the hedgehog pathway (Tamari, Tomita and Hirota, 2011; Li et al., 2011). Similarly, it has been shown that SHH is linked to airway fibrotic disease (Stewart et al., 2003), which is often a sequalae of chronic asthma. This starts to form the basis that Hh signalling is intrinsically linked with both the asthmatic event and any post-asthmatic symptoms/diseases.

Furthering this avenue of research, Furmanski et al (2013) showed that SHH skews the T-cell response to an asthmatic event towards that of a Th2 response, which leads to the

increased recruitment of granulocytes through the release of IL4 and other cytokines. Further, concentration of SHH increases in lung tissue in murine models following allergen (house dust mite: HDM) inhalation. In addition to this epithelial and T-cells from lung samples post-HDM treatment expressed higher levels of SMO, suggesting upregulated SHH signalling. This in tandem with the SHH being present in the epithelium shows that the inflamed epithelial cells can transduce the Hh signal via paracrine signalling to nearby T Cells. This is significant, as an upregulated Hh signal to T cells will result in an increased Th2 response as demonstrated above, leading to the increased recruitment of lymphocytes to the epithelium, specifically an increase in eosinophils and other granulocytes.

Following this, Standing et al. (2017) further showed that SHH increases incrementally in the lung following episodes of allergen inhalation, with a classical Th2 response observed in BAL, lung tissue, and eosinophil recruitment. Using GFP-Gli reporter mice, the lymphocytes and other cells present in allergen-exposed lungs were shown to be responding to Gli-activating signals during the Th2 response (likely due to the upregulation of Hh ligand). Further, genetic inhibition of Gli-mediated transcription in T cells results in a downregulation of Th2 cell response during allergen challenge but shows no significant change in eosinophil recruitment in response to HDM. The same study then showed that interestingly, lung epithelial cells, eosinophils, and phagocytes undergo Hh signalling in response to allergen exposure. Immunofluorescent analyses of lung sections from allergen-treated GFP-Gli reporter mice showed that GFP signal was strongest in E-cadherin+ bronchial and peribronchial cells after allergen exposure. Similarly, there was Gli activity noted in peribronchial SiglecF+ cells, of which are most likely eosinophils as anti-SiglecF antibodies bind specifically to eosinophils, with limited binding affinity for basophils and mast cells. This would suggest that the eosinophils about to cross the air/lung interface, and enter the airspaces are receiving and transducing Hh signals. In support of this, 50% of eosinophils showed Gli activation *in vivo* in allergen-treated GFP-Gli-reporter mice, and eosinophils were also shown to express SHH after HDM-induced Th2 response in WT mice (Standing et al., 2017). As these cells are both capable of receiving a Hh pathway signal and expressing and secreting SHH, it suggests that a positive feedback loop would occur, wherein the initial Hh/Gli signal would cause the cells to potentiate the Hh signal either autocrinally or paracrinally. This is shown in Figure 1.3, a model proposed by Standing et al. (2017), where

secreted SHH binds to receptors on the same cells that secrete it, showing eosinophils as one of these. This idea is further built upon by the finding that lung epithelial cells and innate immune cells, specifically eosinophils, express the Hh signalling pathway components (Standing et al., 2017). These data collectively form the basis for the current investigation, as during an allergic asthma in mice, eosinophils are recruited and are able to respond to Hh signals.

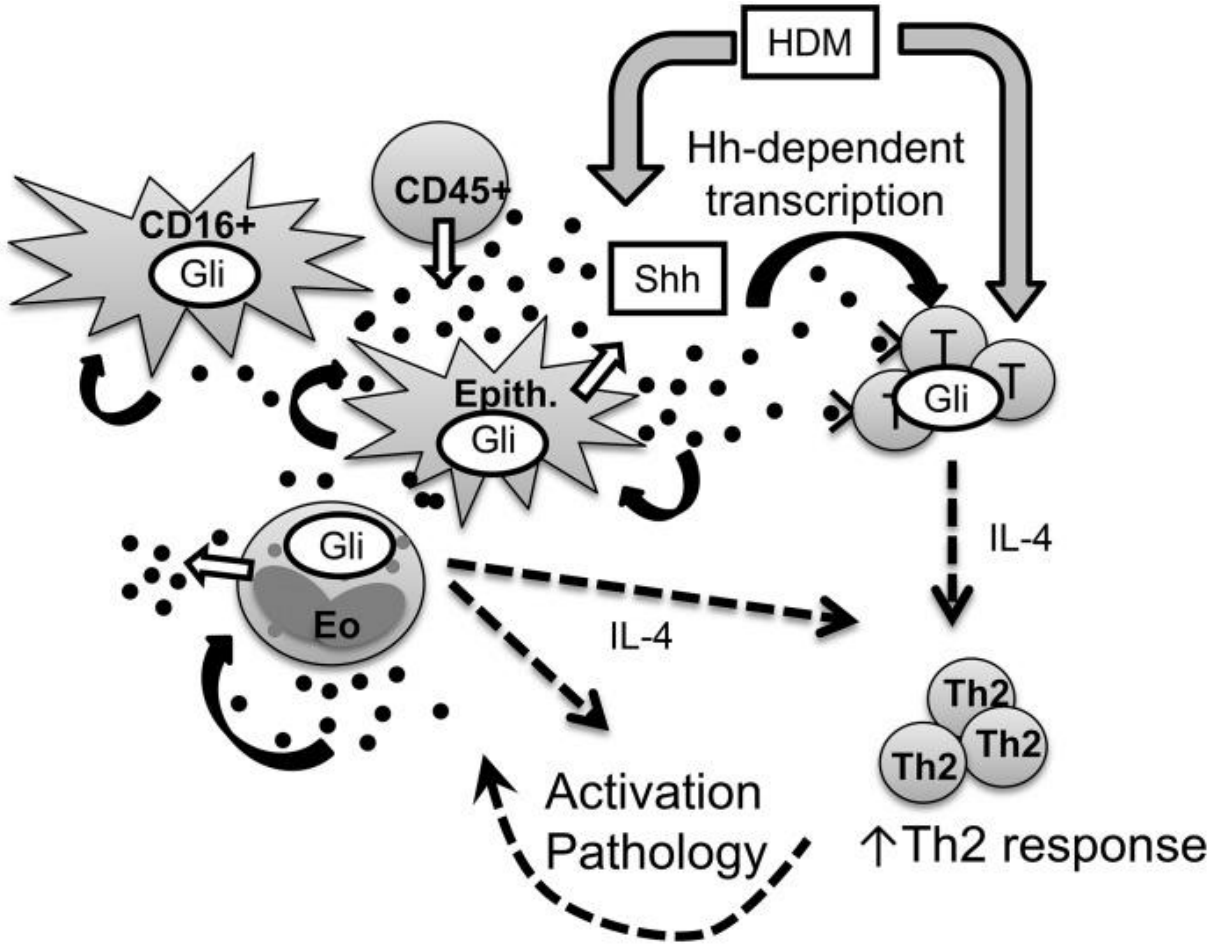

Figure 1.3: Lung cell types that potentiate Th2 responses through the production and response to SHH after allergen inhalation during asthma. HDM; House dust mites; CD16+; Neutrophils/Natural killer cells/monocytes/macrophages; CD45+: Leukocytes; Epith.: Epithelial cells; T: naïve T-cells; Eo: eosinophils (taken from Standing et al., 2017).

More recently, it has been shown that SHh also promotes differentiation of Th2 cells from naïve human CD4 T cells *in vitro* (Yánez et al., 2019), bringing human relevance to previous studies, which were all performed in mouse models. This work suggests that mice and human leukocytes respond similarly to Hh signalling. As the treatment of purified murine eosinophils *ex vivo* with recombinant SHH has already been shown to cause the upregulation of the key Th2 cytokine, IL-4 (Standing et al., 2017), it would be of interest to determine whether human eosinophils upregulated IL-4 expression in response to SHH.

There have been other roles reported for Hh/Gli signalling in asthma immunopathogenesis. SHH signalling has also been shown to induce Epithelial-mesenchymal transition (EMT) in human bronchial epithelial cells (HBECs) following treatment with HDM and transforming growth factor β1 (TGF- β), which leads to *GLI1* translocation and the activation of *Snail1* (E-cadherin adhesion repression protein expression site.) (Zhang et al 2019). Recently, it has been shown that Hh signalling also triggers the release of chemokine ligand 2 (CCL2) from human macrophages (Wang et al., 2019). CCL2 is responsible for the recruitment of monocytes, memory T cells, and dendritic cells to sites of inflammation, such as those of asthma for example. Therefore, the presence of SHH may upregulate the expression of CCL2 in the airway and increase the recruitment of broncho-alveolar leukocytes in asthma, thereby participating in the occurrence and development of asthma. These findings collectively show that there is a significant role for Hh/Gli signalling in asthma immunopathogenesis.

Hh signalling, therefore, can be considered as a potential therapeutic target in allergic disease as several lines of evidence suggest that it is activated during allergic inflammation and that it potentiates Th2 immune responses. Furthermore, a recent paper (published online during the final month of this MSc program) has shown that in murine asthmatic models, the inhibition of SMO causes reduced expression of the Hh pathway in response to inhalation of the occupational allergen papain (Yánez et al., 2021). The paper also demonstrated a decrease in eosinophils when SMO is systemically inhibited. This is significant, as it demonstrates that the eosinophil recruitment, function, and/or the production of eosinophils, is responsive to the Hh pathway. Therefore, further characterisation of the Hh pathway and its effects on eosinophils is required. This recently

published work was only performed on murine models however, and so the human relevance is as yet unknown.

Most of the prior work on Hh in immunity was conducted on T-cells. To understand its role in asthma, and other Th2-driven diseases, we must fully characterise what effect the Hh signal has on all key infiltrating immune cells, including eosinophils. Secondly, most of the work thus far has been conducted in mouse models and the full relevance to humans remains unclear. The main aim of this work is therefore to investigate the role of the Hh signalling pathway in human eosinophil biology. This study will contribute new knowledge to our understanding of the molecular signals involved in the pathophysiology of eosinophil-driven diseases such as asthma.

1.8 In Vitro models of eosinophils

Human eosinophils are difficult to culture. Being a terminally differentiated cell type, they must be harvested from whole blood or plasma for each experiment. This creates ethical concerns, requiring ethical approval to harvest blood from healthy donors. Human Tissue Act (HTA) licensing would also be required to culture, store and manipulate cells. The harvested cells would also being subject to human biological variation.

This project aims to study the effect of Hh signalling on Eosinophils, however it is not easy to obtain a reliable and reproducible source of human primary eosinophils, and the University of Bedfordshire do not hold a HTA license, therefore alternative cellular models of eosinophils were sought.

One alternative is the well documented, commercially available HL-60 cell line (ECACC 98070106; Collins, Gallo and Gallagher, 1977). HL-60 is a promyelocytic leukaemia cell line, comprised of myeloblasts. These myeloblasts are considered multipotent they retain the ability to differentiate into a limited number of cell types. The cell line can be stimulated to differentiate predictably into different leukocyte phenotypes using polar planar compounds such as dimethyl sulphoxide (DMSO), retinoic acid, phorbol myristic acid, and Sodium butyrate. Sodium butyrate is commonly used in literature to differentiate HL-60 myeloblasts into eosinophils over a 10-day culture (Ishihara, 2014). Sodium butyrate causes the histones

of the myeloblasts to become acetylated. This prevents correct DNA folding, altering nuclear superstructures. This allows the specific regions of DNA responsible for eosinophilic differentiation to be expressed, as they are more accessible to transcription factors (Fischkoff et al., 1984).

The HL-60 butyrate-treated cells are not true eosinophils at the end of the differentiation process but are an eosinophil-like cell line that exhibit similar phenotypic properties (size, shape, granularity) and functional capability (EPX degranulation, chemotaxis) that allow the cells to be used as a widely accepted eosinophil model (Fischkoff et al., 1984). However, any work to be done using these cells as a model must first establish how suitable the model is for its use. In the case of this investigation, Hh pathway components must be present, in order to test any effects of Hh signalling on HL-60 cells. The first part of the investigation is to determine exactly this.

2.0 Hypothesis and Aims

2.1 Hypotheses

Prior work into this area has shown that mouse lung eosinophils express Hh signalling molecules, that those eosinophils undergo Gli activation in a mouse model of allergic asthma, and that mouse primary naïve eosinophils produce IL4 in response to SHH (Standing et al., 2017). Thus, I hypothesise that (1) human eosinophils can respond to a Hh signal, (2) that Hh signalling alters cytokine production in human eosinophilic cells, and (3) that Hh signalling could alter eosinophilic cell phenotype and/or other key functions.

2.2 Aims

The aim of this investigation is to assess the effect of Hh/Gli activation and Hh/Gli inhibition on the phenotype and function of human eosinophilic cells. This will be completed in two phases:

1) Establish the HL60-Eos Cell Model

   Objectives:

   - Culture cells with butyrate and assess cell viability
   - Confirm cells adopt eosinophilic phenotype by cytochemistry
   - Confirm cells express Hh pathway molecules by RT-PCR

2) Use the HL60-Eos model to assess phenotype and function of HL60-Eos when cultured with (a) rhSHH or (b) GANT61 Gli inhibitor

   Objectives:

   - Determine rates of cell death/viability in cultures by trypan blue exclusion
   - Assess expression of eosinophil associated and Hh pathway genes by qPCR
   - Measure EPX activity by spectrophotometric assay
   - Measure cytokine production by ELISA

3.0 Methods

3.1 Ethical Approval

Ethical approval was granted by the iBEST local Ethics Committee for the work reported here. (Approval reference: UoB/SLS-iBEST-ERP/LL-02-2019)

3.2 Cell Culture

3.2.1 Standard Cell Culture

The standard HL60 cell line (ECACC 98070106) was resurrected from frozen stock according to supplied protocol as follows: A frozen ampoule of cells was retrieved from liquid nitrogen and thawed quickly in a water bath. Cells were resuspended in 4ml complete RPMI 1640 (Gibco #21875034 supplemented with 20% FBS, 1% penicillin/streptomycin), pelleted and resuspended in complete RPMI containing 10% FBS at a concentration between 1-9x10^5 cells/ml. Cells were maintained at 37°C; 5% CO2 in 25ml flasks with vented lids. Once a stable culture was established, the cells were maintained at 1-3x10^5 cells/ml. These cells were split, and the media refreshed every 3-4 days. Cell number and viability was assessed by trypan blue exclusion counts.

3.2.2 Differentiation of HL60 cells to HL60-Eos

Cells were differentiated from myeloblasts to eosinophilic cells by culturing 3x10^5 cells/ml with 1:1000 Sodium Butyrate (Sigma-Aldrich #B5887-250MG) for 7 days (Fischkoff et al., 1984). Every 2 days, the culture media and Sodium Butyrate was refreshed. Day 7 butyrate cultures were classed as starting point (t=0 hours) samples for experiments, with a starting cell concentration of 6x10^5 cells/ml. On harvesting, samples were centrifuged at 350xg, the supernatant aspirated into a separate tube, and both pellet and supernatant are stored at -80°C. The pellet is stored in 100µL Lysis Buffer (Absolutely RNA miniprep kit, Agilent, #400800).

3.2.3 Cell Culture with SHH or GANT61

HL60 cells were differentiated to HL-60-Eos as in 3.2.2. After 7 days of butyrate exposure, cells were resuspended at 6x10^5 cells/ml in 24 well plates in the presence or absence of 500ng/ml recombinant human SHH (1845-SH/CF, R&D Systems) or 50uM

GANT61 (ab120904, abcam Biochemicals). Samples were taken from cultures 24, 48, 72 and 96 hours after the addition of SHH or GANT61. In preliminary experiments (data not shown), cells were cultured with a range of SHH and GANT61 concentrations, and cell viability counts performed to establish ideal concentrations to use for each molecule.

3.3 Molecular Analysis

3.3.1 cDNA Synthesis

RNA was extracted using a commercially available kit (Absolutely RNA miniprep kit, Agilent, #400800). The cells were first lysed using the lysis buffer in the kit, then the constituents were washed using a range of concentrations of salt buffers and alcohol washed, to separate the RNA from the rest of the cell debris. The isolated RNA was then converted to cDNA using the High-Capacity RNA to cDNA kit (Applied Biosystems) in a thermal cycler (Bio-Rad T100) with the following conditions: 25°C for 10min, 37°C for 120min, 85°C for 5min. DNA concentration was tested post-amplification via micro-volume spectrophotometry (Nanodrop 2000 – ThermoFisher)

3.3.2 RT-PCR

cDNA created in 3.3.1 was subject to PCR with multiple different primer pairs (Table 1) to amplify cDNA from genes encoding: *GAPDH*, *PTCH1*, *SMO*, *GLI1*, *GLI2*, *GLI3*, and *SHH*. Primers were commercially validated Quantitect primer assays (Qiagen, UK) with the exception of *GAPDH* primers, which were as in Fu et al., 2016. The cycling parameters used are as follows: 1) 95°C for 3mins, 2) Denaturation at 95°C for 15sec, 3) Annealing at 58°C for 30sec for *GAPDH* PCR (Table 1) or 60°C for 30 sec for all other primers, 4) Extension at 72°C for 30sec, and 5) Final extension at 72°C for 5 mins. Steps 2-4 were repeated 35 times. The products were then visualised using 2% agarose gel electrophoresis, with 0.1% Ethidium Bromide as an ultraviolet detectable dye. The gel was run for 30mins at a constant 80V.

Table 1: RT-PCR Oligonucleotide DNA Primers used in this study

Gene	Annealing Temp[1]	Source		Sequence (5'-3')
GAPDH	55°C	Fu et al., 2016	F	GACCTGACCTGCCGTCTA
			R	AGGAGTGGGTGTCGCTGT
PTCH1	60°C	Qiagen (Cat. 249900, QT00075824)		Not Provided, Sold Commercially
SMO	60°C	Qiagen (Cat. 249900, QT00050701)		Not Provided, Sold Commercially
GLI1	60°C	Qiagen (Cat. 249900, QT00060501)		Not Provided, Sold Commercially
GLI2	60°C	Qiagen (Cat. 249900, QT00018648)		Not Provided, Sold Commercially
SHH	60°C	Qiagen (Cat. 249900, QT00205625)		Not Provided, Sold Commercially

[1] = Primer annealing temperature selected based on preliminary RT-PCR findings; GAPDH primers were synthesised by Sigma Aldrich

3.3.3 Quantitative PCR (qPCR)

QPCR was performed in triplicate for three independent experiments using primers taken from literature (Table 2) with the following cycle parameters: Hot start at 95°C for 15mins, Denaturation at 95°C for 30s, Annealing at T_a°C for 1 min, Extension at 72°C for 30s, and a final extension at 72°C for 5 mins. Abundance of transcript was quantified by measuring SYBR green fluorescence during extension phases. $\Delta\Delta C_t$ analysis was then performed on the resulting C_t values, to give a fold change in expression between treated and untreated cells. In the first qPCR experiment Gapdh primers were used to amplify a ten-fold serial dilution of HL60 cell cDNA in triplicate to assess operator pipetting technique and efficiency of the housekeeping primers (data not shown).

Table 2: qPCR Oligonucleotide DNA Primers used in this study

Gene	Annealing Temp[1]	Source		Sequence (5'-3')
GAPDH	55°C	Fu et al., 2016	F	GACCTGACCTGCCGTCTA
			R	AGGAGTGGGTGTCGCTGT
GLI1	65°C	Wu et al., 2015	F	CTGGAGCAGCCCCCCCAGT
			R	GGGATGATCCCACATCCTCAGTC
TGF-β	64°C	Xie et al., 2009	F	CCCAGCATCTGCAAAGCTC
			R	GTCAATGTACAGCTGCCGCA
EPX	59°C	Ye et al., 2019	F	CAGGATGCCATGTGAGTGGA
			R	GCTAAGCTGTGCCAAATTCC
IL4	60°C	Qiagen (Cat. 249900, QT00012565)	Not Provided, Sold Commercially	

[1] = Primer annealing temperature selected based on preliminary RT-PCR findings; F = Forward Primer Sequence; R = Reverse Primer Sequence. All primers were synthesised by Sigma Aldrich except *IL4*, which was a Qiagen Quantitect primer assay.

3.4 May-Grünwald Giemsa (MGG) stain

For MGG staining, a sample of the cell culture ($1x10^6$cells/ml) was harvested and then pelleted at 350xg. Most of the supernatant was removed and the pellet was resuspended in around 50µl of the remaining supernatant. 5µl of this suspension was placed towards the frosted end of a Superfrost Ultra Plus (ThermoFisher) microscope slide. Another slide was then used to streak the sample across the slide, forming a thin film. This was then submerged in methanol for 3mins to fix the sample to the slide. Following this, the slide was then placed into the May-Grünwald stain for 5mins. After this, the slide was placed into PBS to wash off remaining stain from the previous step, then placed into the Giemsa stain for 10mins. The slide was then gently rinsed with dH_2O and left to air dry. Once dry, the slides could be viewed under a light microscope (100x).

3.5 Flow Cytometry

10^5 to 10^6 cells were taken from cell cultures, centrifuged at 500g for 5 min. Cell pellets were resuspended in 1x FACS buffer (Gibco PBS pH7.2, 2%FBS, 0.01% Sodium Azide). Samples were then analysed on a flow cytometer (BD Accuri C6). Data were analysed using the BD Accuri software.

3.6 OPD Assay

$1x10^6$ cells were taken from triplicate cultures at 96 hours post SHH/GANT61 treatment, and pelleted. The pellets were resuspended in a solution of 0.05M TrisHCL ph8, with 1mM H_2O_2, 0.2% Triton-x, and 0.1mM o-phenylenediamine (OPD). The absorbance of samples was then measured at A450nm by spectrophotometer after 30mins. A solution as above but without OPD was used to blank the spectrophotometer.

3.7 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed in duplicate for IL4, IL5, TGFβ, and GM-CSF using commercially available kits (Invitrogen/ThermoFisher). Supernatants from cell cultures at 96hr timepoints from SHH and GANT61 experiments (n=3 each) were analysed following the protocol provided in the kits. The absorbance of each plate was measured at 450nm using a spectrophotometer (Multiskan FC, ThermoFisher). Calculation of sample concentrations was performed through the use of a standard curve.

3.8 Statistical analysis

Data were analysed and graphs created using Microsoft Excel. All error bars shown are mean Standard Deviation in both positive and negative directions. Statistical testing used unpaired t-tests. Experiments are n=3 unless otherwise stated. Unpaired t-tests for qPCR data were performed on ddCt data as opposed to Fold Change, to avoid the heteroscedasticity of Fold Change.

4.0 Results: Cell Model Validation

In order to investigate the effect of Hh signalling on eosinophils, an eosinophil model must first be selected. The University is not licensed by the Human Tissue Authority (HTA), thus we are not able to obtain and store or culture primary human cells for research purposes. Ethical permission could have been sought for an alternative source, for example to obtain surplus blood products from the NHS Blood and Transfusion (NHSBT) service, however the NHSBT ceased to offer non-clinical issue surplus products during the pandemic. Collectively this prevented the use of primary eosinophils isolated from whole blood for this project. Therefore, an appropriate commercial human cell line was chosen (HL60). HL60 is a well-documented myeloid leukaemia line that can be differentiated along the myeloid lineage into eosinophil-like cells through the addition of planar compounds. This investigation used Sodium butyrate to selectively differentiate the HL60 cells into an eosinophil-like cell population (HL60-eos).

In order to use the model experimentally, the model must first be valid and meet certain conditions: the cells must exhibit both an eosinophil-like phenotype and genotype, the cells must still be viable at the end of the differentiation period (ensure that the sodium butyrate treatment does not kill the cells), and for our purposes, must express Hh pathway components after differentiation into eosinophils.

4.1 Phenotype of HL60 cells cultured with Sodium Butyrate

HL60 cells were cultured in the presence of sodium butyrate. At intervals during the culture period, cells were taken for phenotypic analysis. Initially, the differentiation process was investigated by cytochemistry using May-Grunwald Giemsa (MGG) staining. MGG is a combination stain, using methylene blue, and other azures to stain basophilic cells and or cell contents, and eosin which stains acidophilic cells and or cell contents. It would be expected, therefore, that the myeloblast cells would stain blueish purple due to their large, dispersed nucleolus, and lack of granules. On the contrary, eosinophils would stain a reddish pink, due to the highly acidophilic nature of the granules. The MGG stain was performed on days 0, 4, 7, and 10 of differentiation. cultures treated with 50µM Sodium Butyrate, and the samples were imaged by light microscopy (figure 4.1).

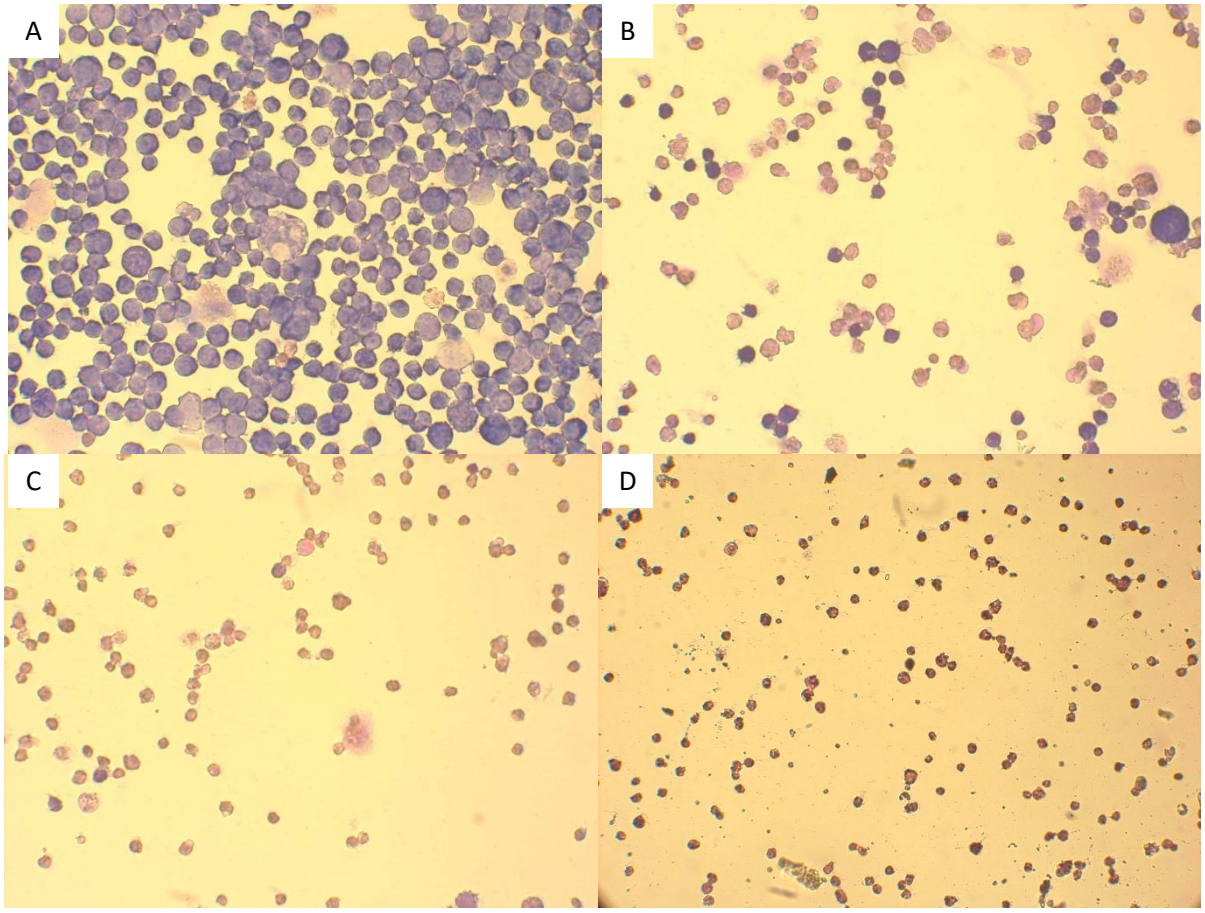

Figure 4.1: Light Microscopy of MGG-stained HL60 cells treated with Sodium Butyrate, at 100x magnification. A) Day 0. B) Day 4. C) Day 7. D) Day 10. Representative of 3 independent experiments.

In figure 4.1A (day 0), the cell population is mostly homogenous, with most of the cells being large and agranular with cells taking on the more bluish-purple methylene blue stain. Figure 4.1B shows a heterogenous mix of cell phenotypes, with a majority of the cells taking on a smaller size and pinker colour. Some of the large purple cells remain, however. By day 7 (figure 4.1C), the cells are almost entirely homogenous, with nearly all cells displaying the pink colour. Figure 4.1D shows a continuation of this homogeneity, with all cells being pink in colour, with none of the larger purple cells present.

This is the first evidence that the cells are an eosinophil-like cell population post-butyrate culture, due to their pinkish stain and smaller size in figure 4.1D when compared to the blast-like undifferentiated cells in figure 4.1A. This is in keeping with previous studies that have used sodium butyrate to differentiate eosinophilic cells (Ishihara, 2014; Fu et al.,

2016). The cell populations' size and complexity were next qualitatively measured by flow cytometry.

The size and complexity of a cell can be measured using the forward and side scatter data generated from flow cytometry. A highly complex cell will scatter more light when passed through a laser due to the large number of intracellular components, whereas a less complex cell will scatter less light. This is measured as light scatter at 90° to the laser beam. A large cell will scatter light more widely when the beam is shone directly at the cell, whereas a smaller cell would have a lower forward scatter profile as it interrupts. These two scatter profiles are measured independently of each other, and the same cell can be measured for both simultaneously and plotted on a scatter graph. This allows distinct populations to be identified by phenotype. In HL60-butryate cultures, an eosinophil-like cell would show a smaller forward scatter (FSC), being that the cells are smaller, and a greater side scatter (SSC), as the cells would be more granular. The inverse would be true for the myeloblast cells.

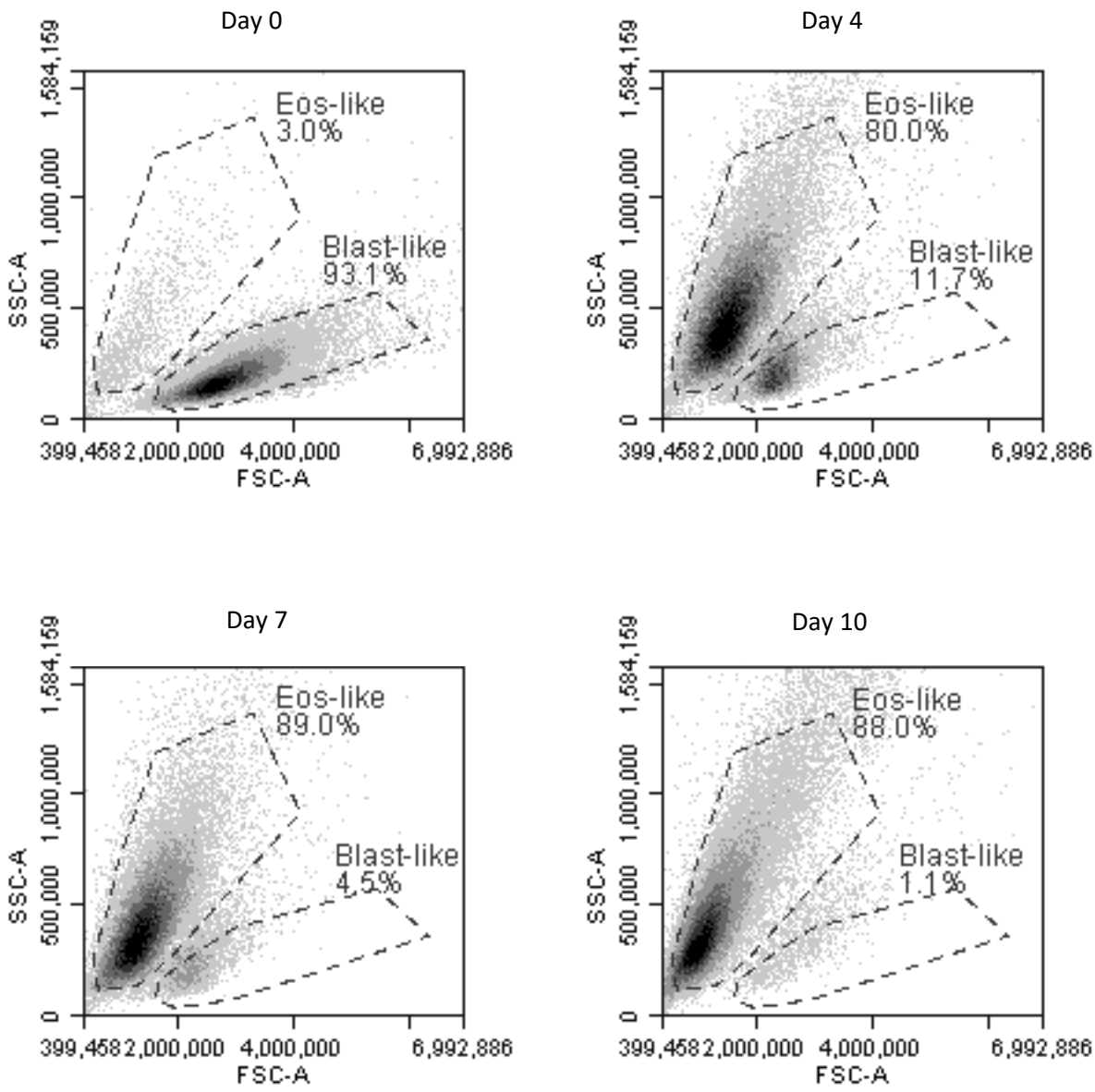

Figure 4.2: Forward scatter (FSC) and Side scatter (SSC) flow cytometry plots of butyrate treated HL60 cells at: A) Day 0, B) day 4, C) Day7, and D) Day 10. Eos-like = exhibits eosinophilic characteristics; Blast-like = exhibits myeloblast characteristics. Gates placed around known likely population centres for each cell type.

Figure 4.2A shows that on day 0, 93.1% of the cell population exists within the "blast-like" gate, with a low complexity (granularity), and a large size. Only 3% of cells express eosinophil characteristics of high complexity and smaller size. By day 4 (figure 4.2B), the

cells have already begun to shift in population, with 80% of cells now showing eosinophil characteristics, and the population of blast-like cells has decreased dramatically to 11.7%. Figure 4.2C continues this trend, with the population of eosinophil like cells further increasing, to 89%, and the blast like cells decreasing to 4.5% as the population nears the homology expected from the differentiation protocol. Day 10 (figure 4.2D) shows a near complete absence of the original blast like population, as only 1.1% of the cells have not differentiated.

4.2 Viability of HL60 cells with Sodium Butyrate

As figure 4.1 shows, through the use of staining, a progression from larger, less granular cells to smaller, more granular cells, and figure 4.2 also shows the same progression through the observation of scatter profiles, the cells are phenotypically eosinophil-like, and homogenous in population. These two characteristics show that the Sodium butyrate treatment does cause the cells to adopt an eosinophilic phenotype, and that the model itself is valid, at least phenotypically. This fits the literature surrounding the use of differentiated HL60 cells as eosinophils (Ishihara, 2014). Following this, it needed to be shown that the butyrate treatment does not lower the proportion of viable cells in the populations. In order to achieve this, trypan blue exclusion cell counts, taken at each time point, were used to calculate any changes in viability (figure 4.3).

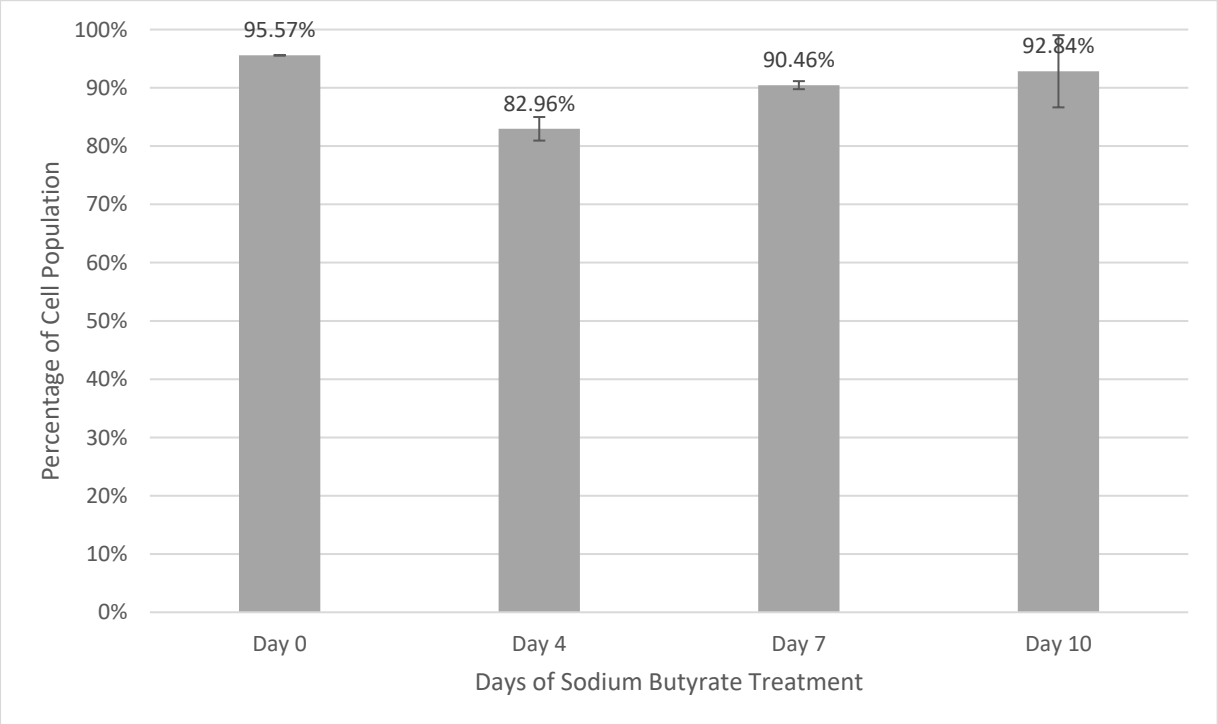

Figure 4.3: Percentage of viable butyrate treated HL60 cells throughout the differentiation process, at day 0, 4, 7, and 10 respectively (n=3). Bars show mean±SD, t-test comparing d0 and d10 p=0.46 (not significant)

Figure 4.3 shows that, although some change is seen in the cell population between day 0 and day 10, the difference is negligible and, more importantly, non-significant (p=0.46). This shows that the cells are not dying due to the butyrate treatment. Having shown this, we can conclude that the butyrate differentiation of HL60 cells into eosinophils is an acceptable model, with regards to phenotype. The next logical step, therefore, was to prove the expression of the Hh pathway components by HL60 cells, as the cells must express these in order to investigate the effects of activating/inhibiting the pathway.

4.3 Hedgehog pathway molecules in HL60 and HL60-eos cells

Having shown the phenotypic validity of the HL60-eos model, the final step was to ensure that the cells do in fact express molecules required for Hh pathway signal transduction. This was achieved through the selective amplification of Hh pathway genes (mRNA, cDNA) through RT-PCR. These genes are: Patched (*PTCH*) and Smoothened (*SMO*), both membrane-bound proteins with *PTCH* being the Hh ligand receptor; and *Gli1-3*, the downstream mediators of the Hh pathway. *SHH* was also tested to see if the ligand is expressed.

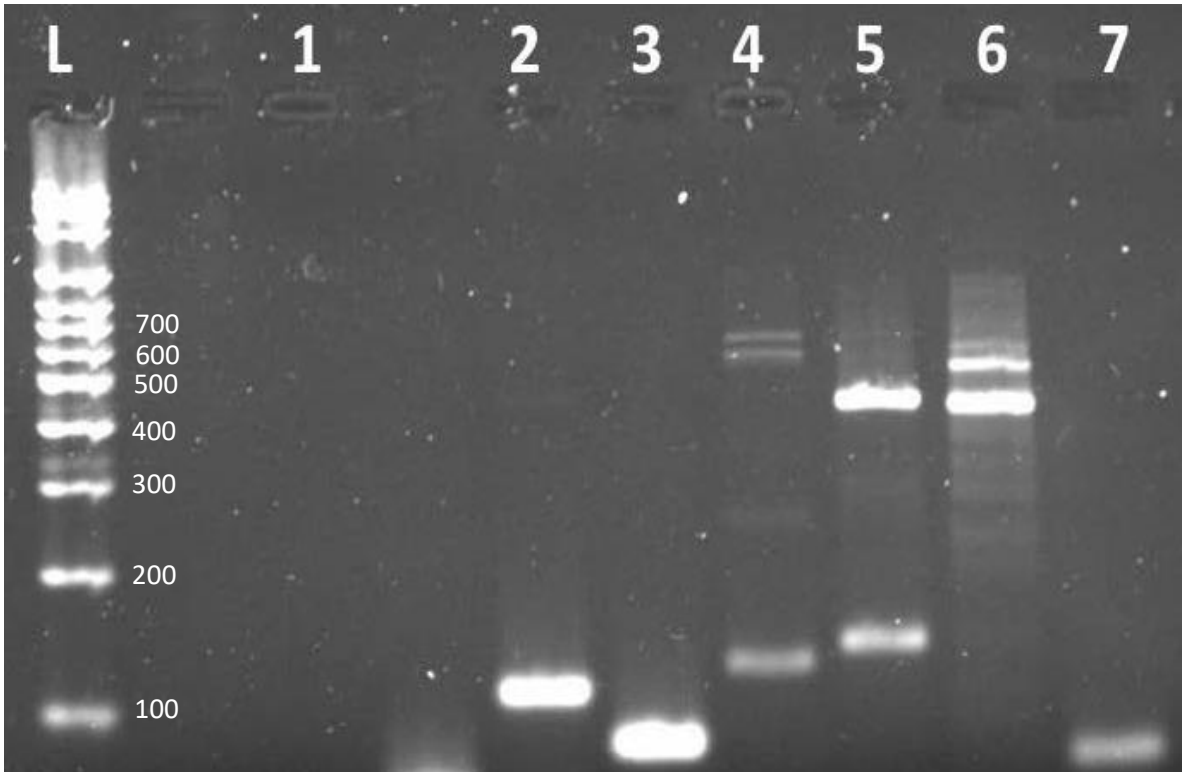

Figure 4.4: Expression of SHH/Gli pathway genes in undifferentiated HL-60 cells. RT-PCR of cDNA from HL-60 cells cultured in standard conditions. L: 100bp ladder; 1: Blank; 2: PTCH (119bp); 3: SMO (89bp); 4: GLI1 (148bp); 5: GLI2 (189bp); 6: GLI3 (79bp expected, not expressed); 7: SHH (89bp)

Figure 4.4 shows expression of all HH/GLI pathway molecules tested, with the exception of *GLI3*. This shows that undifferentiated HL60 cells express molecules required to transduce a Hh signal. Interestingly, *SHH* is also expressed. The HH pathway is also known for maintenance of haematopoietic stem cell niches (Shi et al., 2018), and so this may be related to HL60 being a myeloid leukaemia cell line.

Following this, it was then necessary to ensure that this pathway molecules remain expressed by cells at day 10 of the differentiation process. If the pathway was no longer present by day 10 it would not be possible to investigate any effect SHH/GANT61 might have on the cells. To ensure the pathway remained expressed, RT-PCR was performed using primers specific for *PTCH*, *SMO*, *GLI1*, and *GLI2* genes.

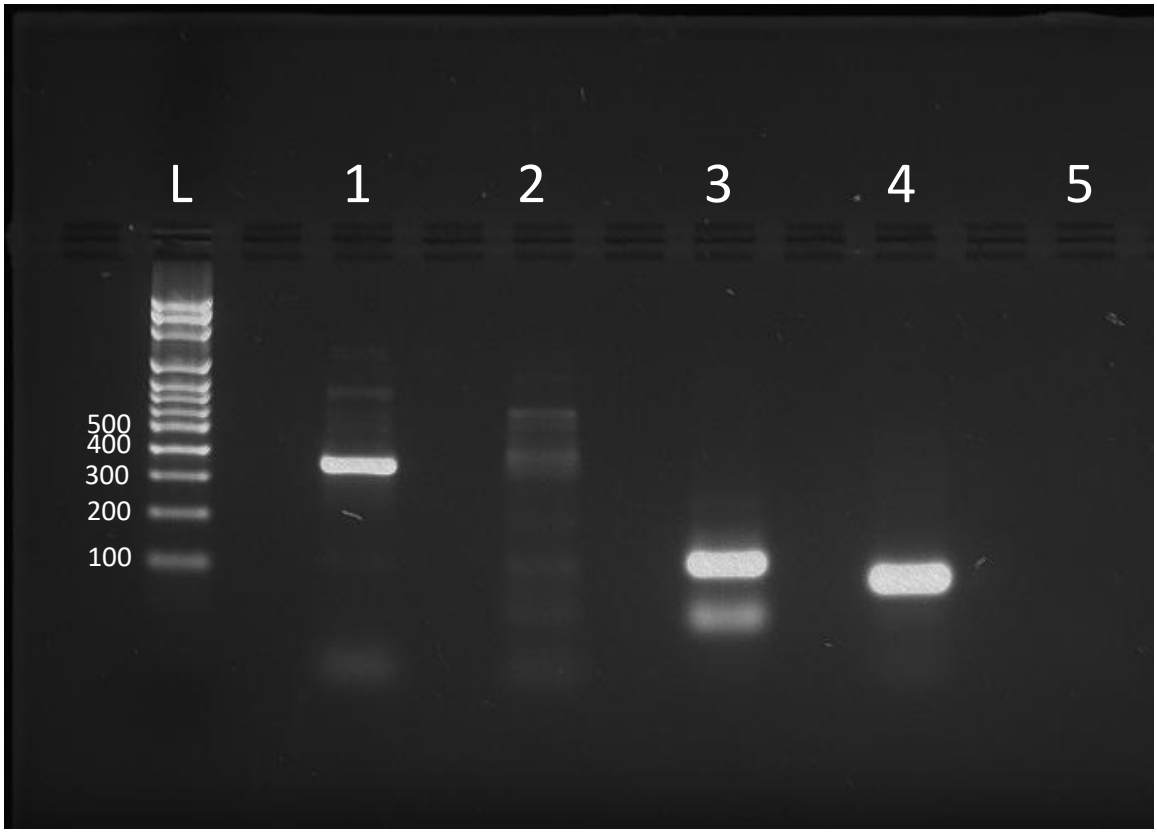

Figure 4.5: Expression of SHH/GLI pathway genes in HL60-eos cells. RT-PCR of cDNA from HL60 cells after 10 days of culture with sodium butyrate (HL60-eos). L: 100bp ladder; 1: GLI1 (344bp); 2: GLI2 (116bp expected, not seen); 3: PTCH (119bp); 4: SMO (89bp)

Figure 4.5 shows strong expression in all the Hh pathway component genes, apart from GLI2. However, *GLI1* is expressed, and it is a major transcriptional activator and is itself a Hh target gene (Carballo et al., 2018). Therefore, the pathway can still be activated and propagate a signal from PTCH to Gli downstream molecules. Therefore, figure 4.5 shows that the Hh pathway genes are still expressed at day 10 of the culture period and that the pathway is present for any investigations on it.

4.4 Conclusion

Since the cells exhibited a change in size and colour over time, shown visually in size comparison and eosin uptake by microscopy in figure 4.1, and the cells also showed a change in size and complexity (granularity) over time shown by scatter profiles in Figure 4.2, the cells can be considered phenotypically eosinophil-like. Following this, the cells were proven to express the HH pathway at both day 0 and day 10, showing that the pathway would be present for any studies performed around day 10 of the culturing with sodium

butyrate. These data, when combined, evidence that the differentiated HL60-eos model is a suitable model for investigations into the HH pathway's effects on eosinophil-like cells.

5.0 Results: Investigation of the effect of Shh signalling on HL60-eos cells

Having shown that the HL60-eos model is a suitable model for *in vitro* eosinophil-like cells, the second phase of the investigation could begin. The aim of this was to assess any changes in cell phenotype or function in the presence of recombinant Human SHH (rhSHH). HL60-eos cells were cultured for 96h with rhSHH, the activator ligand of the Hh pathway. SHH binds to PTCH, triggering a downstream signal cascade, resulting in the Gli-dependent gene expression. 96h was chosen as the endpoint for the experiment as this should be sufficient time for a Hh signal to be received and transduced, and for gene transcription and protein production to have occurred.

5.1 Viability of HL60-eos cultured with SHH

As it was unknown what effect adding rhSHH will have on the HL60-eos cells, the first part of the characterisation process was to check that the cells survive the SHH treatment. The hypothesis for this is that the presence of SHH would not cause cell death, as eosinophils encounter +SHH conditions *in vivo* without triggering cell death. This investigation was performed via trypan blue exclusion staining. Trypan blue is excluded from viable cells by the cell membrane, whereas non-viable cells are stained blue due to the permeable membrane, hence the cell viability can be measured. Viability is calculated as the number of unstained cells as a percentage of the total number of cells counted.

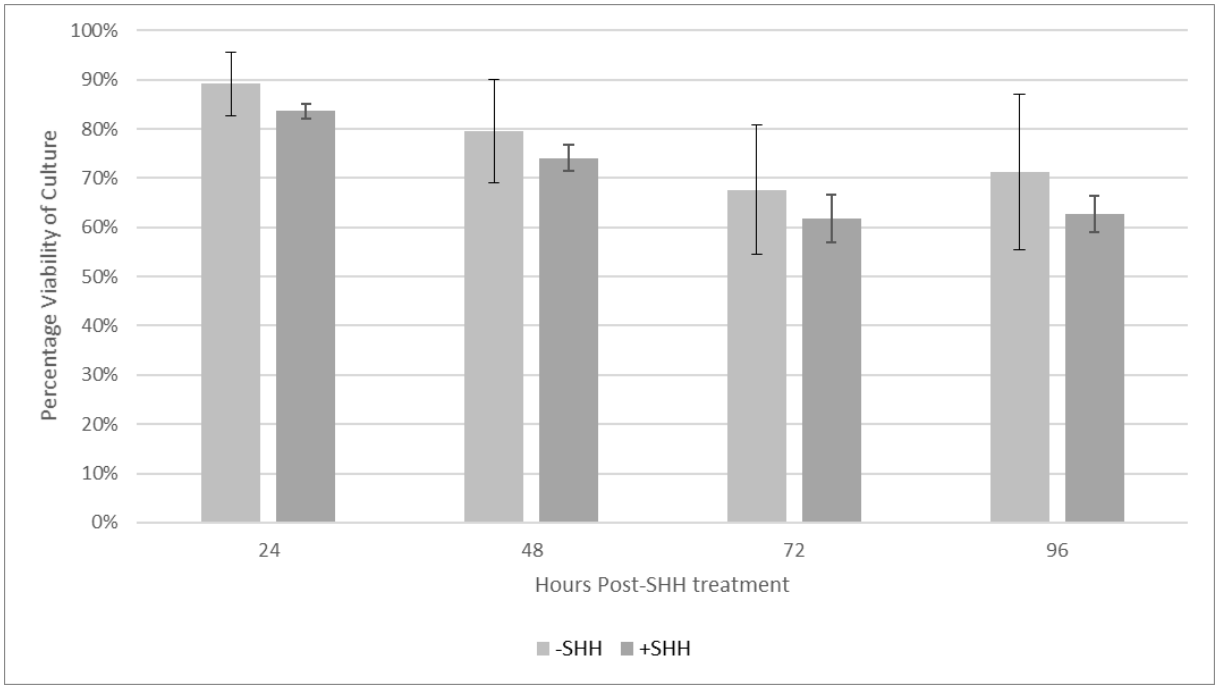

Figure 5.1: Trypan Blue exclusion cell viability counts of HL60-Eos cultures at 24, 48, 72, and 96hr of culture in untreated (-SHH) and SHH-treated (+SHH, 500µg/ml) conditions (n=3). T-tests conducted at all timepoints to compare -Shh to +Shh conditions were not significant (n/s) (p>0.05).

Figure 5.1 shows the proportion of viable cells within the +SHH culture decreases by 21% between 24 hours and 96 hours post-SHH treatment. Similarly, the proportion of viable cells in untreated cultures shows a downward trend, decreasing by 18% between 24hrs and 96hrs. This figure demonstrates that, despite a decrease in viable cell population being shown, there is no catastrophic loss of cell population during the culture process in either experimental condition. The difference between control and SHH-treated cells at 96hrs shows a marginal decrease in the mean number of viable cells from 71.18% in untreated cultures to 66.61% in +SHH cultures. This was determined to be non-significant (p=0.07) via a two-tailed T test. Therefore, the proportion of cells that are viable are comparably the same across both conditions, and that the addition of SHH has no significant effect on the number of viable cells.

5.2 Morphology of HL60-eos with SHH

As figure 5.1 shows that the +SHH treated cells do not undergo a decrease in viability over time compared to untreated cells and confirms that the cells are as viable in +SHH

conditions as they are in -SHH conditions, the investigation into the downstream effects of SHH signalling in these cells could continue. This began with investigation into the cells' morphology. The morphology of the cells in both +SHH and -SHH conditions were compared using the MGG stain described in Chapter 3.4 (Figure 5.2).

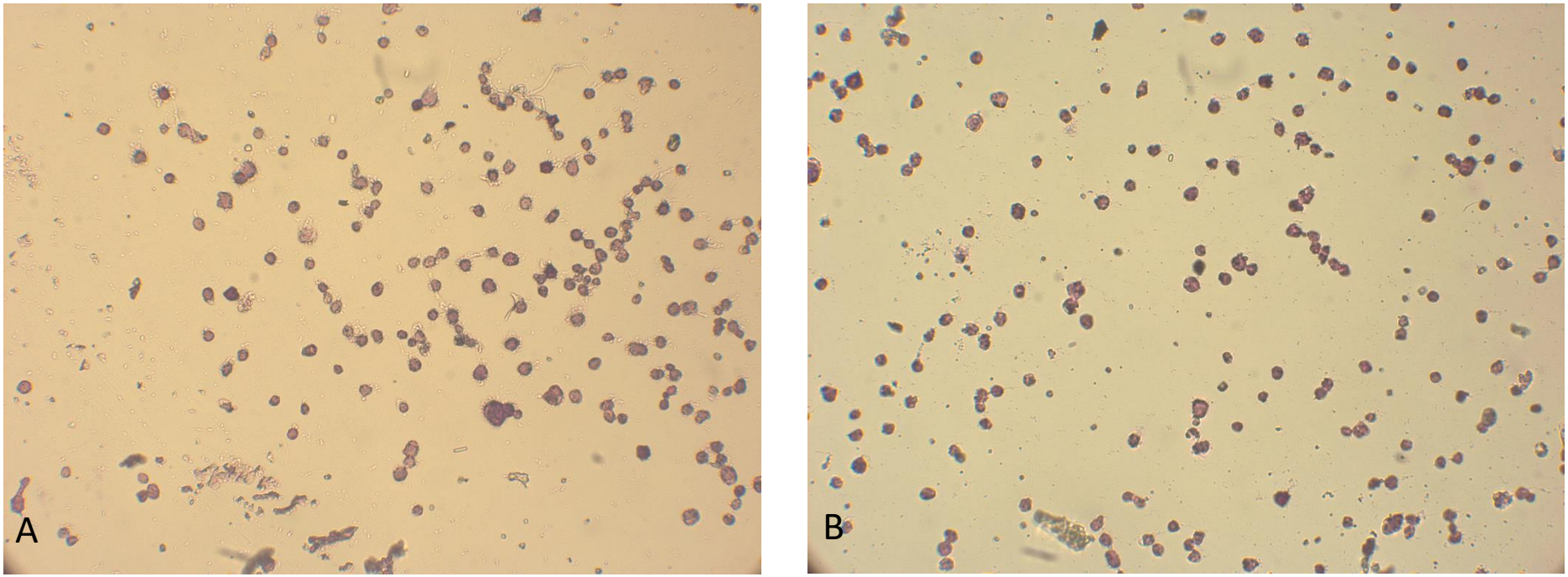

Figure 5.2: Light Microscopy of MGG-stained HL60-eos after 96 hours in -SHH conditions (A), and +SHH conditions (500µg/ml) (B), at 100x magnification. Representative of 5 independent experiments.

Figure 5.2 shows that both SHH treated and untreated cells are similar in morphology at 96 hours, showing similar degrees of homology in both size and colour. Both cell populations were smaller cells than is seen in HL60 at day 0 (Fig. 4.1), with a pinkish colour, showing the expected intake of eosin. These images are reflective of 5 independent experiments that all showed the same morphology. Therefore, we can say that SHH has little effect on cell morphology, at least that is visible to light microscopy.

5.3 Activation of Hh signalling in HL60-eos with SHH

To confirm that Hh signalling had occurred in the +SHH cultures, qPCR was performed with primers specific for human Glioma-associated oncogene 1 (*GLI1*). *GLI1* was chosen as it is a downstream mediator of the Hh pathway, being c-terminally truncated when the pathway is active to form the activator form, Gli1A. *Gli1* is also itself a target gene and therefore an amplifier of the Hh signalling pathway (Ruiz i Altaba, 1997), so *GLI1* expression should be increased in cells undergoing active Hh signalling. As expected, *GLI1* was upregulated in HL60-eos following 96h treatment with SHH (Figure 5.3). All qPCR experiments were performed in triplicate, and this was repeated for three independent experiments. Gene expression is expressed relative to *Gapdh*, a housekeeping gene, and data were analysed using the delta-delta Ct method. Data are presented as fold change between control (-SHH, set to '1') and SHH-treated (+SHH) for each independent experiment.

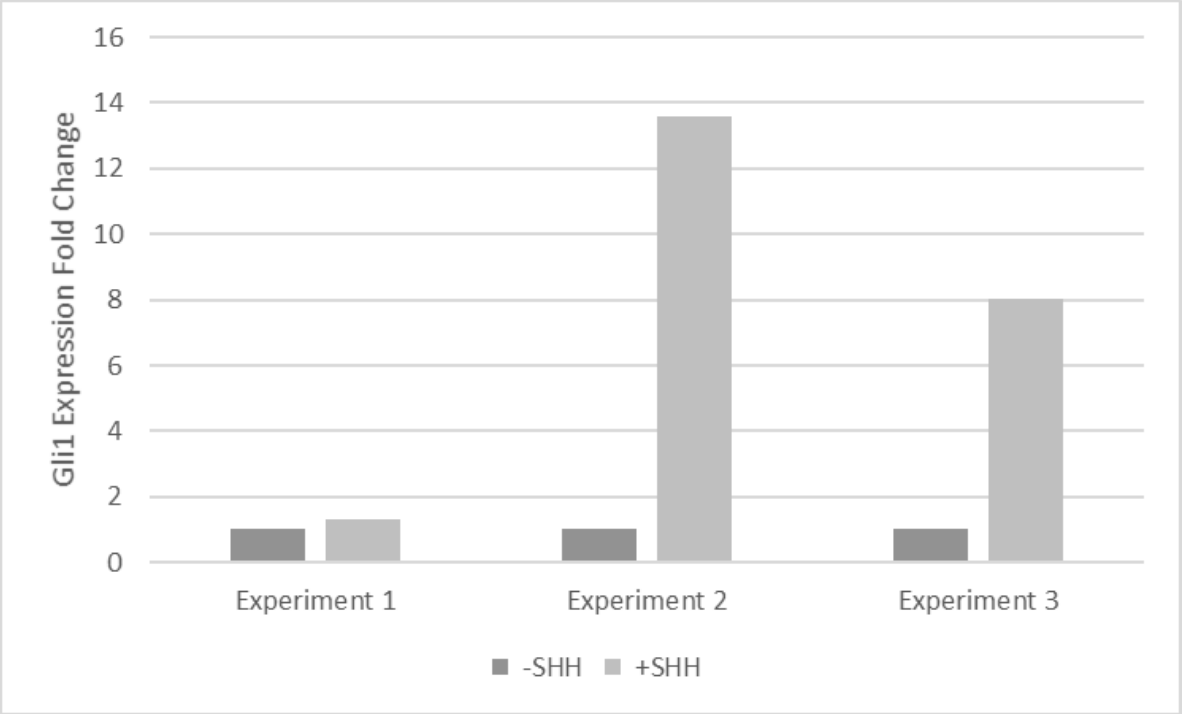

Figure 5.3: GLI1 Gene Expression fold changes in Shh-treated cultures relative to control cultures for three independent experiments. Gli1 expression was analysed relative to Gapdh expression and was determined by ΔΔCt analysis. HL60-Eos cells were cultured with (+SHH, 500µg/ml) and without SHH (-SHH). The experiment was performed three times independently (Experiments 1, 2 & 3) and each qPCR experiment was performed in triplicate. Each experiment is shown independently for transparency.

Figure 5.3 shows a large increase in *GLI1* gene expression for both experiments 2 and 3 (13.5-fold and 8-fold respectively), however experiment 1 shows only a marginal increase in GLI1 expression. While these results aren't significant (Unpaired t test, -SHH ddCt vs +SHH ddCt: p=0.14), there is a strong trend towards *GLI1* expression being increased in the presence of SHH, and given additional repeats, it is likely that the results would become significant, as experiment 1 is likely anomalous. This data suggests that Hh signalling has occurred in the +SHH cultures.

5.4 Expression and Production of Cytokines by HL60-eos with SHH

The next step was to investigate whether SHH treatment induces changes in gene expression within the cells, starting with IL4. IL4 is a cytokine that triggers differentiation of Th0 cells into Th2 cells and is a known mediator of pro-inflammatory functions during asthma and other respiratory diseases (Rao and Avni, 2000). Eosinophils are known to

produce IL4 (Bjerke et al., 1996) and IL4 is also known to upregulate VCAM-1 expression, leading to the recruitment of additional eosinophils into the extracellular space around the site of inflammation (Iademarco, Barks and Dean, 1995). Our previous work showed that *IL4* is a target gene of Hh signalling in T cells (Furmanski et al., 2013), and that *IL4* is upregulated in mouse primary eosinophils following culture with SHH (Standing et al., 2017). We can therefore hypothesise that *IL4* is also upregulated in human eosinophils by Hh signalling. If this hypothesis is proven, we would then further hypothesise that SHH in the lung in asthma is driving eosinophils to produce IL4 and further exacerbate the Th2 response. Therefore, HL60-eos cells were treated with SHH for 96h and expression of *IL4* was assessed by qPCR using primers specific for the human *IL4* gene (Figure 5.4). No significant difference was seen in IL4 expression between untreated and SHH-treated cultures (Figure 5.4a, p=0.33), however there was a trend towards higher *IL4* expression in both experiments. The experiment was conducted three times but *IL4* was not detectable in one of the experiments. As there was variability in the results, IL4 protein expression was assayed in cell culture supernatants at 96h. This was performed by ELISA (Figure 5.4b). ELISA showed that the levels of IL4 produced were very low, with no significant difference in IL4 concentration in Shh+ compared to untreated cultures (Unpaired T test, -SHH concentration vs +SHH concentration: p=0.49). Similarly, the error bars are large indicating variable results, which makes this difficult to interpret. This is likely due to the data being extrapolated from below the standard curve as the level of IL4 in the supernatants was barely detectable.

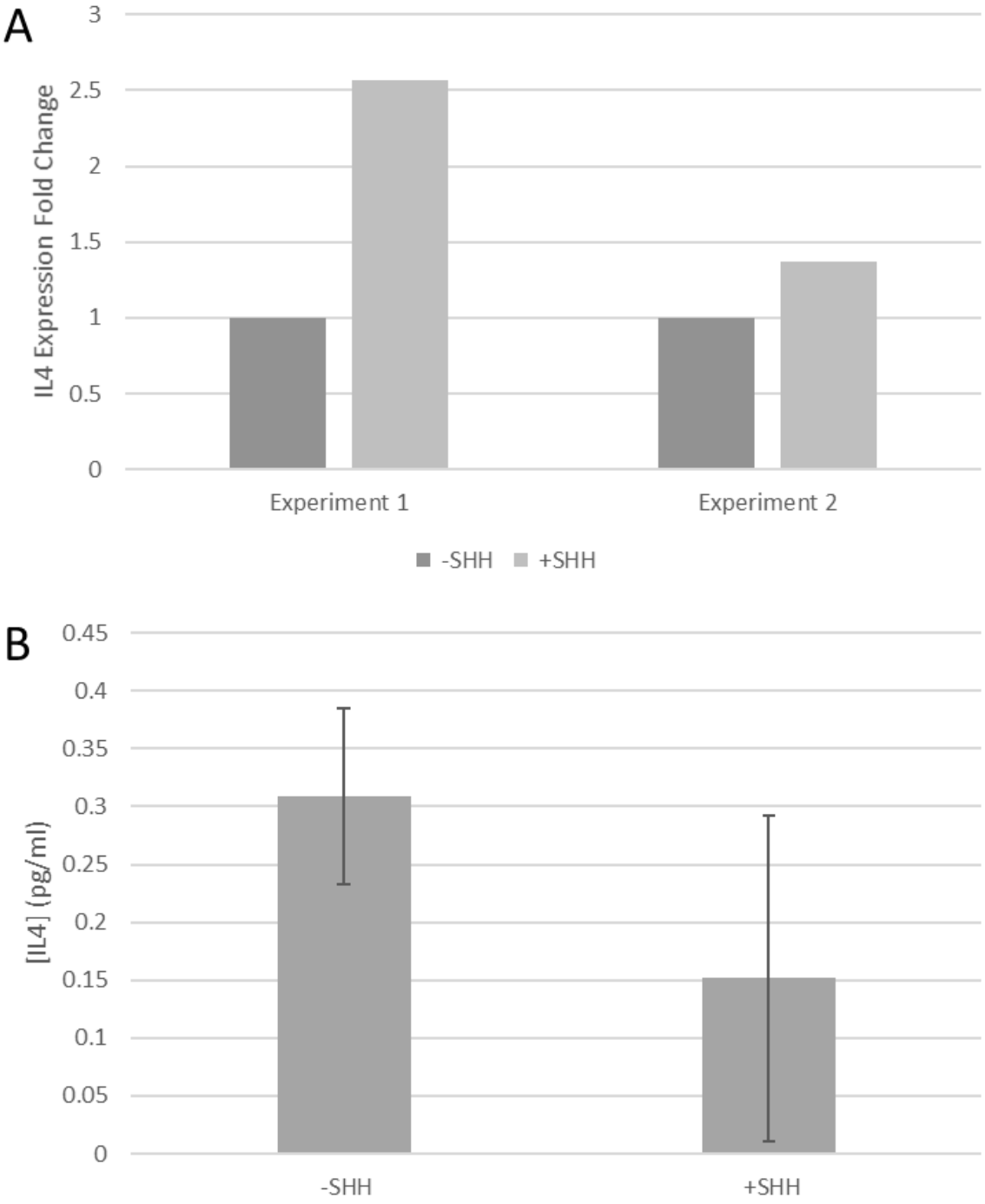

Figure 5.4: Interleukin 4 expression in HL60-eos cells. A, qPCR, IL4 Gene expression fold change relative to Gapdh expression determined by ΔΔCt analysis, performed three times independently (Experiments 1, 2, and 3; 3 not shown as no data) and each qPCR experiment was performed in triplicate. B, Secreted IL4 protein analysis via ELISA, performed in duplicate for each of three experiments. HL60-Eos cells were cultured with (+SHH, 500µg/ml) and without SHH (-SHH) for 96hrs, p>0.05 n/s.

Next, expression of the cytokine TGF-β was investigated. TGF-β is a multifunctional cytokine expressed by eosinophils, among other cells (Minshall et al., 1997). It is responsible for T cell regulation through: the induction of regulatory T cells (Yoshimura and Muto, 2010), the secretion of pro-inflammatory cytokines from T cells, the regulation of other lymphocytes, and in higher concentrations apoptotic effects on granulocytes (Letterio and Roberts, 1998). Furmanski et al. (2013) showed via microarray that TGF-β family member genes are upregulated during active Gli-dependent transcription in CD4+ T-cells, and so there is already some evidence that Hh pathway activation can potentially lead to increased TGF-β expression in immune cells. It has also been shown that TGF-β is increased in airways of people with allergic asthma (Doherty and Broide, 2007) and that eosinophils can produce TGF-β (Ohno et al., 1992). Therefore, HL60-eos cells were treated with SHH for 96h and expression of *TGF-β* was assessed by qPCR using primers specific for the human *TGF-β* gene (Figure 5.5a). A significant difference was seen in *TGF-β* expression between untreated and SHH-treated cultures (Unpaired t test, Control ddCt vs +SHH ddCt: p=0.045) with up to a 7-fold increase in two of three experiments. However, there was variability between the independent experiments with experiment 1 showing a smaller increase. Following the significant increase, TGF-β protein expression was assayed in cell culture supernatants at 96h by ELISA (Figure 5.5b). ELISA showed no significant difference in TGF-β concentration in +SHH conditions compared to untreated cultures (Unpaired T-test, -SHH concentration vs +SHH concentration: p=0.95). The data shows that whilst TGF-β is expressed in both untreated and SHH-treated cells, there is no difference in the levels of protein produced.

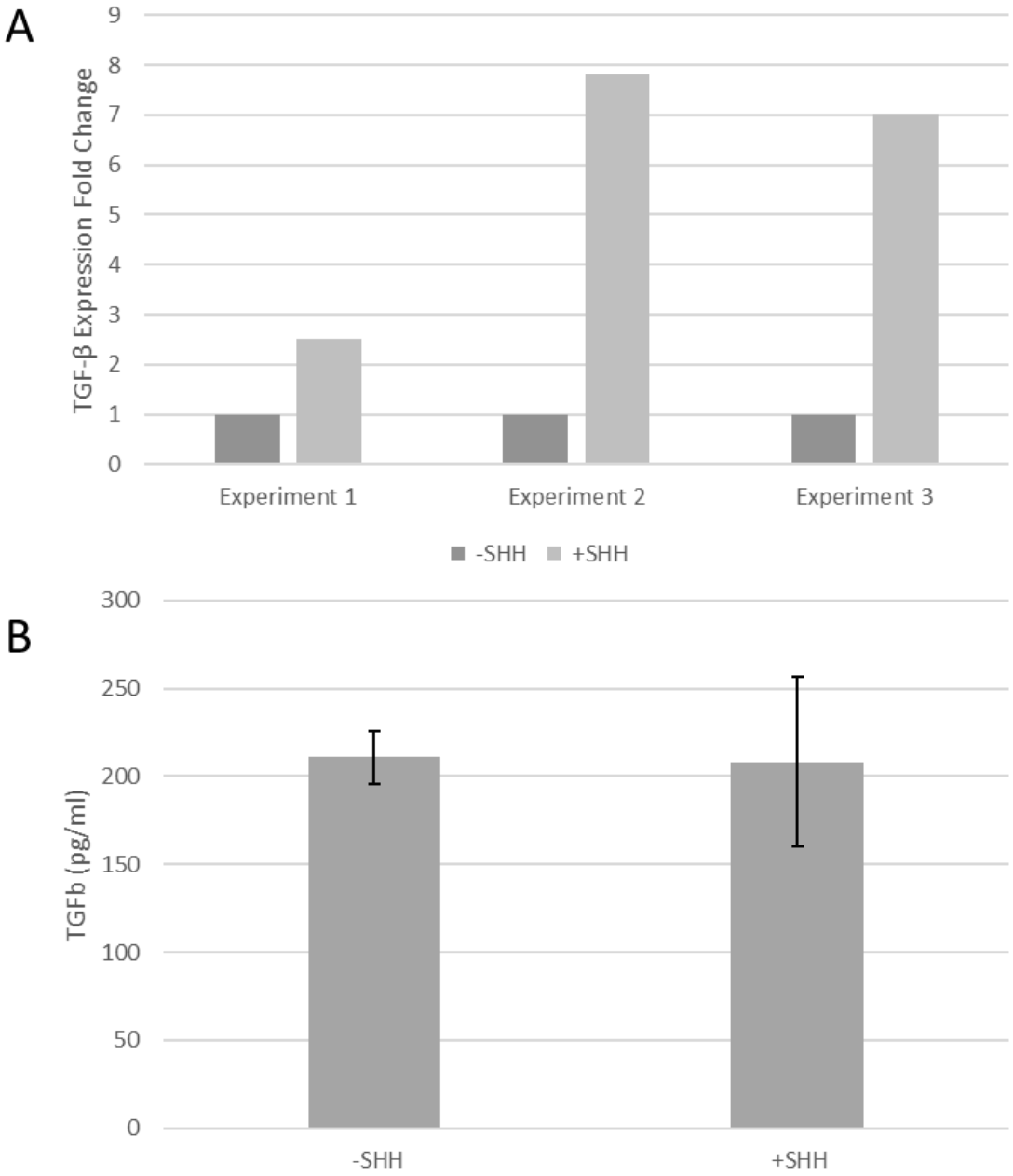

Figure 5.5: Transforming Growth Factor Beta expression in HL60-eos cells. A, TGF-β Gene expression fold change relative to Gapdh expression and determined by ΔΔCt analysis, performed three times independently (Experiments 1, 2 & 3) and each qPCR experiment was performed in triplicate; B, Secreted TGF-β protein analysis via ELISA, performed in duplicate for each of three experiments. HL60-Eos cells were cultured with (+SHH, 500µg/ml) and without SHH (-SHH) for 96hrs, p>0.05 n/s.

The lack of change in TGF-β protein expression was not expected, considering the significant increase in gene expression. It may be that the experiment looked in the supernatant for

TGF-β protein too early or too late during the time course. However, this could be the first evidence that suggests that SHH has a 'priming' effect on the eosinophils, in that SHH could prepare the cells' gene expression profile for TGF-β production. The cells would likely release stored cytokine in response to secretagogues such as IL5. A further study would have to be done to test whether HL60-eos cells would actively secrete more TGF-β after culture with SHH in the presence of an eosinophil activator such as IL5.

Following the investigation into TGF-β, the supernatant concentration of two other cytokines was tested, GM-CSF and IL5. GM-CSF is a protein that stimulates proliferation and maturation of multiple different cell types, most relevant of which is eosinophils (Woolley et al., 1994). It has been shown to stimulate leukocytes to produce pro-inflammatory cytokines, and increased concentrations of GM-CSF has been shown to promote inflammation in asthma, as well as arthritis and other inflammatory diseases (Nobs, Kayhan and Kopf, 2019). The rationale behind the investigation of GM-CSF is that it promotes the production of granulocytes from stem cells, and importantly, that the gene for GM-CSF (*CSF2*) is upregulated by Gli2-dependant transcription in CD4+ T-cells (Furmanski et al, 2013). IL5 is a key mediator in eosinophil activation (Fabian et al., 1992; Stein et al., 2008). Therefore, we investigated whether GM-CSF and/or IL5 production was increased in HL60-eos in the presence of SHH at 96h by ELISA. Neither GM-CSF nor IL5 were detectable in supernatant ELISA, with all samples showing no protein present (data not shown). This is not an error of the ELISA method, as the standard curve still formed correctly, therefore, this can be attributed to the cells not secreting IL5 and GM-CSF into the supernatant during culture, or just not producing the protein at all. This is possibly due to the cells being unstimulated/resting during culture, and so will remain sedentary with regards to degranulation and the secretion of some of the cytokines. This seems likely given IL4 was also detected only at very low levels. Stimulation of the cells could be achieved through the addition of an eosinophil activator or secretagogue such as IL5 and/or C5a, PAF, for example. Another explanation could be that any secreted protein is sequestered by the cells themselves. For example, eosinophils are well known expressors of the IL5 receptor, IL5R. This would mean that, for lower concentrations of protein, the presence of IL5 would be undetectable as it could all be bound to the cells' IL5 receptors. Similarly, supernatants were

stored at -80°C for several weeks before analysis, so it could be that the freezing and/or thawing caused denaturing of the cytokines.

5.5 Expression and Production of Eosinophil Peroxidase (EPX) by HL60-eos with SHH

A major function of eosinophils is degranulation and release of potent cytotoxic enzymes including eosinophil peroxidase (EPX). EPX is found within the granules of eosinophils, or in the intercellular space following their degranulation. EPX is utilised by the eosinophil during degranulation to destroy cell surface membranes, through the creation of hypohalous acids such as Hypobromous acid (Dalen and Kettle, 2001). These acids are oxidising agents and will rapidly destabilise lipid membranes on contact. This is used to destroy pathogens such as helminths in normal conditions but also triggers airway remodelling during asthma and other respiratory diseases (Wang and Slungaard, 2006). We were interested in whether SHH treatment affected the level and function of EPX. EPX should be pre-formed in the cells as it is a hallmark of their eosinophilic identity. Therefore, HL60-eos cells were treated with SHH for 96h and expression of *EPX* was assessed by qPCR using primers specific for the human *EPX* gene (Figure 5.6). SHH-treated HL60-eos cells showed a significant increase in *EPX* expression, with an 8-fold mean increase in expression when compared to the -SHH conditions (Unpaired t test, Control ddCt vs +SHH ddCt: p=0.022). This is therefore statistically most likely due to the addition of SHH. This is, at the time of writing, the first time increased expression of *EPX* in response to a SHH signal has been shown in eosinophil-like cells.

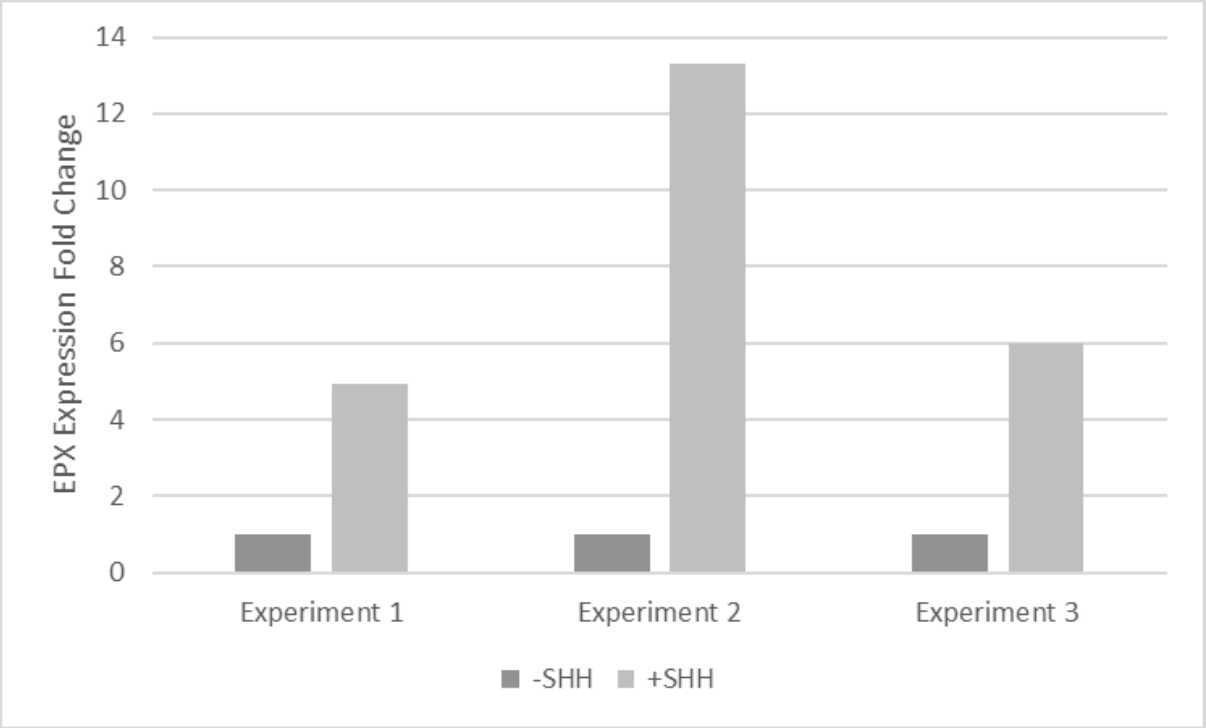

Figure 5.6: EPX Gene Expression fold changes in SHH-treated cultures relative to control cultures for three independent experiments. SHH expression was analysed relative to Gapdh expression and was determined by ΔΔCt analysis. HL60-Eos cells were cultured with (+SHH, 500µg/ml) and without SHH (-SHH). The experiment was performed three times independently (Experiments 1, 2 & 3) and each qPCR experiment was performed in triplicate. Each experiment is shown independently for transparency.

Following this significant upregulation at 96h, we looked earlier in the SHH treatment culture process, to determine when the expression of *EPX* increases (Figure 5.7). A gradual increase in the cells' expression of *EPX* was expected. Figure 5.7 shows that the gene expression levels of *EPX* increase over time, starting with a large decrease at 24 hours to 14% of original expression levels, and show a rising trend with expression peaking in figure 5.8 at 72 hours with a fold change of 2.24. The decrease in expression seen at 24 hours was not expected. However, SHH is a morphogen, meaning that its effect on a cell is based on the concentration available to the cell, and that differing concentrations will have different effects (Roelink et al., 1995). In this case, it could be possible that at 24 hours, the first of the SHH is bound to the HL60-eos cells, causing a decrease in *EPX* expression. As more SHH is bound over time, the expression of *EPX* would increase accordingly, as seen in figure 5.7.

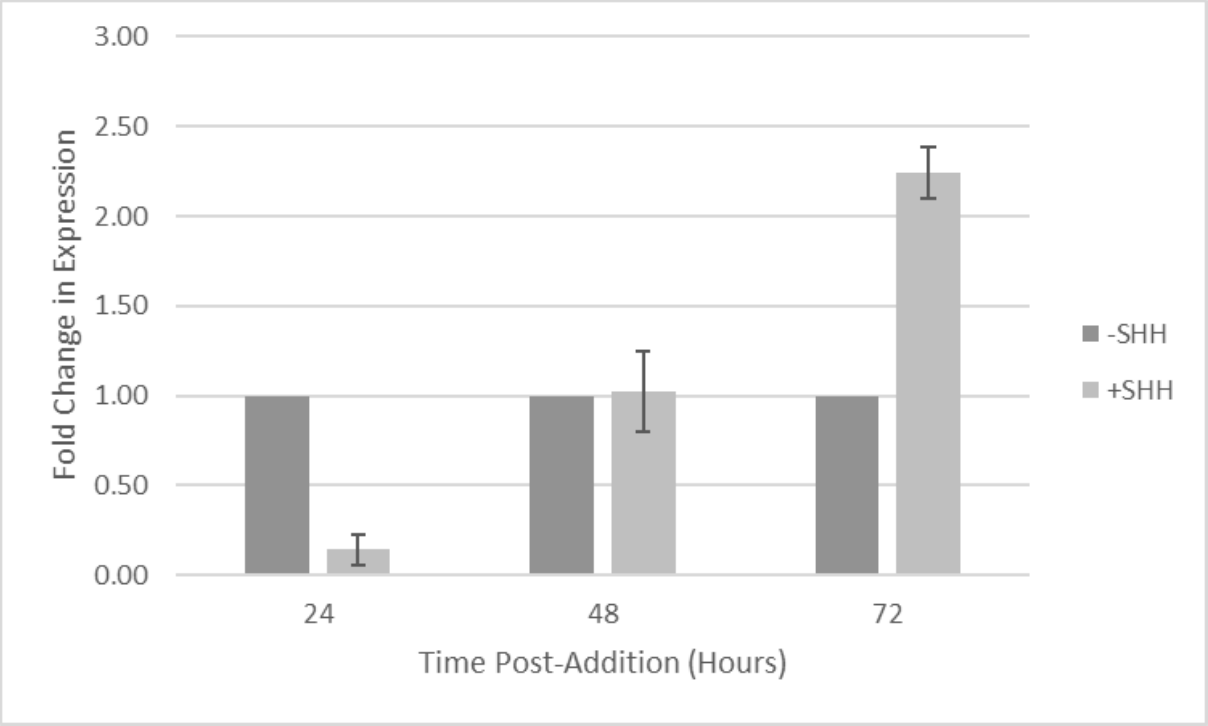

Figure 5.7: EPX Gene Expression mean fold changes in SHH-treated cultures relative to control cultures (set at '1' for each experiment) at earlier time points. SHH expression was analysed relative to Gapdh expression and was determined by ΔΔCt analysis. HL60-Eos cells were cultured with (+SHH, 500µg/ml) and without SHH (-SHH) for 24, 48, and 72 hrs. Each qPCR experiment was performed in triplicate.

If *EPX* expression is upregulated by SHH treatment, then it is possible that the production of the Eosinophil peroxidase enzyme itself is upregulated by SHH signalling. This enzyme is one of the major sources of cellular damage in asthma, so this finding has significant implications for eosinophil biology in the context of asthma, where we know Hh signalling is active. In order to determine whether SHH also increases the expression of Epx enzyme, functional peroxidase studies are needed, which was our next step. To do this, an enzyme-linked colorimetric assay was performed on HL60-eos cell lysates following culture with or without SHH. By using OPD as the substrate, the fold change in peroxidase activity could be measured, allowing for a proxy measurement of the total fold change in peroxidase expression within the granules (Figure 5.8). Figure 5.8 shows no significant change in peroxidase activity (Unpaired T-test, -SHH peroxidase activity vs +SHH peroxidase activity: p=0.15) between -SHH conditions and +SHH conditions. This means that the SHH treated HL60-eos cells do not contain higher levels of pre-formed peroxidases than cells cultured in -

SHH conditions. This is contrary to what is seen in figure 5.6, which shows a significant increase in *EPX* expression (p=0.022). However, the assay is not specifically evaluating EPX activity but assays peroxidase activity in general. Other peroxidase expression, and therefore activity, could remain unchanged in the presence of SHH. Another explanation is that as the proteins are premade and stored in the granules for later release, it is possible that as SHH is added at day 7, there may be little to no effect on the pre-existing granules. Finally, the reaction progresses incredibly quickly, and therefore may not be sensitive enough to determine subtle differences. This assay saturation could be reduced by lowering the concentration of OPD or reducing the number of cells lysed as part of the assay.

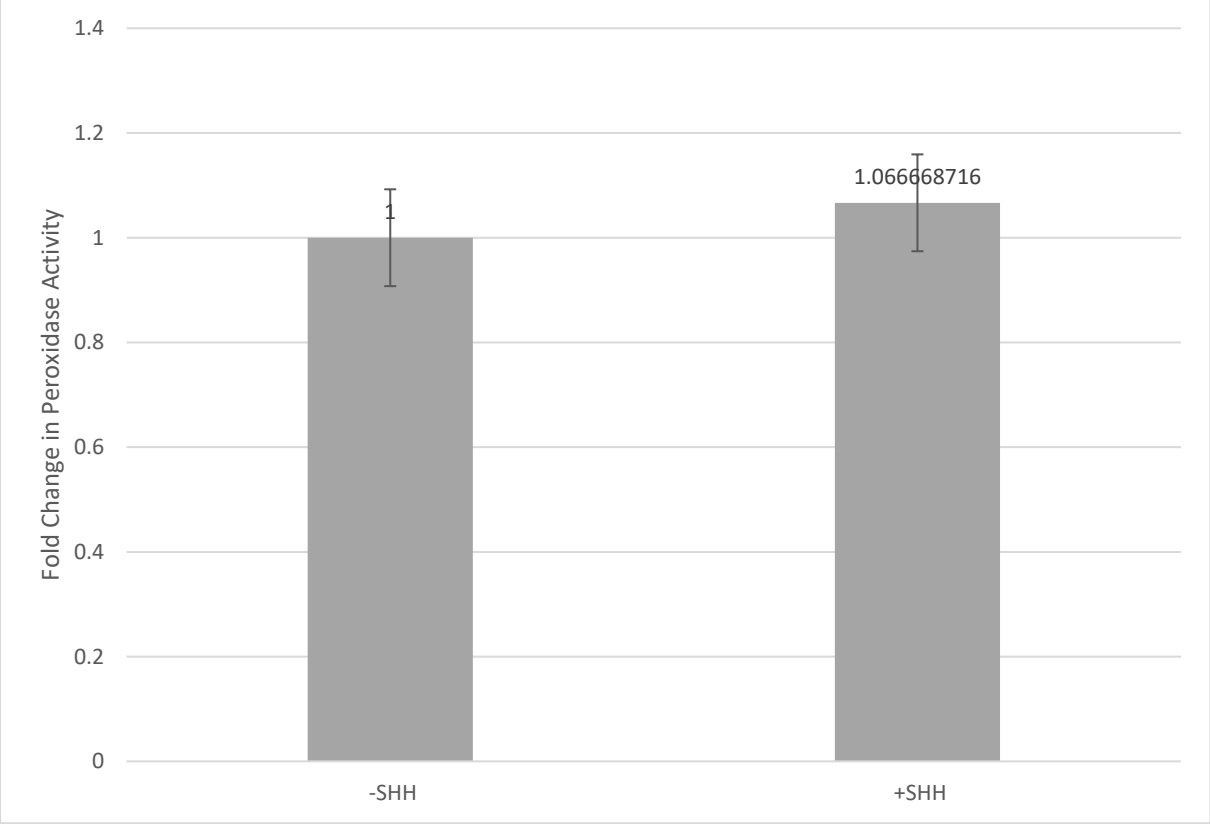

Figure 5.8: Fold change in peroxidase activity determined from OPD assay. HL60-Eos cells were cultured with (+SHH, 500µg/ml) and without SHH (-SHH) for 96 hours, before stimulation with 4µL Cell Stimulation Cocktail (eBioscience, Cat# 00-4975-93) for 1 hour before spectrophotometric analysis at 420nm, n=3, p>0.05 n/s.

5.6 Conclusion

This study has found that, in the presence of SHH, *TGF-β* and *EPX* genes are significantly upregulated (p=0.045 and p=0.022 respectively), with *GLI1* showing a large positive trend (mean fold change increase in Gli1 = 7.6, p=0.14). *IL4* gene expression was not significantly different in the presence of SHH (p=0.33). As these results suggest that EPX is upregulated in response to SHH, then the potential application for this would be a PTCH-binding ligand that competitively prevents the binding of SHH to PTCH, or an anti-SHH antibody that would prevent binding through lowering the concentration of SHH at the site. A simpler method of inhibition would be a SMO inhibitor, which Yanez et al. (2021) have recently shown to decrease SHH signalling and Th2 pathology within murine asthmatic models. This study also shows that there is little change in cytokine protein expression levels despite increased gene expression, suggesting that the expression of genes and expression of proteins are regulated separately within the unstimulated cells. Thus, I conclude that the HH pathway has an impact on the expression of important genes in eosinophil-like cells, but its effect on the protein expression is as of yet undetermined. Future work is therefore needed to ascertain the extent to which the genetic upregulation shown affects the cells when stimulated, perhaps with IL5 or other immunostimuli including PAF or TLR ligands to induce a cellular inflammatory response.

6.0 Results: Investigation of the inhibition of Gli-dependent transcription on HL60-eos cells

Following the validation of the HL60-eos model and the investigation into eosinophil biology in the presence of SHH, a Hh pathway activator, the next objective was to investigate the effects of a Hh inhibitor on HL60-eos cells. In Chapter 4, RT-PCR showed that HL60 cells express SHH mRNA and GLI1, raising the possibility that these cells may be able to undergo autocrine or constitutive Shh signaling in the absence of exogenous rhSHH. To test this, we aimed to inhibit the downstream effects of Hh signaling in the cells and perform molecular assays as in Chapter 5. There are several small molecule inhibitors that interact with various molecules of the Hh pathway. In this case, GANT61 was chosen because it is a direct inhibitor of Gli activity, through binding directly to the GLI protein and preventing binding to promoter regions, thus decreasing the expression of Hh/Gli target genes (Zhang et al., 2016). While GANT61 is an inhibitor of the Hh pathway, it does not act on the same part of the pathway as SHH. SHH is extracellular and binds to the cell surface receptor PTCH, whereas GANT61 acts in the nucleus. Therefore, although SHH and GANT61 act on the same pathway, the results from our experiments with these molecules may not be reciprocally comparable. Of note is that as the GANT61 protein was reconstituted in DMSO, all control '-GANT61' conditions include the same volume/concentration of DMSO as the +GANT61 conditions, in order to standardize the results.

6.1 Viability of HL60-eos cells with GANT61

HL60-eos cells were cultured with GANT61 or DMSO-control for up to 96h. Cell viability was measured by trypan blue exclusion over the days of culture (Figure 6.1).

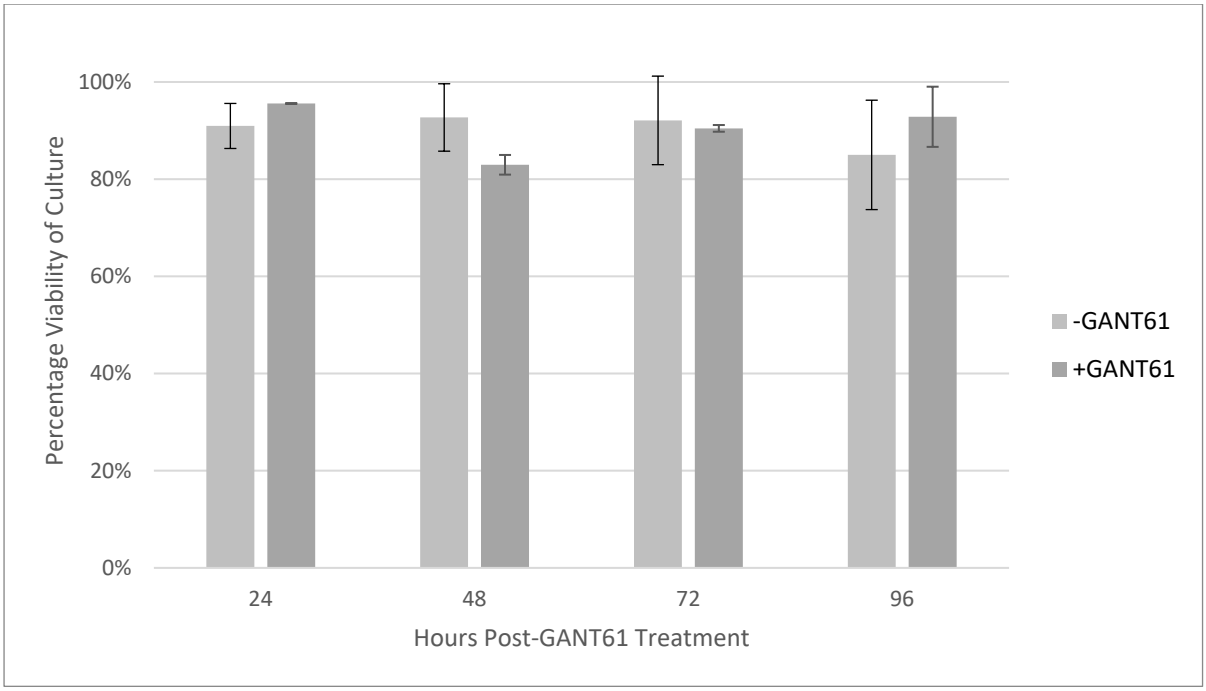

Figure 6.1: Trypan Blue exclusion cell viability counts of HL60-Eos cultures at 24, 48, 72, and 96hr of culture with (+GANT61, 50µM) and without GANT61 (-GANT61), (n=3).

Figure 6.1 shows that the proportion of viable cells in the GANT-treated population only decreases by 3% between 24 and 96 hours, from 96% to 93% respectively, and the largest decrease is between 24 and 48 hours (13%, from 96% to 83% respectively). This figure demonstrates that there is no catastrophic loss of cell population during the culture process. Similarly, there is little difference between the untreated DMSO-control and GANT-treated cells on average, with neither showing an overall trend. Figure 6.1 shows a marginal increase in the proportion of viable cells at 96hrs, from 85% in DMSO-control -GANT61 cultures to 93.29% in +GANT61 cultures. This was determined to be non-significant (p=0.33) via a two-tailed T test. that GANT61 treatment has no significant effect on the proportion of viable cells. Similarly, the untreated DMSO control (-GANT61) data demonstrates that the addition of the small quantity of DMSO has no effect on the cells' viability.

6.2 Morphology of HL60-eos with GANT61

As figure 6.1 shows that the +GANT61 treated cells remain as viable throughout the culture period, the investigation into the downstream inhibition of Gli by GANT61 can continue. This began with studying any changes in cell morphology. The morphology of the cells in both +GANT61 and DMSO-control -GANT61 conditions were compared using the

MGG stain described in Chapter 3.4 and visualised using light microscopy (Figure 6.2). Figure 6.2 shows that GANT61 has little effect on cell morphology that is visible to light microscopy, as both figure 6.2A and figure 6.2B show smaller cells with a pinkish colouration, which aligns with the expected uptake of eosin that would be seen in HL60-eos versus HL60 blast-like cells (Figure 4.2).

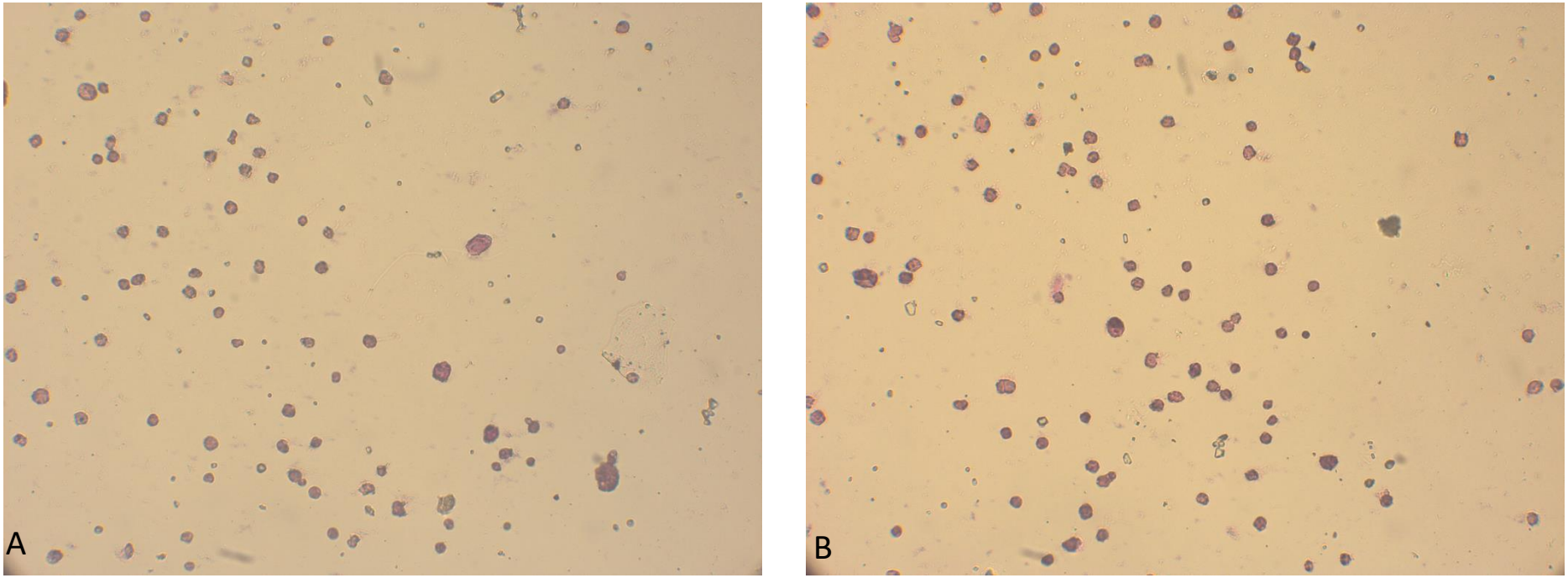

Figure 6.2: Light Microscopy of MGG-stained HL60-eos in DMSO-control -GANT61 conditions (A), and +GANT61 conditions (50µM) (B), at 100x magnification. Representative of 5 independent experiments.

6.3 Inhibition of GLI1 in HL60-eos with GANT61

To confirm that GANT61 treatment inhibited Gli-dependent transcription, QPCR was performed on cDNA created from RNA extracts of the 96hr cell cultures in both +GANT61 and DMSO-control -GANT61 conditions using primers for *Gli1*. GLI1 was chosen for investigation both for its downstream mediator position in the Hh pathway but also for the fact that *GLI1* is inhibited by GANT61 in other cell types *in vivo* (Zhang et al., 2016) and so should be inhibited similarly in HL60-eos, as we know the cells express GLI1 (Figure 4.5). In figure 6.3, *GLI1* expression shows a consistently large decrease in +GANT61 HL60-eos cultures but is not significantly different (p=0.09), presumably due to the variation in the extent of downregulation: 0.5, 0.2, and 0.09-fold changes in three independent experiments respectively (Unpaired t-test of DMSO-control -GANT61 ddCt vs +GANT61 ddCt: 0.09). This is an expected result as GANT61 is a Gli inhibitor, so a downregulation of the level of *GLI1* transcription would be expected.

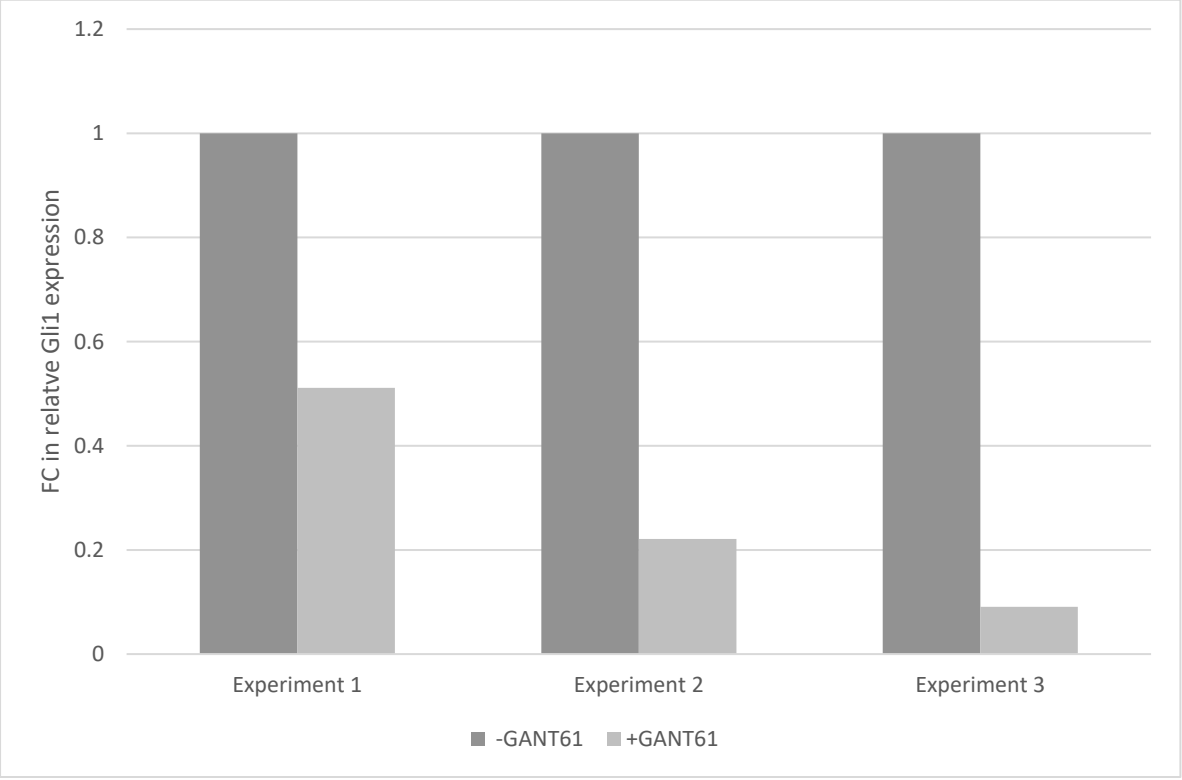

Figure 6.3: GLI1 Gene Expression fold changes in GANT61-treated cultures relative to control cultures for three independent experiments. Gli1 expression was analysed relative to Gapdh expression and was determined by ΔΔCt analysis. HL60-Eos cells were cultured with (+GANT61, 50µMl) and without GANT61 (-GANT61). The experiment was performed three

times independently (Experiments 1, 2 & 3) and each qPCR experiment was performed in triplicate. Each experiment is shown independently for transparency.

6.4 Expression and Production of cytokines by HL60-eos with GANT61

Gene and protein expression levels of IL4, TGF-β and EPX were examined next. These were chosen as genes of interest as these genes are candidate Hh target genes based on our previous work (Standing et al., 2017; present data, Chapter 5.2). As in Chapter 5.2, qPCR was used to identify the levels of gene expression within the cells, and ELISA was used to ascertain the levels of IL4, IL5, TGF-β, and GM-CSF within the supernatant of the cell samples. The rationale for this was that if basal HH signalling is active to some degree in HL60-eos cells, and Hh signals potentiate transcription of our chosen genes, we can test the hypothesis that GANT61 could decrease the expression of these genes in HL60-eos cells, and their subsequent transcription levels.

HL60-eos cells were treated with GANT61 or DMSO-control for 96h and expression of *IL4* was assessed by qPCR using primers specific for the human *IL4* gene (Figure 6.4a). This showed a decrease in *IL4* expression in +GANT61 conditions for one of the replicates, however the other replicate shows no change at all. This was non-significant (Unpaired t-test of DMSO-control -GANT61 ddCt vs +GANT61 ddCt: p=0.57). However, with only two repeats, it is unknown which of these are closer to the actual effect GANT61 has, so further repeats are needed. ELISA was then performed on the cell supernatant (Figure 6.4b). This showed a decreasing trend in the expression of IL4 with a 75% decrease but is not significant (Control IL4 concentration vs +GANT61 IL4 Concentration: p=0.33), likely due to the large variation in the DMSO-control -GANT61 supernatants. This is likely due to the data being extrapolated from below the standard curve as the level of IL4 in the supernatants was barely detectable as before.

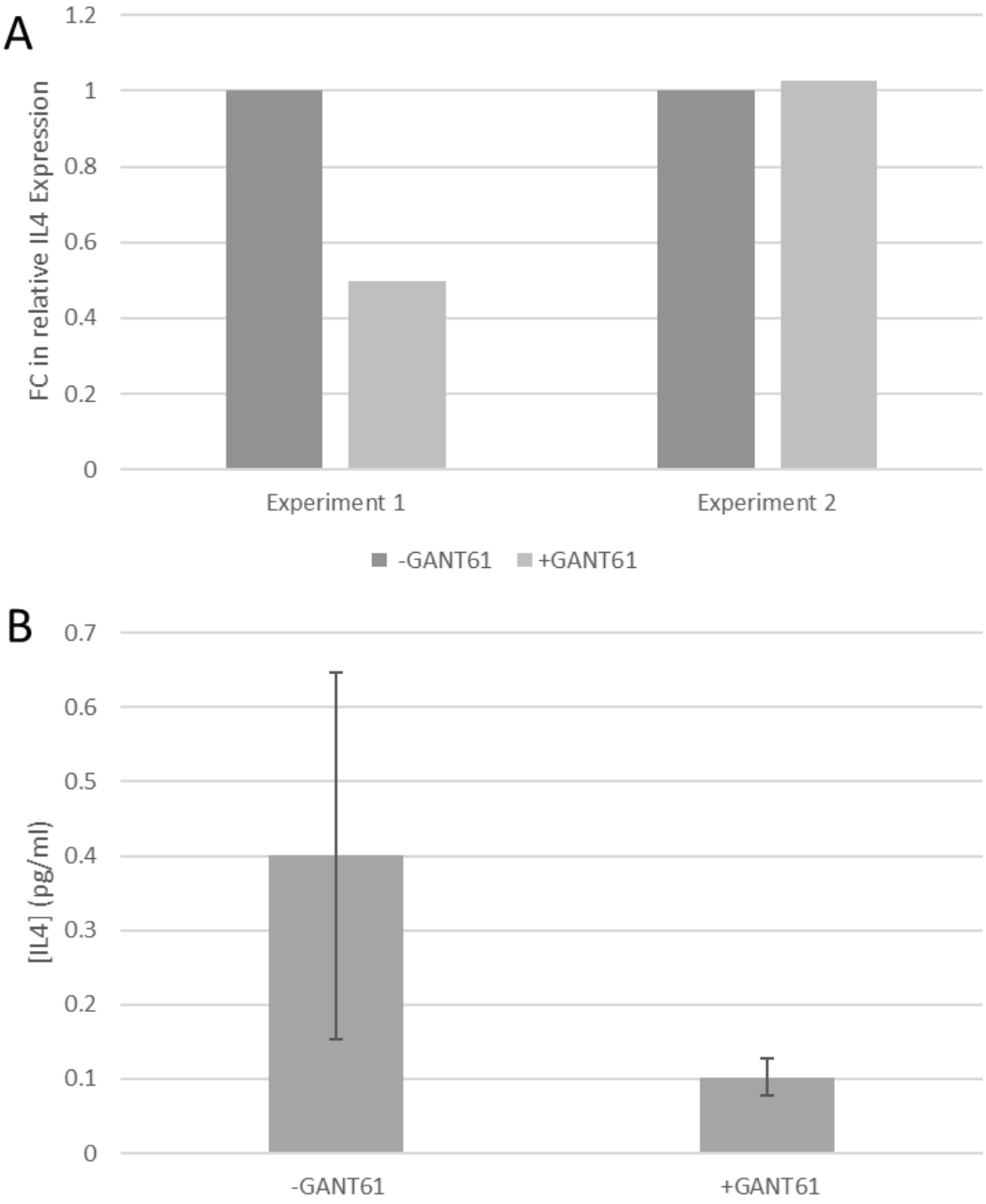

Figure 6.4: Interleukin 4 expression in HL60-eos cells. A, IL4 Gene expression fold change relative to Gapdh expression determined by ΔΔCt analysis, performed three times independently (Experiments 1, 2, 3 not shown as no data) and each qPCR experiment was performed in triplicate. B, Secreted IL4 protein analysis via ELISA, performed in duplicate for each of three experiments. HL60-Eos cells were cultured with (+GANT61, 50μM) and without GANT61 (-GANT61) for 96hrs, p>0.05 n/s.

Following the investigation into IL4, *TGF-β* gene expression was investigated, by qPCR using human *TGFB1* specific primers. No significant difference was seen in *TGF-β* expression between cells treated with GANT61 compared to control cultures (Figure 6.5A; Unpaired t-test of DMSO-control ddCt vs +GANT61 ddCt: p=0.18), however a decreasing trend was seen with an average fold change of 0.6 which signifies a 40% decrease in gene expression. Repeat 2 of this experiment (Figure 6.5A) appears to be an outlier however, and this will affect both the average and the significance of the decrease. For example, the decrease in *TGF-β* expression would likely become significant given another repeat that showed similar decreases to repeats 1 and 3. Having observed this decreasing trend, the next point of investigation was TGF-β protein expression between untreated and GANT61-treated conditions (Figure 6.5B). Despite the decrease in gene expression seen in figure 6.6A, the ELISA results of figure 6.5B show a non-significant increase in protein levels in the supernatant (Unpaired t-test of DMSO-control ddCt vs +GANT61 ddCt: p=0.16). As both the average change in Figure 6.5A, and the increase in figure 6.5B are non-significant, it must be said that GANT61 treatment has no effect on the level of TGF-β produced.

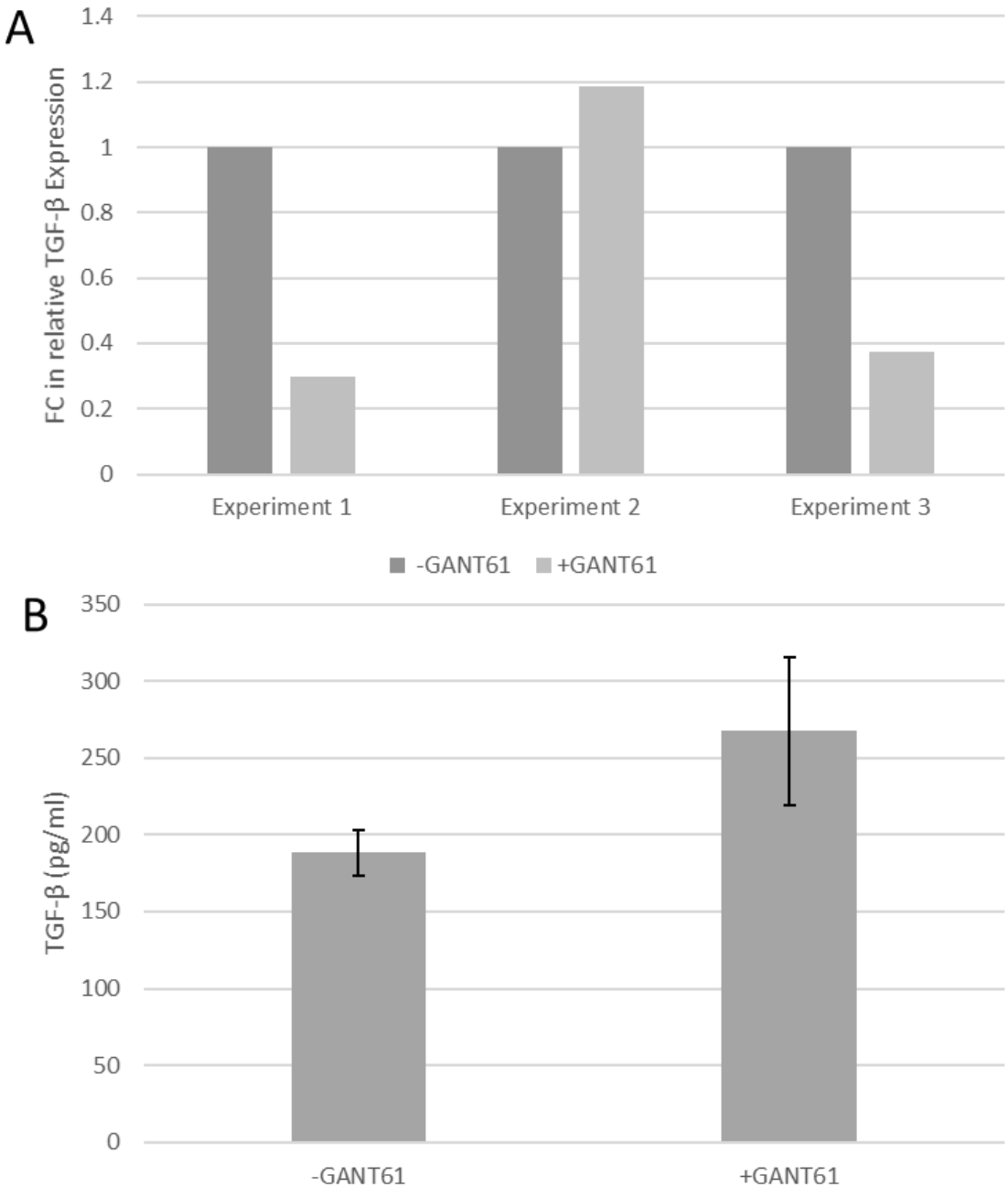

Figure 6.5: Transforming Growth Factor Beta expression in HL60-eos cells. A, TGF-β Gene expression fold change relative to Gapdh expression and determined by ΔΔCt analysis, performed three times independently (Experiments 1, 2 & 3) and each qPCR experiment was performed in triplicate; B, Secreted TGF-β protein analysis via ELISA, performed in duplicate for each of three experiments. HL60-Eos cells were cultured with (+GANT61, 500μM) and without GANT61 (-GANT61) for 96hrs, p>0.05 n/s.

As in Chapter 5, the concentration of GM-CSF and IL5 were also tested in culture supernatants, however neither of the cytokines tested were detected (data not shown). This could be for multiple reasons as discussed in Chapter 5.

6.5 Expression and Production of EPX by HL60-eos with GANT61

Having investigated the target cytokines, we investigated peroxidase concentration and activity, and targeted the *EPX* gene as before. As SHH increased *EPX* gene expression (Figure 5.6), it was hypothesised that GANT61, a GLI1 inhibitor, would inhibit *EPX* expression. For this investigation, HL60-eos cells were treated with GANT61 for 96h and expression of *EPX* was assessed by qPCR using primers specific for the human *EPX* gene (Figure 6.6). Figure 6.6 showed a marginal increase in average *EPX* expression of 19% when cells are treated with GANT61 compared to control cultures, which is non-significant (Control ddCt vs +GANT61 ddCt: p=0.45). This shows that in this case, GANT61 does not cause a change in *EPX* expression. As the difference between experiment 3 and experiments 1 and 2 are so high, it is likely that repeat 3 is an outlier; further repeats would elucidate this.

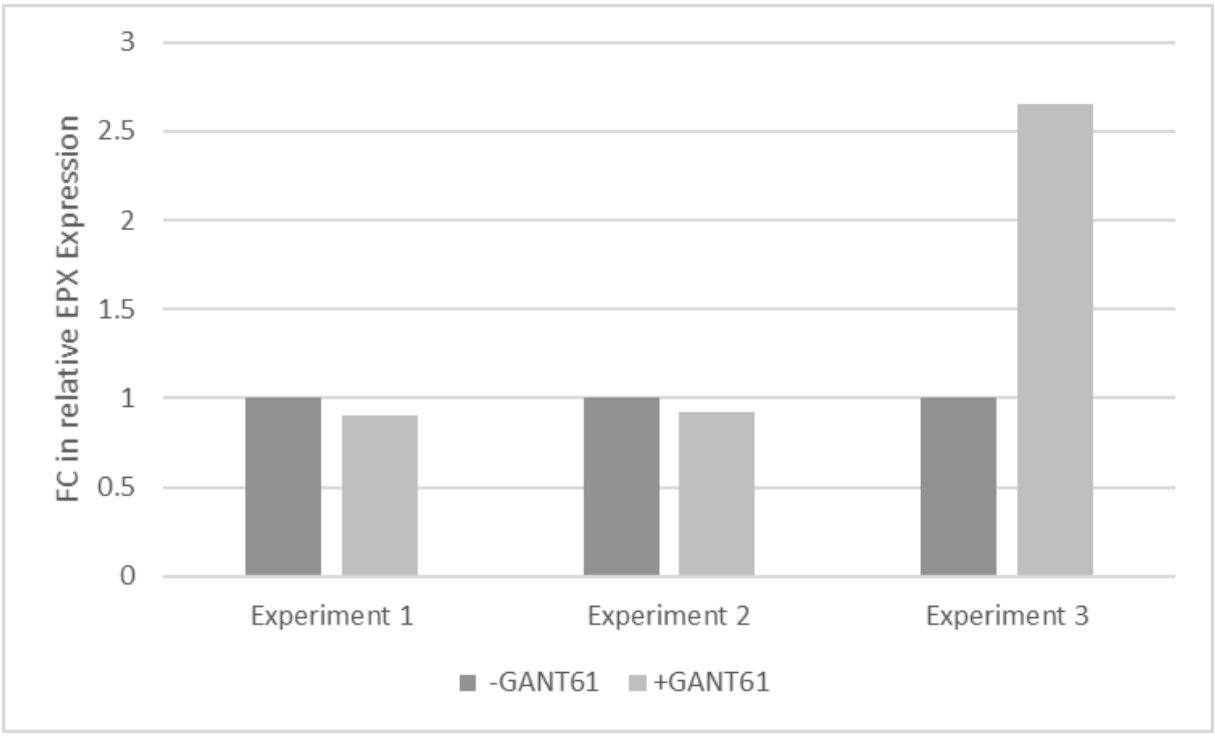

Figure 6.6: EPX Gene Expression fold changes in GANT61-treated cultures relative to control cultures. GANT61 expression was analysed relative to Gapdh expression and was

determined by ΔΔCt analysis. HL60-Eos cells were cultured with (+GANT61, 500μM) and without GANT61 (-GANT61) for 96 hrs. The experiment was performed three times independently (Experiments 1, 2 & 3) and each qPCR experiment was performed in triplicate. Each experiment is shown independently for transparency.

In Figure 5.6, we showed that SHH treatment increases the expression of *EPX*. GANT61 treatment did not decrease expression of *EPX*, (Fig 6.6) nevertheless OPD assays were performed on GANT61 and control treated HL60-eos cells for completeness (Figure 6.7). Fold change in peroxidase activity was measured using OPD as in Chapter 3.6. Limited change in peroxidase activity was expected, as the gene expression levels should be roughly proportional to the protein expression levels. Peroxidase activity showed a non-significant minor decrease in Figure 6.7 (Control peroxidase activity vs +GANT61 peroxidase activity: p=0.78), with the difference being less than 1%. As the change in peroxidase activity between the two samples is non-significant, we must therefore conclude that the +GANT61-treated cells' granules do not contain any higher levels of peroxidases than in the control cells, thus GANT61 has no effect on peroxidase expression levels in eosinophils. Whilst this data is a representation of the expression profile of unstimulated/resting eosinophils, this may not be the case in stimulated cells however, and so further investigation is required on this.

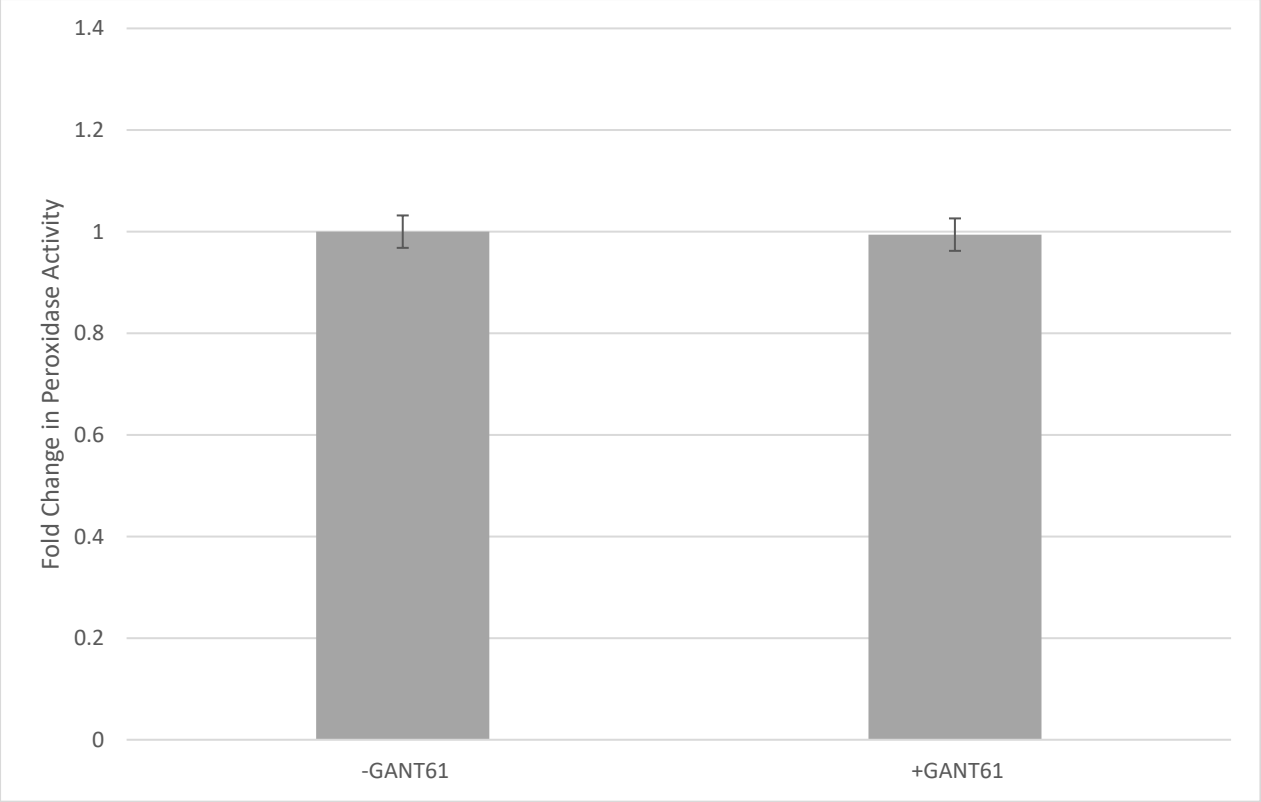

Figure 6.7: Fold change in peroxidase activity determined from OPD assay. HL60-Eos cells were cultured with (+GANT61, 50µM) and without GANT61 (-GANT61) for 96 hours, before stimulation with 4µL Cell Stimulation Cocktail (eBioscience, Cat# 00-4975-93) for 1 hour before spectrophotometric analysis at 420nm, n=3, p>0.05 n/s.

6.6 Conclusion

This study found that, despite decreasing trends seen in gene expression, GANT61 has no significant effect on gene expression levels in resting HL60-eos (*GLI1* p=0.09, *IL4* p=0.57, *TGF-β* p=0.18, *EPX* p=0.45). I theorise that this may be down to experimental error margins, and less the effect of GANT61 on the cells themselves. Importantly, these experiments were also performed in HL60-eos cells without any exogenous SHH. GANT61 treatment should reduce any endogenous Gli-dependent transcription in the HL60-eos cells (as seen in *Gli1* expression, Fig 6.4), however the data collectively suggest that the cells are not undergoing significant autocrine signaling. Similarly, the protein concentrations in supernatants showed no significant changes (IL4 p=0.33, TGF-β p=0.16), but did show some trends that may be more pronounced with further repeats and/or in a stimulated HL60-eos experiment. Finally,

the peroxidase activity showed very little change at all between conditions possibly showing that eosinophil peroxidase activity, and by proxy their peroxidase content, is regulated by a separate downstream mediator that isn't GLI-dependent. Future work is therefore needed to ascertain the extent to which the eosinophil model reacts to GANT61 treatment when cells are stimulated with an eosinophil secretagogue, as outlined in Chapter 5, and in the presence of exogenous SHH, as discussed in Chapter 7.

7.0: Discussion

7.1 Results generated and their applications

Within this investigation, we have shown that SHH treatment causes the significant increase of *EPX* and *TGF-β* gene expression within HL60-eos cells (p=0.022 and p=0.045 respectively). At the time of writing, we believe this has not been previously shown. The discovery of *EPX* being upregulated by SHH is important, as EPX is one of the four most common components of eosinophil granules, the others being MBP, and RNAse 2 and 3. As described in Chapter 1, these granules form within the cytoplasm of the eosinophil and release their contents onto an infiltrating multicellular organism, destroying the organism's cell membrane through catalysed free radical formation (Wang and Slungaard, 2006). In allergic asthma, eosinophils are recruited to the lung and degranulate into the airways, resulting in major cell damage and the release of Damage-Activated Molecular Patterns (DAMPs). This results in the classical pathology of allergic asthma, with high airway sensitivity, increased mucus production, and inflammation. Our finding builds on the current knowledge that murine eosinophils can transduce a Hh signal (Standing et al., 2017), by showing *in vitro* that human eosinophilic cells can undergo Hh signalling, and that one of the consequences of this is upregulation of *EPX* gene expression. Because of this, it is probable that this would result in increased EPX protein production, which is yet to be shown. If this occurs, we could speculate that the concentration of EPX produced by eosinophils would increase, which could exacerbate the damage to local cells caused by EPX as a result of degranulation. This could increase the severity of allergic asthma through increased damage, release of DAMPs and inflammatory sequelae. This could result in the recruitment of, among other cell types, T cells to the site of damage. The T cells would then convert to Th2 cells at an enhanced rate due to the presence of SHH (Furmanski et. al 2013, Standing et al., 2017, Yanez et al., 2019), resulting in increased eosinophil recruitment, potentiating the inflammatory cycle. Notably, SMO-inhibitor treatment in a murine model of occupational allergic asthma showed decreased eosinophil recruitment to the lung (Yanez et al., 2021). It is still not known whether inhibition of Hh/Gli signalling could be therapeutic in human allergic asthma. Therefore, further investigation into this is required, such as the use of 3D culture models, as discussed in the next section: Future Work.

Applications of this discovery are not limited to allergic asthma, as other eosinophilic disorders such as eosinophilic esophagitis, which also involves bystander tissue damage due to the degranulation of eosinophils. It would be interesting to investigate whether there is a link between Hh signalling and eosinophilic esophagitis among other eosinophil-associated diseases, as all share similar pathology involving degranulation, implicating EPX and TGF-β. This could be tested in murine models to begin with. Dosing the mouse with varying concentrations of SHH in the oesophagus as well as an allergen known to trigger eosinophilic esophagitis, followed by esophageal biopsy would show any changes in severity of response, and therefore severity of damage, as a result of SHH treatment. The protein content (EPX, MBP, RNAse2 and 3) or eosinophil prevalence in the esophageal biopsy could then be measured through immunofluorescent staining. Conversely, the opposite could be tested, where a mouse model of eosinophil esophagitis, as described above, is treated with a Hh inhibitor (GANT61 for example) to see if the disease symptoms alleviate. For a human investigation into this, patient biopsies could be investigated for evidence of Hh signalling. For example, downstream Hh/Gli target genes such as GLI1, GLI2, EPX, and TGF-β could be targeted for QPCR analysis of samples, and immunofluorescent staining performed to identify SHH location and a qualitative idea of its presence.

Papaioannou et al. (2019) have investigated the influence of Shh on atopic dermatitis. They show that SHh signalling reduced the pathology of atopic dermatitis, with the severity of disease being determined by the expression levels of SHh. This was then shown to occur through the increase of Treg cell populations, ultimately resulting in increased TGF-β expression. Whilst this appears to show the opposite of the findings presented in this thesis, in that SHH downregulates atopic dermatitis whereas it appears to upregulate an allergic response in eosinophils (and in asthma), the two functions of SHH are not mutually exclusive. SHh is a morphogen, meaning that the effect caused by the presence of SHH might not increase linearly with any change in concentration. SHh responses are highly concentration-specific, and so this may be why the effect is different. This remains to be formally tested. Similarly, the environments being investigated are different, with the skin being optimised as a defensive layer, and this investigation using single cells. Likewise, there will be differences in microenvironmental cues, such as the signals from other cytokines and surrounding skin cells to name a few. Finally, a major factor to compare between the two

findings is that of the model types. This thesis uses a human cell line model with only one cell type in isolation. In contrast, Papaioannou et al. used a mouse model of atopic dermatitis. The difference in model species would create some differences with regards to response to SHh, due to the differing gene expression profiles. Similarly, the mouse model is an in-vivo study, as opposed to the in-vitro system used in this thesis. Therefore, it is also harder to dissect whether the response to SHh is a primary or secondary response in an in-vivo model, as multiple factors could be responsible. The differences of in-vitro and in-vivo investigation on different cell types would therefore also be an explanation of the different effects seen. As highlighted here and in the Papaioannou et al. paper, SHh's functions exhibit a high level of context dependency, as well as being regulated differently due to variance in CD4−+ T cell regulation, therefore secondary factors must be considered when elucidating SHh's effects.

The finding that *TGF-β* transcription in HL60-eos cells is significantly upregulated by SHH treatment ($p=0.045$) is also important as TGF-β is a cytokine with multiple different functions. It is responsible for leukocyte regulation and cytokine secretion from T cells, and in higher concentrations has apoptotic effects on granulocytes (Yoshimura and Muto, 2010; Letterio and Roberts, 1998). TGF-β is also known as a regulator of eosinophil chemotaxis (Li et al., 2006). This means that TGF-β increases recruitment of eosinophils to the site of immune response, as well as modulating the immune response through increased T cell cytokine secretion, triggered as a response to TGF-β. In asthma, this will result in further pathogenesis. The eosinophils will degranulate, damaging surrounding tissues, and the T cells will increase leukocyte recruitment, continuing the asthmatic episode. Furthermore, TGF-β is well documented for its role in triggering fibrosis (Frangogiannis, 2020) and mediating airway remodelling in asthma (Al-Alawi, Hassan and Chotirmall, 2014), as well as being upregulated in the airways of human allergic asthmatics (Vignola et al., 1997). Therefore, our study shows evidence that Hh signalling could play a role in airway remodelling, a direct consequence of chronic inflammation in asthma, through the upregulation of TGF-β. *In vivo* investigations, likely in mice, would be required to confirm this, however.

Furmanski et al. (2013) showed via microarray, that TGF-β family member genes are upregulated during active Gli-dependent transcription in CD4+ T-cells. Other studies have examined the link between Hh/Gli signalling and TGF-β signalling. For example, Furler and Uitternbogaart (2012) showed that GLI2 is a transcriptional regulator of *TGF-β1* in Human CD4+ T cells. Our findings may suggest the same is true in eosinophils, as activated GLI2 is a product of the Hh/Gli pathway activation, therefore any increase in expression of TGF-β because of SHH treatment could involve increased activation of GLI2. However, to confirm this, expression levels of the activator form of GLI2 would need to be analysed. GLI protein expression is not limited to canonical Hh/Gli pathways, as the TGF-β/SMAD pathway also has GLI1/2 as an early gene transcription target (Dennler et al., 2007). Cells could therefore be capable of non-canonical expression of Hh target genes, through the presence of TGF-β. These findings demonstrate that the two pathways have reciprocal synergism with one another, with both pathways being capable of expression of downstream targets of GLI1/2 independent of one another, in turn activating both Hh and TGF-β pathways once again. Our study demonstrates the Hh/Gli pathway aspect of this synergy, through the increased TGF-β expression a result of SHH treatment.

To conclude, we have found that eosinophilic cells upregulate genetic expression of EPX and TGF-β in the presence of SHH. This builds on the research of Furmanski et al. (2013) and Standing et al. (2017) and helps to further characterise the effect of the Hh pathway on eosinophils. These findings have some cross-disease application also, as other eosinophil-associated diseases share some common pathological mechanisms, implicating the Hh pathway in the pathogenesis of these diseases.

7.2: HL60-eos as a model of eosinophils

Throughout this investigation, we used HL60-eos cells as a model for human eosinophils. This is a published protocol that differentiates HL60 to HL60-eos cells by culture with Sodium Butyrate (Fischkoff et al., 1984). The cell line has been used repeatedly throughout the literature, from the use of the HL60-eos cells to characterise the response to hematopoietic growth factors, to the determination of leukotriene expression regulation in the presence of GM-CSF and IL5 in HL60-eos (Fabian, Lass, Kletter and Golde, 1992; Scoggan, Ford-Hutchinson and Nicholson, 1995). The use of the model is well documented, and widely accepted. However, the model is not without fault. Whereas in most papers the cells are reportedly entirely homogenous after differentiation or treated as such, our data shows otherwise (Figures 4.1, 5.3, and 6.3). Our populations are mostly homogenous, with all cells taking on a pinker colour, however there is still some variation in intensity of colour as well as size and shape of the cell, with some cells being larger than the main population of cells. The size and shape changes are especially evident in the flow cytometry analysis in Figure 4.2. Whilst the homogeneity reported in the literature was not achieved, there was still a clear shift from the blast-like HL60 cells to the more eosinophilic HL60-eos cells, with only a small fraction of cells retaining their original morphology. The lack of total homogeneity could be due to the increased passage number, as HL60 were resurrected at passage 7 and kept in culture until passage 30. It is widely recognised that too high a passage number in any cell culture can lead to malperformance of cultures and has the potential to increase genomic instability. Romero-Steiner et al. (1997) reported difficulty in differentiating HL60 cells into granulocytes at passages above 35, however the HL60 cells used in this investigation never passed passage 30, even after differentiation.

Another issue with the model is that the model is a leukaemic cell line. This means that the cells do not have standard signalling profiles and have an increased degree of genetic instability. This means that, whilst the cell line is functionally immortal, there will be a point at which too many mutations may have accumulated, and the cells do not function as required. This would be disastrous if these mutations prevented correct differentiation into eosinophils and may skew results. Similarly, there is the possibility that as these cells are leukaemic, that the HH pathway is signalling abnormally. For example, a mutation causing

constitutive expression of GLI1 would result in constant transcription of Hh target genes. This could mean that adding SHH to the cells would have a minimal additive effect on the HL60-eos cells, as the Gli-dependant genes would be upregulated already. Aberrant Hh signalling has been reported in Acute Myeloid Leukaemia (Long et al., 2011). However, our data from experiments with GANT61 (Chapter 6) treatment of HL60-eos cells shows little difference compared to control cultures, so it seems unlikely that Hh-Gli signalling is aberrantly activated to the extent that it is stimulating leukaemic proliferation in our differentiated HL60-eos cells. If there was aberrant signalling, it would be expected that GANT61 would have more of an effect on the viability of the cells, due to GANT61 being a Gli antagonist. However, this study did not have a non-leukaemic cell population to compare against, so it is not possible to state whether HL60/HL60-eos rampantly express Gli1 or not.

Whilst the HL60 cell line has its drawbacks and weaknesses, it is a well-documented cell line that is still widely used for investigations to this day. Therefore, I conclude that the model is suitable for this short MSc by Research project. However, the best cell type for our experiments would certainly be primary human eosinophils. These would remove the complicating factors that comes with using a leukaemic cell line. However, primary eosinophils have their own drawbacks, such as requiring ethical permission and licensing to obtain, store and culture, and repeated harvesting being required. Variation between donors is another factor to consider when performing these investigations on human primary eosinophils. Future experiments utilising these cells are proposed in Chapter 8: Future work.

7.3 Limitations within the Project

Some of the data presented in Chapters 5 and 6 show outliers and large margins of error within replicates. For example, the qPCR data for GLI1 expression in the presence of SHH shows one of the replicates as having little change, when the other two repeats show a 13-fold and 8-fold increase respectively. Considering it is well known that GLI1 is upregulated during Hh signalling, and the fact that our data shows this for two experiments, we suspect that the third experiment is an outlier. This trend is seen in other figures shown in this investigation as well. In order to determine whether such results are outliers, and to elucidate the true result, more repeats must be completed. Under normal circumstances,

these repeats would have been performed however, due to the unforeseen interruptions caused by the SARS-CoV2 pandemic, laboratories were repeatedly closed causing several stop-start periods during the cell culture process. The priority for any future work, therefore, would be to repeat experiments again, to increase validity.

Along a similar vein, more time points were planned to be investigated. For example, we now know that *TGF-β* is upregulated by HL60-eos cells at 96hrs post-SHH treatment. We are the first study at time of writing to show that the human *TGF-β* gene is upregulated in the presence of SHH in human eosinophilic cells. However, we are still unaware at what point this genetic upregulation happens, whether immediately or slowly over time. We showed a time course like this for *EPX* in figure 5.8 and investigating earlier in the culture period and at every 24hr interval would be of interest for TGF-β, however due to time and budget restraints this became unfeasible. I would postulate that the gene expression changes slowly over the course of the culture, as seen in Figure 5.8. This could be completed, as samples are currently stored at -80°C, however it would require the purchasing of new reagents and due to external logistical issues in the UK, delivery times have increased significantly. Similarly, it would be interesting to test different dose ranges of SHH, as SHH is a morphogen and so may have varying effects on the cells based on the concentration present.

One final point of note is that the functional test for EPX must be more specific as the OPD assay tests for all peroxidases. Therefore, had the assay shown any significant difference, it would have been unclear whether it is EPX being upregulated or the other peroxidases within the cell. A more specific test for this would be to stain EPX within the cells using a membrane-permeable fluorescent dye. This would allow for the exact location of EPX expression to be detected. For more quantifiable results, an EPX ELISA would be ideal. The cells must be made to degranulate prior to testing however, as ELISA cannot test for protein within the cell.

The above will be further discussed in terms of potential future work for this project in Chapter 8.

8.0 Future work

This MSc by Research project started towards the end of 2019 but was severely and repeatedly disrupted when the SARS-CoV2 pandemic led to lockdown and the closure of labs. This meant that most of the planned work was performed in a stop-start manner, which is difficult for tissue culture. Further, external supply and logistics issues with ordering delayed acquisition of consumables. Therefore, whilst this has been a successful and rewarding MSc project, it has been a challenging experience.

This paper has thus far shown, in HL60-eos cells, that SHH significantly upregulates gene level expression of EPX and TGF-β ($p=0.022$ and $p=0.045$ respectively), and that inhibition of Gli activity by treatment with GANT61 has no significant effect on these genes ($p=0.45$ and $p=0.18$ respectively). However, several questions remain:

1. Is the concentration of EPX increased by the presence of SHH?

2. Is EPX a direct target gene of GLI?

3. Does SHH upregulate TGF-β protein expression in HL60-Eos cells and if so, what effect does this increase have on additional cell functions?

4. Do HL60-Eos cells require immunostimulation via eosinophil inducing agents or secretagogues to reveal further effects of SHH or GANT61 treatment?

5. What relevance, if any, do our findings have to primary human eosinophils?

6. Are our observations still true if we culture HL60-eos cells with GANT61 or SHH from the start of the differentiation process?

7. What effect does culturing the cells with both SHH and GANT61 simultaneously have on the target genes?

8. How do other cell types react to SHH or GANT61 for the same or similar genes/proteins?

Future work would involve repeating the QPCR for those genes where the results showed a trend but without statistical significance, for example Gli1 and Il4 in SHH-treated cells, and

EPX and TGF-β in GANT-treated HL60-eos cells. The finding that SHH signaling influences EPX expression could be functionally significant as it is a major eosinophil mediator. Further work therefore should measure EPX protein levels in HL60-eos more specifically than the OPD assay in SHH treated versus control conditions, for example performing ELISA would allow for a quantitative measurement of EPX protein expression, as opposed to the proxy measurement of peroxidase activity. QPCR could also be performed for other granule proteins, such as MBP and RNAse 2 and 3, in order to determine whether the increase in EPX seen in SHH-treated cells is also seen for genes that encode the other granule proteins, or whether SHH is an upregulator of EPX only. Finally, it would be interesting to determine whether EPX is a direct target gene of Gli. This could be investigated through bioinformatic analysis to search for a Gli binding site in the promoter region of the *EPX* gene (consensus GLI sites 5'-GACCACCC-3' (Kinzler and Vogelstein, 1990)). If one is present, then it is likely that GLI is a regulator of *EPX* expression. In order to determine transcription factor binding, a chromatin immunoprecipitation (ChIP) assay could be performed to determine whether GLI can bind to the *EPX* gene. Further, ELISA could be performed for the granule proteins, although this should be conducted on cells that had been triggered to degranulate into supernatant because ELISA can only detect protein in suspension, not intracellularly. So, either the cells must be lysed, which would introduce a lot of contamination from cellular debris, or the granules must be released into the supernatant.

Although not presented or discussed in this thesis, this current study did also attempt to measure the chemotactic ability of the HL60-eos cells after SHH or GANT61 treatment. This proved challenging but with optimization this would be an interesting experiment. Eosinophils exhibit chemotactic behaviour in response to a range of chemoattractants including Eotaxin, an eosinophil-specific chemokine. Eotaxin binds to the eosinophil cell surface receptor CCR3, which in turn triggers a downstream cascade resulting in chemotaxis (Rothenberg et al., 1996). SHH is found in the same areas as eosinophils during an allergic response in murine lung, eosinophils are known to receive and transduce Hh signals (Standing et al., 2017) and Shh is a morphogen: it can determine the pattern of cells through non-uniform concentration gradients. Therefore, I hypothesise that SHH itself could act as a chemoattractant, promoting leukocyte recruitment to the lung during allergy. It is known that SHH acts as a chemoattractant for monocytes and macrophages (Schumacher et al.,

2012), as well as being well documented as behaving as a chemoattractant for neurones during embryogenesis (Dunaeva, Voo, Oosterhoud and Waltenberger, 2009; Hammond, Blaess and Abeliovich, 2009; Charron et al., 2003). The hypothesis could be tested through the use of transwell assays to measure the effect of SHH on the cells' chemotactic ability. SHH would be placed on the opposite side of a membrane to HL60-eos cells, along with a known chemoattractant (Eotaxin) or control. By counting the cells that had crossed the membrane in +/- SHH and +/- Eotaxin conditions, it would be possible to establish the degree to which SHH acts as a chemokine, either solo or in collaboration with Eotaxin with SHH acting as a chemotaxis promoter.

Whilst this study has helped to characterise the Hh pathway's effects on eosinophils, it has only done so on resting cells, i.e., cells that have had no immune activating stimuli to produce immune mediators such as cytokines. To trigger this in eosinophils, cells are normally cultured with IL5, PAF or C5a among other stimuli, for anywhere between 24 and 96 hours before harvesting (Reimert, Skov and Poulsen, 1998; Plager et al., 2009; Elsner, Höchstetter, Kimmig and Kapp, 1996) In terms of this study, that would mean treating the cells with IL5 and/or PAF or a mitogen at the same time as the SHH or GANT61 treatment. This should stimulate the cells' immune functions, resulting in a higher expression of cell surface receptors, increased cytokine production, and trigger the expression of genes and proteins that would otherwise remain unexpressed due to the dormant state of the cell. This would allow for a more complete characterization of the HL60-eos response to activation (+SHH) or inhibition (+GANT61) of Hh signalling.

Following this, it would be of interest to co-treat HL60-eos cells with IL5, SHH, and GANT61 simultaneously, to see to what degree SHH and GANT61 interact. For example, we now know from this study that SHH upregulates EPX production. If, hypothetically, we were to treat the cells both with SHH and GANT61 and found that the cells EPX expression is lowered to baseline levels, then this would be evidence that GANT61 directly inhibits EPX production during Hh signalling. This would then suggest a clinical application for GANT61 as a prophylactic anti-inflammatory for use by people with eosinophilic asthma, for example.

Arguably, the most important set of experiments would be to perform all of these experiments, but on primary human cells. Primary human eosinophils from blood or bone

marrow derived eosinophils would be closer to a true in vivo representation of an eosinophil. Harvesting fresh eosinophils from whole blood would remove the need for an extended culture period, requiring only the culture with SHH or GANT61. This would allow not only for a faster turnaround from culture to data but would also be the closest representation of an in vivo eosinophil possible. This would require many repeats due to the variable nature of human biology. However, the University of Bedfordshire does not have the HTA licensing required for this, neither does our research group have external ethical permission for this; this will be sought in the future. Whilst a human model for this is rather difficult to develop, recent advances in microfluidics and small-scale tissue culture have led to the development of so-called "organ on a chip" technologies (OOACs) (Haeberle and Zengerle, 2007). These are biomimetic microfluidic-sustained culture plates that have the capacity to mimic entire organs. In the context of modelling the effect of Hh/Gli activation or inhibition in allergic asthma, a small sample of lung tissue could be taken from a patient with acute allergic asthma and suspended on culture media. This would mimic the interface between air, tissue, and extracellular fluid that would exist in the natural tissue. Varying concentrations of SHH and/or inhibitors could then be added to the culture media and flowed over the lung sample. The tissue could then be removed from the OOAC and stained to measure cell damage/death. Similarly, the culture media could be tested for cytokine (IL4, IL5, etc) and enzyme release (EPX, MBP, RNAse 2 and 3) by local eosinophils. The number of leukocytes that have undergone differentiation as a result could also be determined, through the use of flow cytometry, and specific fluorophore-conjugated antibodies for unique differentiated cell markers (CD15+/CD16- for eosinophils, for example). The air-interfacing surface of the tissue could also be washed, and the fluid collected, to identify and characterise any cells that may have undergone chemotaxis. Through this, the OOACs would allow for as close to a human model as possible without the need for an actual patient, or an animal model as a replacement.

In conclusion, this study has begun to characterise the role of the Hh pathway in eosinophils by building on previous work performed on T cells and in vivo mouse models, however there is still many pieces of the puzzle left to characterise, and improvements to the model to be made before the work can have clinical applications.

9.0 Appendix

9.1 Data Management Plan

Project title
The Role of Hedgehog Signalling in the Biology of Eosinophils
Student name and Research Institute
Name: Lewis Lochhead **Research Institute: iBEST**
Supervisor(s)
Note: the Director of Studies is the Data Steward
*Give the names and affiliations for all of your supervisors and place a * next to the name of the Data Steward: this person provides you with guidance on how to manage your data.* **Dr Anna Furmanski*** **Dr Eleftheria Diakogiannaki**
Project description: aim, objectives, main research methods used (2-3 sentences will suffice)
Aim/Objective: To assess the effect of Hedgehog activation and Hedgehog inhibition on the phenotype and function of human eosinophilic cells **Main research Methods: PCR (both quantitative and reverse transcription), Cytokine assays, Functional component assays, cell culture of leukaemia cell line**

Compliance

When you submit your DMP you are confirming that you have read and understood all of the legislative, policy and contractual requirements that apply to your project.

University policy requirements
Provide a brief summary (2-3 lines) of the main University policy requirements regarding the management of your research data. **Ethical permission was granted, and all work performed complies with the guidelines set out below**
University policy or guidance
University of Bedfordshire GDPR Guidance for Researchers (BREO sign-in required)
University of Bedfordshire Research Ethics Policy
University of Bedfordshire Internet-Mediated Research Guidelines (BREO sign-in required)
University of Bedfordshire Intellectual Property Policy
University of Bedfordshire Compliance with the Concordat to Support Research Integrity

Legal requirements
Provide a brief summary (2-3 lines) of any discipline-specific legislative requirements regarding the management of your research data and list legislation that is specifically relevant to your research, or enter 'Not Applicable' in this section.
UK Legislation or framework
Not Applicable
Contractual requirements
Provide a summary of any contractual requirements regarding the management of your research data or enter 'Not Applicable' in this section. If your funder has a data policy you should include the URL to the policy in this section. You can find out whether your funder has a data policy from the funder's website or by contacting Innovation and Enterprise Services.

Name of funder	**Data policy URL**
Not Applicable	Not Applicable

Gathering data – see 'Assessment of Existing Data' and 'Information on New Data' in the RDM Checklist. Cross-reference with your Data Protection Impact Assessment.

Description of the data
3.1.1 Types of data – existing and/or new
• *Types of secondary data used (literature review)* – ***Secondary data will only be used for the background knowledge, introduction, and for basing the hypothesis on. There is no need for the use of secondary data within the research itself*** • *Types of data generated in the project (primary data set)* – ***Images (Agarose Gels), raw numerical data (QPCR Ct values, Absorbance readings, Cell numbers), Graphs (Flow cytometry, QPCR)***
3.1.2 Format and scale of the data
• *Types of files you will be creating (e.g. Excel, Word, etc.)* ***Excel, Word, Publisher, Powerpoint, JPEG/TIF, Rotor-gene experiment files)*** • *Types of files to be used for long-term archiving* – ***All of the above-mentioned file types will be used for long term archiving***
Data collection methods
• *How will you collect your data?* ***Using equipment provided, such as the Rotor-gene QPCR unit, and the BD Accuri C6 Flow cytometer.*** • *Will you collect data from other sources? If so, include citations of these sources* ***Data will not be collected form other sources, with only figures being cited for background information and to form the basis of my hypothesis***

Development of original software
• *Describe scripts, libraries, plug-ins, software tools, applications, etc. that you will develop: what programming language will be used, how you will address dependencies in your code, etc.* ***Not Applicable*** • *Is the software you're developing for this project only or will other versions be released later?* ***Not Applicable***

Working with data – see 'Quality Assurance of Data' and 'Backup and Security of Data' in the RDM Checklist. Cross-reference with your Data Protection Impact Assessment.

Short- and medium-term data storage arrangements
• *Where will the data be stored during the project (short- and medium-term)?* ***On the equipment producing the data, in my lab book, and on USB drives*** • *How will you create backups?* ***Multiple USB drives will hold copies of my Data, as well as on cloud storage*** • *How will you ensure all files are secure?* ***Multiple password layers (Device, Software, and File level) as well as USB encryption***
Control of access to data and sharing with collaborators (beyond your supervisory team)
• *Who will have access to data?* ***Just me and my Supervisors*** • *How will you control access to data?* ***I keep the copies of the data on my person (USBs), and unless directly shared with supervisors, all original copies are held solely by myself also*** • *Do you plan to transfer data to another institution? If so, how will you do so securely?* ***No***
File organisation and version control
• *How will your files be organised?* ***Folders and sub-folders for each different data and file type*** • *What naming conventions will you use for different versions of the files? (e.g. 'Literature Review – draft 1 – unchecked' or 'Literature Review 1-2-2')* ***"Description dd/mm/yy Version"*** • *How will you keep track of different versions of the files?* ***Version will be noted at the end or, with regards to final versions, noted within the title. Similarly, last edited time will be tracked for all files, for ease of archiving***
Documentation that will accompany the data
• *What documents do you and others need to be able to understand and reuse your data?* ***All data come with labels and titles that make the data legible, however some images will lack labels. Therefore, my lab book notes would be needed to understand my raw images. Processed images however (e.g., for figures) will be understandable without a lab book, as labels and legends will be included*** • *How will these documents be written?* ***Lab book contains all lab notes, gel layouts, and images of the gels***

- *How will they accompany the data files?* ***Lab book will be handed in at the point of submission***

Archiving data – see 'Preparation of Data for Sharing and Archiving' in the RDM Checklist

Selection of data to be retained and deleted at the end of the project

- *Which data will you keep? (e.g. for publications from the thesis)* ***All data will be kept as archived files on USB, as well as cloud storage***
- *Which data will you delete?* ***None***
- *How will you delete/destroy personal data securely?* ***No personal data is being used***
- *If following the funder's guidelines, how will you fulfil their requirements on data preservation?* **Not applicable**

Data preservation strategy and retention period

- *Note: you cannot keep your data on the University server indefinitely;*
- *Where will you store the data after your project is over?* ***USB drives as well as on cloud storage***
- *How long will you store it for?* **Indefinitely**

Maintenance of original software

- Are you going to archive a binary executable, installer or package file to accompany your data? **No**
- Will you submit your code to a language-specific network? **No**
- Will you share your code through a source code repository? **No**
- Will you continue to develop your code after the project is over? If so, who will have rights to edit the code? **No**

Sharing data – see 'Preparation of Data for Sharing and Archiving' in the RDM Checklist

Justification for any restrictions on data sharing

- *Note: after publishing your research, you will need to share the data underpinning it as openly as possible unless there are compelling reasons not to do so.*
- *If there are, what are these reasons?* **None**
- *What restrictions will you impose on sharing your data?* **All use of my data must be accompanied by an accurate citation/reference**

Arrangements for data sharing

- *How will you share data with other researchers at the end of your project?* ***Data will be stored on the university's repository for thesis submissions.***
- *What research data archive or repository are you planning to use?* ***The University of Bedfordshire's repository (*****http://lrweb.beds.ac.uk/libraryservices/repository)**

Implementation – see 'Responsibilities' in the RDM Checklist

Review of the Data Management Plan
• *How often will you review your data plan? Provide key dates for reviews (e.g. pre-PP2, post-PP2,etc* **Pre-MiPP (May 2020), as well as when new file/data types are created (~Once fortnightly)** • *Who is responsible for keeping the plan up to date?* **Myself**
Special resources required for the project
• *Are there any resources you need that the University cannot provide? (e.g. storage space, specialist software, etc.)* **No**
Further training needs
• *Do you need any additional training to complete this plan?* **No**

9.2 Ethics Approval

Approval Reference:
UoB/SLS-iBEST-ERP/LL-02-2019

UNIVERSITY OF BEDFORDSHIRE

Research Ethics Scrutiny (Postgraduate Research Students)

When completing this form please ensure that you read and comply with the following:

Researchers must demonstrate clear understanding of an engagement with the following:

1. *Integrity* - The research has been carried out in a rigorous and professional manner and due credit has been attributed to all parties involved.
2. *Plagiarism* - Proper acknowledgement has been given to the authorship of data and ideas.
3. *Conflicts of Interest* - All financial and professional conflicts of interest have been properly identified and declared.
4. *Data Handling* - The research draws upon effective record keeping, proper storage of date in line with confidentiality, statute and University policy.
5. *Ethical Procedures* - Proper consideration has been given to all ethical issues and appropriate approval sought and received from all relevant stakeholders. In addition the research should conform to professional codes of conduct where appropriate.
6. *Supervision* - Effective management and supervision of staff and student for whom the researcher(s) is/are responsible
7. *Health and Safety*- Proper training on health and safety issues has been received and completed by all involved parties. Health and safety issues have been identified and appropriate assessment and action have been undertaken.

The **Research Institutes** are responsible for ensuring that all researchers abide by the above. It is anticipated that ethical approval will be granted by each Research Institute. Each Research Institute will give guidance and approval on ethical procedures and ensure they conform to the requirements of relevant professional bodies. As such Research Institutes are required to provide the University Research Ethics Committee with details of their procedures for ensuring adherence to relevant ethical requirements. This applies to any research whether it be, or not, likely to raise ethical issues. Research proposals involving: vulnerable groups; sensitive topics; groups requiring gatekeeper permission; deception or without full informed consent; use of personal/confidential information; subjects in stress, anxiety, humiliation or intrusive interventions must be referred to the University Research Ethics Committee.

Research projects involving participants in the NHS will be submitted through the NHS National Research Ethics Service (NRES). The University Research Ethics Committee will normally accept the judgement of NRES (it will never approve a proposal that has been rejected by NRES), however NRES approval will need to be verified before research can commence and the nature of the research will need to be verified.

Where work is conducted in collaboration with other institutions ethical approval by the University and the collaborating partner(s) will be required.

The **University Research Ethics Committee** is a sub-committee of the Academic Board and is chaired by a member of the Vice Chancellor's Executive Group, appointed by the Vice-Chancellor and includes members external to the University

Research Misconduct: Allegations of Research Misconduct against staff or post graduate (non-taught) research students should be made to the Director of Research Development.

October 2014

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UNIVERSITY OF BEDFORDSHIRE

Research Ethics Scrutiny (Annex to RS1 form)

SECTION A To be completed by the candidate

Registration No: 1601323

Candidate: Lewis Lochhead

Degree of: Masters By Research

Research Institute: iBEST

Research Topic: Hedgehog-triggered Gli-activated eosinophils differ in structure and function to non Gli-activated eosinophils.

External Funding: N/A

The candidate is required to summarise in the box below the ethical issues involved in the research proposal and how they will be addressed. In any proposal involving human participants the following should be provided:

- clear explanation of how informed consent will be obtained,
- how will confidentiality and anonymity be observed,
- how will the nature of the research, its purpose and the means of dissemination of the outcomes be communicated to participants,
- how personal data will be stored and secured
- if participants are being placed under any form of stress (physical or mental) identify what steps are being taken to minimise risk

If protocols are being used that have already received University Research Ethics Committee (UREC) ethical approval, then please specify. Roles of any collaborating institutions should be clearly identified. Reference should be made to the appropriate professional body code of practice.

This project uses human cell lines that are commercially available. Thus, the cells would have been ethically sourced by the company that provided them and would have all required licenses at the time of purchase.
The cells being used do not fall under the Huan Tissue Act (HTA), as they are a small population of single cell-lines.

1. Any external sources of information (journals, books, etc.) will be correctly cited, to ensure that credit is given to the original authors. Likewise, any research will be noted in the iBEST Laboratory notebook, to ensure accurate and robust record keeping.
2. As above, external sources will be cited and credited where appropriate, and all other work shall be my own.
3. There are no conflicts of interest within nor around this project.
4. All data obtained during this project will be stored according to the RGS guidelines on correct data handling, with the format of: "date(YYYYMMDD)"_"file summary". The data will be stored on university computers, under personal account drives.
5. All research will conform to the Good Laboratory Practice (GLP) that iBEST set out as guidelines to ethical and professional conduct. All samples and reagents are commercially available, with all licenses present at time of purchase.
6. Research will be supervised by the Director of Studies (Dr Furmanski), and will be consolidated on during, at a minimum, monthly formal meetings, of which a summary of this meeting will be recorded on PebblePad.
7. Health and safety requirements have been met, with the appropriate forms completed, including a risk assessment. These forms are available on request.

cell line
Human

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Answer the following question by deleting as appropriate:

1. Does the study involve vulnerable participants or those unable to give informed consent (e.g. children, people with learning disabilities, your own students)?
   No

   If **YES**: Have/will Researchers be DBS checked?
   No

2. Will the study require permission of a gatekeeper for access to participants (e.g. schools, self-help groups, residential homes)?
   No

3. Will it be necessary for participants to be involved without consent (e.g. covert observation in non-public places)?
   No

4. Will the study involve sensitive topics (e.g. sexual activity, substance abuse)?
   No

5. Will blood or tissue samples be taken from participants?
   No

6. Will the research involve intrusive interventions (e.g. drugs, hypnosis, physical exercise)?

   No

7. Will financial or other inducements be offered to participants (except reasonable expenses)?
   No

8. Will the research investigate any aspect of illegal activity?
   No

9. Will participants be stressed beyond what is normal for them?
   No

10. Will the study involve participants from the NHS (e.g. patients) or participants who fall under the requirements of the Mental Capacity Act 2005?
    No

If you have answered yes to any of the above questions or if you consider that there are other significant ethical issues then details should be included in your summary above. If you have answered yes to Question 1 then a clear justification for the importance of the research must be provided.

*Please note if the answer to Question 10 is yes then the proposal should be submitted through **NHS research ethics approval procedures** to the appropriate **NRES**. The UREC should be informed of the outcome.

Checklist of documents which should be included (Tick as appropriate):

Project proposal (with details of methodology) & source of funding	✓
Documentation seeking informed consent (if appropriate)	N/A
Information sheet for participants (if appropriate)	N/A
Questionnaire (if appropriate)	N/A

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Applicant declaration
I understand that I cannot collect any data until the application referred to in this form has been approved by all relevant parties. I agree to carry out the research in the manner specified and comply with the statement of ethical requirements on page 1 of this form. If I make any changes to the approved method I will seek further ethical approval for any changes.

Signature of Applicant: ..... Date: 25/10/19

Signature of Director of Studies: ~~.....~~ Date: 25/10/19 .

This form together with a copy of the research proposal should be submitted to the Research Institute Director for consideration by the Research Institute Ethics Committee/Panel

Note you cannot commence collection of research data until this form has been approved

SECTION B To be completed by the Research Institute Ethics Committee:

Comments: No specific ethical issues associated with HTA and/or human participants ~~identified~~ within research proposal presented. Wider research ethical issues have been addressed.

Approved

Signature Chair of Research Institute Ethics Committee: .....

Date: 12/11/2019

This form should then be filed on the student's record

If in the judgement of the committee there are significant ethical issues for which there is not agreed practice then further ethical consideration is required before approval can be given and the proposal with the committees comments should be forwarded to the secretary of the UREC for consideration.

There are significant ethical issues which require further guidance

Signature Chair of Research Institute Ethics Committee:

Date:

This form together with the recommendation and a copy of the research proposal should then be submitted to the University Research Ethics Committee

Approval Reference:

UoB/SLS-iBEST-ERP/LL-02-2019

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10.0 Bibliography

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